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ECOLOGY OF BRYOZOAN EPIPHYTISM ON THE KELP *AGARUM FIMBRIATUM* HARV.

BY

KATHLEEN MARY DURANTE 

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

DEPARTMENT OF ZOOLOGY

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We, the undersigned, certify that the research presented in Chapter II of this Ph.D. thesis (published as Durante, K. M., Chia, F. -S. 1991. Epiphytism on *Agarum fimbriatum*: can herbivore preferences explain distributions of epiphytic bryozoans? Mar. Ecol. Prog. Ser. **77**: 279-287) was designed and executed solely by the primary author, Kathleen M. Durante. We grant permission for a version of this publication to be included as Chapter II of this thesis.

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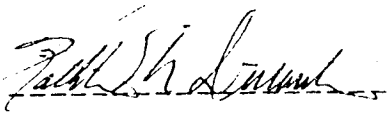
Signed: 

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ECOLOGY OF BRYOZOAN EPIPHYTISM ON THE KELP *AGARUM FIMBRIATUM* HARV. submitted by KATHLEEN MARY DURANTE in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Date: 22 June '94

I dedicate this thesis to my father,

William Virginio Durante
(1929-1988)

who opened my eyes to the wonders of the ocean.

ABSTRACT

Secondary metabolites manufactured by marine algae serve as defenses against herbivory and fouling, but those herbivores acquiring resistance to algal chemical defenses can both tap underexploited food resources while receiving associational protection from algal defensive compounds. In contrast to the many studies addressing how plant defensive chemicals affect herbivores, this thesis addresses how chemical defense in a marine plant may affect organisms other than those using the plant as food. In particular, I focused on the relationship between a group of epiphytic bryozoans and their preferred host plant, a chemically defended kelp.

In Barkley Sound on Vancouver Island, blades of the kelp *Agarum fimbriatum* are heavily colonized by the epiphytic bryozoans *Lichenopora novae-zelandiae* and *Tubulipora* spp. Not only do larvae of these bryozoans preferentially settle on *A. fimbriatum*, but they also select young blade tissue near the intercalary meristem. Substratum selectivity together with kelp growth patterns explain why juvenile bryozoan colonies are concentrated on young blade tissue and adult colonies on older blade tissue.

This age specific pattern in the distribution of epiphytic bryozoans also mirrors, however, a gradient in polyphenolic content. Polyphenolics, known to be effective herbivore deterrents, are concentrated most highly in young blade tissue nearest the meristem. Because polyphenolics are water soluble, they can (and do) leach from kelp tissues into the surrounding seawater, where they are accessible for detection by bryozoan larvae. In settlement bioassays, bryozoan larvae settled on substrata made with water soluble compounds extracted from young blade tissue, but avoided settling on those made with extract from old blade tissue. Young tissue extract had a higher polyphenolic content than old tissue extract, and its ability to induce bryozoan larval settlement contradicts reports that polyphenolics have anti-fouling properties.

Polyphenolics were also heavily concentrated in structures anchoring the kelp to the substratum (holdfast and stipe), and in reproductive sorus tissue. Consistent with theoretical predictions, tissues in *Agarum fimbriatum* most important for maintaining plant fitness were also those receiving the highest allocation of anti-herbivore defenses. By associating with polyphenolic-rich tissues in this kelp, bryozoans appear to gain a refuge from herbivory-related costs, as well as a continually renewable substratum that is generally free of competing epiphytic species.

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CHAPTER 1

General introduction

Sessile marine invertebrates colonize a variety of substrata, including living plants and animals. The association of a sessile invertebrate with a particular substratum may occur because (1) the invertebrate larva has selected that substratum for a location to settle and metamorphose to the sessile stage, (2) individuals settling on that substratum survive to reproduce more often than those selecting other substrata, or (3) both. In this thesis, I examine why, in Barkley Sound on Vancouver Island, certain bryozoans belonging to the genera *Lichenopora* and *Tubulipora* grow in high densities on blades of the kelp *Agarum fimbriatum*. For many reasons, blades of *A. fimbriatum* would seem to be unlikely locations to support bryozoan growth and reproduction, but the most puzzling reason is that tissues of this kelp contain unusually high levels of polyphenolic compounds (Steinberg 1985), which are thought to kill or at least deter settling invertebrate larvae (Conover & Sieburth 1966, Sieburth 1968). The main focus of this study was to determine why *A. fimbriatum* is a preferred substratum for these epiphytic bryozoans, and what role polyphenolics play in the epiphytism.

Plant secondary chemicals

Some chemicals found in living organisms have no apparent metabolic function, yet can be energetically very expensive to synthesize and store (Gulmon & Mooney 1986, Bazzaz et al. 1987). These compounds,

termed secondary metabolites, are present in great diversity among plant taxa (Swain 1977, Jones & Firn 1991), yet explanations for the evolution of secondary chemical diversity in plants remain controversial (Tallamy & Krischik 1989). Many claim that secondary metabolites function as defenses against herbivory (reviewed in Rhoades & Cates 1976, Rhoades 1979, Feeny 1992, Hay & Steinberg 1992), and the evidence supporting this hypothesis is consistent with theoretical predictions. (1) Chemically defended plants should be grazed less than palatable species (e.g., Steinberg 1985, Targett et al. 1986, Paul & Hay 1986). (2) As predicted by McKey (1974), within a plant, structures most vulnerable to damage by herbivores, such as reproductive structures and young or meristematic tissue, should receive a disproportionately high allocation of secondary chemicals (e.g., Steinberg 1984, Johnson & Mann 1986, Paul & Van Alstyne 1988a, Pfister 1992). (3) Herbivores feeding on chemically defended plants should suffer increased mortality or reduced fitness (see Rhoades 1979, Feeny 1992). Regardless of the extensive evidence implicating herbivory as the major selective force in the evolution of plant secondary chemicals, other factors, such as UV light and stress have also been suggested as potential selective agents (see discussion in Tallamy & Krischik 1989).

Many animals acquire resistance to potent toxins and antifeedants, and they become specialist consumers of chemically well-defended plants. By preferentially consuming tissues of a chemically defended plant, these herbivores specialize on a food resource that other organisms generally avoid, and therefore the food resource is not likely to disappear rapidly. Through coevolution, certain plants can, however, alter their chemical defenses in response to levels of herbivory (see Rhoades 1979, Spencer 1988). Some marine algae (Van Alstyne 1989) and terrestrial plants

(Zangerl 1990, Zangerl & Berenbaum 1990) can respond to herbivory on a much shorter time scale, by increasing chemical synthesis or translocating stored defensive chemicals to the site of wounding. Such inducible defenses (reviewed in Harvell 1990) enable plants to avoid producing chemical defenses when they are not at risk of being grazed.

Vicarious defense

Because the cost of providing chemical defense can be prohibitively expensive (Gulmon & Mooney 1986, Bazzaz et al. 1987), some organisms cheat by forming symbiotic associations with chemically defended species. In such relationships, the chemical defenses of one organism also protect the cheater, giving the cheater vicarious protection, termed associational defense (*sensu* Hay 1986), from herbivory, predation, colonization, or disease. Vicarious protection can occur, for example, (1) when mimics acquire aposematic coloration (see examples in Wickler 1968) or chemical signals (Stowe 1988) similar to that of defended species, (2) when herbivores sequester defensive chemicals from their plant prey and are subsequently avoided by predators (e.g., Paul & Van Alstyne 1988b, Hay et al. 1989, Hay et al. 1990b), (3) when animals live on or near defended plants and are hidden from or avoided by predators (e.g., Hay et al. 1990a), and (4) when, by growing near chemically defended plants (Hay 1986, Littler et al. 1986) or animals (Littler et al. 1987), plants find a refuge from herbivory.

Epiphytism on marine algae

Marine algae are known to produce hundreds of secondary metabolites (Scheuer 1978-1983, Fenical 1982), yet we understand the ecological significance of only a few of these compounds (Hay & Fenical 1988). In

addition to facing the risk of herbivory, marine plants also must contend with the possibility that their tissues may be colonized by plant or animal epiphytes. Sessile invertebrates, particularly clonal species that grow laterally and produce colonies with large surface areas, commonly foul the surfaces of marine algae (reviewed in Seed & O'Connor 1981, Seed 1986). Although they do not consume macrophyte tissue, these suspension feeding invertebrates may impose serious costs to their underlying plant substratum. For example, in fast moving currents, kelp blades with heavy epiphyte loads may experience drastic increases in drag forces, even to the point of being ripped from the stipe (Dixon et al. 1981). Fronds of heavily encrusted seaweeds are known to suffer reduced photosynthesis (Oswald et al. 1984, Cancino et al. 1987). Because marine algae lack roots, photosynthesis and other metabolic pathways depend on the direct uptake of nutrients from the surrounding seawater (Dring 1982), and invertebrate epiphytes can create physical barriers to nutrient uptake processes (Hurd et al. 1994).

Although both natural products chemistry (Scheuer 1978-1983, Fenical 1982) and epiphytism (Seed & O'Connor 1981, Seed 1986) are well understood, only a few studies have addressed whether sessile epiphytic invertebrates are affected by chemical defenses in their host algae. Tannins present in the epiphyte-free growing tips of the brown alga *Sargassum natans* have been shown to precipitate protein in the tentacles of a hydroid that commonly grows on older tissues of the same alga (Sieburth & Conover 1965). Also, young algal tissue of both *S. natans* and *S. fluitans* killed bacteria normally associated with older tissue of the same plants (Conover & Sieburth 1964). These studies on *Sargassum* suggest that tannins may affect distributions of epiphytic organisms (including bacteria)

by preventing colonization of young tissues.

Hay and his colleagues have discovered that certain crustaceans and molluscs are preferentially associated with chemically defended plants (reviewed in Hay & Fenical 1988, Hay 1991, 1992). These mobile animals also consume tissues of their host plant, however, so it is not clear how important epiphytism is relative to herbivory in the dynamics of these ecological relationships. In one study on freshwater organisms, larvae of sessile rotifers that settle preferentially on carnivorous plants initiate settlement behavior in response to chemical signals from glandular trichomes on their host plant (Wallace 1978). Although the chemicals that these rotifer larvae use as cues to initiate settlement may not necessarily be defensive, the plant itself provides a refuge from predation to the rotifers, as potential predators are consumed by the plant while leaving the rotifers undisturbed (Wallace 1978). I predict that sessile marine invertebrates that colonize chemically defended seaweeds would also benefit from plant defenses in cases when plant enemies also harm the invertebrates.

For sessile invertebrates to grow on chemically defended algae, however, at least one of the following conditions must be true: (1) the animals are resistant to plant toxins, (2) the animals possess a mechanism to block the passage of chemicals from plant to animal tissues, or (3) the plant secondary chemicals are contained where they are never exposed to animal tissues in the first place. Because, during normal metabolic processes, marine algae exchange dissolved substances with the surrounding seawater, it seems unlikely that secondary chemicals would remain isolated inside plant cells, although plant cell membranes could possibly prevent the escape of large non-polar molecules. Several studies have confirmed that bryozoans encrusting on marine algae exchange

metabolites and dissolved gases with underlying algal tissues (De Burgh & Fankboner 1978, Muñoz et al. 1991, Hurd et al. 1994), and thus the bryozoan tissues are permeable to at least small organic molecules. Given the enormous diversity in colony morphology and degree of calcification among marine bryozoans, however, bryozoan surfaces contacting plant tissues certainly must differ in their permeability to plant chemicals. The importance of algal secondary chemistry to invertebrate epiphytes depends on (1) whether chemicals are released from plant surface tissues, (2) whether animal membranes at the substratum-epiphyte interface are permeable to released chemicals, (3) toxicity of plant compounds to their animal epiphytes, and (4) the evolutionary tradeoffs between potential negative effects of plant chemicals on animal physiology, and the ecological benefits of being associated with chemically defended substrata.

Antiherbivore compounds

Defensive compounds synthesized by plants belong to one of two major functional classes: (1) toxins, which act on the physiological processes of animals, and (2) digestibility reducers, which either form chemical complexes with plant proteins, rendering the plant tissue indigestible by proteolytic enzymes, or bind with the digestive enzymes themselves (Rhoades & Cates 1976, Reese 1979). In the tissues of temperate marine brown algae, the most common digestibility reducing compounds are polymers of the tannin-like molecule phloroglucinol, called polyphloroglucinols, polyphenolics, or phlorotannins (reviewed in Ragan & Glombitza 1986). The digestibility reducing function of polyphenolics is currently under debate, however, and the incidence and effectiveness of polyphenolic defenses may depend on geographic latitude (for discussion,

see Estes & Steinberg 1988, Steinberg 1989, Van Alstyne & Paul 1990, Steinberg & van Altena 1992, Targett et al. 1992). As for most digestibility reducing defenses, the efficacy of polyphenolics in reducing herbivory depends on their concentration in algal tissues (Geiselman & McConnell 1981, Anderson & Velimirov 1982, Steinberg 1984, 1985, 1988, Van Alstyne 1988, Denton & Chapman 1991, Winter & Estes 1992), and this trend can be explained by evidence that polyphenolics reduce digestibility of algal food in a dose-dependent manner (Tugwell & Branch 1992). In the intertidal brown alga *Fucus distichus*, production of polyphenolic defenses was found to be inducible by mechanical damage, (i.e., simulated herbivory) (Van Alstyne 1988), but Lowell et al. (1991) found a significant decrease in polyphenolic content in experimentally damaged fronds of *Ascophyllum nodosum* relative to undamaged fronds. The discrepancy in results from these two studies may have been due, among other things, to the location of mechanical damage (wing tissue in *F. distichus* vs. central frond axis in *A. nodosum*) or the period of incubation following experimental injury (two weeks for *F. distichus* vs. four for *A. nodosum*).

Although considerable evidence supports the hypothesis that digestibility reducing tannins are non-specific defenses against herbivory, chemical diversity of tannins is high, and these molecules may be specific for chemical substrates other than food proteins and enzymes (Zucker 1983). Brown algal polyphenolics have toxic effects on the larvae of certain marine invertebrates (Conover & Sieburth 1966), and may also function as (1) antibiotics preventing the formation of a bacterial film on algal surfaces (Sieburth & Conover 1965, Sieburth 1968), (2) allelopathic agents hindering growth of other algae, and (3) antifoulants to macroalgae (Conover & Sieburth 1964, Sieburth & Conover 1965, Anderson & Velimirov 1982).

Tannins from terrestrial plants also cause systemic toxicity and ulceration in herbivore digestive systems (Bernays et al. 1989). Hence, for sessile epiphytic invertebrates, the risk of being poisoned by chemicals leaching from their host algae may be independent of whether the chemicals are toxins or digestibility reducers. Being water soluble, polyphenolics can leach from tissues of marine algae and diffuse into the surrounding seawater (reviewed in Ragan & Glombitza 1986). Under certain conditions, (e.g., low diffusion rates near an algal surface), perhaps polyphenolics could be present in high concentrations at the algal surface. As many sessile invertebrates lack true circulatory or excretory systems, and must exchange metabolites with the surrounding seawater via epidermal cells, they would likely be defenseless against an onslaught of chemicals capable of crossing cell membranes.

Bryozoan epiphytism on *Agarum fimbriatum*

My thesis examines the relationship between defensive chemicals produced by a marine brown alga and the epiphytic bryozoans using that alga as their preferred substratum. The Bryozoa include approximately 20,000 fossil and living species (Boardman et al. 1983), and constitute a major animal phylum of which many species are exclusively epiphytic on marine algae. In Barkley Sound, on Vancouver Island, British Columbia, Canada, several species in the bryozoan genera *Lichenopora* and *Tubulipora* (Cl. Stenolaemata: O. Cyclostomata) colonize a north temperate kelp, *Agarum fimbriatum* Harvey 1862 (Cl. Phaeophyta: O. Laminariales). *A. fimbriatum* is a dominant understory kelp that grows in shallow subtidal regions along the west coast of North America, and, together with its congener *A. cribrosum*, is a low preference food item for

generalist herbivores feeding on macroalgal prey (Vadas 1977). On blades of *A. fimbriatum*, these bryozoans are found in high densities, yet they are rare on other substrata in the same habitats. The reason why this epiphytic relationship is so unusual is that *A. fimbriatum* tissues contain some of the highest concentrations of polyphenolic compounds found in temperate brown algae (Steinberg 1985). Because brown algal polyphenolics appear to have generally toxic effects on small fouling organisms and invertebrate larvae (Sieburth and Conover 1965, Conover & Sieburth 1966, Sieburth 1968), that these stenolaemate bryozoans appear to have selected a polyphenolic-rich alga as their preferred substratum is most unusual.

The distribution of stenolaemate bryozoans on blades of *Agarum fimbriatum* appears to be non-random, with larger bryozoan colonies occurring on older kelp tissue, and vice-versa (see Fig. I-1 for explanation of kelp morphology). In this kelp species, polyphenolic content is generally higher in younger blades than in older blades (Steinberg 1985), but nobody has investigated the possibility that polyphenolic content varies along a gradient of tissue age within a blade. Theory predicts that young, meristematic tissues (vulnerable to herbivory because plants cannot grow without them) should be more heavily defended against herbivory (McKey 1974). At least one marine brown alga allocates more polyphenolic defenses to young tissue near its apical meristem (Van Alstyne 1989), so I would expect that in *A. fimbriatum*, younger blade tissue near the intercalary meristem would have higher polyphenolic content. My observations on bryozoan distributions together with evidence supporting differential within-plant allocation to chemical defenses in marine algae (Steinberg 1984, Van Alstyne 1989) prompted me to ask whether the spatial patterns exhibited by epiphytic bryozoans on blades of *A. fimbriatum* could

be explained by patterns of chemical defense in the underlying algal tissue.

Epiphytic bryozoans colonizing undefended substrata are at a higher risk than those on chemically defended plants because (1) herbivores grazing algal tissues could inadvertently damage or ingest epiphytic animals, (2) small pieces of algae ripped from the whole plant by herbivores inevitably wash up on shore, or are carried by currents to habitats where the algal tissue and its accompanying epiphytes soon die, and (3) blades severely damaged by herbivory may not recover, and the decomposing organisms on the dying algal tissue would also likely smother and kill epiphytic bryozoans. Because *Agarum fimbriatum* is more likely to provide a refuge from herbivory related damage than other temperate kelps (Vadas 1977), bryozoans may improve their chances for survival by selecting that alga as a substratum. If, as for other marine algae (e.g., *Sargassum natans* and *S. fluitans*; Conover & Sieburth 1964, Sieburth & Conover 1965), polyphenolics in *A. fimbriatum* function as antifoulants, then specialist bryozoan epiphytes may ensure a habitat with little or no competition for space. In many subtidal encrusting communities, space available for colonization is rare, and competition for that limited resource is intense (Jackson 1983, Buss 1986, Sebens 1986). Encrusting invertebrates that can grow on plants improve their chances of finding suitable adult habitat, and, if they can also tolerate an undesirable plant where no other organisms can grow, then they greatly improve their potential to grow and reproduce successfully.

Bryozoan larvae & settlement preference

Like the majority of marine bryozoans, stenolaemates produce non-feeding (i.e., lecithotrophic) larvae that are planktonic for a short period (ca

minutes to hours) before settling and metamorphosing on a suitable substratum (Nielsen 1970, Zimmer & Woollacott 1977) (see Fig. I-2). Because choice of substratum may determine whether an organism survives to reproduce, the dispersing larvae play a critical role in securing safe habitat for future growth and reproduction of sessile adults, and selective pressure for the evolution of larval discrimination in sessile marine invertebrates should be intense.

Many marine invertebrate larvae can recognize suitable habitat and discriminate among potential substrata (reviewed in Meadows & Campbell 1972, Crisp 1974, 1976, Pawlik 1992a, b), yet we are only beginning to understand the mechanisms they invoke for substratum recognition. Recently, nerves associated with larval sensory structures were found to be stimulated when exposed to chemicals from the animal's preferred substratum (Arkett et al. 1989), strongly supporting the idea that invertebrate larvae use chemicals from their habitat as cues to initiate settlement and metamorphosis (reviewed in Burke 1983, Pawlik 1992a, b).

Although some studies have addressed why certain bryozoans preferentially settle and grow on marine algae (Ryland 1959, Ryland & Stebbing 1971, Stebbing 1971, 1972, Ryland 1974), none has tested the possibility that bryozoan larvae use algal secondary compounds as chemical cues to locate their preferred substrata. Crisp and Williams (1960) found that the larvae of epiphytic bryozoans settled in response to aqueous extracts from their preferred substrata, fucoid algae, but it is not clear whether defensive compounds were present in the extracts. Fucoid algae do contain polyphenolic compounds (Steinberg 1985, Van Alstyne 1988, 1989), but, given that the recommended solvents for extracting polyphenolics are alcohol and acetone (Ragan & Glombitza 1986), aqueous

extractions of fucoids may yield low polyphenolic content. Substances that slough from the surface of *Agarum fimbriatum* blades can repel herbivorous urchins, (Vadas 1977), however, so polyphenolics may leach from algal surface cells in sufficiently high concentrations to be of ecological significance.

Because epiphytic bryozoans growing on *Agarum fimbriatum* are substratum specific, their larvae probably have acquired mechanisms to distinguish algae from inert substrata, and *A. fimbriatum* from other kelps. Also, as *A. fimbriatum* is reported to produce unusually high levels of water soluble polyphenolic compounds (Steinberg 1985), and as invertebrate larvae commonly use chemical cues to identify their preferred substratum (reviewed in Pawlik 1992a, b), I address whether larvae of the bryozoan epiphytes on *A. fimbriatum* use polyphenolic compounds as settlement inducing cues. Because secondary metabolites in seaweeds presumably evolved in response to herbivory rather than to fouling, however, it is difficult to imagine how epiphytic organisms could have acquired recognition of these compounds unless they also benefit in some way from plant chemical defenses.

A note on terminology

To clarify any ambiguity in the terminology used to describe relationships between algae and the sessile invertebrates using those algae as living substrata, I need to describe the different terms used throughout the literature, and to define those I will be using throughout this thesis. The term "algal epifauna" has been used commonly to describe those animals growing on the surfaces of algae (e.g., Seed & Boaden 1977, Hayward 1980, Seed & O'Connor 1981, Oswald & Seed 1986). In contrast,

Osman (1977) describes "epifaunal communities" as sessile invertebrates that grow on some type of hard substratum, (i.e., rock or shell), consistent with the definition of "epifauna" as benthic animals living on the surface, as opposed to within, the sea bed (Allaby 1985). Animals growing on algae have also been termed "epizooic" (De Burgh & Fankboner 1978), although "epizoic" (sic) is generally applied to non-parasitic animals that attach themselves to the outer surfaces of other animals (Allaby 1985). "Epiphyte" commonly refers only to those plants using other plants for physical support (Allaby 1985).

The word "epiphytic" is derived from the Greek prefix, "epi", meaning on or upon, and root "phyton", meaning plant, and therefore its conservative definition should be "upon plants". Also, as the root "faunal" describes animals, then the word epifaunal should mean "upon animals" (see Gove 1986). To avoid confusion, I will use the term epiphyte only to describe organisms living on the surface of plants. Hence, epiphytic bryozoans, as used throughout this thesis, are bryozoans that grow on the surface of plants (see also Ryland & Stebbing 1971), and epiphytes of the kelp *Agarum fimbriatum* may include both animals and plants, although the focus of this study was exclusively those epiphytes belonging to the Bryozoa.

Organization of the thesis

The central focus of my thesis is the relationship between chemical defense and bryozoan epiphytism in the kelp *Agarum fimbriatum*. The thesis is organized in paper format, and each of the four major chapters (II, III, IV, V) is or will be published as a separate manuscript. Although I have made every effort to minimize duplication, some repetition of background

material was necessary to maintain clarity in each chapter.

In Chapter II, I report results from a study (published as Durante & Chia 1991) on the relationship between the stenolaemate bryozoan *Lichenopora novae-zelandiae* and its algal substratum *Agarum fimbriatum* from Dixon Island, in Barkley Sound, British Columbia. In this study, I examined spatial relationships of bryozoan colonies along kelp blades, substratum selection by bryozoan larvae, and the risk of herbivory of blade tissue from *A. fimbriatum*. Larval selection was studied using laboratory choice experiments, in which larvae (Fig. I-2c) were offered equal size disks of blade tissue, cut from several kelp species. In these experiments, larval preference was determined by counting numbers of newly settled bryozoan ancestrulae (Fig. I-2d) on each choice substratum, with the preferred substratum having the highest number of settled bryozoans. The attractiveness of *A. fimbriatum* tissues to herbivores was determined by offering, in non-choice experiments, clean and bryozoan-epiphytized kelp disks to the generalist herbivorous gastropod *Tegula pulligo*. Results from this study enabled me to focus on the nature of the epiphyte-host plant relationship, including: (1) why the bryozoans appear to prefer plant tissue that is more heavily defended against herbivory, and (2) why *A. fimbriatum* may not deter fouling by *L. novae-zelandiae*.

Chapter III documents results of a long term census in which I quantified the distribution of bryozoans along blades of *Agarum fimbriatum*, which were collected approximately bimonthly from September 1990 to May 1993. I specifically examined temporal patterns of bryozoan abundance, and the relative positions of post-larval ancestrulae, juveniles, and adults (see Fig. I-2). Also, as the kelp *A. fimbriatum* is a living substratum, I measured growth of kelp blades in both the laboratory

and the field, and used this growth information to interpret bryozoan spatial patterns.

Chapter IV addresses bryozoan larval preferences for tissues of their preferred kelp substratum, *Agarum fimbriatum*, and those of other co-occurring kelps. In closed containers in the laboratory, larvae of *Lichenopora novae-zelandiae* (Fig. I-2c) and *Tubulipora* spp. were released naturally from parental brood chambers (Fig. I-2a, b), and then were able to select from among several test substrata. In paired or block experimental designs, larvae were tested for their ability to discriminate kelp substrata based on the following factors: (1) kelp species, (2) age of kelp tissue, (3) bacterial cues, and (4) chemical cues.

In chapter V, I examine within plant variation in polyphenolic chemical defenses in the kelp *Agarum fimbriatum*. To measure polyphenolic content in kelp tissues, I homogenized small amounts (ca 100-500 mg) of *A. fimbriatum* tissue in 80% methanol, and then used a standard colorimetric assay (Folin & Denis 1915, Swain & Hillis 1959, AOAC 1990) to determine the concentration of polyphenolics in the extract. Polyphenolic concentration in plant tissues was then calculated based on the wet mass of the sample tissue and the wet/dry ratio of adjacent tissue to yield a concentration expressed as the % polyphenolics per unit dry mass of kelp tissue. Tissue samples used for these analyses were collected from two sites in Barkley Sound and at several times of the year.

Within individual kelp plants, I measured polyphenolic content in tissues from regular intervals along the vegetative blade, from patches of reproductive sori, from the holdfast, and from proximal and distal regions of the stipe (see Fig. I-1). Patterns of polyphenolic distribution were then compared to known spatial patterns of epiphytic bryozoans. Also, phenolic

leaching from cut pieces of kelp tissue was documented experimentally.

Chapter VI is a general summary of the thesis, in which I discuss how my results contribute to current knowledge of chemical ecology, plant-animal interactions, settlement behavior and substratum selection by marine invertebrate larvae, and the ecology of polyphenolic defenses in brown algae. Also, as dissertations frequently generate more questions than answers, and this thesis is no exception, I elaborate on the new ideas created during this work, and on the future directions of research on the ecology and evolution of chemical communication between epiphytes and their host plants.

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Figure I-1: Sporophyte of the subtidal kelp *Agarum fimbriatum*. The kelp is attached to the benthos by a branched holdfast and a tough rubbery stipe. As new tissue is formed at the intercalary meristem, older tissue is pushed distally, analogous to a conveyor belt, and the oldest senescing tissue erodes from the distal tip of the blade. Hence, from the meristem to the distal tip, blade tissue exhibits a gradient in age from young to old. The blade is simple with one central midrib, is ruffled or bullate in appearance, and may have darker patches of reproductive sori.

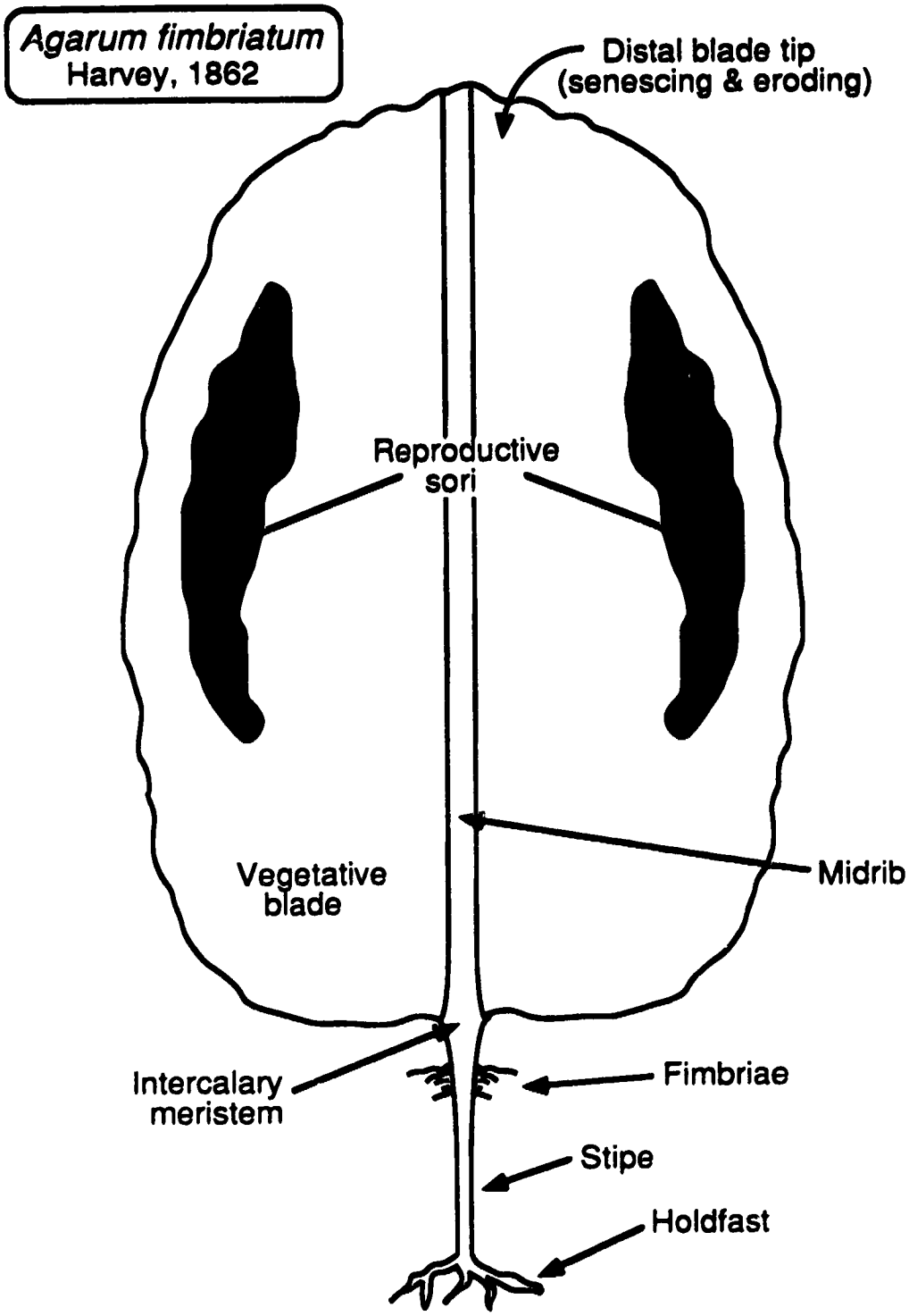
Agarum fimbriatum
Harvey, 1862
Distal blade tip
(senescing & eroding)
Reproductive
sori
Vegetative
blade
Midrib
Intercalary
meristem
Fimbriae
Stipe
Holdfast

Figure I-2: Scanning electron micrographs (SEMs) showing external morphology of the stenolaemate bryozoan *Lichenopora novae-zelandiae.* (a) Sexually mature adult colony; z = zooid tubes, o = ooeciostome, cn = cancelli, scale bar = 500 µm. (b) Close up view of ooeciostome (o), indicated by arrow, through which brooded larvae will emerge, scale bar = 200 µm. (c) Ciliated larva, fixed after being released from adult brood chamber, scale bar = 20 µm. (d) Newly settled ancestrula on blade tissue of the kelp *Agarum fimbriatum,* scale bar = 40 µm. SEM photographs taken by Mr. G. D. Braybrook, University of Alberta.

CHAPTER II

Epiphytism on *Agarum fimbriatum*: can herbivore preferences explain distributions of epiphytic bryozoans?[1]

Introduction

Symbioses are widespread throughout aquatic and terrestrial habitats (Davenport 1955). In marine benthic habitats, many invertebrates display remarkable specificity in their associations with other animals or algae (e.g. Ross 1965, Greene 1970, Ross 1971, Bloom 1975, Stoner 1980, Osman & Haugsness 1981, Olson 1983, Keough 1986, Paul & Van Alstyne 1988). These associations are thought to arise not by coincidence, but via natural selection for adaptive consequences of the symbioses. For sessile invertebrates, permanently affixed to a substratum for their entire adult life, associations or symbioses are formed only when dispersing larvae settle on or near their partners. Thus, if strong selection for symbioses occurs among sessile invertebrates, then habitat selection by dispersing larvae must be the mechanism for bringing associated species together. Most of the previous studies examining ecological and evolutionary associations between species concentrate on only two associated organisms. In this study, I examined an unusual tripartite association among an epiphytic bryozoan, its kelp substratum, and herbivorous snails.

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In Barkley Sound, on Vancouver Island in Canada, I observed a very close association between the bryozoan *Lichenopora novae-zelandiae* (Class Stenolaemata: Order Cyclostomata) and its algal substratum, the subtidal kelp *Agarum fimbriatum* (Class Phaeophyceae: Order Laminariales). Repeated attempts to locate this bryozoan species on other algal substrata and rocks, and among fouling organisms on floating docks were unsuccessful. On *A. fimbriatum*, I observed that younger *L. novae-zelandiae* were concentrated on younger regions of the algal tissue. Many species of bryozoans are known to settle on the surfaces of marine algae and seagrasses (Stebbing 1972, O'Connor et al. 1979, Seed & O'Connor 1981, Oswald et al. 1984, Keough 1986), and may even derive nutrition from their living substrata (De Burgh & Fankboner 1978). Although the planktonic larvae of certain bryozoans settle preferentially on older microbial films (Mihm et al. 1981, Maki et al. 1989), the necessary distributional consequence of such a preference (younger bryozoans found on older plant tissue) is rarely observed in the field. Rather, age distributions of epiphytic bryozoans (as inferred from relative colony sizes) appear to reflect the age of the plant substratum: younger bryozoans are found on young plant tissue, and older bryozoans are found on older plant tissue (Stebbing 1972, Roland 1980, Oswald et al. 1984). If, as numerous studies suggest, bryozoan larvae settle preferentially on older microbial films and on younger plant tissue, then there is a puzzling discrepancy in the physical characteristics of substrata that induce bryozoan settlement. Older plant tissue would have to be covered by an older microbial film than is present on younger regions of the same plant, yet the larvae of certain epiphytic bryozoans prefer younger tissue harbouring younger microbial films. These contrasting reports of bryozoan larval preferences suggest that microbial films,

although important cues for settlement on inert substrata such as glass (Mihm et al. 1981), wood (Brancato & Woollacott 1982), and plastic (Mihm et al. 1981, Maki et al. 1989), may have comparatively little influence when larvae are also exposed to cues associated with living substrata. The work presented here focuses on why epiphytic bryozoans settle only on certain algal species and only on certain regions of the algal thallus. In particular, I considered herbivory to be a potentially important selective force directing the settlement of epiphytic bryozoans.

The association of *Lichenopora novae-zelandiae* with a particular kelp species, and its non-random distribution along kelp blades, prompted me to ask whether selective settlement by the larvae of these bryozoans could explain the observed distribution, and, if so, why this particular distribution might be considered beneficial. Given that *Agarum* spp. are reported to be avoided by generalist herbivores such as urchins (Vadas 1977, Larson et al. 1980), I proposed that either the same mechanism deterring herbivores from this kelp species may also be attracting epiphytic bryozoans, or there has been selection for a substratum that, historically, herbivores (with potential for incidental damaging or killing epiphytic organisms) have avoided. Here, to address the above questions about an observed symbiotic association, I present data on the distribution of the bryozoan *L. novae-zelandiae* along its kelp substratum *Agarum fimbriatum*, results of bryozoan larval selectivity experiments, and results of herbivore preference tests among and within algal food items.

Materials and Methods

Location

All field work was conducted using SCUBA in 10 to 15 m of water at Dixon Island in Barkley Sound, Vancouver Island, British Columbia, Canada [48° 51.24' N, 125° 7' W] between May 1990 and February 1991. The subtidal site is characterized by a gently sloping cobble substratum, and is well protected from storm-induced wave action. Herbivore feeding experiments and bryozoan settlement experiments were conducted in running seawater aquaria.

Epiphyte censuses

The diversity and abundance of epiphytic bryozoans on *Agarum fimbriatum* were measured by taking samples using a transect line marked in 1 m intervals, placed through a dense subtidal bed of *A. fimbriatum* in a randomly determined compass direction. The plant whose holdfast was closest to each transect mark was removed from the substratum and carefully placed inside a sealed polyethylene bag until 10 plants had been collected. The *A. fimbriatum* samples were immediately returned to the laboratory and subsampled in the following manner. Based on a preliminary survey of *A. fimbriatum* sizes at Dixon Island, the average length of this species during August 1990 was ca 120 cm (unpublished data). In members of the Laminariales, including *A. fimbriatum*, new blade tissue is added from an intercalary meristem between the stipe and the blade (Bold & Wynne 1985); thus, plants were divided into three 40 cm sections in order to sample young, medium, and old *A. fimbriatum* tissue. Within each algal age category, a cork borer was used to remove 10 disks

(area of each disk was 3.03 cm^2). In this subsampling procedure, four disks were cut from each of the left and right halves of the blade, and two were cut from the central midrib. The disks were placed in 70% ethanol for storage and later examined for the presence of bryozoans. Epiphytic animals were not loosened or dislodged during preservation in ethanol, although pigments from the algae were extracted by the solvent. To check the accuracy of the subsampling protocol, an additional *A. fimbriatum* was collected, and all *L. novae-zelandiae* ancestrulae (one zooid), juveniles or small adults (asexually growing zooids not yet forming a complete circle on the substratum), and adults (circular colonies) were carefully counted.

Bryozoan settlement experiments

To test whether the planktonic larvae of *Lichenopora novae-zelandiae* actively choose their substrata, I offered a choice of common kelp species at my field site to *L. novae-zelandiae* larvae in a laboratory settlement experiment. This experiment also tested larval preferences between young and old tissue of the bryozoan's commonly used substratum, *Agarum fimbriatum*. For all algal species tested, tissue age categories were determined by measuring the length of the blade from meristem to distal tip, and dividing it into three equal length sections, with the youngest category being that nearest to the meristem, the oldest being nearest to the distal tip, and the intermediate being in between. Substrata for choice experiments (young *A. fimbriatum*, old *A. fimbriatum*, *Nereocystis luetkeana*, and *Macrocystis integrifolia*) were prepared by cutting circular disks (1.6 cm in diameter) from each algal type. For algal species other than *A. fimbriatum*, intermediate age tissue was selected from the central portion of each frond. Following removal of all epiphytes from the

surfaces of the disks, the choice substrata were placed under a jet of running seawater for ca 24 h until mucous discharge from wounded algal tissue ceased. One disk of each choice substratum was placed in each of 10 replicate glass dishes. To avoid bias from unidirectional light in the laboratory, the algal disks were randomly assigned to one of four positions within the dishes.

To obtain bryozoan larvae, adult colonies of *Lichenopora novae-zelandiae* were carefully excised from *Agarum fimbriatum*, and placed in sealed glass dishes containing 1 μm filtered seawater. The dishes were kept cool (ca 10° C) by partially submerging them in a flow-through seawater aquarium, and were shaded by covering them with an opaque black plastic bucket for 6 h. Upon removal from darkness, the dishes were placed on ice and illuminated by a 100 W incandescent bulb, similarly to methods described by Reed (1987). As larvae began swimming out of parental brood chambers, they were pipetted into dishes containing choice substrata. Larvae were not counted individually, but rather were distributed evenly among the 10 dishes by pipetting aliquots of a larval suspension into each dish. The experimental dishes were sealed, partially submerged in a flow-through seawater aquarium, covered with an opaque board, and left undisturbed during the course of the experiment. *L. novae-zelandiae* ancestrulae that had settled on each choice substratum were counted after 24 h and again after 72 h. These data were analyzed using a nonparametric Friedman test for randomized block experimental designs (Zar 1984).

Herbivore feeding experiments

During my field collections and surveys, the dominant herbivore observed feeding on *Agarum fimbriatum* was the prosobranch gastropod

Tegula pulligo. In a previous study at this field site, *T. pulligo* was observed on both *Macrocystis integrifolia* and *A. cribrosum*, and was found to exert considerable grazing damage on *M. integrifolia* (Sharp 1974). As marine prosobranch gastropods have been used with great success in numerous studies addressing palatability of algae and the presence of plant secondary metabolites (e.g. Geiselman & McConnell 1981, Targett & McConnell 1982, Steinberg 1984, 1985, Johnson & Mann 1986), I chose to use the abundant *T. pulligo* for all herbivore feeding experiments presented here. Snails ranged from 1.8 to 2.9 cm in shell diameter, and were collected exclusively from the surfaces of *M. integrifolia* and *A. fimbriatum*. During transport from the field to the laboratory, snails were kept submerged in seawater and shaded from sunlight. In the laboratory, *T. pulligo* were fed *ad libitum* the algae from which they were collected.

I conducted several experiments to test the preferences of snails for the three common kelp species present near my field site, *Macrocystis integrifolia*, *Nereocystis luetkeana*, and *Agarum fimbriatum* (Pace 1975, personal observation). Although *Tegula pulligo* is present on a number of different algal surfaces, I was specifically interested in comparing rates of food consumption between *A. fimbriatum* (*Agarum* spp. are reported to be highly unpalatable; Vadas 1977, Larson et al. 1980, Steinberg 1985) and other more palatable algae. Feeding time among these experiments ranged from 24 to 68 h; this variation was largely due to the prolonging feeding times when low seawater temperatures reduced grazing rates of snails.

First, I compared grazing by *Tegula pulligo* among the above common algal species. To test rates of grazing on these algae, I placed 0.5 to 1.2 g blotted wet weight of algae (intermediate age tissue in all cases) and one *T. pulligo* into 16 replicate flow-through plastic containers (10.5 x 10.5 x 6 cm)

which were covered on three sides by 2 mm Nitex™ mesh. The snails were assigned to treatments randomly, and the mean shell diameter of snails was not found to be different among the three treaments (Kruskal-Wallis Test, $0.25 < p < 0.50$). Four of the containers in each algal treatment were designated as controls, in which snails were confined in Vexar™ bags within the plastic containers, allowing them to fertilize the algae but preventing them from grazing. As each replicate only contained one of the three algal species in this non-choice experiment, a total of 48 replicate containers were used. The containers were submerged in a running seawater aquarium for 48 h, after which all snails were removed and the algal pieces blotted dry and reweighed. Data on the mass of algae consumed in each replicate were adjusted based on growth of algae in the controls. Differences between algal treatments were tested using a Kruskal-Wallis test, followed *a posteriori* by a nonparametric analogue to Tukey's multiple comparisons procedure (Zar 1984).

Second, given evidence that defensive compounds in algae tend to be concentrated more heavily in meristematic and younger tissues (Phillips & Towers 1982, Johnson & Mann 1986, Paul & Van Alstyne 1987, Hay et al. 1988), I conducted a second experiment to test the effect of algal age on the consumption of *Agarum fimbriatum* by *Tegula pulligo*. In addition, because population size and age structures of epiphytic organisms tend to covary with the age of the living substratum (Seed & O'Connor 1981), I designed this experiment to include colonized and cleaned algae as two levels of a second factor. Thus, the four treatments of *A. fimbriatum* tissue in this two-factor experiment were: young colonized, young clean, old colonized, and old clean. Fifteen replicate plastic freezer containers served as the experimental chambers for each treatment, of which five

were designated as controls for algal growth. Algae for clean treatments were scraped with a stainless steel probe and examined under a dissecting microscope to insure that all macroscopic epiphytic organisms were removed. Algae were blotted dry, weighed, and added to containers with two *T. pulligo*. After 24 h, algae were removed from the containers, blotted dry, and reweighed. After adjusting the data for algal growth in the control replicates, the mass of algae consumed was compared among the four treatments using a nonparametric two-factor ANOVA based on ranks (Zar 1984).

Third, to evaluate the potential risk to *Agarum fimbriatum* of consumption by *Tegula pulligo* at the field site, I tested whether snails maintained on diets of different kelp species consumed similar amounts of young and old *A. fimbriatum* tissue. Using snails collected exclusively from the surfaces of either *A. fimbriatum* or *Macrocystis integrifolia*, and maintained on constant diets of these respective algae, this feeding experiment was designed as above, with two levels of two factors: *A. fimbriatum* diet and fed young *A. fimbriatum*; *A. fimbriatum* diet and fed old *A. fimbriatum*; *M. integrifolia* diet and fed young *A. fimbriatum*; *M. integrifolia* diet and fed old *A. fimbriatum*. I placed 0.8 to 3.0 g of young or old *A. fimbriatum* and 1 *T. pulligo* into each of 48 containers, with 12 replicates of each treatment. Eight replicates served as controls for algal growth with snails confined in Vexar™ bags. After submerging the cages in running seawater aquaria and allowing snails to feed for 68 h, the algae were blotted dry and reweighed. As above, these data were analyzed using a nonparametric two-factor ANOVA (Zar 1984).

Results

Epiphyte censuses

The distribution of *Lichenopora novae-zelandiae* along a blade of *Agarum fimbriatum* collected during a period of high bryozoan abundance in May of 1990 (personal observation) showed clear differences in the locations of ancestrulae, juveniles, and adults (Fig. II-1). Although random settlement of larvae could have resulted in the observed adult bryozoan distribution (i.e. the algal substratum must have been around long enough for the bryozoans to grow to adult size), newly settled ancestrulae were clearly present in higher numbers on young algal tissue. As ancestrulae bud new zooids to form juvenile colonies, the alga continues to grow, shifting the bryozoans distally along the blade. Considering the dynamics of algal growth (new tissue being added at the intercalary meristem and transported distally) in concert with clonal growth of bryozoans, the location of the juvenile mode halfway between the meristem and the distal tip of the blade simply reflects the distal movement of ancestrulae as they undergo budding and colony formation.

During September 1990, the abundance of adult bryozoan colonies began to decline, probably due to seasonal senescence and erosion of the distal tips of *Agarum fimbriatum* blades. The distribution of juveniles and ancestrulae along 10 blades of *A. fimbriatum* was qualitatively the same as that described above for a blade collected during the previous spring (Fig. II-2A). In November 1990, when very few adult bryozoans remained on *A. fimbriatum*, there was no new settlement, yet the algae continued to grow. As a result, juvenile and adult bryozoans were concentrated on the older 50% of the blade (Fig. II-2B).

Bryozoan settlement experiments

Laboratory experiments testing the choice of settling bryozoan larvae for different species of subtidal algae revealed a high preference for young *Agarum fimbriatum* tissue over all other choice algae (Fig. II-3). After 24 h, the difference in the number of larvae settling among the 4 choice substrata was significantly different (Table II-1, Fig. II-3A). After allowing the remaining competent larvae to settle for an additional 48 h, the high preference for young *A. fimbriatum* tissue became even more evident (Fig. II-3B), with a mean of >133 larvae settling on the young *A. fimbriatum* choice in each dish. Again, the Friedman test showed a highly significant difference among the four algal choices (Table 1).

Herbivore feeding experiments

When offered *Agarum fimbriatum, Nereocystis luetkeana,* or *Macrocystis integrifolia* as food items, *Tegula pulligo* consumed significantly different amounts of each algal species (Fig. II-4). Consumption of *N. luetkeana* was not significantly different from that of *M. integrifolia,* but both were consumed significantly more than *A. fimbriatum* (Fig. II-4).

The experiment testing effects of *Agarum fimbriatum* age, and presence or absence of epiphytic bryozoans, on consumption of *A. fimbriatum* by *Tegula pulligo* showed significant effects of both factors (Table II-2). Cleaned *A. fimbriatum* was consumed in higher amounts than colonized *A. fimbriatum,* and, regardless of the presence of epiphytes, older algae were consumed more than younger algal tissue (Fig. II-5).

Algal consumption was influenced by the age of *Agarum fimbriatum* offered during the experiment but not by the diet on which the *Tegula*

pulligo were maintained in the laboratory prior to experiments (Table II-3). Although both groups of snails consumed very small quantities of young *A. fimbriatum* tissue (< 0.12 g in 68 h), snails raised on this alga consumed higher amounts of old *A. fimbriatum* tissue than did snails raised on *Macrocystis integrifolia* (Fig. II-6).

Discussion

Adaptive consequences to bryozoans

Larvae of many sessile invertebrates are known to settle preferentially on substrata where the risk of mortality is low (e.g. Grosberg 1981, Moyse & Hui 1981, Young & Chia 1981, Sebens 1983, Johnson & Strathmann 1989, Young 1989). The association of the bryozoan *Lichenopora novae-zelandiae* with an algal substratum that is less likely to be consumed by herbivores suggests that the bryozoan may obtain refuge from herbivory-related costs, such as loss of habitat, and inadvertent consumption during grazing. The substratum selection experiments *L. novae-zelandiae* larvae clearly show that, regardless of post-settlement factors, active larval choice alone can explain the eventual distributions of this species among algae and along an algal thallus. The actual mechanisms directing these bryozoan larvae to their preferred substratum are currently under investigation, yet several lines of evidence lead me to suspect larvae may be responding to differences in anti-herbivore chemical defenses.

Palatability of temperate brown algae to grazers is strongly negatively correlated with levels of polyphenolic compounds, known as phlorotannins (Ragan & Glombitza 1986). Because *Agarum fimbriatum*

tissues contain among the highest known percentages of total polyphenolics among northeast Pacific brown algae (Steinberg 1985), this feature of the alga's biochemical composition may serve as a beacon for attracting organisms that, for a variety of reasons, may be specialist consumers on *A. fimbriatum*. Plant anti-herbivore defenses in terrestrial systems are often exploited by specialist herbivores as cues for locating preferred food (Whittaker & Feeny 1971). Not only do the larvae of *Lichenopora novae-zelandiae* prefer settling on a kelp species known to contain unusually high levels of polyphenolic compounds, but they also preferentially select younger tissues, which, together with reproductive structures, are often the most well defended regions of the alga (Phillips & Towers 1982, Johnson & Mann 1986, Paul & Van Alstyne 1987, Hay et al. 1988).

Although phenolic compounds are thought to have some antifouling properties (Sieburth & Conover 1965), phenolics in brown algae are contained in sealed vesicles called physodes (Ragan 1976). In the epiphytic association studied here, potential toxic effects of contacting polyphenolic compounds may be minimized by the heavy calcification of *Lichenopora novae-zelandiae* colonies, as well as by biological membranes surrounding physodes. The absence of large encrusting bryozoan colonies on the surfaces of *Agarum fimbriatum* in my field surveys may be due to polyphenolic toxicity in organisms with membranous or less heavily calcified body walls, such as the common epiphytic bryozoan *Membranipora membranacea*. Alternatively, larger encrusting colonies may fracture during expansion of the blade by the superficial meristoderm (Bold & Wynne 1985), or may simply have insufficient morphological plasticity to adhere to the ruffled and perforated surface of *A. fimbriatum*

blades.

Adaptive consequences to algae

By comparing grazing on colonized and cleaned algae, I revealed the intriguing possibility that *Lichenopora novae-zelandiae* colonies may offer some protection against herbivory by *Tegula pulligo*. Although it is generally assumed *a priori* that epiphytic organisms impose great costs on host algae, very few studies have demonstrated reduced photosynthetic or growth rates due to the presence of epiphytic organisms (but see Oswald et al. 1984). In fact, an empirical study showed that, although the bryozoan *Membranipora tuberculata* greatly reduced the incident light reaching photosynthetic tissues of its algal substratum *Gelidium rex*, net growth of *G. rex* was unaffected by the presence of bryozoans (Cancino et al. 1987).

Because the experiments comparing herbivory on bryozoan colonized tissue with that on manually cleaned tissue did not include an additional treatment to control for cleaning effects, however, it is possible that the cleaning process itself may have rendered the tissue more palatable. For the cleaned treatments, bryozoan colonies were removed by scraping the surface of the algal tissue with a metal probe. Although care was taken to prevent injury to the kelp tissue during this process, cell walls may have inadvertently been ruptured, and perhaps the separation of bryozoan colonies from the algal tissue also damaged surface cells. During rupture of cells at the surface of *Agarum fimbriatum*, significant quantities of polyphenolics, which deter grazing in proportion to their concentration in algal tissues (see Steinberg 1988), could have leached from the tissue. If polyphenolics had leached from cleaned treatments in sufficient quantities to render the tissue more palatable, then the increased grazing could have

resulted from the cleaning artifact, not simply from the absence of bryozoan colonies. This experiment could have been improved by scraping both cleaned and colonized algae to ensure any damage to algal cells was the same for both treatments.

Factors determining whether epiphytic bryozoans harm or benefit their algal hosts depend not only on the percentage of photosynthetic tissue colonized, but also on the morphology and physiology of the bryozoans themselves. Although my investigation did not include physiological analyses of the epiphytism, any nitrogen limitation in *Agarum fimbriatum* may in part be alleviated by the absorption of nitrogenous wastes from epiphytic bryozoans. Seasonal pulses in growth rates of the subtidal kelp *Laminaria longicruris* have been shown to follow peaks in dissolved nitrate in surrounding seawater (Chapman & Craigie 1977). One study on the interaction between herbivorous zooplankters and their algal prey showed that the increase in algal production due to nitrogen fertilization by herbivores equalled the biomass consumed by herbivores (Sterner 1986).

I noted that the colonial morphology of *Lichenopora novae-zelandiae* and other stenolaemate bryozoans might render them less harmful epiphytes. Encrusting stenolaemate bryozoans are characterized by relatively small size and heavily calcified body walls (Hayward & Ryland 1985). Consequently, they are not likely to form sheet-like colonies that cover large percentages of photosynthetic area on host algae. In addition, because these bryozoans remain small in area and relatively prostrate on the algal surface, they probably do not cause increased hydrodynamic drag on the host algae, nor would they render the entire blade more fragile and subject to loss from the stipe, as occurs with heavy fouling (Dixon et al.

1981). The holdfast of *Agarum fimbriatum* is smaller and less firmly attached to the rock substratum than those of other kelps such as *Pterygophora californica, Macrocystis integrifolia,* and *Eisenia arborea* (personal observation), which would render *A. fimbriatum* particularly vulnerable to dislodgement if drag forces were to be increased by epiphyte load.

Concluding remarks

The association of the epiphytic bryozoan *Lichenopora novae-zelandiae* with its host algal substratum *Agarum fimbriatum* appears to benefit the bryozoan by providing a continually renewable substratum that is less likely to be consumed by generalist herbivores. Both the preferential settlement of *L. novae-zelandiae* larvae on younger regions of *A. fimbriatum,* and the avoidance of younger tissues by herbivorous snails support the possibility that this association may be derived from herbivory-related costs. Because the presence of *L. novae-zelandiae* colonies may have reduced grazing on *A. fimbriatum* by herbivorous gastropods, the relationship between this small stenolaemate bryozoan and its algal substratum may be mutualistic.

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Figure II-1: *Lichenopora novae-zelandiae* settling on *Agarum fimbriatum*. Positions of newly settled ancestrulae, juvenile colonies, and adult colonies of bryozoans along a frond of kelp, as observed during May 1990.

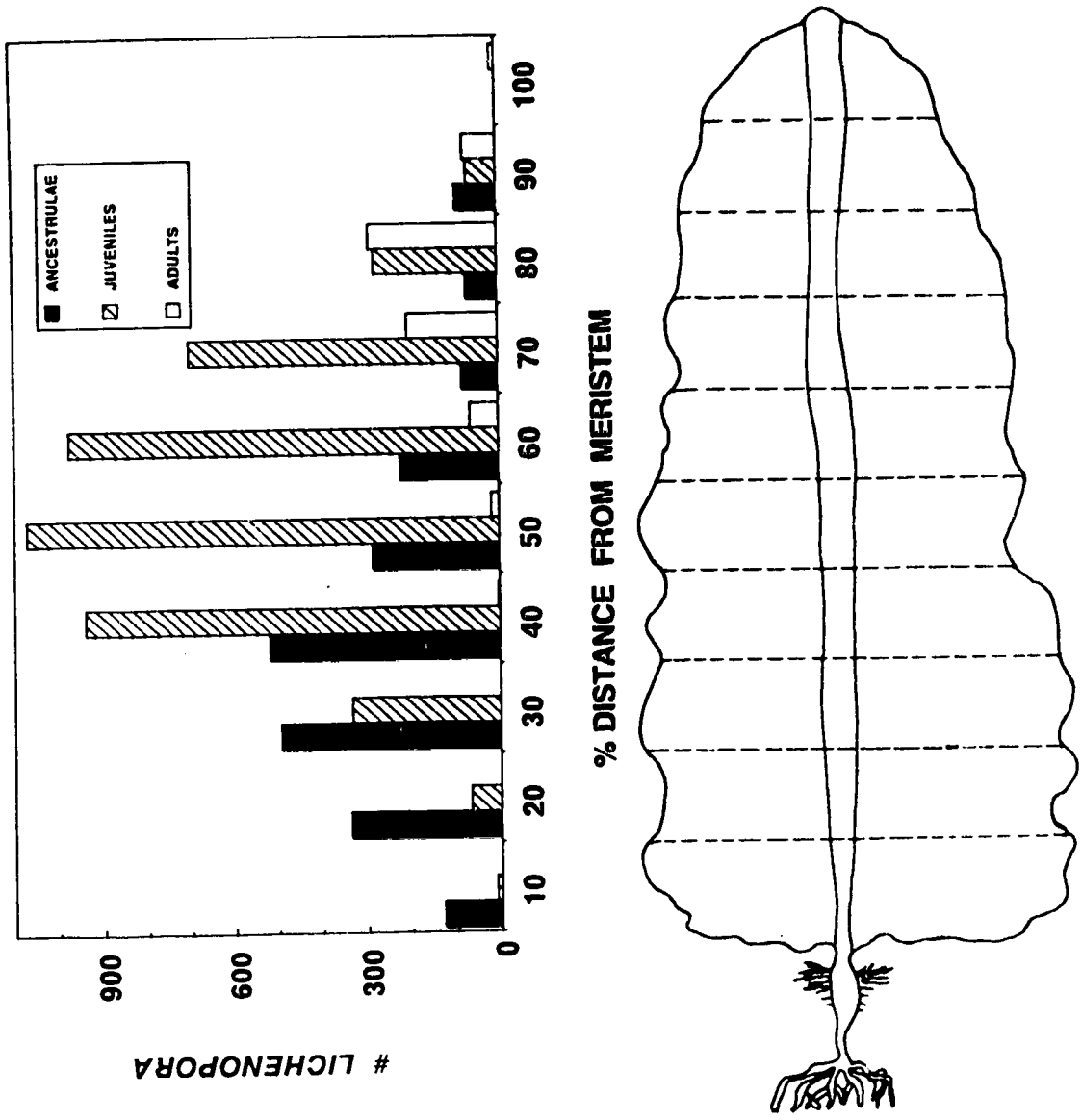
ANCESTRULAE
JUVENILES
ADULTS
LICHENOPORA
0
300
600
900
10
20
30
40
50
60
70
80
90
100
% DISTANCE FROM MERISTEM

Figure II-2: *Lichenopora novae-zelandiae* settling on *Agarum fimbriatum*. Densities of juvenile (including ancestrulae) and adult colonies of bryozoans along fronds of kelp collected during (A) September and (B) November 1990. Data are means ± SE, n = 10 plants

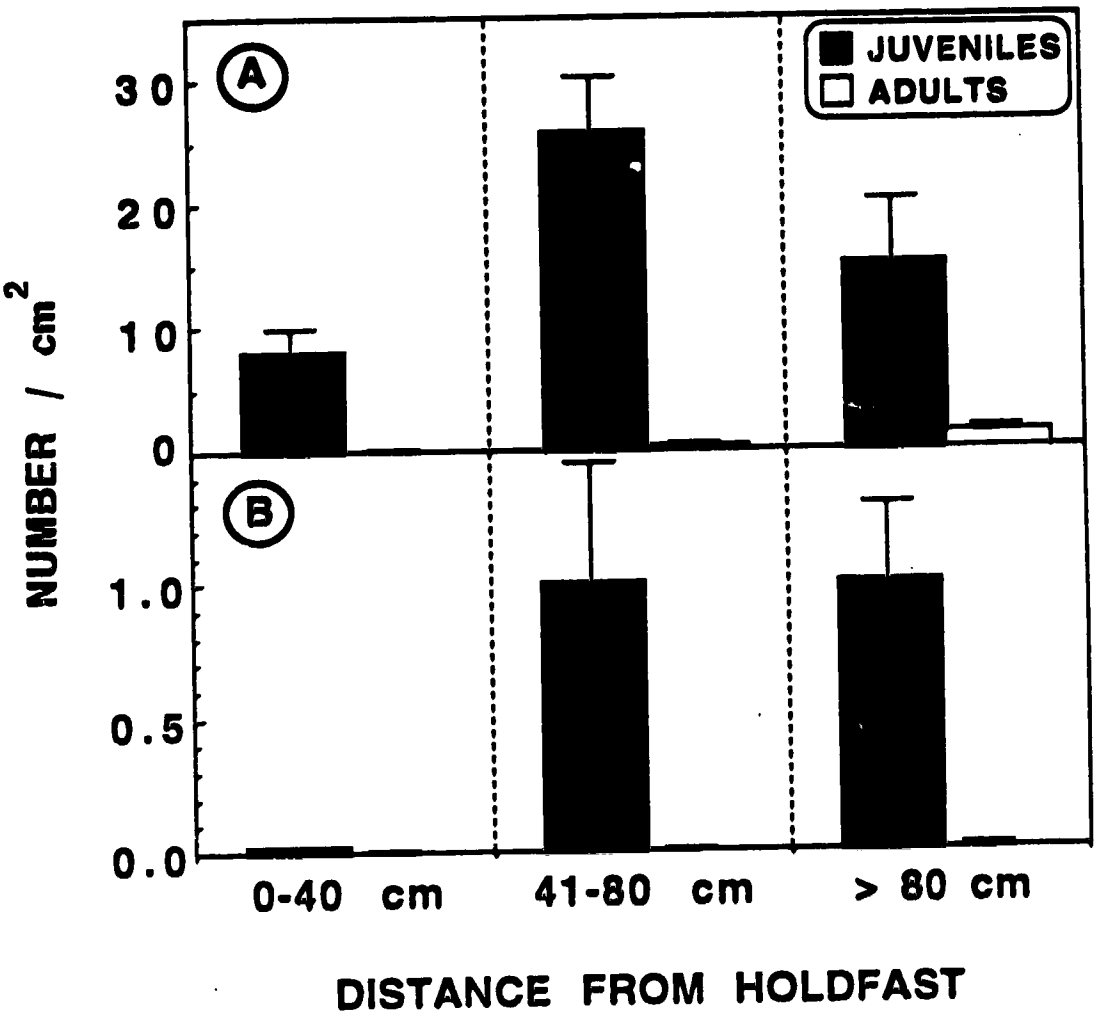
JUVENILES
ADULTS
A
B
30
20
10
0
1.0
0.5
0.0
NUMBER / cm2
0-40 cm
41-80 cm
> 80 cm
DISTANCE FROM HOLDFAST

Figure II-3: Settlement preferences of *Lichenopora novae-zelandiae* larvae for four algal substrata: young *Agarum fimbriatum*, old *A. fimbriatum*, *Nereocystis luetkeana*, and *Macrocystis integrifolia*. Newly settled ancestrulae were counted (A) after 24h, and again, (B) after 72 h from the start of the experiment. Data are means ± SE for 10 blocks

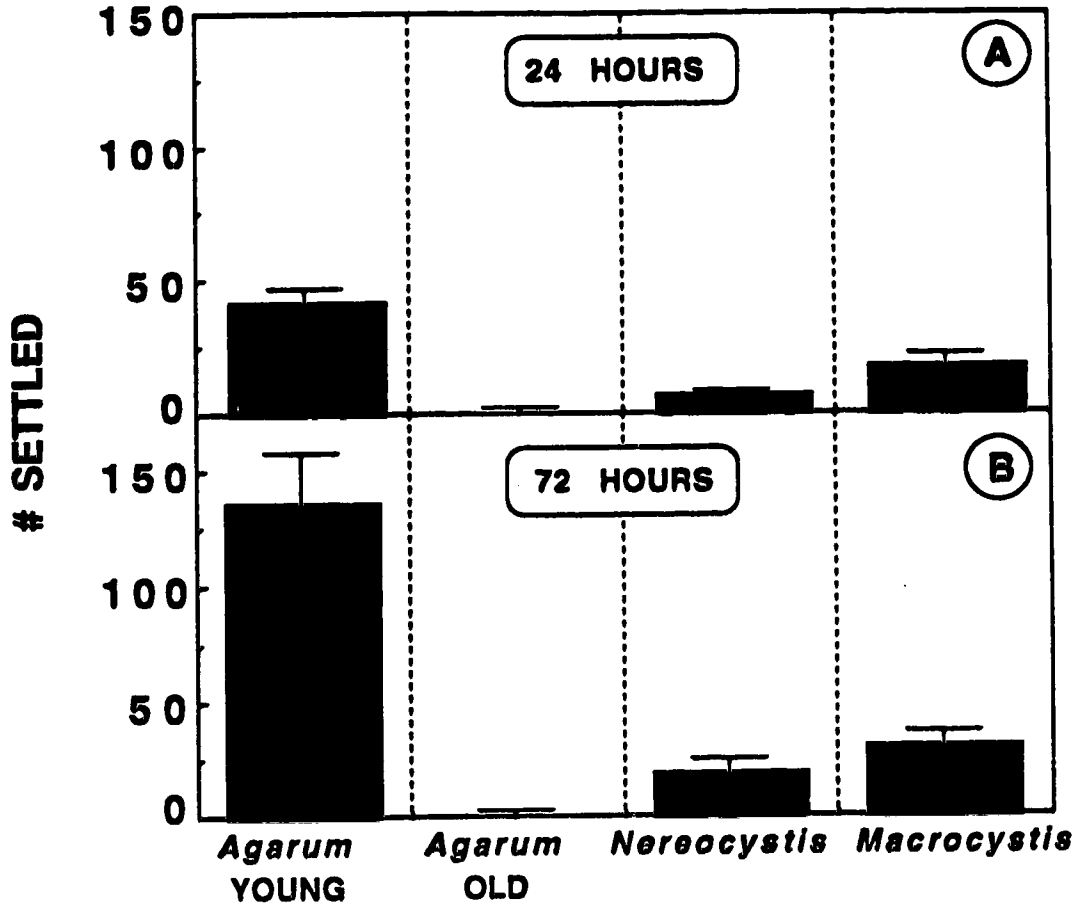
150
100
50
0
24 HOURS
A
150
100
50
0
72 HOURS
B
SETTLED
Agarum
YOUNG
Agarum
OLD
Nereocystis
Macrocystis

Figure II-4: Mass of intermediate age *Agarum fimbriatum, Nereocystis luetkeana,* and *Macrocystis integrifolia* consumed by *Tegula pulligo* over 48 h. Data are means ± SE of 12 replicates. Prior to analysis, data were adjusted based on mass changes in control algae. Horizontal bars beneath histogram denote separate groups as determined by a nonparametric Tukey-type multiple comparisons procedure

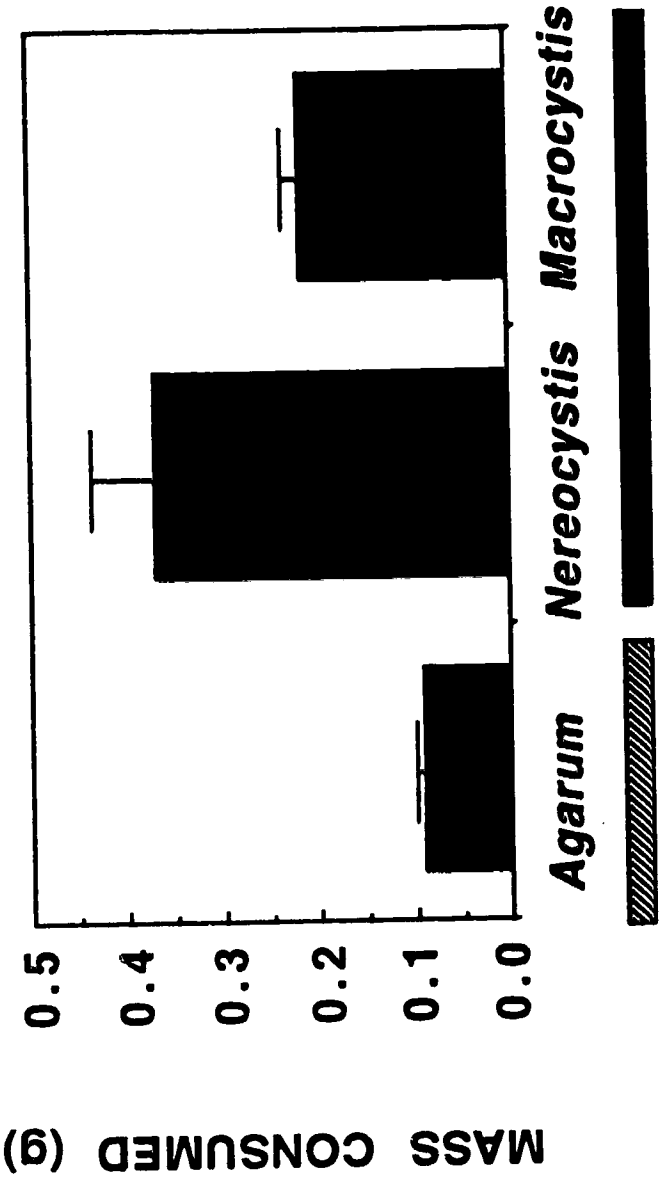
MASS CONSUMED (g)
0.5
0.4
0.3
0.2
0.1
0.0
Agarum
Nereocystis
Macrocystis

Figure II-5: *Agarum fimbriatum* consumed by *Tegula pulligo*. Mass of young (hatched bars) and old (filled bars) kelp consumed by snails over 24 h. Both colonized algae (i.e. algae with resident epiphytic organisms) and cleaned algae were offered to the snails. Data are means ± SE of 10 replicates. Prior to analysis, data were adjusted based on mass changes in control algae

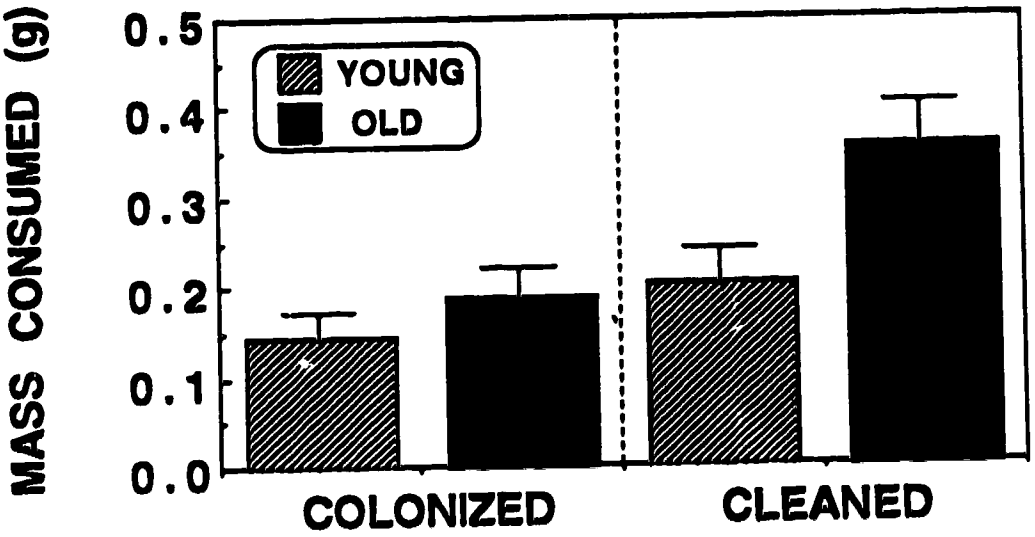
MASS CONSUMED (g)
0.5
0.4
0.3
0.2
0.1
0.0
YOUNG
OLD
COLONIZED
CLEANED

Figure II-6: *Agarum fimbriatum* consumed by *Tegula pulligo*. Mass of young (hatched bars) and old (filled bars) kelp consumed over 68 h by snails that were raised on diets of either *A. fimbriatum* or *Macrocystis integrifolia*. Data are means ± SE of 10 replicates Prior to analysis, data were adjusted based on mass changes in control algae

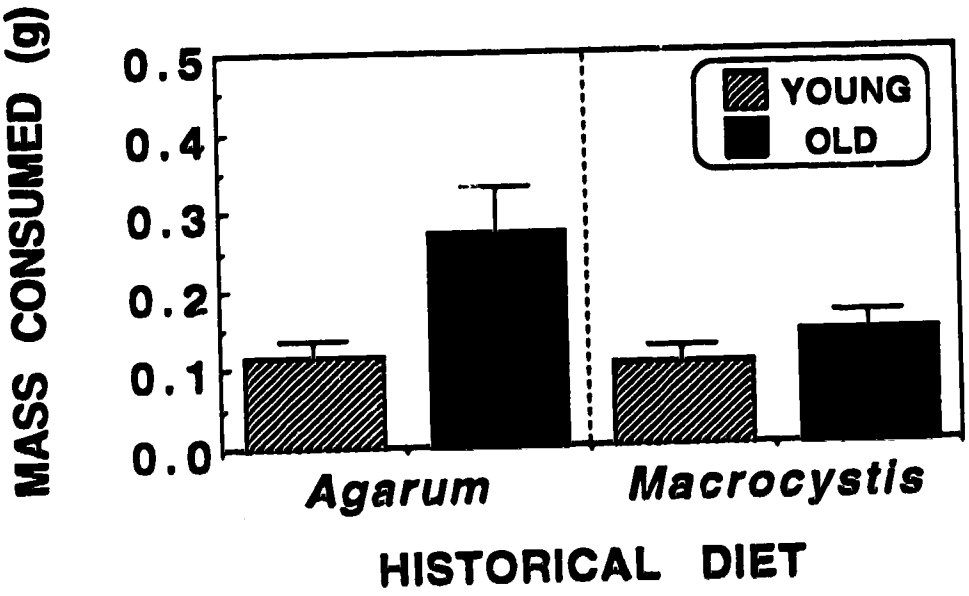
MASS CONSUMED (g)
0.5
0.4
0.3
0.2
0.1
0.0
YOUNG
OLD
Agarum
Macrocystis
HISTORICAL DIET

Table II-1: Friedman's analysis of variance by ranks, applied to settlement of *Lichenopora novae-zelandiae* larvae on young *Agarum fimbriatum*, old *A. fimbriatum*, *Nereocystis luetkeana*, and *Macrocystis integrifolia*

Elapsed time	df	n	X^2_R	Significance
24 HOURS	3	10	24.212[a]	$p < 0.001$
72 HOURS	3	10	24.840	$p < 0.001$

[a] X^2 statistic corrected for the presence of tied ranks

Table II-2: Results of a nonparametric two-factor ANOVA testing effects of *Agarum fimbriatum* age and colonization by *Lichenopora novae-zelandiae* on consumption by *Tegula pulligo*. The test statistic, H, is compared with $X^2_{\alpha, df}$

Source	df	SS	H	Significance
Age	1	688.9	5.041	$0.01 < p < 0.025$
Colonization	1	828.1	6.059	$0.01 < p < 0.025$
Interaction	1	115.6	0.846	$0.25 < p < 0.50$

Table II-3. Results of a nonparametric two-factor ANOVA testing effects of algal age and historical diet of *Tegula pulligo* on consumption of *Agarum fimbriatum*. The test statistic, *H*, is compared with $X^2_{\alpha, df}$

Source	df	SS	*H*	Significance
Age	1	756.9	5.538	$0.01 < p < 0.025$
Historical Diet	1	207.0	1.515	$0.10 < p < 0.25$
Interaction	1	70.2	0.514	$0.75 < p < 0.90$

CHAPTER III

The association of bryozoan epiphytes with the kelp *Agarum fimbriatum* (O. Laminariales): age-specific variation and kelp growth patterns

Introduction

Marine macroalgae often dominate intertidal and subtidal benthic communities (reviewed in Norton 1986), and their contribution to primary production may be enormous (Smith 1981), even exceeding that of temperate grasslands and forests (Mann 1973). The importance of macroalgae to benthic communities is twofold, however, because in addition to being metabolically active participants in the dynamics of nutrient recycling, algae also increase spatial heterogeneity in habitats where space is typically a limiting resource. Many species of sessile invertebrates use macroalgae as living substrata to which they are permanently affixed for their entire adult life (reviewed in Hayward 1980, Seed & O'Connor 1981, Seed 1986). Although certain physiological responses of marine algae to their epiphytes are well documented (Wing & Clendenning 1971, Dixon et al. 1981, Probyn & Chapman 1983, Oswald et al. 1984, D'Antonio 1985, Cancino et al. 1987a, 1987b, Muñoz et al. 1991, Hurd et al. 1994), the effect of host algal growth on the population dynamics of epiphytic organisms is unknown. In this chapter, I explore this possibility by describing temporal and spatial patterns in populations of epiphytic bryozoans that colonize a marine brown alga, and by comparing bryozoan distributions with growth patterns of their underlying algal substratum.

Last, I discuss how bryozoans growing on plants may be subject to different evolutionary pressures on their life history patterns than those growing on non-living substrata.

Study organisms

The kelp *Agarum fimbriatum* (O. Laminariales) is a single-blade species inhabiting shallow subtidal regions along the west coast of north America from southern C lifornia to southern Alaska (Druehl 1970), although it has not been reported between Puget Sound in Washington and Point Conception in California (Abbott & Hollenberg 1976). Blade tissue of this kelp is bullate (Abbott & Hollenberg 1976), and, depending on habitat (C. L. Hurd, personal communication), can be fragile, tearing easily relative to the rubbery blades of other kelps. Among temperate marine brown algae, *A. fimbriatum* is unusually well defended against herbivory with elevated concentrations of carbon-based polyphenolic compounds (Steinberg 1984, 1985). Marine herbivores that commonly graze a number of benthic algae avoid feeding on tissues of *A. fimbriatum* and those of a congener, *A. cribrosum* (Vadas 1977, Steinberg 1985). From late spring to early fall, blades of *A. fimbriatum* in Barkley Sound on Vancouver Island in Canada are heavily colonized by bryozoans in the genera *Lichenopora* and *Tubulipora* (Stenolaemata: Cyclostomata).

Colonies of encrusting stenolaemate bryozoans are typically smaller than those of gymnolaemates (McKinney 1993). Athough they lack defensive heterozooids (e.g., avicularia and vibracula) (Silén 1977), stenolaemate colonies are enriched with high calcite content (Sandberg 1977), and often possess spinous skeletal protrusions (Boardman 1983), structural features that may provide some protection from pr dators.

Individual zooids in the colonies are contained within calcareous tubes, but the degree of calcification is variable (Silén 1977). In Barkley Sound, adult colonies of *Lichenopora* and *Tubulipora* rarely exceed ~0.5 cm in diameter, and, when removed from their algal substratum, are quite brittle and fracture easily (personal observation). *Tubulipora* colonies are particularly fragile because their zooidal tubes are tall and slender, and they lack the reinforcing calcification of cancelli, found in more robust genera, such as *Disporella* and *Lichenopora* (Osburn 1953). Because of their inability to bend, it is not surprising that colonies of *Lichenopora* and *Tubulipora* are found in more protected habitats where wave forces, currents, and surge are reduced. To survive on algae in more exposed habitats, bryozoan colonies must be able to undulate when the underlying algal substratum moves (Ryland 1970).

Like other brooding bryozoans, stenolaemates house their developing embryos in specialized zooids called gonozooids (Ström 1977), or ovicells. Development in stenolaemates is unique, however, in that embryos undergo an asexual division process known as embryonic fission (Harmer 1893) or polyembryony, resulting in the production of numerous genetically identical embryos. Within ovicells, embryos develop into ciliated lecithotrophic (i.e., non-feeding) larvae, which, when fully developed, will swim out into the surrounding seawater and become planktonic for a short time (ca 1 d). Upon reaching suitable substrata, larvae settle and metamorphose into sessile ancestrulae (i.e., one-zooid juveniles), and later produce a colony through asexual budding (Nielsen 1970). Although early post-metamorphic ancestrulae, older juveniles, and adult colonies of *Lichenopora* and *Tubulipora* are easily distinguished by observation under a dissecting microscope, their small size limits field

identification to the generic level only. Older juvenile and adult colonies can be distinguished in the field on the basis of colony shape and color; *Lichenopora* colonies are circular and light purple in color and *Tubulipora* colonies are wide fan-shaped and white to beige in color.

In protected shallow subtidal habitats around Barkley Sound (ca 0-30 m depth), *Lichenopora novae-zelandiae* are absent or rare on substrata other than *Agarum fimbriatum* (Durante & Chia 1991, Chapter II). Members of the genus *Tubulipora* are found on a variety of substrata, including rocks, empty shells, and other algae, but their population densities reach highest proportions on blades of *A. fimbriatum* (personal observation). In this chapter, I present data from field surveys and growth experiments, which describe temporal patterns of bryozoan abundance and distribution on blades of the kelp *A. fimbriatum*, and growth patterns of kelp tissue.

Materials and Methods

Field surveys

During approximately bimonthly field surveys conducted between September of 1990 and February of 1993, I collected blades of the kelp *Agarum fimbriatum* using SCUBA from 10-15 m depth at several sites in Barkley Sound on the west coast of Vancouver Island, British Columbia [48° 51.24' N, 125° 7' W]. Because the kelp blades were up to seven orders of magnitude larger in surface area than their epiphytic bryozoans, I subsampled kelp blade tissue to quantify characteristics of bryozoan populations. Tissue was subsampled by cutting disks (19.65 mm in diameter) from the blades using a cork borer. Depending on the particular

survey, blade tissue was either divided arbitrarily into ten or three equal sized relative age categories (Fig III-1a), or cut into 10 cm increments along the midrib (Fig. III-1b), and then five or ten subsamples were taken from each of the blade tissue categories (Fig. III-1). For most censuses, blades were divided into three age categories (Fig. III-1a), but occasionally more detailed sampling was possible when time and resources permitted. Following subsampling, freshly cut kelp disks were immediately transferred to vials with 70% ethanol for storage, and bryozoans were later identified and counted using a dissecting microscope. Although during storage, ethanol solubilized pigments from the kelp tissue, it did not appear to dislodge, decalcify, or otherwise damage epiphytic bryozoans.

To describe bryozoan population structure, each colony that I counted was classified either as an ancestrula (i.e., one-zooid stage), a juvenile (i.e., small fan-shaped colony with > 1 zooid, but without ovicells), or an adult (i.e., large circular or wide fan-shaped colony, which may or may not possess ovicells) (Fig. III-2). For the May 1993 sample only, adult colonies were further classified into immature (i.e., no ovicells) or mature (i.e., with ovicells) categories. In my samples, bryozoans in the genera *Lichenopora* and *Tubulipora* were the most common epiphytic organisms living on *Agarum fimbriatum*. Occasionally, other bryozoan species such as *Membranipora membranacea* and *Tricellaria occidentalis*, were found growing on *A. fimbriatum*, but these species occured so rarely that they are not addressed further in this thesis. Within *Lichenopora*, only *L. novae-zelandiae* was found growing on *A. fimbriatum*, but at least three species of *Tubulipora*, including *T. tuba*, *T. pacifica*, and *T. pulchra* colonized this kelp. Species of *Tubulipora* are distinguished by the morphology of the ooeciostome (Osburn 1953, Ross 1970), an opening leading into the

brood chamber through which swimming larvae will emerge when released from the parent colony. Unfortunately, colonies of *Tubulipora* are not identifiable to the species level until they are sexually mature, and damage to the delicate calcareous tubes and ooeciostomes can render specimens unidentifiable. Therefore, I pooled all *Tubulipora* observations into a single species category, *Tubulipora* spp., although I distinguished specimens into three age categories, as described above.

Statistical analyses

Data from field surveys conducted from 1991 to 1993 were analysed using randomized block design analyses of variance (hereafter referred to as ANOVAs) followed *a posteriori* with Student-Newman-Keuls (hereafter referred to as S-N-K) multiple comparisons procedures. All statistical tests were applied at a signficance level (i.e., α) of 0.05. Although numbers of bryozoan colonies per blade of algae often exceeded 8,000 (see Fig. 1 in Durante & Chia 1991, Fig. II-1 in Chapter II), sample size was determined by the number of algal blades collected, not by the number of bryozoan colonies. Consequently, small sample sizes of 5 and 10 required that the data be transformed prior to analysis. Using a widely accepted and robust nonparametric prodedure (Conover 1980), I applied parametric tests to rank-transformed data, which enabled the application of multiple comparisons procedures. I used ANOVAs to test whether location along kelp blades significantly affected the distribution of post-metamorphic life history stages of bryozoans. S-N-K tests identified which location(s) along the blades had significantly higher or lower bryozoan densities. All statistical analyses throughout the study were conducted with the assistance of SuperANOVA™ (version 1.0) or Statview™ SE+Graphics (version 1.04)

software for Macintosh™ microcomputers.

Kelp growth measurements

Although they are diverse in morphology and life history characteristics, kelps (Order Laminariales) exhibit most of their growth at a single meristematic region intercalary between the stipe and blade (Bold & Wynne 1985). As new tissue grows at the intercalary meristem, older tissue is pushed distally away from the meristem, and the blade itself appears to be like a belt of moving tissue with increasingly older tissue present towards the distal tip (Mann 1972). Because of this characteristic growth morphology, growth rates of kelps can be easily measured by punching small holes in the tissue and measuring how far the holes move from the meristem (Parke 1948); all kelp growth data presented in this thesis were obtained using this method.

Laboratory growth measurements

In November 1990, I collected nine blades of *Agarum fimbriatum* from the Dixon Island site, and immediately transported them to flowing seawater aquaria at the Bamfield Marine Station, where they were held for the duration of the growth study. As described above, five holes, several cm apart, were punched through the midrib of each blade, starting several cm distal to the meristem. The distance between the intercalary meristem and the first hole, the distances between each of the five holes, and the total blade length were recorded seven times over 180 days.

***In situ* growth measurements**

Using SCUBA, I monitored growth of 20 *Agarum fimbriatum*

individuals at Dixon Island between January and May 1991. Experimental blades were selected by placing a transect line in a randomly determined direction through a large subtidal bed of *A. fimbriatum*. Blades whose holdfasts were attached closest to 1 m interval marks along the transect line were tagged by encircling the stipe with a numbered plastic hose clamp. At 10 to 15 m depth near Dixon Island, water motion from currents, tides, or waves is minimal, and the loosely fitting tags did not appear to cause chafing damage to the stipes. Immediately following tagging, a cork borer was used to punch two holes (0.6 cm in diameter) in the midrib of each plant, several cm distal to the meristem. The following measurements were then recorded for each tagged plant: stipe length, blade length, meristem to hole #1 distance, and hole #1 to hole #2 distance. These measurements were repeated three times over the course of the experiment.

Results

Seasonality of bryozoan abundance

Population density of *Lichenopora novae-zelandiae* and *Tubulipora* spp. varied seasonally from 1990 to 1993, with peaks in abundance occurring from late spring to early fall (Figs. III-3 & III-4). For both species, densities of immature colonies, (including ancestrulae and juveniles) were always higher than those of mature adult colonies, perhaps reflecting demographic differences among life history stages or differential post-settlement mortality. Because the number of immature colonies approximates the production of new individuals in the population more

closely than the number of mature colonies, for purposes of comparing seasonal differences among years and species, I will refer to immature stages only in this and the following paragraph.

During 1990, mean peak densities of *Lichenopora novae-zelandiae* colonies were extremely high (> 15 colonies/cm^2), but then declined to < 0.5 colonies/cm^2 in 1991 (Fig. III-3). During the same two seasons, patterns in abundance of *Tubulipora* spp. were reversed: in 1990, *Tubulipora* spp. densities only reached a peak of <0.2 colonies/cm^2, but they reached 0.9 colonies/cm^2 in 1991 (Fig. III-4). In 1992, densities of both bryozoan genera peaked again during June-September: ca 2.75 colonies/cm^2 for *L. novae-zelandiae* and ca 1.6 colonies/cm^2 for *Tubulipora* spp. (Figs. III-3 & III-4). In May 1993, virtually no colonies of *Tubulipora* spp. were present on the sampled blades, but densities of *L. novae-zelandiae* colonies rose to ca 0.4 colonies/cm^2 (Figs. III-3 & III-4). Field observations from the summer of 1993 suggested, however, that the comparatively low densities of bryozoan colonies during the May 1993 sample were due to sampling early in the annual season of bryozoan reproduction, rather than to unusually low numbers of individuals in the population. By mid summer of 1993, nearly every blade of *Agarum fimbriatum* at the Dixon Island site was heavily epiphytized by *L. novae-zelandiae* and *Tubulipora* spp.

Effects of algal age on bryozoan distributions

Because the bryozoans *Lichenopora novae-zelandiae* and *Tubulipora* spp. are attached to a living and growing substratum, time differences potentially could alter the spatial relationship between bryozoans and algae. To avoid confounding effects of kelp growth, which varies seasonally (Mann 1972, Chapman & Craigie 1977), effects of algal tissue age on

bryozoan distributions were analyzed only within samples, (i.e., blades collected from the same location at the same time). Also, for samples collected when bryozoan population densities were zero or statistically indistinguishable from zero, analyses of bryozoan distributions would have been impractical, and thus were not conducted.

August 1991: San José Islets

Blades collected from the San José Islets in August 1991 were divided into 10 equal sized sections (Fig. III-1a), and the bryozoan densities in each of these 10 sections are shown in Figs. III-5 and III-7. Although variation in the data for *Lichenopora novae-zelandiae* was high due to low colony densities (< 0.4 colonies/cm^2), examination of the mean densities along kelp blades showed that ancestrulae were present mostly on the youngest 50% of the blade, and virtually absent from older tissue (Fig. III-5). Juvenile colonies exibited a normal distribution along the blades, reaching peak densities on intermediate aged tissue (Fig. III-5). Adult colonies were virtually absent from tissue younger than 50% along the blade, but were in highest abundance on older tissue (Fig. III-5).

Distributions of *Tubulipora* spp. on the same kelp blades were less variable between 10% sections, and the tendency for ancestrulae to be associated with young kelp tissue is evident (Fig. III-7). Juvenile colonies appear on tissue as young as 20% of the blade length from the meristem, but are in highest abundance on the older tissue from 60 - 100% of blade length (Fig. III-7). Adults were present only on tissue older than 60% of blade length (Fig. III-7). Overall, colony densities on kelp blades in this sample were higher for *Tubulipora* spp. than for *L. novae-zelandiae* (Figs. III-3 & III-4), and, for both species, colony densities of younger bryozoans

were higher than those of older bryozoans (Figs. III-5 & III-7).

To test whether algal age had an effect on bryozoan density, census data were pooled into five equal-sized relative age categories instead of the original 10. This reduced Type II errors in the analysis (Sokal & Rohlf 1981), caused by small sample size or low bryozoan densities in each of 10 categories. ANOVAs (Table III-1) and S-N-K tests on *Lichenopora novae-zelandiae* data showed that densities of ancestrulae in the four youngest algal age categories were not significantly different from each other, but were, as a group, significantly higher than those in the oldest age category (Fig. III-6). Densities of juvenile colonies of the same species, although peaking on intermediate aged tissue, were not significantly different among the five relative age categories (Fig. III-6, Table III-1). Adult colony densities on the youngest three age categories were significantly lower than those on the oldest three age categories (Fig. III-6). Densities of ancestrular, juvenile, and adult colonies of *Tubulipora* spp. were significantly different among the five algal age categories (Fig. III-8, Table III-1). Ancestrular densities in the youngest three categories were significantly higher than those in the oldest two categories (Fig. III-8). Juvenile colony densities in the oldest three categories were higher than those in each of the two other categories, and juvenile densities were lowest in the younger of the two youngest age categories (Fig. III-8). Adult *Tubulipora* spp. densities in each age category were significantly lower than those in the adjacent older category (Fig. III-8).

July 1992: Dixon Island

On kelp blades collected in July 1992 from Dixon Island, ancestrulae, juveniles, and adults of *Lichenopora novae-zelandiae* were significantly different among the three algal age categories (Fig. III-9, Table III-1).

Ancestrular densities were highest on young algal tissue, intermediate on medium-age tissue, and lowest on old tissue (Fig. III-9). Juvenile colony densities were highest on young and medium age algal tissue, and lowest on old tissue (Fig. III-9). The pattern of adult colony densities was opposite to that of ancestrular colonies; adults were absent on young tissue, present in low density on medium age tissue, and most dense on old tissue (Fig. III-9).

Overall densities of *Tubulipora* spp. colonies along the same kelp blades were low, and the distributions of ancestrulae were not significantly affected by algal age (Fig. III-10, Table III-1). At 0.0547, the P-value for the ANOVA on juvenile colonies was, when rounded to two significant digits, equal to the significance of the test, ($\alpha = 0.05$) (Table III-1), and the histogram indicates a tendency for juvenile colonies to be associated with older blade tissue (Fig. III-10). Although densities of adult colonies were lower than those of either ancestrulae or juveniles, the significance of the ANOVA in this case can probably be attributed to adults being absent from young and medium age tissue (Fig. III-10, Table III-1).

September 1992: Dixon Island

The distributions of all three life history stages of *Lichenopora novae-zelandiae* that settled on blades of *Agarum fimbriatum* collected from Dixon Island in September 1992 were significantly affected by algal age (Fig. III-11, Table III-1). Ancestrulae were found in highest abundance on the young and medium age tissue, juveniles on the medium tissue, and adults on the medium and old tissue (Fig. III-11).

Tubulipora spp. densities on the same blades were less affected by algal age than were colonies of *L. novae-zelandiae*. Neither ancestrulae nor

adult colonies of *Tubulipora* spp. exhibited significant distributional patterns with respect to algal age (Fig. III-12, Table III-1). The distribution of juvenile colonies was, however, affected by algal age; juveniles were in highest abundance on medium and old age tissue, and were sparse on young tissue (Fig. III-12, Table III-1).

May 1993: Dixon Island

On blades of *Agarum fimbriatum* collected from Dixon Island in May 1993, *Tubulipora* colonies were completely absent (Fig. III-4), but colonies of *Lichenopora novae-zelandiae* were abundant, and their distributions are addressed in more detail below. For each blade in this census, bryozoan colonies were counted at 10 cm increments beginning at the meristem and continuing distally for the entire blade length (see Fig. III-1b). The distributions of four life history stages of *L. novae-zelandiae* along these blades are shown in the scatterplots in Fig. III-13. Ancestrulae peak in abundance on the youngest tissue adjacent to the meristem, and gradually decline to near zero abundance 100 cm along the blades (Fig. III-13). Juvenile colonies peak in abundance on intermediate aged tissue located 40 cm distal to the meristem, but are in high abundance from 30 to 100 cm from the meristem (Fig. III-13). Immature adults (i.e., with adult colony morphology, but lacking ovicells) are in highest abundance 90 cm distal to the meristem, their abundance tapering gradually on tissues proximal and distal to the 90 cm section, but are virtualiy absent from the youngest 40 cm of the blades (Fig. III-13). Mature adult colonies are also absent from the youngest 40 cm of the blades, and they gradually increase in abundance from 50 cm to the distal ends of the blades (Fig. III-13). Because these blades were not of the same length, however, data for the distal-most sections

represent fewer than five blades, and thus variation about the means in these sections is higher (Fig. III-13).

Variation in the distributional data for all four life history stages does not appear to be independent from the means (i.e., the data are heteroscedastic), but, for the statistical analyses below, data were rank-transformed rather than log-transformed, the latter being a recommended transformation for heteroscedastic data (Sokal & Rohlf 1981). As above, the reason for applying the rank transformation in this case was that it is impossible to ensure normality and homoscedasticity in the data when the sample size is as small as five. For testing the effects of algal age on colony abundance for this data set, positions of colonies were reclassified into three relative age categories, as in the previous analysis. ANOVAs showed that distributions of all four bryozoan life history stages were significantly affected by algal age (Fig. III-14, Table III-1). Ancestrulae were in highest abundance on young tissue, significantly lower abundance on medium age tissue, and lowest abundance on old tissue (Fig. III-14). Density of juvenile colonies was highest on young and medium aged tissue, and lowest on old tissue (Fig. III-14). There were significantly more immature adult colonies on medium and old age tissue than there were on young tissue (Fig. III-14). Mature adult colonies were absent from young tissue, in low abundance on medium age tissue, and peaked in abundance on old tissue (Fig. III-14).

Between-plant variation

Use of randomized block design ANOVAs in the above analyses enabled me to examine effects of the individual blades, (i.e., blocks) on bryozoan distributions. There did not appear to be any consistent patterns in the effects of blocks; even for the same five blades, block effects could

differ among the three or four bryozoan life history stages examined. These block effects, however, are consistent with my observations of bryozoan abundance in the field. I noted that, during the onset of bryozoan reproduction (which varied temporally between years), bryozoan distributions among individual plants in the kelp beds were patchy; adjacent blades could have considerably different numbers of epiphytic bryozoans. High between-blade variation in bryozoan abundance would cause significant block effects, whereas low variation would result in no significant block effects. When bryozoan larvae were abundant during peak periods of reproduction, newly settled ancestrulae were also abundant on young tissue of nearly every available blade (personal observation), and thus between-plant variation in the distribution of ancestrulae would have been low.

Kelp growth measurements

Laboratory growth measurements

Blades of *Agarum fimbriatum* cultured in laboratory aquaria exhibited growth patterns characteristic of those of kelps in the field. Total blade length declined during the first two months of the study in November and December, but then increased gradually from January through the end of the study in March (Fig. III-15). Vadas (1968) found that growth in *A. fimbriatum* was highest from ca January to May, and thus blades exhibiting little growth in late fall might tend to decrease in length due to erosion at the tip. Nearly all of the growth in the blades occurred between the meristem and the first hole punched in the midrib, with tissue lengths between the other four holes remaining virtually the same (Fig. III-15). Statistical comparison of growth among the five tissue sections showed

that tissue nearest the meristem grew significantly more than any of the other sections, and tissue between the first and second holes grew less than meristem tissue, but still significantly more than all other tissue distal to the second hole (Fig. III-16, Table III-2).

In situ growth measurements

Due to unexpectedly intense grazing by red sea urchins, *Strongylocentrotus franciscanus*, only nine of the original 20 blades of *Agarum fimbriatum* were present at the end of this growth study. Growth in the remaining nine blades was similar, however, to that in blades cultured in laboratory aquaria. Blade length increased slightly and stipe length decreased slightly over the course of the experiment, and length of tissue between the meristem and the first punched hole increased more than tissue between the first and second holes (Fig. III-17). A paired Student's *t*-test showed that blade tissue nearest to the meristem grew significantly more than tissue between the first and second punched holes (Fig. III-18).

Discussion

Bryozoan distributions on *Agarum fimbriatum*

Spatial patterns

Spatial distributions of epiphytic organisms on living plant substrata are rarely random, and many environmental or substratum-specific factors have been implicated in directing where invertebrate epiphytes settle and grow (reviewed in Hayward 1980, Seed & O'Connor 1981, Seed 1986). Data

from my field censuses strongly show that population density of the bryozoans *Lichenopora novae-zelandiae* and *Tubulipora* spp. along blades of their kelp substratum is influenced by algal age. Although newly settled ancestrulae, juveniles, and adults of these bryozoans are partitioned among different regions of the kelp blades, only the ancestrulae provide information about where initial epiphytic colonization might have occurred. The tendency for reproductively mature colonies to be associated with older blade tissue yields no clues about locational preferences along the blades. If bryozoan larvae settled randomly with respect to location along a blade, then the resulting distribution of adult colonies would be no different from the distributions described in this study, and this can be explained soley by kelp growth patterns. As blades of *Agarum fimbriatum* grow and add new tissue at the intercalary meristem, tissue adjacent to the meristem (along with its associated epiphytes) is pushed distally. Due to this growth pattern, by the time bryozoan colonies have reached sexual maturity, they will have been pushed all the way to the distal end of the blade.

The significant concentration of bryozoan ancestrulae on young blade tissue suggests that larvae settle there preferentially, although alternate hypotheses must be considered. It is possible that non-random distributions of newly settled bryozoans were caused by random settlement followed by high post-settlement mortality on medium and old aged blade tissue (see Keough & Downes 1982). These and other hypotheses relating to bryozoan settlement on *A. fimbriatum* are addressed in more detail in Chapter IV. Here, as algal tissue age may be correlated with a number of confounding physical and biological factors, I discuss below the potential causes of age-specific distributions of bryozoans on *A. fimbriatum* and the

evolutionary consequences of an epiphytic existence.

Site differences and seasonal variation

Most of the kelp blades included in this study were collected from my main study site at Dixon Island, but a smaller number of blades from other sites (e.g., San José Islets) were also examined to determine whether the age-specific distribution of bryozoan epiphytes was a site-specific anomaly within Barkley Sound. During seasonal peaks in bryozoan abundance, when densities of colonies on kelp blades were sufficiently high to detect within-blade spatial patterns, bryozoan colonies were distributed similarly on blades collected at different locations. The age-specific distribution of bryozoan epiphytes was not an unusual condition found only on kelp blades from Dixon Island, but rather appeared to occur at all locations where I collected or observed *Agarum fimbriatum*.

Although within-plant distributions of bryozoans were qualitatively similar for all collections during periods of peak bryozoan abundance, in some cases bryozoan colony densities were not significantly different among kelp age categories. In these cases, variation in bryozoan colony densities was increased by two possible mechanisms: (1) overall colony densities were low, or (2) colony densities across the population were patchy. Towards the end of the bryozoan spawning period in the fall, the numbers of larvae available for settlement were declining, hence reducing the density of ancestrulae on young kelp tissue. Because the larvae of stenolaemate bryozoans likely settle quite close to their parent colony (C. McFadden, personal communication), as larval production in the population begins to decline, there will be patches of low larval density interspersed with patches of high larval density. This patchiness likely

increases variation in settlement across the population, and my data support this assumption. In September 1992, for example, variation in the distribution of ancestrulae was high relative to that of juvenile and adult colonies. During peak spawning periods, however, larval settlement was more uniform across the population, and thus variation in ancestrular distributions was lower. The overall densities of *Lichenopora novae-zelandiae* ancestrulae in May 1993 were the same or lower than those in September 1992 at the same location, yet variation was lower during May 1993, when larval availability was likely much higher. One reason why the distribution of ancestrulae is more uniform at the onset of larval production in late spring than at the end of the spawning period might be that the timing of larval production is constrained by food availability and temperature, whereas the end of spawning may occur, for example, when (1) the kelp substratum dies or is damaged, or (2) the bryozoans die. Unpredictable, density independent factors leading to the end of spawning will result in patchy settlement patterns.

Physical differences

Certain epiphytic organisms select living substrata, not necessarily for their biological properties, but rather because the substrata are in locations where physical conditions, (e.g., silt content and water motion, O'Connor et al. 1979; available surface area, Stoner 1980) are preferable or optimal. As a number of bryozoan species produce phototactic larvae (reviewed in Ryland 1974), photic gradients along large algal substrata may influence settlement choice. The long fronds of the giant kelp *Macrocystis pyrifera* (> 30 m, North 1971) are exposed to a wide range of light conditions between their benthic holdfasts and pelagic blades, and these differences in

light have in part been implicated in directing settlement of the bryozoan *Membranipora membranacea* onto the younger kelp blades near the water surface (Bernstein & Jung 1979). At my study sites, blades of *Agarum fimbriatum* were always found lying prostrate along the bottom rather than extending vertically into the water column. Because of this horizontal blade position, and the generally low levels of water motion at the study sites, it is unlikely that light or hydrodynamic conditions change appreciably along blades. For some epiphytic organisms, surface topography on their algal hosts is an important physical cue influencing settlement on a small scale (reviewed in Seed & O'Connor 1981, Seed 1986). Although surface topography was not addressed in this study, I observed no pattern in bryozoan settlement or abundance with respect to the bullae on blades of *A. fimbriatum*.

Chemical constituents of algae

Among North American temperate kelps, *Agarum fimbriatum* is unusually well defended against herbivory with an arsenal of tannin-like molecules called polyphenolic compounds or phlorotannins (Ragan & Glombitza 1986, Steinberg 1984, 1985, Chapter V). Polyphenolics in vegetative blade tissue of *A. fimbriatum* from Barkley Sound are concentrated most heavily in young tissue nearest the meristem (Chapter V). Polyphenolics from other brown seaweeds have been shown to function as antibiotics, reducing or eliminating bacteria and microalgae normally associated with the algal surface (Sieburth & Conover 1965, Sieburth 1968).

Considerable controversy exists over whether bryozoan larvae settle preferentially on microorganismal films, which develop on surfaces

immersed in seawater after glycoproteins have adsorbed (Davis et al. 1989), or whether they are deterred by them. A recent study has shown that attractiveness of a microorganismal film to settling bryozoan larvae is linked to specificity for particular bacterial strains (Maki et al. 1989), but there are many other examples of substratum specificity by bryozoan larvae that appear unrelated to bacterial cues (see Ryland 1974). Because ancestrulae of *Lichenopora novae-zelandiae* and *Tubulipora* spp. are found nearly exclusively on young blade tissue of their kelp substratum, their larvae must be settling on algal tissue that, due to its recent growth and polyphenolic content, is least likely to have been colonized by bacteria (see Chapters IV & V).

Substratum growth and availability of space

When settlement rates of epiphytic species are high and the availability of new plant substrata is low (perhaps worsened by reduced plant growth), crowding may lead to competition for space among epiphytes. For many epiphytic species, a common way to avoid other epiphytic competitors on growing plants is to settle preferentially on the youngest plant tissues (reviewed in Ryland 1974, Hayward 1980, Seed & O'Connor 1981, Seed 1986). Space available for colonization is not necessarily a limiting factor for epiphytic organisms, however. Dirnberger (1990) recently showed that spirorbid polychaetes settled preferentially on the young bases of growing seagrass blades, but he claimed that their settlement preference was an avoidance of epiphytic algae associated with the distal (older) end. By demonstrating that settlement did not increase when more space was created experimentally, he concluded that space necessary for settlement was not limiting in that system (Dirnberger 1990).

By selecting the youngest possible tissue on an individual plant, epiphytes maximize their potential lifespan on that plant, and this idea is supported by evidence from the literature. For example, bryozoans that colonize fucoid algae settle near the branch tips rather than proximally near the holdfast (Hayward & Harvey 1974). As fucoid algae have apical meristems, however, the tissue where bryozoans are settling is nearest the meristem. Bryozoans that grow on the kelp *Laminaria digitata* exhibit similar distributions along blades, and their larvae preferentially choose tissue near the intercalary meristem at the time of settlement (Stebbing 1972). If the lifespan of the plant is much greater than that of the epiphyte, however, then careful selection of plant tissues may be unnecessary.

To assess all costs and benefits for settling on a particular region of a plant, one needs to know whether loss of older tissue is predictable, (and hence could function as a selective pressure), and how likely older tissue is to disappear before the epiphytes can reproduce (see Keough 1986). For those epiphytic organisms exhibiting semelparity (i.e., single reproductive effort, see Stearns 1976), selection of young substrata may be more critical because, in order for fitness of the epiphytes to be maintained, the plant substrata must survive long enough for the epiphytes to reach sexual maturity. The dominant epiphytic organisms on blades of the giant kelp *Macrocystis pyrifera* (i.e., three bryozoan species, and one spirorbid polychaete) reproduce well within the six month lifespan of their substratum (Bernstein & Jung 1979). Although the kelp *Agarum fimbriatum* is perennial, its blades can become severely eroded at the distal tip during the winter, if new growth at the meristem is reduced due to low light levels (Vadas 1968). Consequently, blades can nearly disappear before growth begins again (personal observation), resulting in tissue loss

analogous to that of deciduous trees. My observation that stipes of *A. fimbriatum* became slightly smaller over a four month period (Fig. III-17) may have been an artifact of the measuring method. Stipe length was determined by measuring from the intercalary meristem down to the closest hapteron on the holdfast. If, over the course of the study, haptera were produced from tissues on the stipe further from the substratum than previously observed haptera, then the recorded stipe length would have been smaller.

In Barkley Sound, the stenolaemate bryozoans that grow on *Agarum fimbriatum* are most abundant during the summer and early fall, when the blades exhibit their highest annual growth. During the winter months, when blades are small, bryozoans are absent or rare, and my attempts to locate reproducing colonies of *Lichenopora novae-zelandiae* and *Tubulipora* spp. elsewhere at this time were unsuccessful. My observations of rapid population growth in these bryozoans suggests that they are able to reach sexual maturity within one growing season of their kelp substratum. Specimens of *L. novae-zelandiae* and *T. tuba* have been dredged from areas as deep as 60-230 m (Osburn 1953), hence it is possible that populations of these bryozoans overwinter on alternate substrata in deeper water. Or, although they have never been described from a marine bryozoan, perhaps asexually produced resting stages, (such as the statoblasts produced by freshwater phylactolaemate bryozoans (Wood 1983)), could maintain bryozoan populations in marine systems when ephemeral substrata disappear.

Evolutionary consequences of epiphytism

Macroalgae influence biological processes in benthic communities by

fixing carbon as an edible food source for herbivores (grazing reviewed in Lubchenco & Gaines 1981, Hawkins & Hartnoll 1983), and by exchanging nutrients, gases, and metabolites with surrounding seawater (Dring 1982). The effects of both herbivory and nutrient dynamics may be amplified, however, from an epiphytic perspective. Because they generally lack the complete regenerative potential of plants, sessile invertebrates that cannot detach from their algal substrata could suffer an even greater risk to herbivory than the algal tissues themselves. Whole or partial blades removed during grazing may carry with them individual animals or colonies. Depending on flow conditions, such organisms may be doomed to wash up on shore, sink into deep water where food availability is below sustenance levels, or float indefinitely in surface waters where a variety of physical stresses may kill them. Also, in the process of feeding on algae, herbivores and omnivores may damage, kill, or ingest epiphytic animals. By colonizing living algae, sessile animals may be exposing themselves to unnecessarily high risk of herbivory related mortality. In addition, the direct contact between plant and animal tissues that occurs in epiphytic associations provides a venue for the exchange of primary and secondary metabolites. A number of algal secondary compounds are known to have toxic or antifouling effects on animals (Sieburth 1968, Fenical 1982, Davis et al. 1989), and these negative effects would certainly be enhanced by the proximity of plant and animal tissues in epiphytic associations.

In order for epiphytic relationships among macroalgae and sessile invertebrates to have persisted through evolutionary time (see McKinney & Jackson 1989), however, the risk of mortality due to the association must have been outweighed by associational benefits. Unlike inert hard substrata such as rock or shell, macroalgae provide a continually renewable

surface for colonization. Sessile invertebrates that can tolerate the potential dangers associated with a living substratum also enjoy an environment where competition for space is reduced relative to that on non-living substrata (see Seed & O'Connor 1981). Thalli of most benthic macroalgae are elevated above the surrounding primary substratum and hence are not in the boundary layer where fluid velocity approaches zero (Vogel 1981). Sessile invertebrates on algal thalli might then benefit from higher water motion and thus increased flux of suspended particulate food. Newly settled bryozoans may be small enough, however, to be contained within the boundary layer at the algae-seawater interface, so hydrodynamics may not be important to bryozoans at the time of settlement (C. L. Hurd, personal communication).

Epiphytic organisms that are associated with chemically protected marine algae may, by association, reap the benefits of their partner's defenses (reviewed in Hay 1992). Some epiphytic invertebrates maintain a physiological connection with their algal hosts, enabling them to fertilize the underlying algal substratum with nitrogenous waste (Hurd et al. 1994). In other cases, carbon-based exudates from algal tissues may be taken up and metabolized by epiphytic animals (De Burgh & Fankboner 1978). Perhaps associations in which epiphytes exchange metabolites with their host algae and both partners benefit from the physiological exchange might better be described as episymbiotic rather than epiphytic.

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Figure III-1: Sampling protocols for blades of the kelp *Agarum fimbriatum*. In both methods, blades were divided into equal length increments along the midrib (in black), and a blade section for each increment was obtained by making a slice (dashed line) perpendicular to the midrib. Subsamples (shaded circles) were cut from each slice using a cork borer. In method A, increments were obtained by dividing the blade into three (or 10 in Aug. 1991 sample) equal sized sections (young, medium, old). In method B, each blade section was exactly 10 cm in height, and hence the number of sections per blade was variable. Location of the intercalary meristem, where most blade tissue is generated, is indicated by the cross-hatched oval.

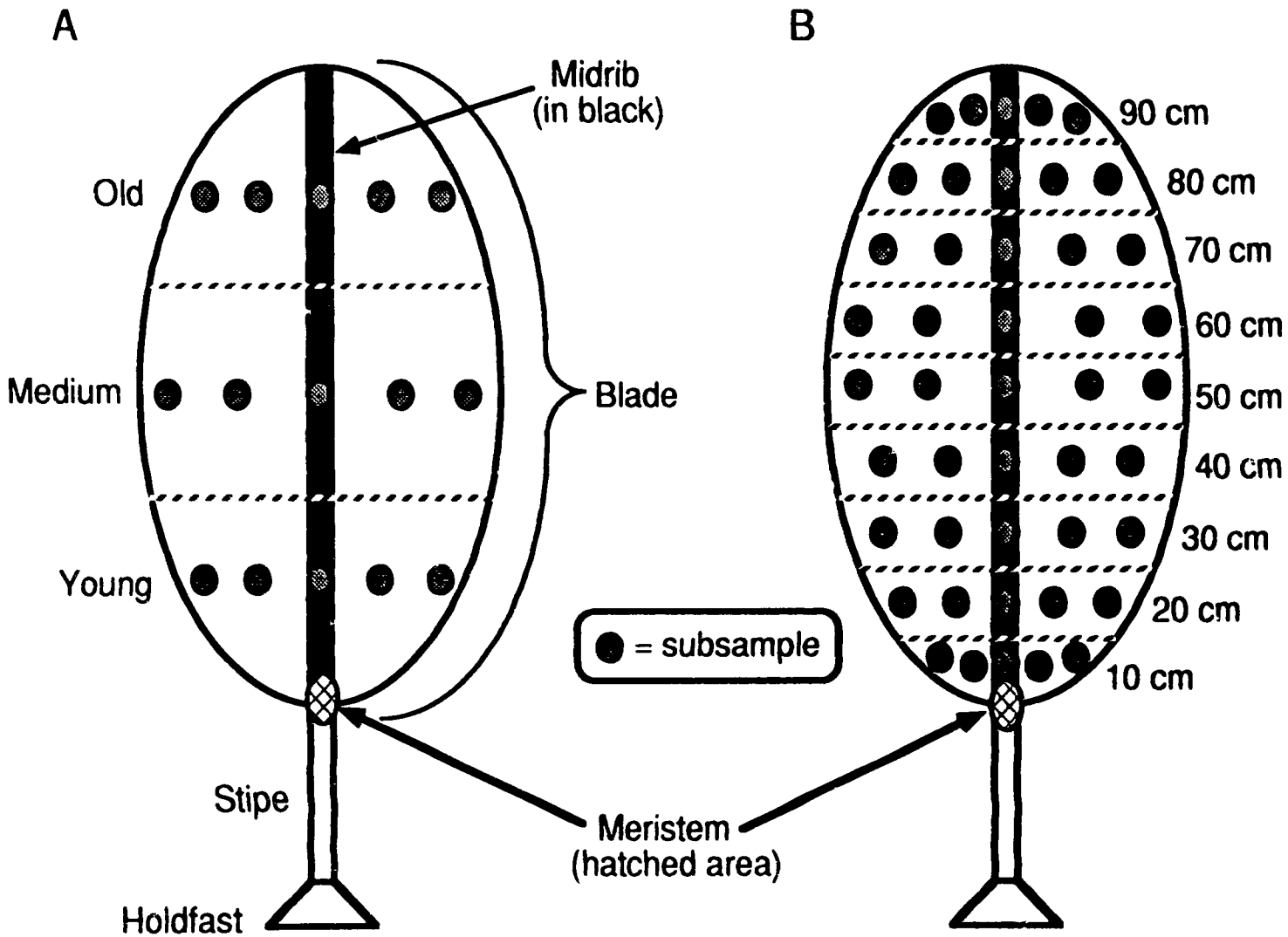
A
B
Midrib
(in black)
Old
Medium
Young
Blade
Stipe
Holdfast
= subsample
Meristem
(hatched area)
90 cm
80 cm
70 cm
60 cm
50 cm
40 cm
30 cm
20 cm
10 cm

Figure III-2: Criteria used to distinguish among ancestrulae, juveniles, and adults of bryozoans in the genera *Lichenopora* and *Tubulipora*. The ancestrula, the founding zooid of the colony, is present immediately following metamorphosis up to the point when asexual budding begins. Juveniles have >one zooid, but are fan shaped and lack brood chambers (i.e., ovicells). In *Lichenopora* juveniles, the 1° disk of the ancestrular zooid is still visible. Adult *Lichenopora* colonies are circular, and the ancestrular disk is generally obscured by the addition of new zooids to the colony. The presence of ovicells in *Lichenopora* is indicated only by a characteristic opening, called an ooeciostome (in black), accompanied by swelling in the central region of the colony, (ovicell represented by shaded circle). Adult colonies of *Tubulipora* may be fan-shaped or circular, and their ovicells are identified by both a unique morphology and an ooeciostome.

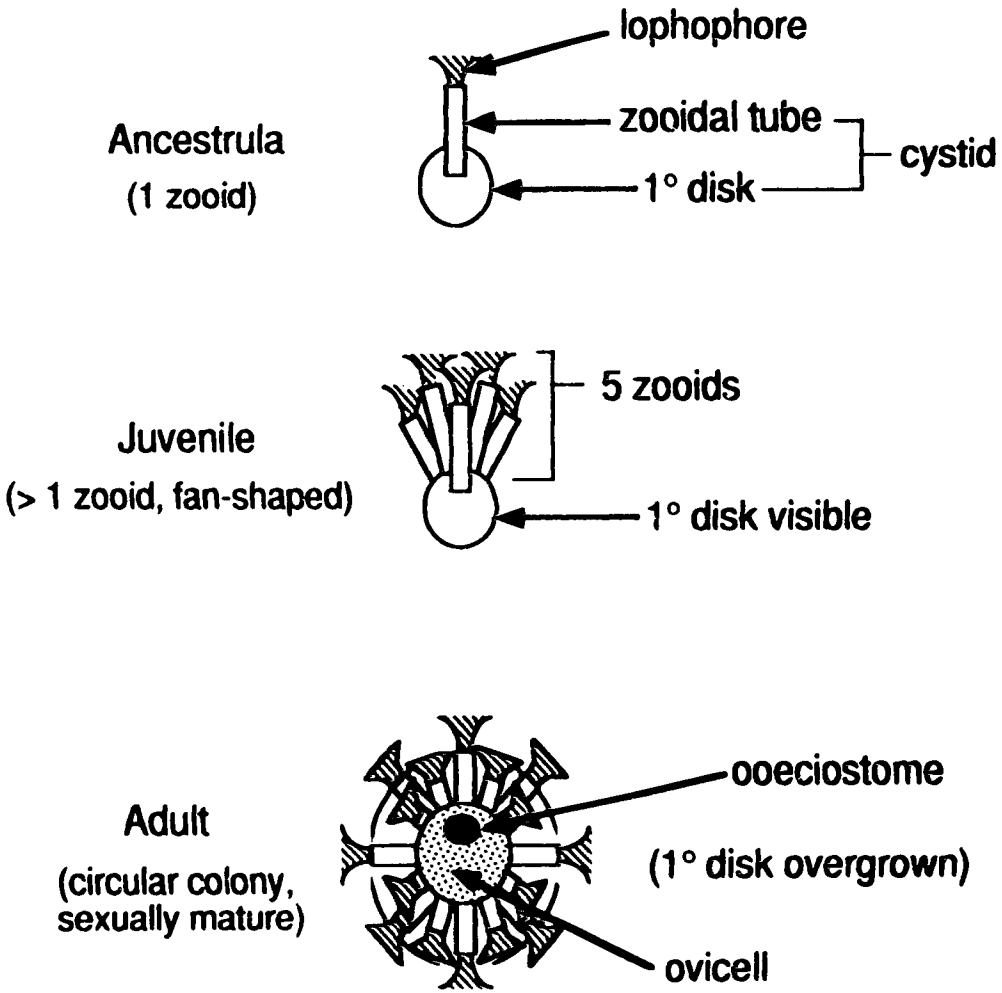
Ancestrula
(1 zooid)
lophophore
zooidal tube
1° disk
cystid
Juvenile
(> 1 zooid, fan-shaped)
5 zooids
1° disk visible
Adult
(circular colony,
sexually mature)
ooeciostome
(1° disk overgrown)
ovicell

Figure III-3: Abundance of *Lichenopora novae-zelandiae* on blades of *Agarum fimbriatum* collected from September 1990 to May 1993. Densities of sexually immature (open circles, i.e., ancestrulae & juveniles) and mature colonies (solid circles) are expressed as the number of colonies per cm^2. Five to ten blades were collected on each sampling date, and data are mean densities over entire blade ± standard errors. Where error bars appear absent, standard errors are less than the diameter of the plot symbols.

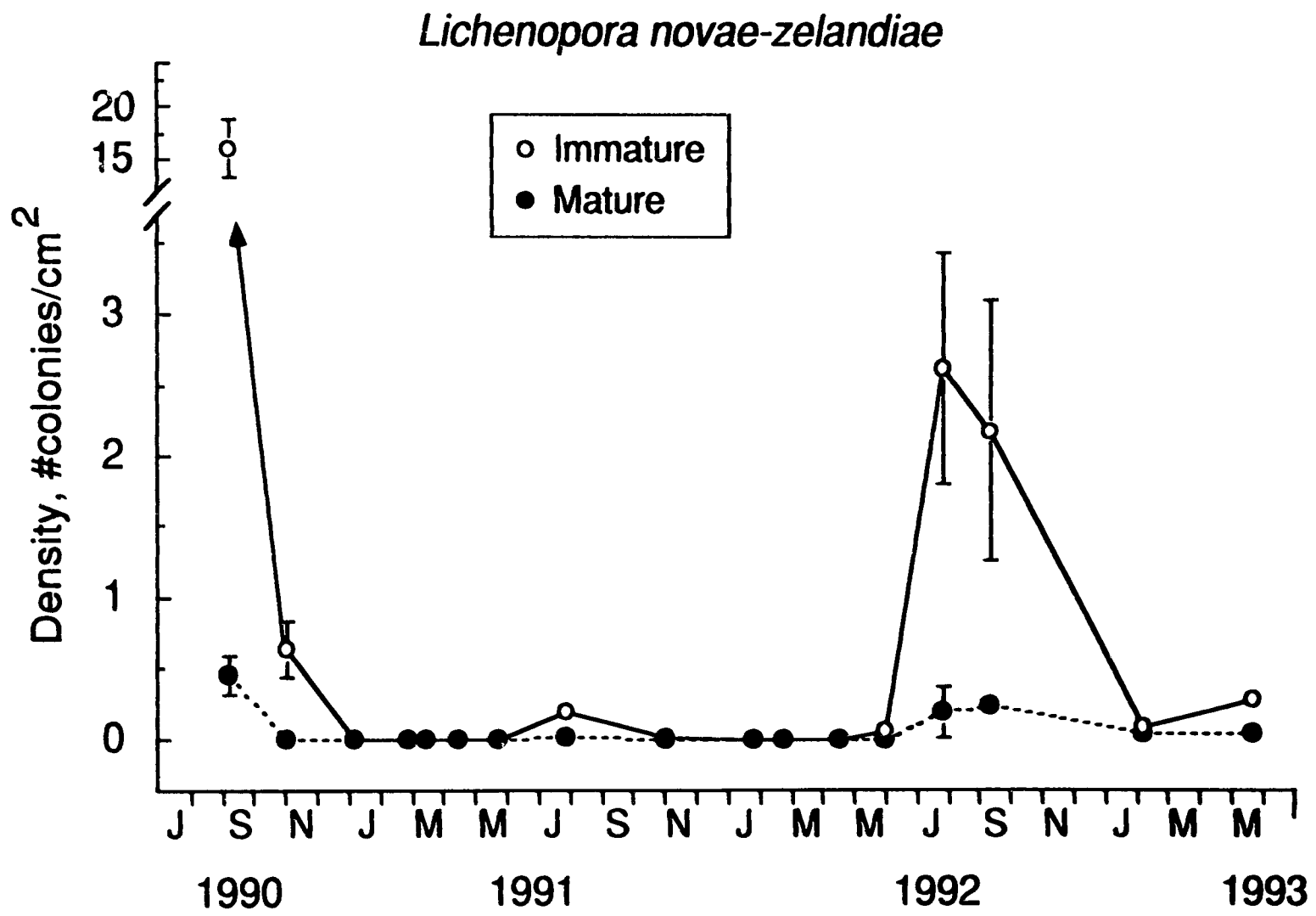
Lichenopora novae-zelandiae
Immature
Mature
Density, #colonies/cm2
20
15
3
2
1
0
J S N J M M J S N J M M J S N J M M
1990
1991
1992
1993

Figure III-4: Abundance of *Tubulipora* spp. on blades of *Agarum fimbriatum* collected from September 1990 to May 1993. Densities of sexually immature (open circles, i.e., ancestrulae & juveniles) and mature colonies (solid circles) are expressed as the number of colonies per cm^2. Five to ten blades were collected on each sampling date, and data are mean densities over entire blade ± standard errors. Where error bars appear absent, standard errors are less than the diameter of the plot symbols.

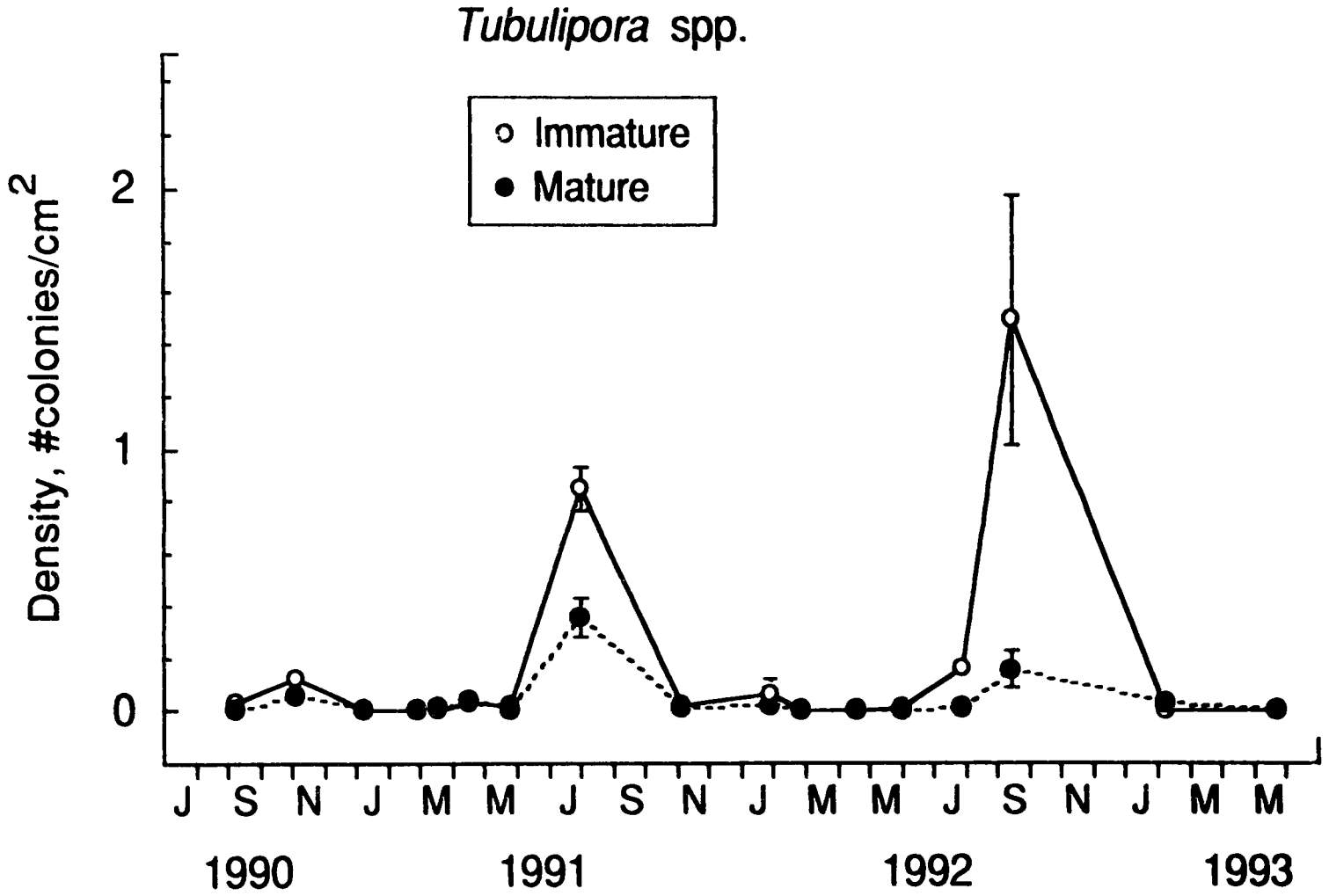
Tubulipora spp.
Immature
Mature
Density, #colonies/cm^2
0
1
2
J S N J M M J S N J M M J S N J M M
1990
1991
1992
1993

Figure III-5: Distribution of *Lichenopora novae-zelandiae* along blades of the kelp *Agarum fimbriatum* collected from the San José Islets in August of 1991. Scatterplots show densities (expressed as #colonies/cm^2) of ancestrular, juvenile, and adult colonies of bryozoans at 10% increments along kelp blades. Data plotted are means ± standard errors for five blades.

Lichenopora novae-zelandiae, Aug. 1991, San José Islets

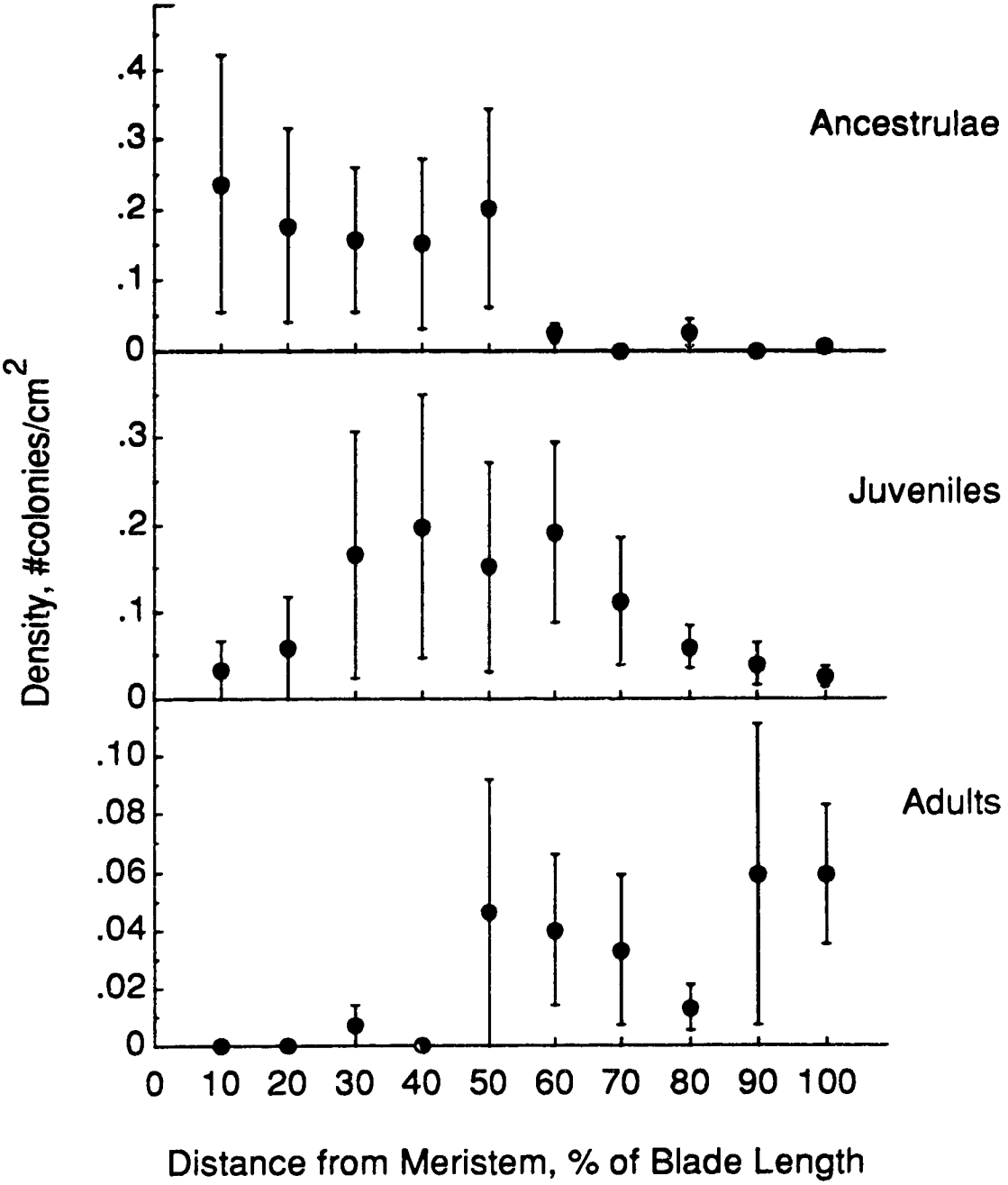

Figure III-6: Densities of *Lichenopora novae-zelandiae* colonies in five age categories of the kelp *Agarum fimbriatum* collected from the San José Islets in August 1991. For statistical analyses, data from Figure III-5 were rank-transformed and pooled into five categories (meristem, young, medium, old, and senescing), representing sequentially older algal blade tissue. For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), transformed data were analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of untransformed data. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

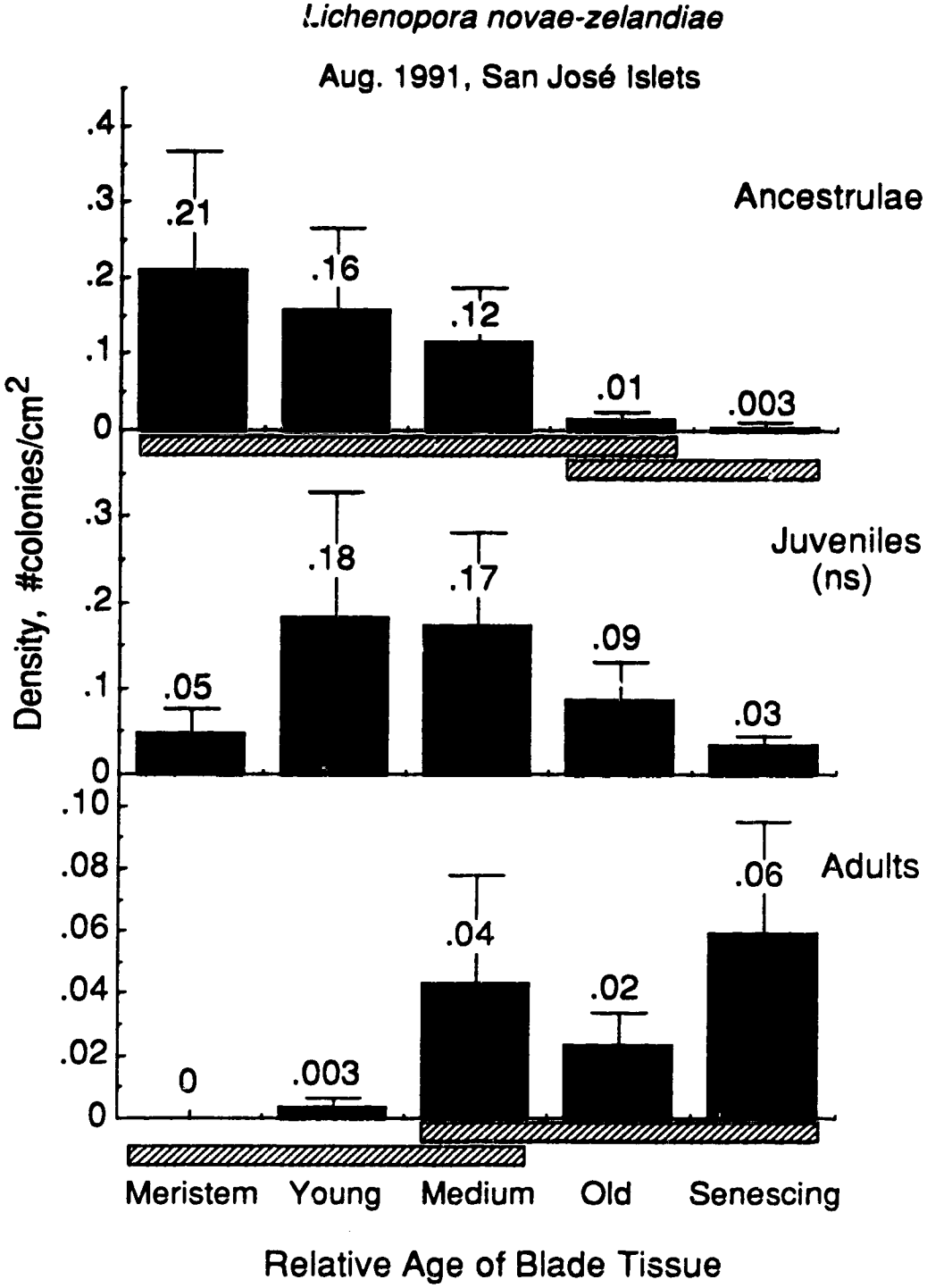
Lichenopora novae-zelandiae
Aug. 1991, San José Islets
Ancestrulae
Juveniles
(ns)
Adults
Density, #colonies/cm2
.4
.3
.2
.1
0
.21
.16
.12
.01
.003
.3
.2
.1
0
.18
.17
.09
.05
.03
.10
.08
.06
.04
.02
0
.04
.02
.06
0
.003
Meristem
Young
Medium
Old
Senescing
Relative Age of Blade Tissue

Figure III-7: Distribution of *Tubulipora* spp. along blades of the kelp *Agarum fimbriatum* collected from the San José Islets in August 1991. Scatterplots show densities (expressed as #colonies/cm^2) of ancestrular, juvenile, and adult colonies of bryozoans at 10% increments along kelp blades. Data plotted are means ± standard errors for five blades.

Tubulipora spp., Aug. 1991, San José Islets

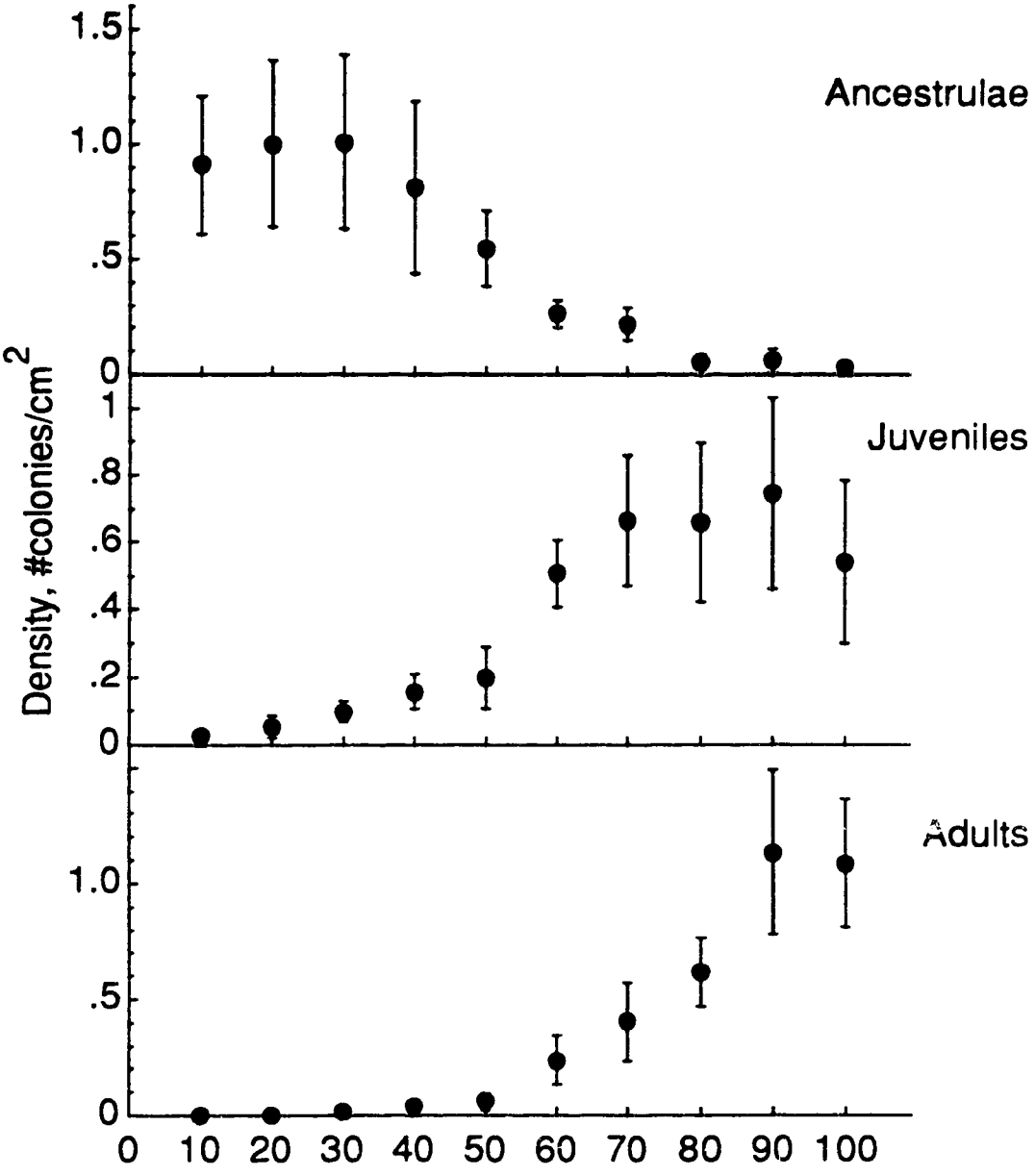

Figure III-8: Densities of colonies of *Tubulipora* spp. in five age categories of the kelp *Agarum fimbriatum* collected from the San José Islets in August 1991. For statistical analyses, data from Figure III-7 were rank-transformed and pooled into five categories (meristem, young, medium, old, and senescing), representing sequentially older algal blade tissue. For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), transformed data were analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of untransformed data. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

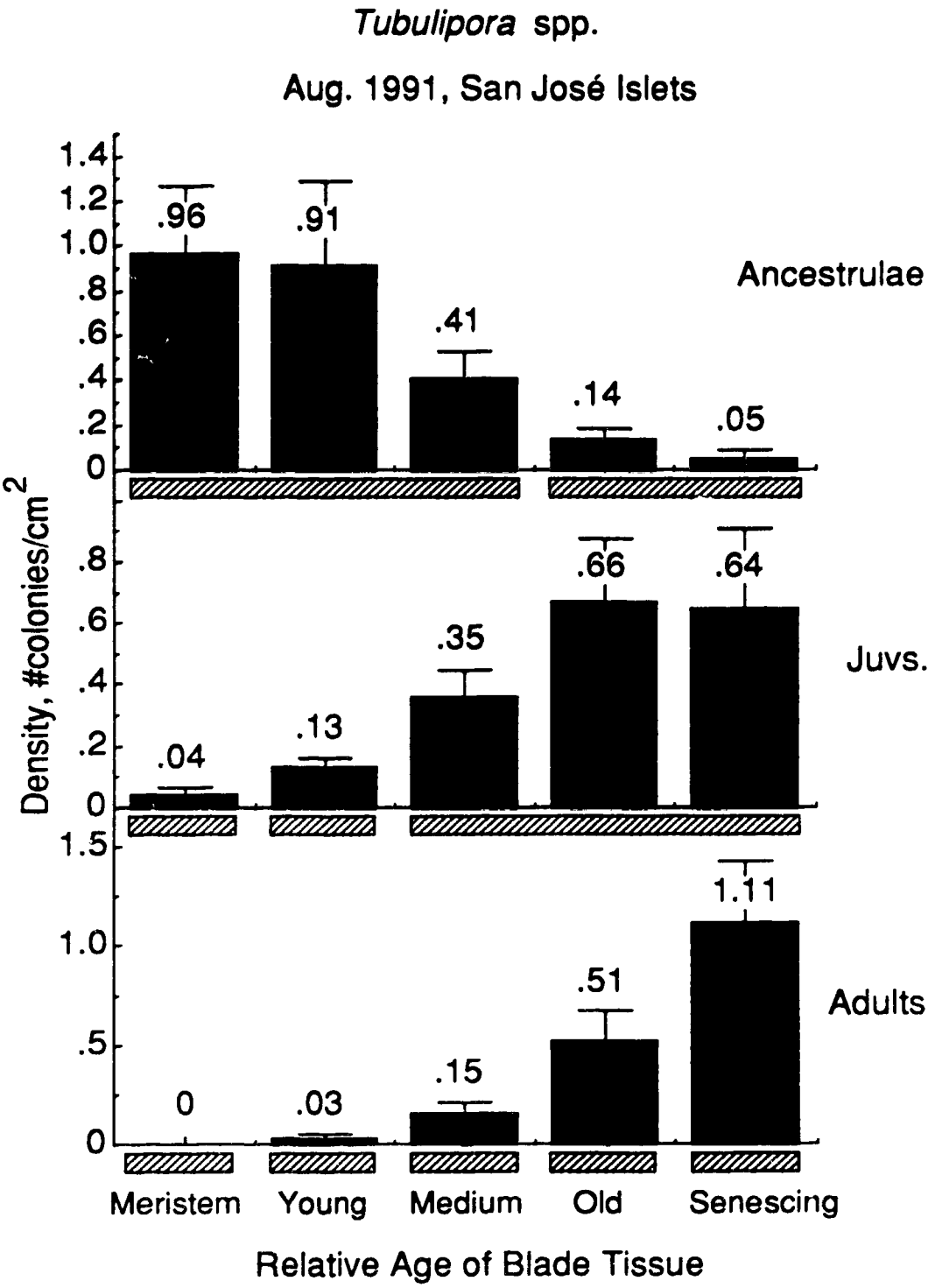
Tubulipora spp.
Aug. 1991, San José Islets
Density, #colonies/cm^2
Ancestrulae
Juvs.
Adults
.96
.91
.41
.14
.05
.04
.13
.35
.66
.64
0
.03
.15
.51
1.11
Meristem
Young
Medium
Old
Senescing
Relative Age of Blade Tissue

Figure III-9: Densities of *Lichenopora novae-zelandiae* colonies in three age categories of the kelp *Agarum fimbriatum* collected from Dixon Island in July 1992. For sampling, kelp blades were divided into three relative age categories, young, medium, and old, representing sequentially older algal blade tissue (see Figure III-1a). For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), data were rank-transformed and analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of untransformed data. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

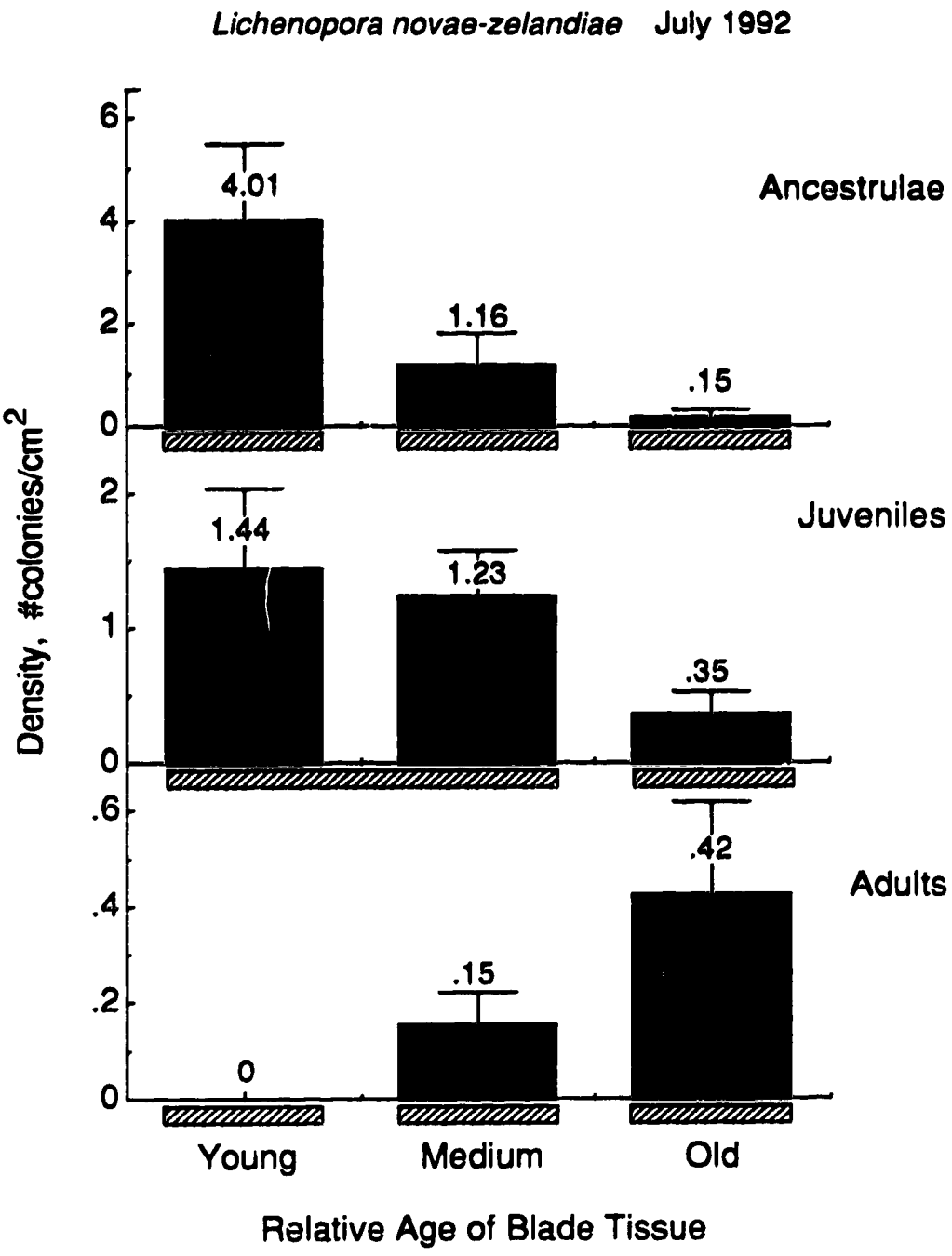
Lichenopora novae-zelandiae July 1992
Density, #colonies/cm^2
6
4
2
0
2
1
0
.6
.4
.2
0
4.01
1.16
.15
1.44
1.23
.35
.42
.15
0
Ancestrulae
Juveniles
Adults
Young
Medium
Old
Relative Age of Blade Tissue

Figure III-10: Densities of colonies of *Tubulipora* spp. in three age categories of the kelp *Agarum fimbriatum* collected from Dixon Island in July 1992. For sampling, kelp blades were divided into three relative age categories, young, medium, and old, representing sequentially older algal blade tissue (see Figure III-1a). For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), data were rank-transformed and analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of untransformed data. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

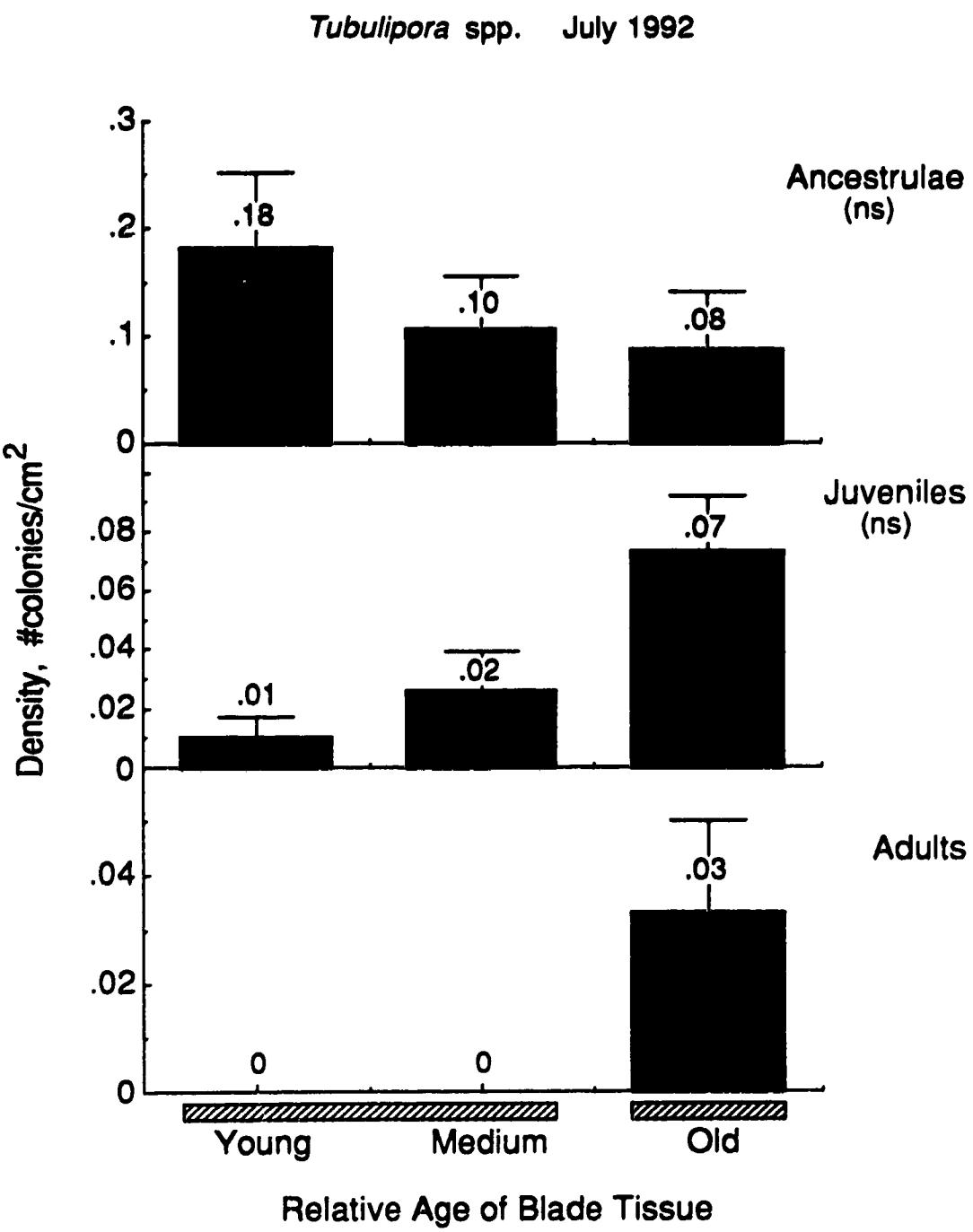
Tubulipora spp. July 1992
Density, #colonies/cm2
.3
.2
.1
0
.18
.10
.08
Ancestrulae
(ns)
.08
.06
.04
.02
0
.07
.02
.01
Juveniles
(ns)
.04
.02
0
.03
0
0
Adults
Young
Medium
Old
Relative Age of Blade Tissue

Figure III-11: Densities of *Lichenopora novae-zelandiae* colonies in three age categories of the kelp *Agarum fimbriatum* collected from Dixon Island in September 1992. For sampling, kelp blades were divided into three relative age categories, young, medium, and old, representing sequentially older algal blade tissue (see Figure III-1a). For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), data were rank-transformed and analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of four blades. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

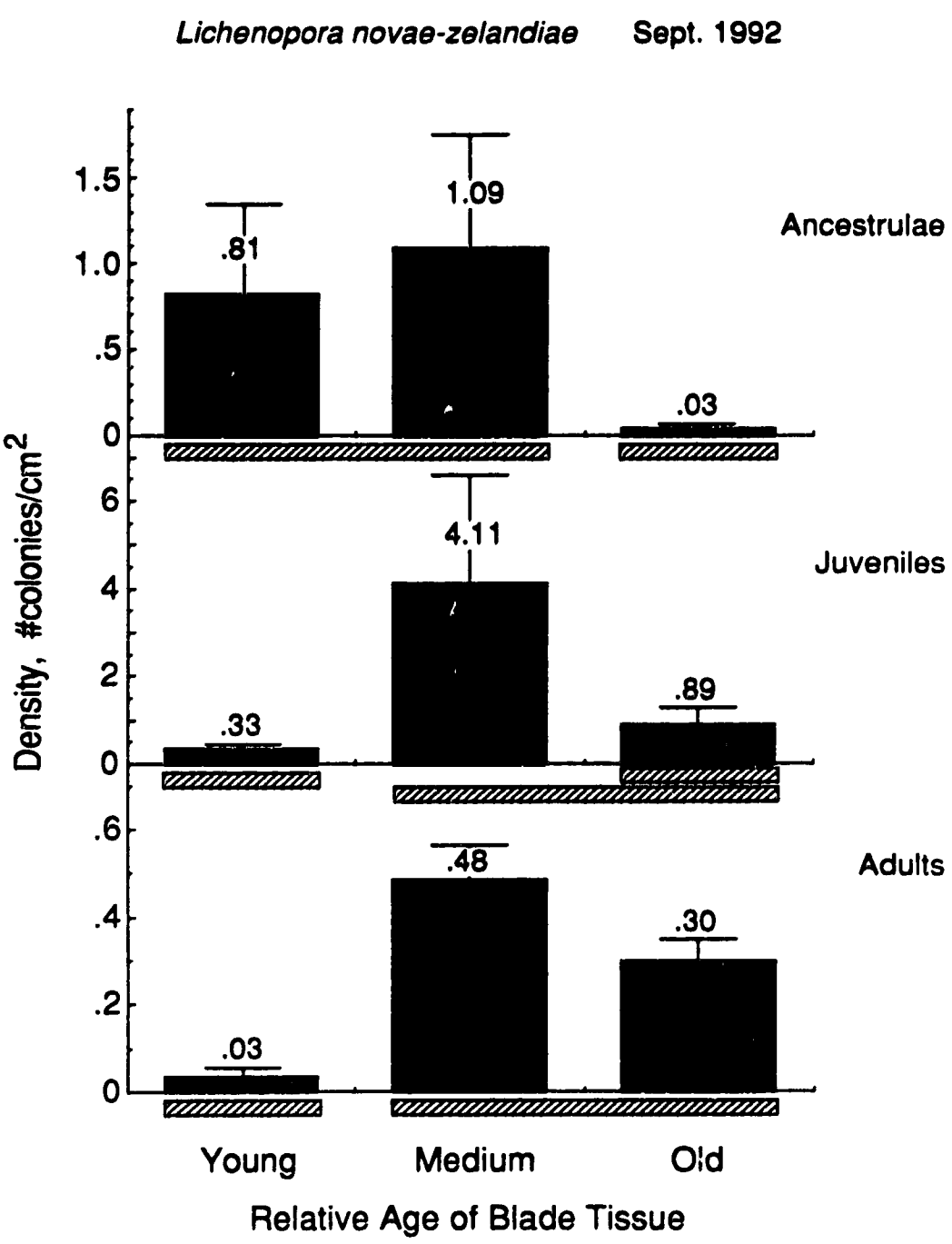
Lichenopora novae-zelandiae Sept. 1992
Density, #colonies/cm^2
1.5
1.0
.5
0
6
4
2
0
.6
.4
.2
0
.81
1.09
.03
4.11
.33
.89
.48
.30
.03
Ancestrulae
Juveniles
Adults
Young
Medium
Old
Relative Age of Blade Tissue

Figure III-12: Densities of colonies of *Tubulipora* spp. in three age categories of the kelp *Agarum fimbriatum* collected from Dixon Island in September 1992. For sampling, kelp blades were divided into three relative age categories, young, medium, and old, representing sequentially older algal blade tissue (see Figure III-1a). For each of three bryozoan age categories, (ancestrulae, juveniles, and adults), data were rank-transformed and analyzed using a randomized block design ANOVA, followed *a posteriori* with a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of four blades. Numbers above histogram bars indicate mean values. Horizontal hatched bars beneath histograms represent groups identified by S-N-K procedures.

Tubulipora spp. Sept. 1992

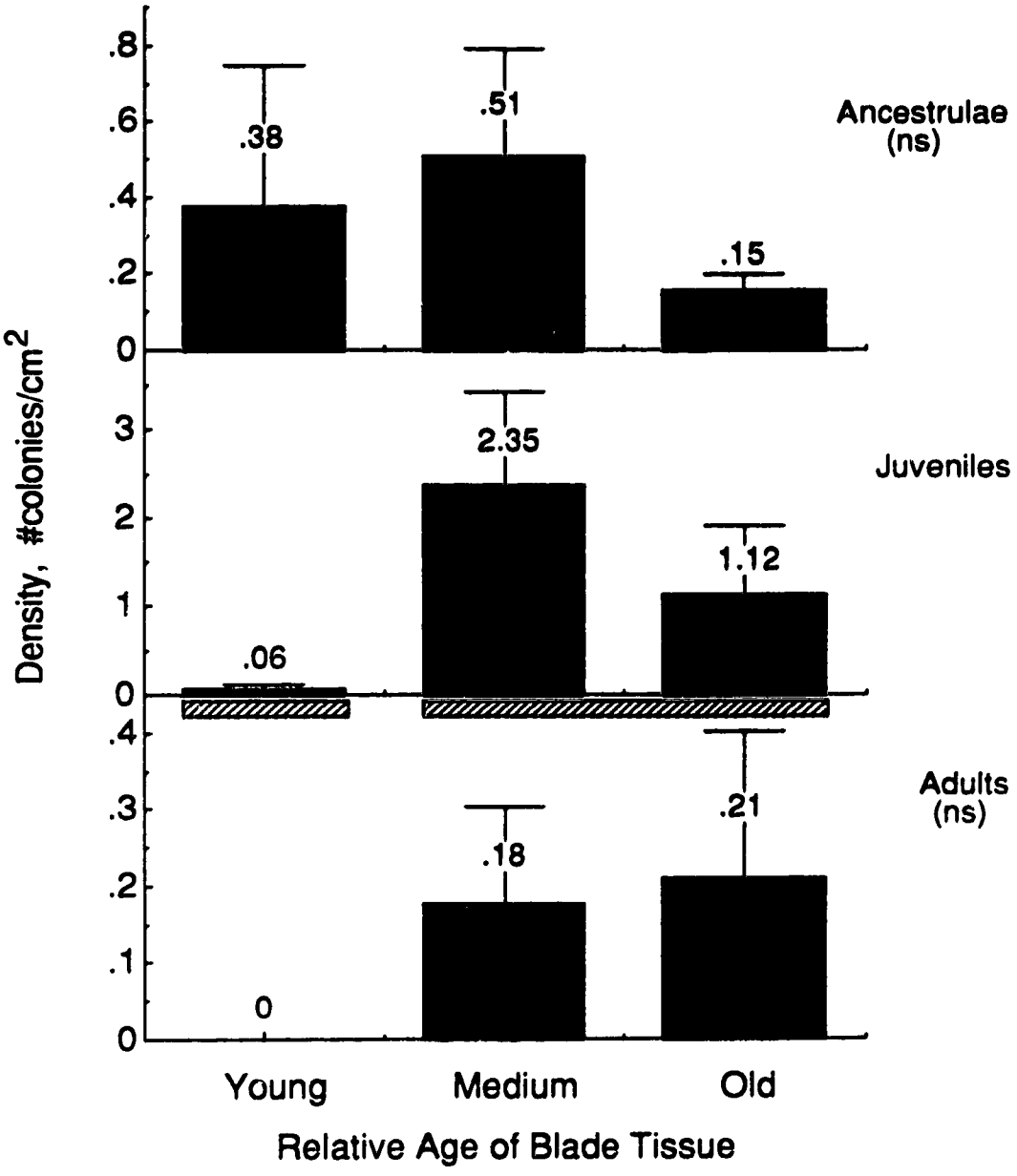

Figure III-13: Densities of *Lichenopora novae-zelandiae* colonies along blades of the kelp *Agarum fimbriatum* collected from Dixon Island in May 1993. Kelp tissue was subsampled at 10 cm increments from the intercalary meristem (see Figure III-1b), and colonies were categorized into one of four age groups: ancestrulae, juveniles, immature adults, and mature adults (See Figure III-2). Data are means ± standard errors from five blades.

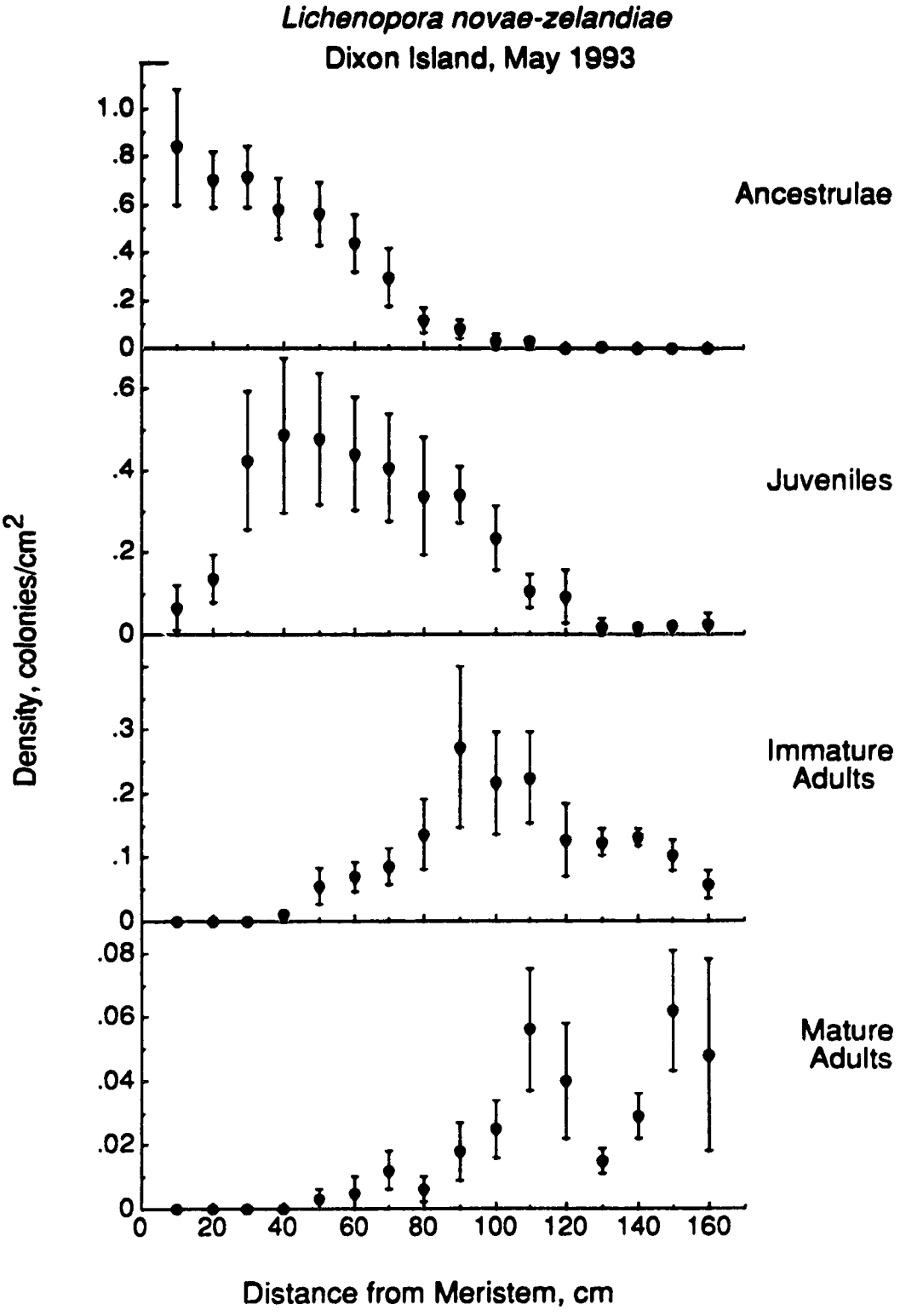
Lichenopora novae-zelandiae
Dixon Island, May 1993
Density, colonies/cm2
Ancestrulae
Juveniles
Immature Adults
Mature Adults
1.0
.8
.6
.4
.2
0
.6
.4
.2
0
.3
.2
.1
0
.08
.06
.04
.02
0
0
20
40
60
80
100
120
140
160
Distance from Meristem, cm

Figure III-14: Densities of *Lichenopora novae-zelandiae* colonies in three age categories of the kelp *Agarum fimbriatum* collected from Dixon Island in May 1993. For analysis, data were pooled into three relative age categories of the kelp tissue, (young, medium, old). For each of four bryozoan age categories, (ancestrulae, juveniles, immature adults, mature adults), rank-transformed data were analyzed using a randomized block design ANOVA, followed *a posteriori* by a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Shown on the histograms are true means ± standard errors of untransformed data. Numbers above histogram bars indicate mean values, and hatched bars beneath the solid histogram bars indicate groups identified by S-N-K tests.

Lichenopora novae-zelandiae May 1993

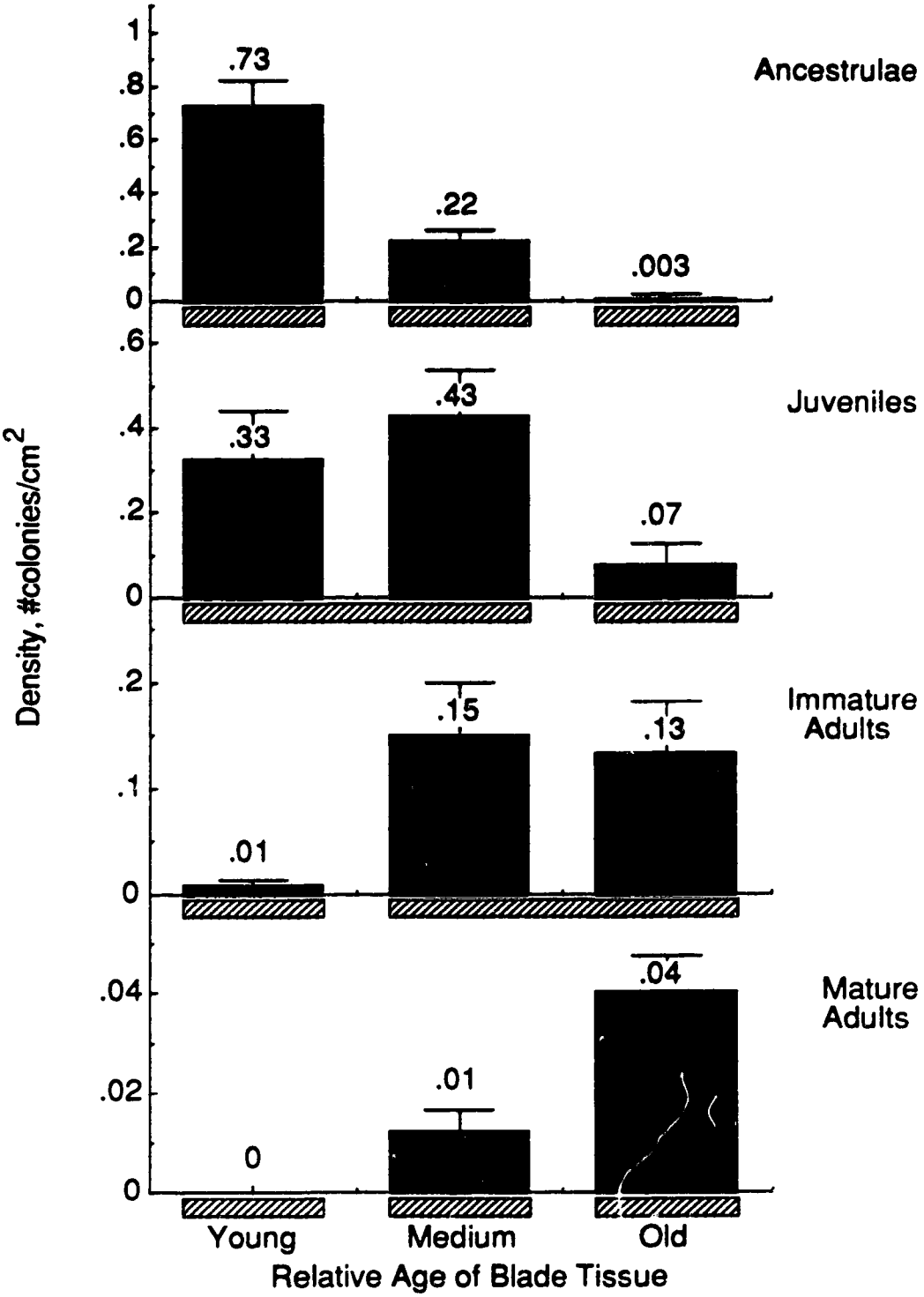

Figure III-15: Length of tissue sections from blades of the kelp *Agarum fimbriatum* (n=9) cultured in laboratory tanks from November 1990 to March 1991. Schematic drawing of kelp blade above graph indicates location of tissue samples. Data are plotted on a log scale to show more clearly the directional patterns in kelp tissue growth. Total blade length (open circles) declines over the course of the experiment, tissue away from the meristem (triangles and squares) does not change size appreciably, but tissue at the intercalary meristem (solid circles) increases in size throughout the study.

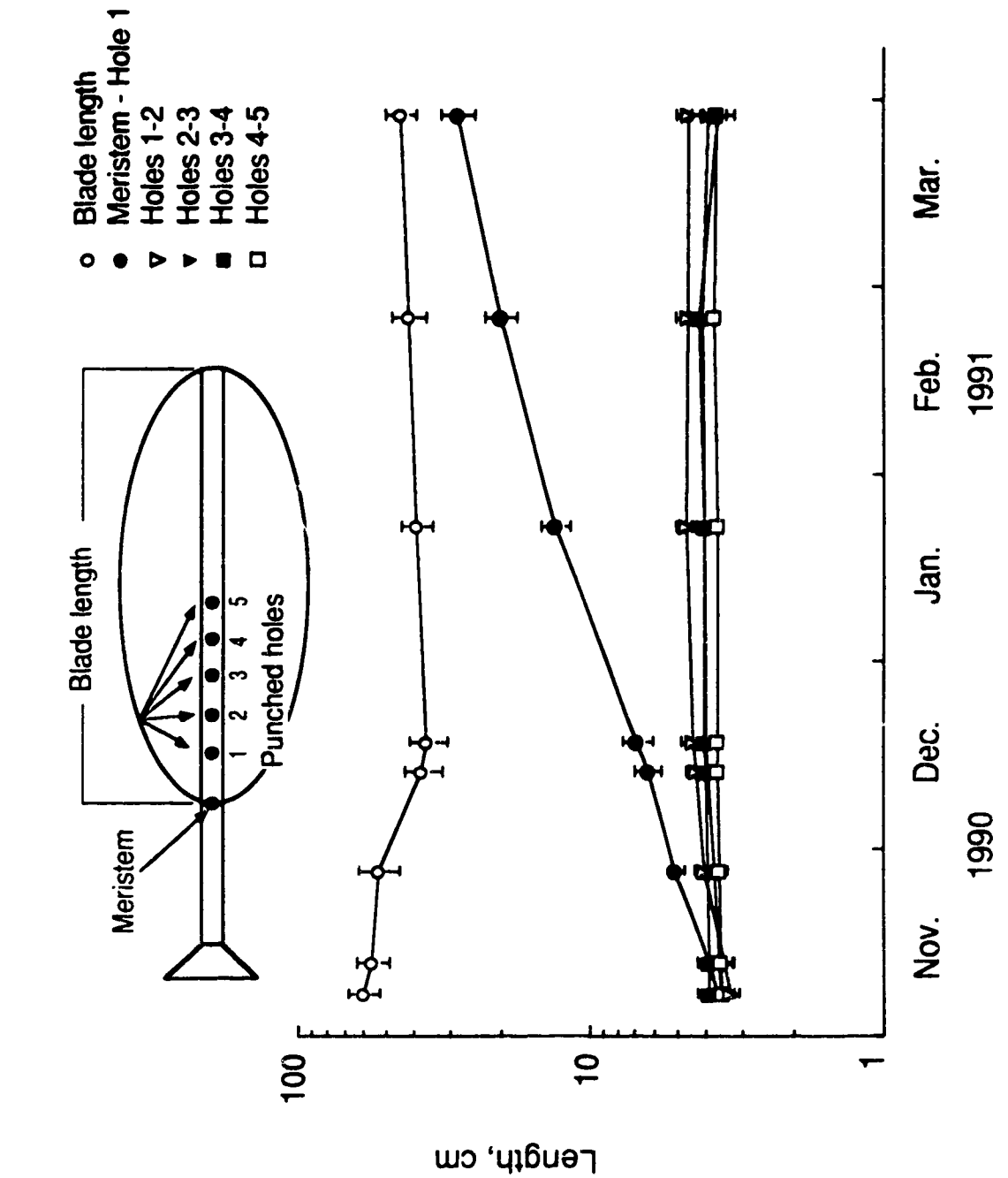
Blade length
Meristem - Hole 1
Holes 1-2
Holes 2-3
Holes 3-4
Holes 4-5
Blade length
Meristem
1 2 3 4 5
Punched holes
100
10
1
Length, cm
Nov.
Dec.
Jan.
Feb.
Mar.
1990
1991

Figure III-16: Growth in five tissue sections along blades of the kelp *Agarum fimbriatum* cultured in laboratory tanks from November 1990 to March 1991. Locations of tissue sections are explained in previous figure. Log-transformed data were analyzed using a randomized block design ANOVA followed *a posteriori* by a Student-Newman-Keuls (S-N-K) multiple comparisons procedure. Data shown are means ± standard errors of nine blades, plotted on a log scale. Horizontal hatched bars beneath histogram indicate groups identified by the S-N-K test.

Agarum: Growth in Laboratory Tanks
7 Nov '90 to 30 Mar '91

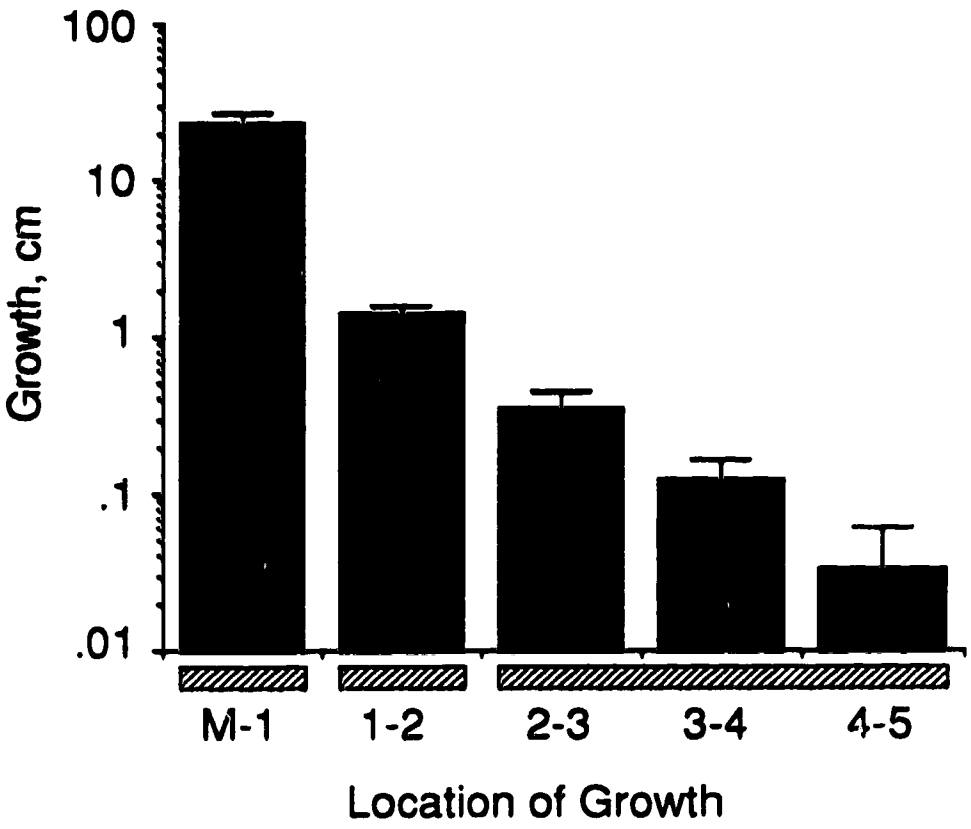

Figure III-17: Length of tissue sections from blades of the kelp *Agarum fimbriatum* measured *in situ* at Dixon Island on three occasions from January to April 1991. See Figure III-15 for explanation of where growth was measured on blades. Means ± standard errors of 9 blades are plotted on a log scale to show more clearly the directional patterns in kelp tissue growth. (Where standard error bars appear to be missing, plot symbols are larger than error bars). Blade length (open circles) and length of tissue between holes 1 and 2 (open triangles) increased slightly, and stipe length (solid squares) decreased slightly over the course of the experiment. Tissue between the meristem and hole 1 (solid circles), however, greatly increased in length relative to other tissues.

Growth in *Agarum fimbriatum*

Dixon Island: January - April 1991

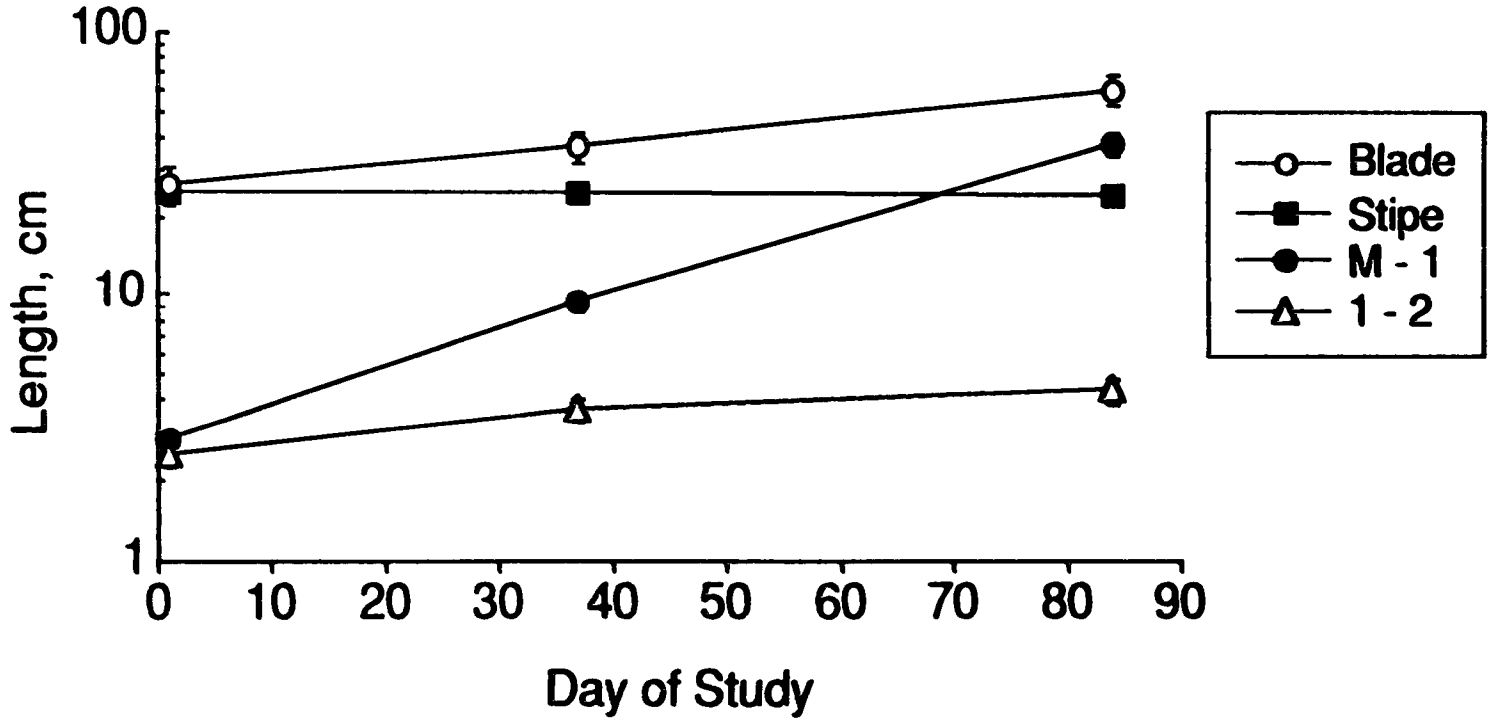

Figure III-18: Growth in two tissue sections along blades of the kelp *Agarum fimbriatum* measured *in situ* at Dixon Island from January to April 1991. See Fig. III-15 for locations of tissue sections. Log-transformed data were analyzed using a paired *t*-test. Growth between the meristem and hole 1 was significantly higher than that between holes 1 and 2 (paired t value = 9.115, df = 8, $P < .0001$). Data shown are means ± standard errors of nine blades, plotted on a log scale.

Agarum: Growth at Dixon Island
January - April 1991

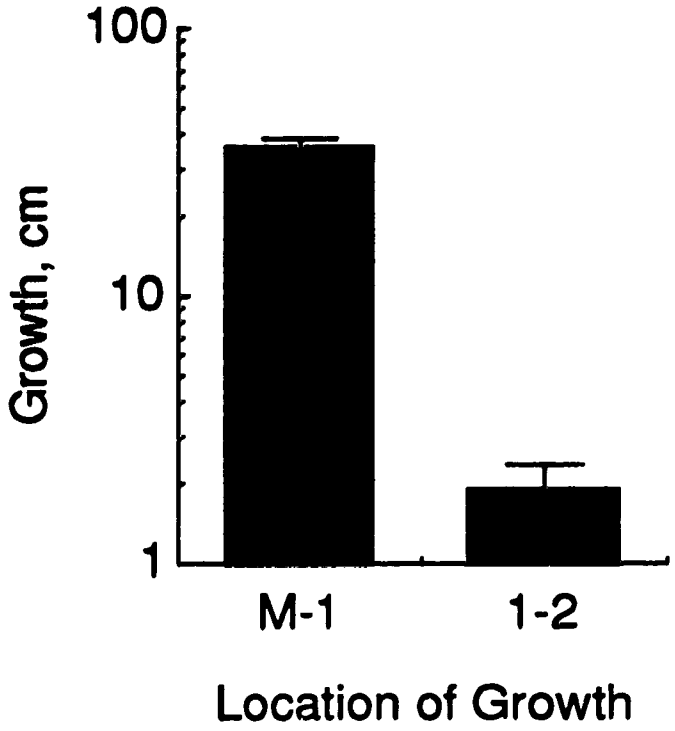

Table III-1: Results of randomized block design ANOVAs testing the main effect of algal tissue age on density of epiphytic bryozoans on blades of the kelp *Agarum fimbriatum*. (*L. n-z* = *Lichenopora novae-zelandiae*, *T.* spp. = *Tubulipora* spp.)

Date	Site	Bryozoan species	Bryozoan Life History Stage	df	F	P value
August 1991	San José Islets	*L. n-z*	ancestrulae	4	4.416	.0135
	San José Islets	*L. n-z*	juveniles	4	.654	.6324
	San José Islets	*L. n-z*	adults	4	6.199	.0033
	San José Islets	*T.* spp.	ancestrulae	4	25.106	< .0001
	San José Islets	*T.* spp.	juveniles	4	18.299	< .0001
	San José Islets	*T.* spp.	adults	4	58.021	< .0001
July 1992	Dixon Island	*L. n-z*	ancestrulae	2	17.792	.0011
	Dixon Island	*L. n-z*	juveniles	2	27.074	.0003
	Dixon Island	*L. n-z*	adults	2	36.719	< .0001
	Dixon Island	*T.* spp.	ancestrulae	2	1.530	.2737
	Dixon Island	*T.* spp.	juveniles	2	4.272	.0547
	Dixon Island	*T.* spp.	adults	2	14.545	.0022
September 1992	Dixon Island	*L. n-z*	ancestrulae	2	10.243	.0267
	Dixon Island	*L. n-z*	juveniles	2	11.861	.0208
	Dixon Island	*L. n-z*	adults	2	17.375	.0107
	Dixon Island	*T.* spp.	ancestrulae	2	.640	.5739
	Dixon Island	*T.* spp.	juveniles	2	11.442	.0221
	Dixon Island	*T.* spp.	adults	2	2.241	.2224
May 1993	Dixon Island	*L. n-z*	ancestrulae	2	107.143	< .0001
	Dixon Island	*L. n-z*	juveniles	2	15.926	.0016
	Dixon Island	*L. n-z*	immature adults	2	15.792	.0017
	Dixon Island	*L. n-z*	mature adults	2	60.000	< .0001

Table III-2: Results of a randomized block design ANOVA testing the effect of tissue location on growth in laboratory reared blades of the kelp *Agarum fimbriatum*. Blocks in the analysis are individual algal blades (n = 9), and data were log-transformed prior to analysis. Shown in the ANOVA table are the degrees of freedom (df), test statistic (F), and P-value for each of the two main effects.

Source	df	F	P-value
Tissue location	4	256.331	< .0001
Block	8	3.202	.0088
Residual	32		

Chapter IV

Substratum selection by larvae of the epiphytic marine bryozoans *Lichenopora novae-zelandiae* (Busk 1875) and *Tubulipora* spp. (Class Stenolaemata: Order Cyclostomata)

Introduction

Bryozoans frequently form specific epiphytic associations with seaweeds (Ryland 1959, 1962, 1974, Seed & Boaden 1977, Bernstein & Jung 1979, O'Connor et al. 1979, 1980, Hayward 1980, Seed & O'Connor 1981, Yoshioka 1982, Oswald & Seed 1986, Seed 1986, Hurlbut 1991), but the adaptive reasons for their choice of substrata, and the factors involved in host plant recognition are poorly understood. Like other marine invertebrates that settle associatively (i.e., settlement of one species on another, *sensu* Crisp 1974), certain epiphytic bryozoans are suspected to settle in response to cues, probably of a chemical nature, emanating from their host plants (reviewed in Pawlik 1992a). The larvae of many bryozoan species also respond, however, to factors such as light (Ryland 1960, 1974, 1977), surface rugosity (Walters & Wethey 1991, Walters 1992), surface wettability, and microbial films (Mihm et al. 1981, Brancato & Woollacott 1982, Maki et al. 1989). In this study, I used a bioassay-guided approach to determine which properties of an algal substratum are most important in directing the settlement of its epiphytic bryozoans.

In shallow subtidal regions around Barkley Sound, on Vancouver Island, Canada, blades of the kelp *Agarum fimbriatum* Harvey 1862 (Class Phaeophyta: Order Laminariales) are heavily colonized by encrusting

bryozoans of two stenolaemate genera, *Lichenopora* and *Tubulipora* (Durante & Chia 1991, Chapters II & III). *L. novae-zelandiae* appears to occur only on *A. fimbriatum*. Species of *Tubulipora*, (including *T. tuba*, *T. pulchra*, and *T. pacifica*) occur in highest densities on *A. fimbriatum*, but they are occasionally found on other substrata. Evidence reported in Durante & Chia (1991) suggested that, when selecting a substratum on which to settle, larvae of *L. novae-zelandiae* choose *A. fimbriatum* over other co-occurring kelps, and they select young blade tissue of their preferred kelp species over old blade tissue. As the larvae of many sessile marine invertebrates actively choose specific substrata at the time of settlement (reviewed in Meadows & Campbell 1972, Crisp 1974, 1976, Pawlik 1992a, b), and as *L. novae-zelandiae* larvae appear to settle preferentially on their host alga, the settlement choices of these bryozoan larvae appear at least partially responsible for the observed patterns in bryozoan distribution.

Along blades of *Agarum fimbriatum*, these epiphytic bryozoans exhibit a striking spatial pattern, with newly settled ancestrulae concentrated on young blade tissue nearest the intercalary meristem, and older bryozoans found on older kelp tissue (Chapters II & III). Interestingly, the spatial patterns of these bryozoan epiphytes mirror the distribution of polyphenolic defenses along blades of their algal substratum. Polyphenolics in *A. fimbriatum* are most heavily concentrated in young blade tissue and decline in concentration towards the distal, older end of the blade (Chapter V). *A. fimbriatum* is an unusual choice of substratum because its tissues contain some of the highest concentrations of polyphenolic compounds found in north temperate kelps (Steinberg 1984, 1985). Although polyphenolic compounds effectively deter marine

herbivores such as gastropods (Steinberg 1984, 1985, 1988, Taniguchi et al. 1991, Winter & Estes 1992), amphipods (Denton & Chapman 1991), and urchins (Anderson & Velimirov 1982, Steinberg 1988), and thus polyphenolic rich algae are resistant to grazing, they are also toxic to some bacteria and small organisms, such as the larvae of many marine invertebrates (reviewed in Ragan & Glombitza 1986). Therefore, bryozoans growing on polyphenolic rich algae must have acquired either a biochemical method of detoxifying these chemicals, or a prophylactic to prevent contact with polyphenolics leaching from their algal substratum.

Some herbivores preferentially consume and live upon seaweeds that use secondary metabolites as defenses against herbivory (Hay et al. 1989, 1990). By associating with chemically defended plants, these animals receive vicarious protection from predation because their predators avoid plant secondary metabolites. That epiphytic bryozoans settle specifically on a polyphenolic rich kelp has raised the intriguing possibility that the bryozoans select their host plant because it provides for them a refuge from predation or herbivory-related costs. If algal chemical defense is an important criterion for determining where these bryozoans live, then this specific habitat requirement should select for bryozoan larvae that use algal secondary metabolites for substratum recognition. In this chapter, I explore the possibility that larvae of these epiphytic bryozoans exploit polyphenolic defenses of their host alga by using these defensive chemicals as cues for larval settlement and metamorphosis.

Materials and Methods

Collection and maintenance of organisms

Plants and animals used in these experiments were collected between April 1991 and July 1993 from several subtidal sites (ca 5-20 m depth) in Barkley Sound on Vancouver Island, British Columbia [48° 51.24' N, 125° 7' W]. Colonies of the epiphytic bryozoans *Lichenopora novae-zelandiae* and *Tubulipora* spp. were collected using SCUBA by removing whole or partial blades of their algal substratum, *Agarum fimbriatum*. Also, whole, undamaged algal blades of several kelp species were collected to use as experimental substrata or to obtain algal chemical constituents for making artificial substrata. During transport to the laboratory (ca 1-2 h), kelp and bryozoans were held in cooled seawater aquaria aboard the research vessel, and upon arrival, were immediately transferred to running seawater aquaria at the Bamfield Marine Station.

General experimental protocol

Larval settlement experiments were conducted both in flowing seawater and in closed containers with still seawater, and all offered larvae a choice of substrata, (2-6 choices, depending on experiment). Neither surgical removal of larvae from colonies nor light induction of larval release (Reed 1987) yielded enough larvae for experiments, so larvae were obtained "naturally" by placing 5-6 sexually mature colonies into each experimental container. In general, little information could be gained from replicates containing fewer than 10 ancestrulae, but if colonies were at their seasonal peaks in fecundity, they could produce many hundreds of larvae over several days. Hence, individual replicates in experiments conducted

during these peak times often contained over 500 ancestrulae.

Over the course of an experiment, dishes with colonies and substratum choices were left undisturbed, and bryozoan larvae could emerge from parental brood chambers and settle on any available surface. After settling on a suitable substratum, bryozoan larvae undergo extensive metamorphosis, and the immediate post-settled juvenile stage (i.e., ancestrula) is identifiable by its distinctive morphology and pattern of calcification. Once a larva has undergone metamorphosis, it is permanently cemented to the substratum, so, in all of these experiments, larval choice was determined by counting numbers of ancestrulae on each choice substratum. Experiments were scored after 3-7 days, the experimental period being constrained by the fecundity of bryozoan colonies and by the integrity of the substrata being tested, particularly of artificial substrata which contained chemicals that could leach out or degrade over time. Many of the experiments described below tested larval preferences among young and old blade tissue. In all cases, these descriptive age terms refer to the youngest third of the blade near the intercalary meristem, and the oldest third near the blade tip.

Several experiments were conducted to test whether tissue subsamples from different kelp species and from different locations on the same kelp blades were distinguishable when presented to larvae "out of context", (i.e., removed from the whole plant). If larvae can distinguish among tissue choices under these conditions, then one can rule out the possibility that larvae need to orient with respect to the alga's gross morphology. That is: larval selection of algal tissues would depend on some property of the tissue itself, rather than its location on the algal thallus. Some of these experiments were conducted in still seawater, in glass dishes, and others

were conducted in flowing seawater, in large aquaria. In the still seawater experiments, I made the test substrata by removing disks of tissue from intact blades using a cork borer, (diameter = 19.7 mm). In some experiments, it was necessary to mark the disks for identification, and this was done by making small cuts in the tissue disks using either a sharp scalpel blade or a fractured capillary tube. Before tissue disks were used in experiments, they were cleaned of epiphytic organisms by wiping them with a Kimwipe™, and then allowed to heal for at least 24h under a jet of running seawater. Experimental dishes, holding vessels, and dissecting tools used to set up these experiments were all of embryological quality (*sensu* Strathmann 1987), (i.e., they were never exposed to soap, fixatives, or other contaminants, and were never stored with contaminated glassware). Freshly filtered seawater (hereafter referred to as FSW) used in contained experiments was obtained from the Bamfield Marine Station seawater system and subsequently filtered through a 1 μm pore size filter bag. Because filtration removed detritus and phytoplankton from the seawater, it was necessary to add 0.5-1.0 ml of cultured microalgal food (1:1 mixture of Tahitian *Isochrysis* sp. and *Rhodomonas* sp.) to containers in which adult bryozoans were held in FSW. Larvae of these bryozoans are non-feeding, so the microalgal food was added only for adults. For the uncontained experiments, flowing seawater was supplied by the Bamfield Marine Station seawater system, and experimental substrata had to be clamped to prevent them from being washed down the drainpipe. Consequently, substrata were made by attaching strips of algae to glass microscope slides, which were held in place in a wood rack. In these experiments, adult colonies received a daily supplement of microalgal food (~300 ml), which was gradually flushed from the aquaria by flowing

seawater.

Variation in settlement among dishes (i.e., blocks) was high due to variation in the number of larvae released from colonies used in the experiments. Although stenolaemate larvae are competent to settle immediately upon release from parental brood chambers, it was not possible to assess *a priori* how many larvae a particular colony would produce. Unlike certain gymnolaemate species which brood single large embryos in distinctive ovicells, bryozoans in the genera *Lichenopora* and *Tubulipora* brood their larvae commonly in completely covered calcified chambers, so it was not possible to see these larvae until they emerged from parental brood chambers. Nonetheless, to predict whether a colony was likely to produce larvae during an experiment, I selected colonies based on several criteria. (1) Larger colonies tended to produce more larvae, (2) the physical presence of brood chambers indicated that the colonies were at least sexually mature, and (3) fouled or unhealthy appearing colonies generally did not produce larvae. Stressed and dying colonies of *L. novae-zelandiae* immediately lose their purple colored exterior cuticle, healthy colonies of this bryozoan were easily identified by their purple hue. Also, the purple pigment granules tended to be concentrated more heavily around ooeciostomes (openings into the brood chambers), which facilitated identification of reproductively mature individuals. Colonies of *Tubulipora* have a colorless cuticle, so their reproductive status was determined by the presence of morphologically distinct brood chambers and ooeciostomes. Unhealthy or dead colonies of both *Tubulipora* and *Lichenopora* tended to be fouled with bacteria and microalgae, and these were rejected for use in experiments.

Statistical analyses

Because of the high between-block variation in these experiments, count data were log transformed to achieve normality and homoscedasticity prior to analysis. Then, transformed data were analyzed using randomized block design analyses of variance (ANOVAs), (which enabled treatments within dishes to be grouped together), followed by Student-Newman-Keuls multiple comparisons procedures (Zar 1984). All statistical analyses in this chapter were conducted with the assistance of SuperANOVA™ (version 1.0) or Statview™ SE+Graphics (version 1.04) software for Macintosh™ microcomputers.

Experiments with *Tubulipora* spp.

Three experiments were conducted to test the preference of settling *Tubulipora* larvae for several kelp substrata.

Algal species & tissue age

In the first experiment, disks of young and old blade tissue from three kelp species, *Agarum fimbriatum*, *Macrocystis integrifolia*, and *Nereocystis luetkeana*, were offered to larvae of *Tubulipora tuba* in a laboratory choice experiment. Disks were excised from healthy blades and then marked for identification by making two small circular cuts, the relative positions of which coded for particular species and age treatments (Fig. IV-1b). In each of five replicate dishes, the six kelp disks were randomly arranged around the dish perimeter, and 5-6 reproductively mature colonies of *T. tuba* were placed in the dish center (Fig. IV-1b). Over the experimental period, larvae emerged from parental brood chambers and could select among the six choice substratum disks. After three days, newly settled ancestrulae on each disk were counted.

Agarum fimbriatum* vs. *Eisenia arborea

In the second experiment, strips of intermediate age blade tissue from two kelp species, *Agarum fimbriatum* and *Eisenia arborea* were attached to glass microscope slides (25 x 75 mm) using elastic bands, and alternately arranged in a wood rack (Fig. IV-2b). The wood rack was submerged in a running seawater aquarium with adult colonies of *Tubulipora* spp. that were growing epiphytically on blade tissue of *A. fimbriatum*. After six days, the rack was removed from the source of bryozoan larvae and scored by counting the numbers of ancestrulae on each strip.

Young vs. old *Agarum fimbriatum* tissue

In the final experiment with *Tubulipora*, strips of young and old tissue from *Agarum fimbriatum* blades were attached to glass slides as above, and then one strip of each age was placed in a wood rack (Fig. IV-3b). Ten replicate wood racks were submerged in an aquarium with reproductively mature colonies of *Tubulipora* spp. (Fig. IV-3b), and left undisturbed for seven days, after which the experiment was scored as above.

Experiments with *Lichenopora novae-zelandiae*

To determine whether larvae of the bryozoan *Lichenopora novae-zelandiae* exhibited preferential settlement for particular locations on their host alga, *Agarum fimbriatum*, I conducted a series of laboratory choice experiments.

Young vs. old tissue of *Agarum fimbriatum*

Knowing that *L. novae-zelandiae* larvae selected young *A. fimbriatum* tissue when it was offered as one of four algal choices (Durante & Chia 1991, Chapter II), I first tested whether the larvae could

discriminate between young and old blade tissue of *A. fimbriatum*. In each of 10 replicate dishes containing 250 ml of FSW and adult bryozoan colonies, tissue disks (one of each age category) which had been cleaned of epiphytes and allowed to heal were arranged around the perimeter of the dish in a randomly determined position (Fig. IV-4b). After 3 days, and again after 6 days, I scored the experiment by counting the number of bryozoan ancestrulae on the tissue disks.

Effect of bacterial cues on larval choice

To determine whether bacterial films on the surface of *Agarum fimbriatum* influence bryozoan substratum choice, I tested larval choice in an experiment with two factors: tissue age and the presence/absence of bacterial cues. As described for the above experiments, this experiment was also a block design, with a young and old algal tissue disk present in each dish along with 250 ml of FSW and adult bryozoans (Fig. IV-5b). Because I was specifically interested in the effects of bacterial films, I did not wipe the surface of the disks as part of the pre-treatment cleaning. Instead, I gently removed any epiphytes from the algal disks with a dissecting tool, leaving the surface microfauna intact. For 24 hours prior to the start of the experiment, young and old algal disks were held in isolated containers and allowed to heal under a jet of flowing seawater. Of 30 replicate dishes, 15 randomly selected replicates received a 40 μg/ml dose of the antibiotics dihydrostreptomycin and penicillin-G, and the remaining 15 dishes were left untreated as controls. The antibiotic dose used here is reported to be safe for use with invertebrate larvae, but is also effective at killing marine bacteria (Strathmann 1987). After 3 days, the experiment was scored by counting the number of settled ancestrulae on each algal disk.

Chemical cues from young & old algal tissue

To determine whether bryozoan larvae could identify their preferred substratum, young *Agarum fimbriatum* tissue, without using physical cues from the intact alga, I set up an experiment with artificial substrata. The test substrata were made by combining aqueous extracts from young and old *A. fimbriatum* tissue with melted agar in a method modified from Targett et al. (1986). The resulting solid substrata contained aqueous components of algal tissue, but the agar matrix prevented water soluble compounds from immediately leaching into the surrounding seawater.

Algal tissues used for extraction were cleaned of epiphytes, and then 25 g of tissue were homogenized in a blender with 250 ml FSW (including wash volume), to yield a 10% (w/v) homogenate. Beakers containing homogenates were packed in ice and the chilled homogenates were allowed to stand for ca 1 hour before filtration. Following coarse filtration and low speed centrifugation, filtrates were further purified by removing remaining suspended material with a .45 µm Millipore™ filter. The purified extracts were refrigerated overnight before mixing with agar. Substrata were prepared by mixing a 1:1 ratio of algal extract to a 4% (w/v) agar-FSW solution, and pouring this mixture into molds constructed with 15 ml polystyrene centrifuge tubes (see Fig. IV-6 for diagram of protocol). After the extract-agar mixtures solidified in their cylindrical molds, they were gently removed, and the lines etched by the .5 ml gradations on the centrifuge tubes were used for scoring marks to slice the cylinders into equal size disks. Slices from young and old extract mixtures were adhered to opposite ends of microscope slides using a drop of melted 4% agar-FSW solution. The slides were arranged in 30 replicate dishes, (their orientations relative to a 360° scale determined using a random number

table), each of which also contained 250 ml FSW and adult bryozoans. After 6 days, the number of bryozoan ancestrulae on each agar-extract disk was counted.

Leachate from young algal tissue

To test whether bryozoan larvae detect water-borne cues leaching from their preferred algal substratum, I set up an experiment that presented larvae with solid agar mixtures of leachates from living and heat killed young *Agarum fimbriatum* tissue. To make leachates, I removed the youngest 10 cm of the blade by slicing perpendicular to the midrib, and sliced this section in half along the midrib (see Fig. IV-8 for diagram of protocol). One half of the young section (20.84 g wet weight) was immersed in a jar containing 2 l of FSW. The other algal half was dried at 80° C for 24 hours and then immersed in 2 l of FSW for leaching. After 3 d, the plant tissues were removed from the jars containing FSW and the remaining leachates filtered through a Kimwipe™ to remove particulate matter. The filtered leachates from fresh and dried algae were mixed in a 1:1 ratio with 4% (w/v) agar in FSW and the leachate-agar liquids were used to coat opposite ends of glass microscope slides. Once the solutions had solidified on the slides, the slides were randomly arranged in 12 replicate dishes, each containing 200 ml FSW and adult bryozoans. After 4 days, the number of bryozoan ancestrulae on the agar-leachate coatings was counted.

Solidified vs. adsorbed aqueous extracts; heating/freezing effects

I set up an additional experiment to test whether agar substrata made from aqueous extract of young *Agarum fimbriatum* tissue induces settlement of bryozoan larvae more than substrata made with a FSW control, and whether the chemical cues in the extract adsorb to surfaces or immediate dissolve in the surrounding seawater. Also, to test whether the

chemical cues affecting larvae are heat stable, test substrata were both oven-dried and frozen. To make the agar substrata, first I made a 10% (w/v) homogenate of young *A. fimbriatum* in FSW, which was subsequently filtered to remove particulate matter. Then, the algal fitrate was mixed in a 1:1 ratio with 4% (w/v) agar in FSW and this mixture applied in equal volume aliquots to microscope slides. Control agar substrata were made by substituting FSW for the algal filtrate, and a third treatment applied undiluted filtrate directly to the glass slides. The slides, each containing one replicate of each treatment (Fig. IV-10b), were firs' oven dried at 80° C for approximately one hour and then held frozen at -15° C for two days until the start of the experiment. The slides were randomly arranged in 10 dishes, each containing 250 ml of FSW and adult bryozoans, and the experiment was scored after 4 days.

Dose dependent responses to chemical cues

To determine whether larvae of *Lichenopora novae-zelandiae* exhibit dose-dependent settlement responses to chemical cues, I conducted a larval choice experiment with three aqueous extract-agar treatments: extract from old tissue, extract from young tissue, and a mixture of young and old extracts. If larval responses are dose dependent, then the mixture of cues from preferred and non-preferred substrata should induce settlement at a level intermediate between those of the unmixed cues. Extracts of young and old *Agarum fimbriatum* tissue were made as described above and Millipore™ filtered to .45 μm to remove all particulate matter. These extracts were made several days ahead of time, however, so they were held at -80°C and defrosted immediately before the start of the experiment. The three extract treatments, (young, old, and 1:1 young:old) were mixed 1:1 with 4% (w/v) agar in FSW and applied to coverslips, which were

subsequently glued to microscope slides using a drop of the 4% agar solution (Fig. IV-11b). The 20 slides were randomly arranged in dishes, each containing 250 ml FSW and adult bryozoans, and the experiment was scored after 3 days.

Heat denaturation of chemical cue?

To determine whether the chemical cues involved in directing settlement of *Lichenopora novae-zelandiae* larvae were inactivated by boiling, I offered larvae a choice of three agar-extract substrata made with aqueous extract of young tissue, boiled extract, and FSW control. This experiment also used previously frozen aqueous extracts which were mixed with agar after defrosting, and the extract for the boiled treatment was heated to 100° C on a hot plate prior to mixing with agar. As above, the three treatments were applied to coverslips and then glued to microscope slides with agar (Fig. IV-12b). The slides were randomly arranged in 20 replicate dishes, each containing 250 ml FSW and adult bryozoans, and the experiment was scored after 3 days.

Effects of phenolic monomers on larval settlement

Because young blade tissue from *Agarum fimbriatum* is known to contain elevated levels of polyphenolic compounds (Chapter V), and because polyphenolic compounds are water soluble, I conducted an experiment to test whether bryozoan larvae are attracted to the purified phenolic monomer phloroglucinol. Test substrata for this experiment were constructed by mixing purified phloroglucinol (Sigma) with 2% (w/v) agar in FSW, in concentrations within the range of polyphenolic content found in *A. fimbriatum* (~ .09 - 1.5 % wet weight, unpublished data). While warm, the phloroglucinol-agar solutions were poured into small polystyrene molds (10 x 35 mm petri dishes) and allowed to cool. Final

phloroglucinol concentrations in the three agar substrata were 10 mg/ml, 1 mg/ml, and a 0 mg/ml control, and one replicate of each treatment was randomly arranged in each of 30 dishes containing 250 ml FSW and adult bryozoan colonies. The experiment was scored after 7 days.

Results

Experiments with *Tubulipora*

Algal species and tissue age

When presented with a choice of six excised tissue disks from two age categories of three kelp species (see protocol in Fig. IV-1b), larvae of *Tubulipora tuba* preferred settling on *Agarum fimbriatum* over the other two kelp species, (*Macrocystis integrifolia* and *Nereocystis luetkeana*), and they preferred young blade tissue from *A. fimbriatum* over old (Fig. IV-1a). An ANOVA revealed significant effects of algal species ($F_{2, 20} = 6.848$; $P = .0054$) and algal age ($F_{1, 20} = 5.146$; $P = .0345$) on bryozoan larval settlement. There was no significant interaction between these two main effects.

Agarum fimbriatum vs. *Eisenia arborea*

Despite being released from adult colonies in an aquarium with flowing seawater, larvae of several species of *Tubulipora* were retained long enough in the aquarium to settle on tissue strips excised from *Agarum fimbriatum* and *Eisenia arborea*. More larvae settled on tissue from the more common host substratum, *A. fimbriatum*, than on tissue from *E. arborea* (Fig. IV-2a) ($F_{1, 4} = 25.769$; $P = .0071$), but the position of tissue strips within the aquarium was also a significant factor affecting

larval choice ($F_{4, 4} = 9.861$; P = .0239). As indicated in Fig. IV-2b, algal strip positions with higher numbers in the rack were closer to the seawater-air interface than those with lower numbers, and larval settlement increased towards the top of the rack (Fig. IV-2c). Although there was no replication of racks in this experiment, and thus it was not possible to determine whether position effects were independent of the rack itself, higher settlement towards the water surface could also indicate that (1) larvae were attracted to higher light levels at the surface (i.e., positive phototaxis), or (2) larvae settled further from the source of flow (i.e., negative rheotaxis), which was located at the bottom of the cylindrical aquarium.

Young vs. old *Agarum fimbriatum* tissue

In a similar experiment in which racks holding young and old tissue strips from *Agarum fimbriatum* were replicated 10 times (Fig. IV-3b), larvae of *Tubulipora* spp. chose the young choice over the old (Fig. IV-3a) ($F_{1, 8} = 62.196$; P = .0001), and this choice was independent of position in rack, (i.e., top vs. bottom). Within the range of means tested, there was no significant interaction between tissue age and position in rack.

Experiments with *Lichenopora novae-zelandiae*

Young vs. old tissue of *Agarum fimbriatum*

When larvae of *Lichenopora novae-zelandiae* were offered a choice of young and old tissue disks excised from their host substratum *Agarum fimbriatum* (Fig. IV-4b), they overwhelmingly preferred the young choice (Fig. IV-4a). The ANOVA revealed a significant effect of algal tissue age after 3 days ($F_{1, 9} = 15.721$; P = .0033) and after 6 days ($F_{1, 9} = 250.794$; P = .0001). As larvae for this experiment were obtained by natural release from parent colonies (Fig. IV-4b) rather than by pipetting known numbers of

larvae into each dish, experimental variation in settlement among dishes can be explained by natural variation in the fecundity of adult colonies used to supply larvae. When larval populations differing greatly in size exhibit the same settlement choices in a randomized and replicated experiment, one can refute the alternate hypothesis that treatment effects were caused by a density dependent anomaly.

Effect of bacterial cues on larval choice

Settlement preference of the larvae of *Lichenopora novae-zelandiae* for young *Agarum fimbriatum* tissue over old ($F_{1, 111} = 261.545$; $P = .0001$) was unaffected by the presence of antibiotics (Fig. IV-5). Larvae settled equally with respect to orientation on the kelp disks (i.e., top surface vs. undersurface), supporting the view that stenolaemate larvae lack photoreceptors (Nielsen 1970, Ryland 1974, Zimmer & Woollacott 1977) and thus cannot exhibit phototaxis. There were no significant interactions among algal tissue age, antibiotic treatment, or disk orientation).

Chemical cues from young and old tissue

When exposed only to solidified agar mixtures of filtered homogenates made from young and old blade tissue of *Agarum fimbriatum* (see protocol in Fig. IV-6), larvae of *Lichenopora novae-zelandiae* settled in higher numbers on the artificial substratum choice that was made from young tissue homogenate (Fig. IV-7), and thus the treatment effect (i.e., homogenate "flavor") was significant ($F_{1, 29} = 6.561$; $P = .0159$).

Leachate from young algal tissue

Leachates made with fresh and oven-dried blade tissue from *Agarum fimbriatum* (see protocol in Fig. IV-8) did not differ significantly in their attractiveness to settling larvae of the bryozoan *Lichenopora novae-zelandiae* (Fig. IV-9), although the effect of leachate choice was just beyond

the significance level of the test at .08. Strangely, this nearly significant effect of leachate source on larval choice was counterintuitive: larvae settled more on substrata made with leachate from dried kelp tissue than those made with fresh tissue.

Solidified vs. adsorbed aqueous extracts; heating/freezing effects

When presented with aqueous extract from *Agarum fimbriatum* mixed with agar, extract painted onto a glass surface, and a FSW control mixed with agar (see protocol in Fig. IV-10b), larvae of *Lichenopora novae-zelandiae* settled in highest abundance on the extract-agar choice (ANOVA: $F_{2, 18} = 17.409$; $P = .0001$) (Fig. IV-10a). There was no significant difference in larval settlement between the other two choices, although the mean settlement on the painted extract choice was 61% higher than than on the FSW control (Fig. IV-10a), indicating that at least some constituents of the aqueous extract were retained on the glass surface long enough to be detected by settling larvae. Because larvae were able to distinguish the extract-agar treatment from the control even after the artificial substrata had been oven-dried at 80° C and subsequently frozen at -15° C for two days, settlement inducing compounds present in aqueous extracts of *A. fimbriatum* are clearly heat stable under the conditions tested.

Dose dependent responses to chemical cues

As predicted, *Lichenopora novae-zelandiae* larvae that were offered a choice of young, old, and combined extracts from their kelp substratum mixed with agar (see protocol in Fig. IV-11b) settled in highest abundance on extract derived from young tissue, in lowest abundance on that from old tissue, and in intermediate abundance on a combined young-old choice (ANOVA: $F_{2, 38} = 10.944$; $P = .0002$) (Fig. IV-11a). Settlement on the old and old-young combined categories were not significantly different, but

both treatments received lower settlement than the young choice (Fig. IV-11a).

Heat denaturation of chemical cue?

In the experiment testing whether the chemical inducer of larval settlement present in aqueous extracts of *Agarum fimbriatum* was proteinaceous and thus denaturable by boiling, choice substrata made with unboiled young extract, boiled extract, and a FSW control were mixed with agar and offered to larvae (see protocol in Fig. IV-12b). In 30 replicates of this array of choices, larvae of *Lichenopora novae-zelandiae* settled equally on the unboiled and boiled extract treatments, and significantly less on the FSW control(ANOVA: $F_{2, 58} = 8.328$; $P = .0007$) (Fig. IV-12a).

Effects of phenolic monomers on larval settlement

In 30 experimental replicates, all adult colonies and larvae of *Lichenopora novae-zelandiae* that were exposed to artificial choice substrata made with the monomer phloroglucinol died during the experiment. In this and previous experiments using phloroglucinol (unpublished data), seawater in containers turned an amber color less than 24 h following introduction of phloroglucinol. This color reaction occurred both when phloroglucinol was mixed with agar, and when it was dissolved in seawater.

Discussion

Larval choice

A specific association between a sessile marine invertebrate and a particular substratum (e.g., host plant) can be initiated by at least two mechanisms: (1) post-settled juveniles suffer differential mortality, after which only those individuals on one substratum type have survived, or (2) planktonic larvae actively choose where they settle, and they select only one substratum type as a settlement site (see Keough & Downes (1982) for discussion of settlement vs. recruitment). The stenolaemate bryozoans living in a specific epiphytic association with the kelp *Agarum fimbriatum* appear to initiate the epiphytism when their planktonic larvae actively choose to settle on blade tissue of this kelp species. In both contained (i.e., still-water) experiments with *Tubulipora tuba*, and flowing seawater experiments with a mixed species assemblage, including *T. tuba*, *T. pulchra*, and *T. pacifica*, bryozoan larvae selected isolated blade tissue from *A. fimbriatum* over tissue excised from other kelp species. These experiments demonstrated not only that substratum specificity is mediated by larval choice, but also, as the larvae responded to excised disks or strips of algal tissue, larval discrimination likely occurs on a small scale, with some chemical or physical properties of the algal tissue being more important for host plant recognition than whole plant morphology or orientation in the environment.

As larvae of *Tubulipora* are small with limited energetic resources, their ability to locate and choose among several choice substrata, particularly while in flowing seawater, is convincing evidence that larval choice plays a major role in forming this bryozoan-kelp association. When

invertebrate larvae use some signal from their preferred substratum as a cue to initiate settlement and metamorphosis, the true relevance of that cue can only be supported by experiments showing that larvae remain responsive to the cue under hydrodynamic conditions similar to those they would experience in the field (e.g., Butman et al. 1988, Pawlik et al. 1991). The sites where bryozoan-encrusted *A. fimbriatum* were collected for this study are protected from the direction of prevailing winds and ocean swells, and hence water motion in the immediate vicinity of the kelp beds was minimal. Although the flow in my experimental aquaria was gentle, (ca 1-5 l/min), bryozoan larvae released into the flowing seawater would nonetheless have to settle soon after being released from parent colonies to avoid being swept down the drain. That bryozoan larvae could locate and settle upon small tissue strips (ca 25 x 75 mm) of their preferred substratum under these conditions is remarkable.

Algal age & microorganismal films

Commonly, the spatial patterns of animal epiphytes on marine plants are well correlated with age gradients along thalli of their host plants. On the articulated green alga *Halimeda tuna*, bryozoan colonies are found concentrated on the oldest thallus articles, which in this alga are nearest the bottom, i.e., proximal (Llobet et al. 1991). On the same plants, however, numerous species of epiphytic hydroids partition regions of the alga into species specific spatial niches, with certain species found only on young articles, and others on median age and old articles (Llobet et al. 1991). Larvae of the tube-building polychaete *Spirorbis spirillum* preferentially settle on newly produced young blade tissue of their algal host, the seagrass *Thalassia testudinum*, and their locational preference appears to be

mediated by some quality of the plant tissue rather than location or orientation of the substratum (Dirnberger 1990). Along blades of the kelp *Laminaria digitata*, larvae of two other spirorbid polychaetes and a bryozoan also settle preferentially on the youngest kelp tissue nearest the intercalary meristem (Stebbing 1972).

Larvae of both *Lichenopora* and *Tubulipora* chose young blade tissue from *Agarum fimbriatum* over old, and this young tissue preference was maintained by *T. tuba* larvae even on the non-preferred kelp *Nereocystis luetkeana*. Kelp tissue samples excised from the same blade, but differing in relative age (i.e., distance from the intercalary meristem), can nonetheless be distinguished by several features recognizable to bryozoan larvae. First, any surface submerged in seawater is almost immediately fouled by glycoproteins followed by a succession of microorganisms, macrophytes, and invertebrates (Mitchell & Kirchman 1984, Davis et. al. 1989). Surfaces immersed in seawater for longer periods would be covered with a more advanced successional stage community of fouling organisms than those immersed for shorter periods. Therefore, new kelp tissue recently produced at the intercalary meristem would have a relatively young community of epiphytic microorganisms compared to older tissue located further from the meristem. Not only would older kelp tissue likely have a greater biomass of epiphytes, but it may also have a qualitatively different epiphyte community, dominated by a different guild of organisms, characteristic of late successional stage fouling communities.

When making generalizations about epiphyte distribution, however, it is important to consider both the overall kelp morphology and orientation as well as the nature of the epiphytes being studied. In one study on the large bull kelp *Nereocystis leutkeana*, the vertical position of the kelp

stipes in the water column was found to be more important than physical and chemical properties of the kelp tissue itself in determining spatial patterns of small algae growing epiphytically on the kelp stipes (Markham 1969). Because the epiphytes in Markham's (1969) study were algae, their light requirements for photosynthesis may be the most important factor in determining their distribution. As stipes of *Agarum fimbriatum* are relatively small and flimsy compared to those of kelps growing in fast-moving water, their blades are usually flopped over, lying prostrate along the bottom rather than being suspended vertically in the water column. Due to this horizontal orientation, it is highly unlikely that light gradients, (other than shadows cast by neighboring organisms), are present along blades of *A. fimbriatum* so light cues would probably not influence the settlement of invertebrate epiphytes, particularly stenolaemate bryozoans, which have non-phototactic larvae.

In laboratory experiments, larvae of the cheilostome (Class Gymnolaemata) bryozoan *Bugula neritina* have been shown to select artificial substrata covered with a microbial film over unfilmed control substrata (Mihm et al. 1981), but apparently this larval response is specific only for certain species of marine bacteria (Maki et al. 1989). Brancato and Woollacott (1982) tested the effect of microbial films on larval settlement of three species of *Bugula* from Woods Hole, and they found that bryozoan larvae offered a choice of filmed and unfilmed substrata always preferred the filmed choice. In their experiments, however, filmed treatments were made by immersing sterile wood tongue depressors in freshly collected aerated seawater for three days. Relative to the time a blade of *Agarum fimbriatum* would be exposed to seawater in a single growing season, the period for which Brancato and Woollacott's (1982) substrata were allowed

to accumulate a microorganismal film is quite short. It is possible that "young" blade tissue of *A. fimbriatum* is exposed to seawater only for a few days before being colonized by larvae of *Lichenopora* and *Tubulipora*. At least one species of *Bugula, B. flabellata,* is repelled by filmed substrata (Crisp & Ryland 1960), however, so it is clear that bryozoan larval responses to films are highly variable, and thus we should not assume *a priori* that the age of a microorganismal film is indicative of its attractiveness to settling bryozoan larvae.

In my study, I tested the importance of microorganismal filming to bryozoan colonization of *Agarum fimbriatum* by offering young and old kelp tissue disks to bryozoan larvae under two treatment regimes, one with antibiotic treated seawater, and one with untreated control seawater. Treating the kelp substrata with a dose of antibiotics reported by Strathmann (1987) to be effective at killing marine bacteria made no difference to the settlement choices of *Lichenopora novae-zelandiae* larvae, suggesting that bacterial cues were unimportant in attracting larvae to young *A. fimbriatum* tissue.

Microtopography of algal surface

A second feature possibly enabling bryozoan larvae to distinguish *in vitro* young and old tissues from a kelp blade is the microtopography of cells at the blade's surface. At the microscopic level, surface texture of seaweeds can differ greatly between tissue types, due to cell structure or growth unique to tissue types (e.g., reproductive vs. vegetative tissue in kelps, Martínez & Correa 1993). In kelps, whose primary meristem is intercalary between the stipe and the blade, a secondary meristem, called the meristoderm, is also present at the blade surface (Bold & Wynne 1985).

If, as with other plant meristems, the meristoderm produces small cells through mitosis that gradually elongate as the tissue ages, then different cell sizes at the blade surface may produce differences in rugosity, or bumpiness, perceptible to bryozoan larvae. The experiments discussed below, although not directly addressing potential differences in surface rugosity between young and old blade tissue, eliminated rugosity as a factor affecting larval settlement by removing textural cues altogether. Substrata for these experiments were constructed with homogenates or leachates of kelp tissue rather than intact algal tissue, and in the process of homogenizing kelp tissue, meristoderm cells and multicellular aggregates were broken up, and if not, were certainly removed by centrifugation or filtration prior to their use in artificial substrata. In the absence of textural cues directing their settlement among choice substrata, bryozoan larvae could only resort to a third possible mechanism for identifying their preferred substratum: chemical signals present in or emanating from the kelp tissues.

Chemical induction of larval settlement

Over 30 years ago, Crisp and Williams (1960) first suggested that extracts of algae can, under certain conditions, induce settlement of epiphytic bryozoans. Despite the vast literature on the larval biology of bryozoans accumulated since then (included in several excellent reviews and books, e.g., Larwood (ed.) 1973, Ryland 1974, 1976, Woollacott & Zimmer (eds.) 1977, Pawlik 1992a) the role of plant chemistry in the settlement of epiphytic bryozoans has been overlooked. Research on the commercially important abalone *Haliotis rufescens* has revealed that chemical

constituents from its algal prey species induce settlement of the abalone larvae (Morse & Morse 1984), but for sessile invertebrate epiphytes, the chemical signals involved in larval recognition of plant substrata remain unknown.

Results from my study showed that chemicals present in the aqueous homogenates of *Agarum fimbriatum* induce larval settlement of the bryozoan *Lichenopora novae-zelandiae*. When offered a choice of artificial substrata made with extracts from young and old *A. fimbriatum* tissue, bryozoan larvae settled on the choice made with extract from their preferred region of the algal blades, young tissue nearest the meristem. And, when offered a third substratum choice made with a mixture of young and old extracts, settlement densities were intermediate between those on young and old choices, suggesting that larval responses to the inducers are dose-dependent.

Chemical nature of the inducer

Numerous attempts to test bryozoan larval responses to organic extracts of *Agarum fimbriatum* (unpublished data) failed to identify bioactive compounds in these extracts, so it appeared that the compounds responsible for inducing larval settlement were either (1) unstable when extracted using an organic solvent, (2) not lipid soluble, (3) extractable only from living algal tissues or cells, or (4) active only when combined with other compounds. The experiment testing the settlement inducing potential of leachates from fresh and oven-dried blade tissue was inconclusive, with leachate source not significantly affecting larval choice. Repeating the experiment with a third control choice made from FSW might reveal preference for algal leachates over non-algal controls. I used a

similar improved design in the experiment testing larval preferences among substrata made with aqueous extract from young *A. fimbriatum* tissue or FSW (for controls). Larvae settled on substrata containing the algal extract more than the controls, which indicated that they were attracted to chemical constituents from their host plant, rather than deterred by chemicals present in an alternate choice. The water soluble settlement inducers remained bioactive following both boiling and freezing to -80° C, evidence supporting the view that they are non-proteinaceous because proteins would normally denature under such conditions.

Although the chemicals inducing larval settlement in these experiments were not purified or their structures identified, several lines of evidence support the hypothesis that polyphenolic compounds in *Agarum fimbriatum* direct the settlement of its epiphytic bryozoans. Young tissue near the intercalary meristem of *A. fimbriatum*, which has the highest concentration of polyphenolic compounds along the blade (Chapter V), is also the region of the blade most preferred by larvae of *Lichenopora novae-zelandiae* and *Tubulipora* spp. In aqueous extracts of kelp tissue used to make artificial substrata, polyphenolic content was twice as high in those made from young tissue as it was in those made from old tissue (Chapter V). Also, polyphenolics are regularly excreted from healthy brown algae (Ragan & Glombitza 1986, Chapter V), and their solubility in water under natural conditions would make them potentially accessible to larvae of bryozoan epiphytes.

Although the evidence from experiments using aqueous extracts from *Agarum fimbriatum* suggest that polyphenolics may function as settlement inducers, substrata made with the purified monomer phloroglucinol failed to induce bryozoan settlement, and in fact were toxic

to adult and larval bryozoans. Potential explanations for the toxicity in this experiment could be: (1) phloroglucinol broke down into toxic by-products, (2) phloroglucinol monomers joined with others to form toxic polymers, or (3) phloroglucinol itself is toxic to bryozoans. As purified phloroglucinol may be unstable when in solution and exposed to light, it could have degraded over the course of the experiment. Due to energy constraints, however, it is unlikely that chemical synthesis of phenolic monomers into toxic polymers would have occurred spontaneously. In algal tissues, phloroglucinol monomers are generally present in low concentrations or are completely absent (Ragan & Glombitza 1986), so bryozoans may never, under natural conditions, be exposed to phloroglucinol. Hence, my experimental substrata made with phloroglucinol in concentrations similar to natural concentrations of total phenolics (including polyphenolics) in brown algae could very well have leached toxic levels of phloroglucinol into the seawater.

Alternatively, it is important to consider whether other water soluble compounds associated with kelp tissue might also exhibit age specific gradients, and thus could be implicated in directing settlement of bryozoan epiphytes. In examining whether a compound might induce bryozoan settlement, there are two questions that should be addressed: (1) does the compound provide any selective advantage to the bryozoans, and (2) is the compound present at the same concentrations in other macroalgae. Metabolites associated with areas of rapid growth (e.g., the meristem) could potentially be candidates for these settlement inducing cues, but compared to other sympatric kelps, *Agarum fimbriatum* do not grow particularly fast. If only those chemicals leaching from kelp meristems were attracting bryozoan larvae, then we would expect the large, annual, rapidly growing

kelps (e.g., *Nereocystis luetkeana* and *Macrocystis integrifolia*) to produce the most powerful larval attractants. My experiments showed, however, that bryozoan larvae preferred tissue disks from *A. fimbriatum* over those from *N. luetkeana* and *M. integrifolia*, so it appears that some compounds either unique to *A. fimbriatum*, or present in significantly higher concentrations in that host plant are responsible for inducing bryozoan larvae to settle. Other experimental evidence presented here showed that bryozoan larvae can distinguish among varying concentrations of extracts from their host plant. It is not unreasonable to suggest, then, that polyphenc ic compounds, present in aqueous extracts of *A. fimbriatum* tissue, might affect bryozoan larval choice.

This study reveals the first evidence that brown algal secondary metabolites may function as interspecific chemical signals with organisms other than herbivores. In a seawater medium, water soluble secondary metabolites will, to a certain extent, leach from kelp tissues into the surrounding seawater. Although sessile invertebrate epiphytes do not consume kelp tissue, they may nonetheless suffer from exposure to compounds leaching from their underlying algal substratum. That colonies of *Lichenopora novae-zelandiae* and *Tubulipora* spp. choose to settle on polyphenolic rich algal substrata indicates that these bryozoans have either acquired physiological resistance to polyphenolics or are physically shielded from polyphenolic-laden leachates. Experimental evidence presented here supports the former possibility, because bryozoans preferentially settle upon substrata made with polyphenolic rich extracts. While apparently harmless to certain stenolaemate bryozoans, polyphenolic compounds in *Agarum fimbriatum* may effectively deter colonization by less resistant epiphytic species, and this may in part explain

the rare occurrence of *M. membranacea* on *A. fimbriatum* even though it is abundant on other kelp species in Barkley Sound (personal observation).

Plant chemical signals that benefit the receiving organism, (i.e., kairomones, Brown et al. 1970, Whittaker & Feeny 1971) may also include scents attracting pollinators to flowers, parasites to hosts, or herbivores to plant prey. For plants producing defensive chemicals, the impact of the receiving organism on the fitness of the signaling plant may determine whether the plant responds to the selective pressure by changing or augmenting its chemical defenses. The cost of epiphytism to *Agarum fimbriatum* may be related to the size of the colonizing organisms. The stenolaemate bryozoans colonizing *A. fimbriatum* are small in diameter relative to many encrusting gymnolaemate species, and thus may pose little risk to the plant's nutrient physiology or to its ability to capture light for photosynthesis. In contrast, colonies of the encrusting gymnolaemate *Membranipora membranacea* can reduce light reaching the underlying algal tissues by up to 44-56% (Cancino et al. 1987, Muñoz et al. 1991). Although their physical presence on kelp blades may reduce the flux of nutrients, metabolites, and gases exchanged between algal tissues and the surrounding seawater, bryozoans also excrete nitrogenous waste. In nutrient uptake experiments, Hurd et al. (1994) demonstrated that bryozoan-derived nitrogen can supplement algal requirements under conditions of nitrogen limitation, and that it accumulates in kelp tissues beneath bryozoan colonies.

Further understanding of how algal secondary metabolites interact with epiphytic organisms will require careful attention to both chemical and ecological details of the associations, and this is probably best accomplished in collaborative research endeavors. Standard assays for

measuring total polyphenolic content in marine algae have revealed many interesting patterns, and generated countless hypotheses about the role of these ubiquitous molecules in marine benthic communities. Because polyphenolics have such enormous chemical diversity, and small changes in chemical structure could profoundly change their biological activity, research in this area could only benefit from increased knowledge of polyphenolic structure.

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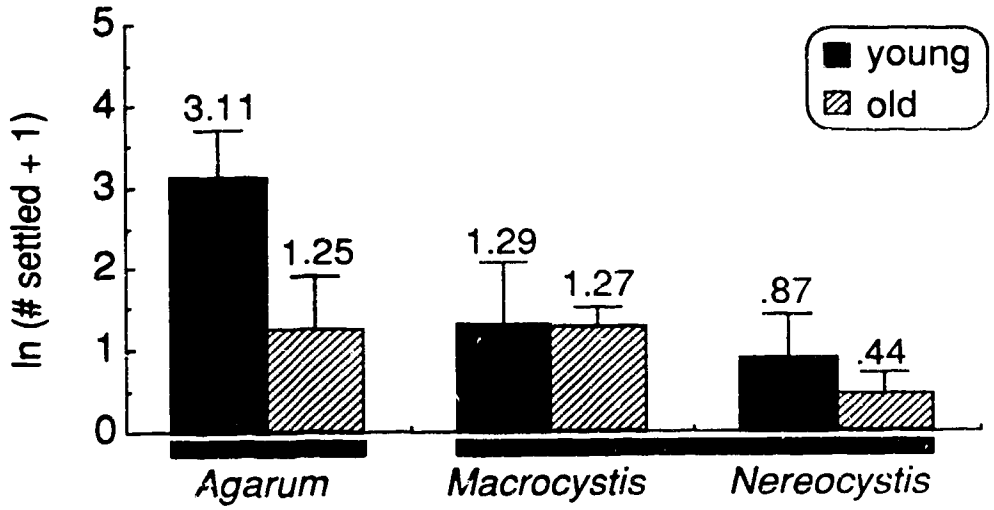
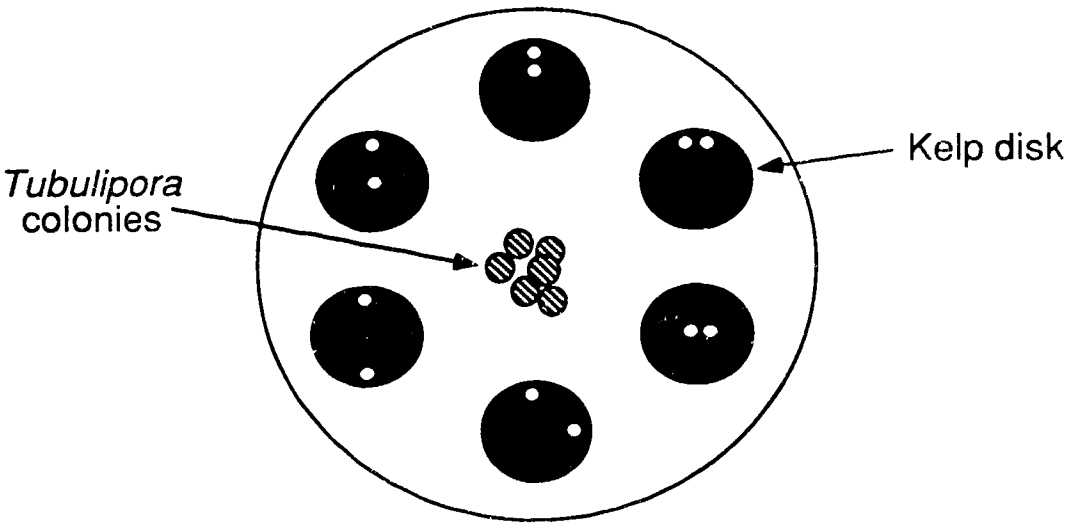
Figure IV-1: (a) Settlement of *Tubulipora tuba* larvae onto disks of kelp blade tissue from *Agarum fimbriatum, Macrocystis integrifolia,* and *Nereocystis luetkeana.* Plotted are means (values above histogram bars) and standard errors of numbers of ancestrulae (log transformed) settled on each of the six substratum choices after exposure for 3 d to larvae released naturally from adult colonies. Tissue of *A. fimbriatum* was preferred over tissue of the other two kelp species (Student-Newman-Keuls test, indicated by horizontal bars beneath histogram), and young tissue (solid bars) was preferred over old (hatched bars). (b) Diagram of one experimental replicate showing six kelp disks (black circles) arranged around perimeter of dish and reproductively mature *T. tuba* colonies (hatched circles) in center of dish. On each disk, species and age were identified by the relative positions of two circular cuts, made with a fractured capillary tube.

(a) Settlement of *Tubulipora tuba* larvae
19-22 July 1991



(b)



Experimental replicate

Figure IV-2: (a) Settlement of *Tubulipora* spp. larvae onto strips of tissue excised from blades of the kelps *Agarum fimbriatum* and *Eisenia arborea*. Plotted are means (values above histogram bars) and standard errors of numbers of ancestrulae (log transformed) settled on each of the two substratum choices after exposure for 6 d to larvae released naturally from adult colonies. (b) Algal strips were attached to 25 x 75 mm glass slides using two elastic bands, and the slides held in a wood rack suspended in an aquarium with running seawater. Vertical position in rack is indicated by numbers 1-10, with 10 being closest to the air-water surface. Over the experimental period, larvae were released from brood chambers of adult colonies, also present in the aquarium. (c) Scatterplot showing settlement on *A. fimbriatum* (solid circles) and *E. arborea* (open circles) in each position of the rack. More larvae settled on *A. fimbriatum* than on *E. arborea* (a), and settlement generally increased towards the top of the rack (a, c).

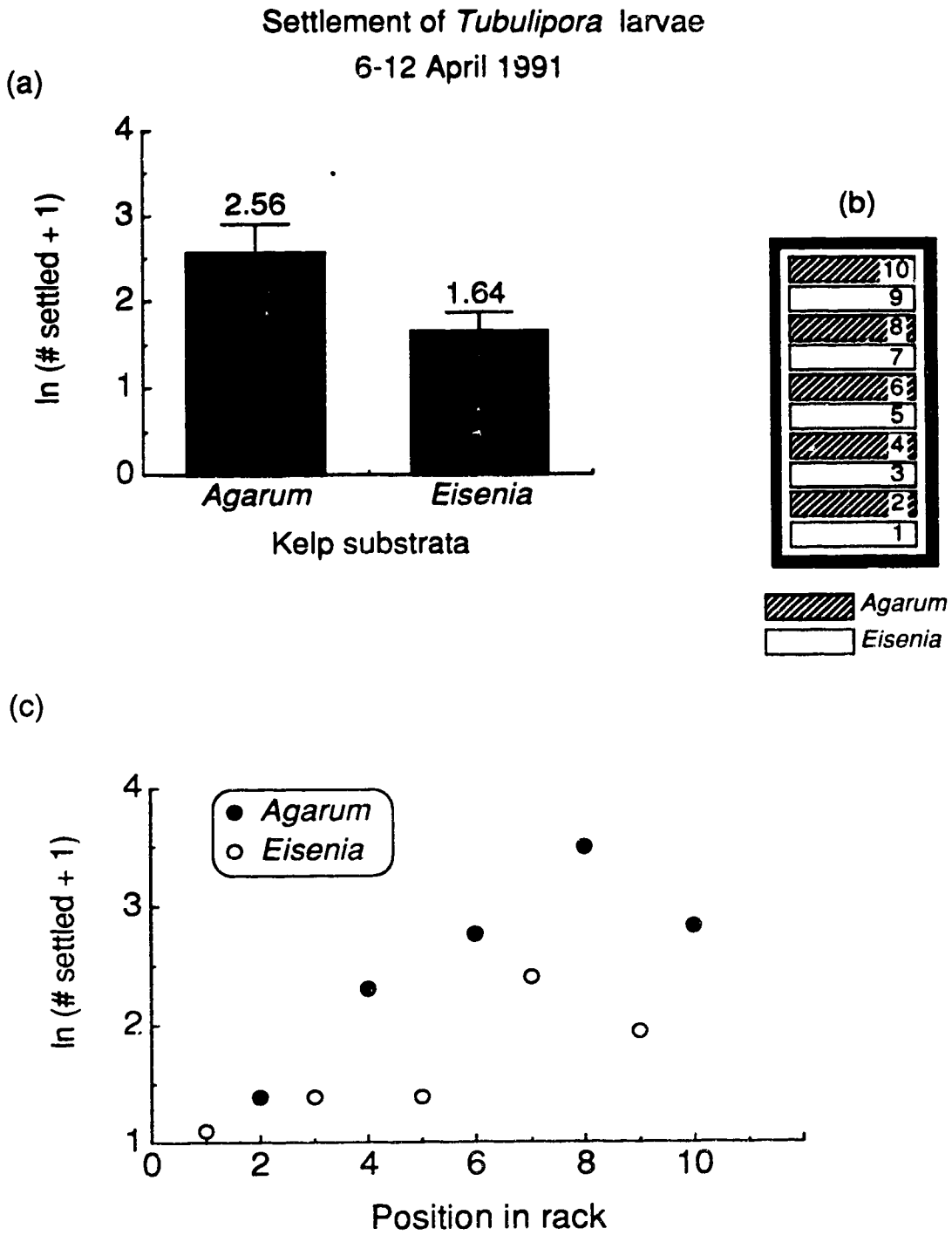
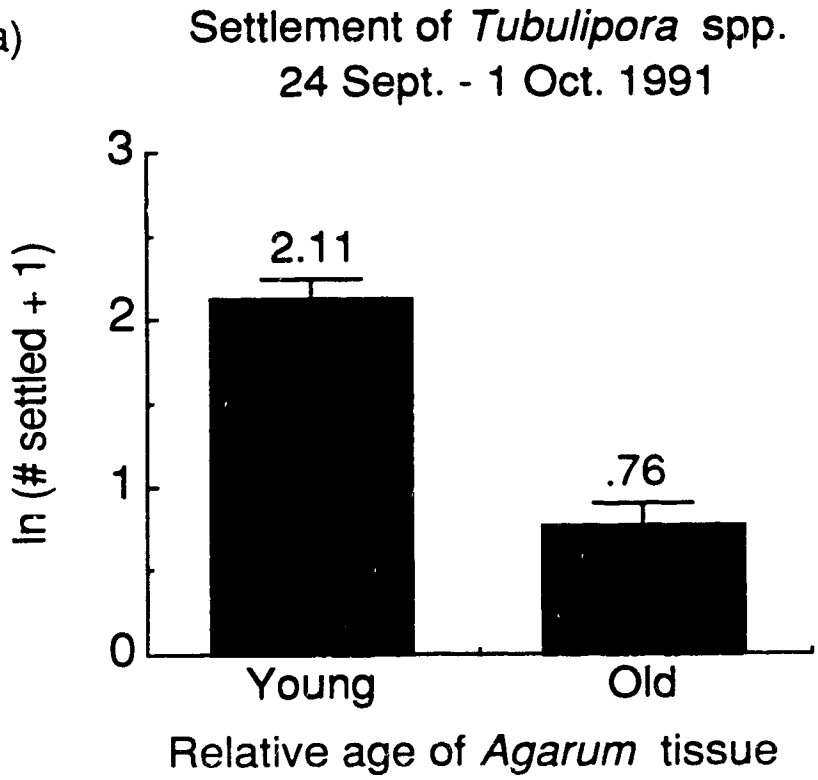

Settlement of *Tubulipora* larvae
6-12 April 1991
(a)
ln (# settled + 1)
4
3
2
1
0
2.56
1.64
Agarum
Eisenia
Kelp substrata
(b)
10
9
8
7
6
5
4
3
2
1
Agarum
Eisenia
(c)
ln (# settled + 1)
4
3
2
1
0
2
4
6
8
10
Agarum
Eisenia
Position in rack

Figure IV-3: (a) Settlement of *Tubulipora* spp. onto young and old strips of blade tissue from *Agarum fimbriatum*. Plotted are means (values above histogram bars) and standard errors of numbers of ancestrulae (log transformed) settled on each of the two substratum choices after exposure for 7 d to larvae released naturally from adult colonies. (b) Diagram of rack (i.e., block) showing placement of young (solid rectangle) and old (hatched rectangle) tissue strips, and the random arrangement of 10 racks submerged in an aquarium.

(a)


Settlement of *Tubulipora* spp.
24 Sept. - 1 Oct. 1991
ln (# settled + 1)
3
2
1
0
2.11
.76
Young
Old
Relative age of *Agarum* tissue

(b)

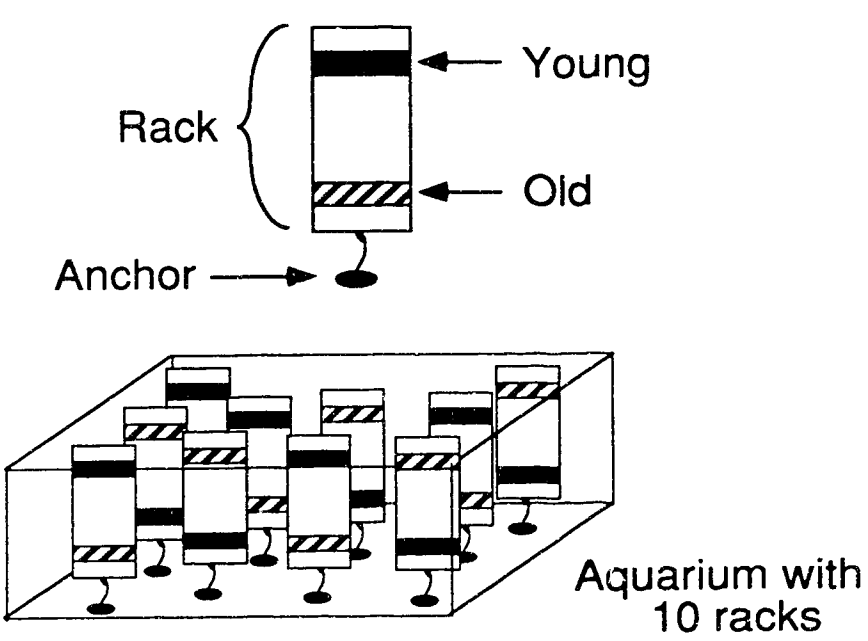
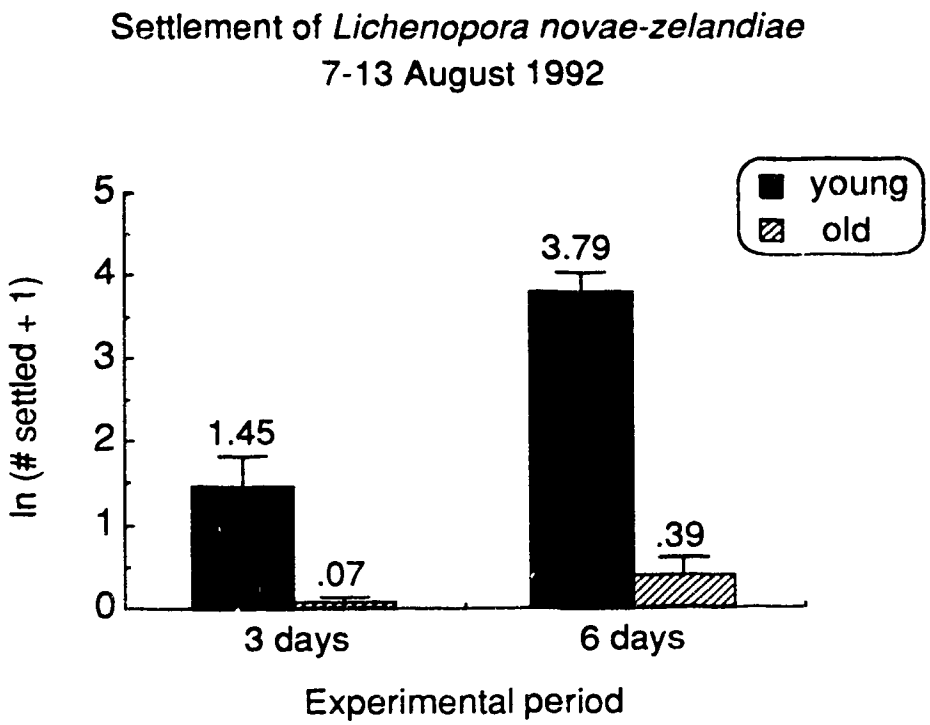

Rack
Young
Old
Anchor
Aquarium with
10 racks

Figure IV-4: (a) Settlement of *Lichenopora novae-zelandiae* onto young (solid bars) and old (hatched bars) tissue disks excised from the kelp *Agarum fimbriatum*. Plotted are means (values above histogram bars) and standard errors of numbers of ancestrulae (log transformed) settled on each of the two substratum choices after 3- and 6-day experimental periods. Settlement increased from 3 to 6 days, but the treatment effects did not change with experimental period; more bryozoans settled onto young disks than old disks. (b) Diagram showing one experimental replicate. Young and old tissue disks are represented by black circles, distinguished only by the locations of small triangular excisions from the disk margins. Relative positions of treatments within dishes was determined randomly. Adult bryozoan colonies were placed in the dish centers, (5-6 colonies/dish), and they released planktonic larvae over the course of the experiment.

(a)


Settlement of *Lichenopora novae-zelandiae*
7-13 August 1992
young
old
ln (# settled + 1)
5
4
3
2
1
0
1.45
.07
3.79
.39
3 days
6 days
Experimental period

(b)

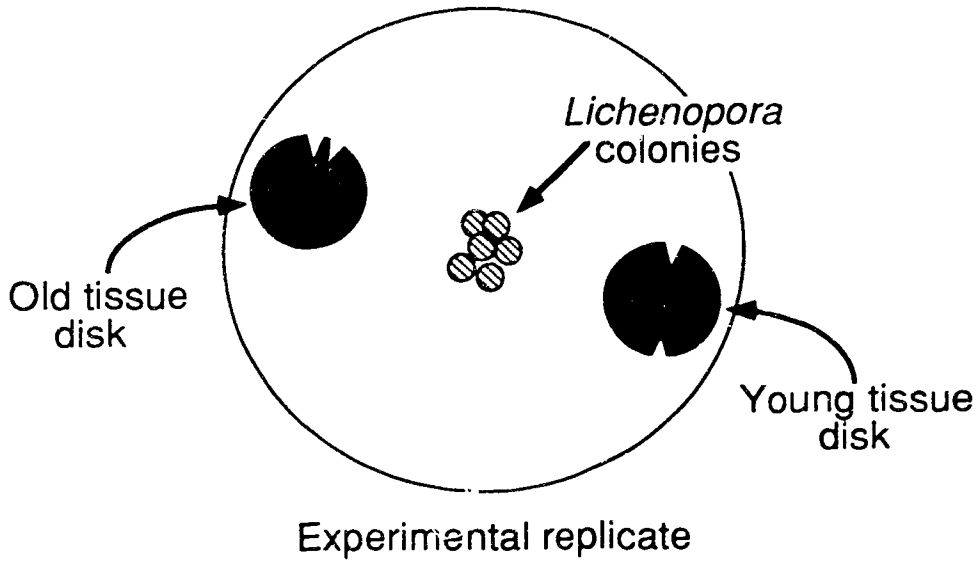
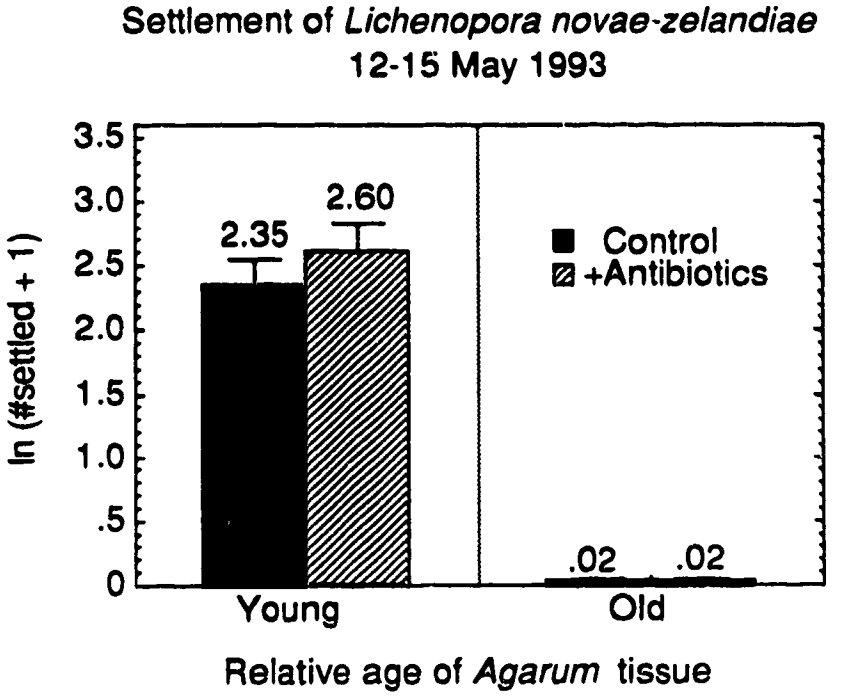

Lichenopora colonies
Old tissue disk
Young tissue disk
Experimental replicate

Figure IV-5: (a) Settlement of *Lichenopora novae-zelandiae* larvae onto young and old tissue disks excised from the kelp *Agarum fimbriatum*. Plotted are the means (values above histogram bars) and standard errors of numbers of ancestrulae (log transformed) settled on each of the two substratum choices. Half of the replicates were treated with antibiotics (hatched bars) and the remaining dishes were left untreated as controls (solid bars). An ANOVA showed that there was no effect of antibiotic treatment on larval choice; more larvae settled on young tissue disks than on old regardless of treatment. (b) Diagram of two experimental replicates, one control treatment and one antibiotic treatment. Young and old kelp disks are represented by black circles, distinguished by triangular marks at the disk margin. Adult bryozoan colonies (5-6 colonies/dish) were placed in dish centers, and they released planktonic larvae over the duration of the experiment.

(a)


Settlement of *Lichenopora novae-zelandiae*
12-15 May 1993
ln (#settled + 1)
3.5
3.0
2.5
2.0
1.5
1.0
.5
0
2.35
2.60
.02
.02
Control
+Antibiotics
Young
Old
Relative age of *Agarum* tissue

(b)

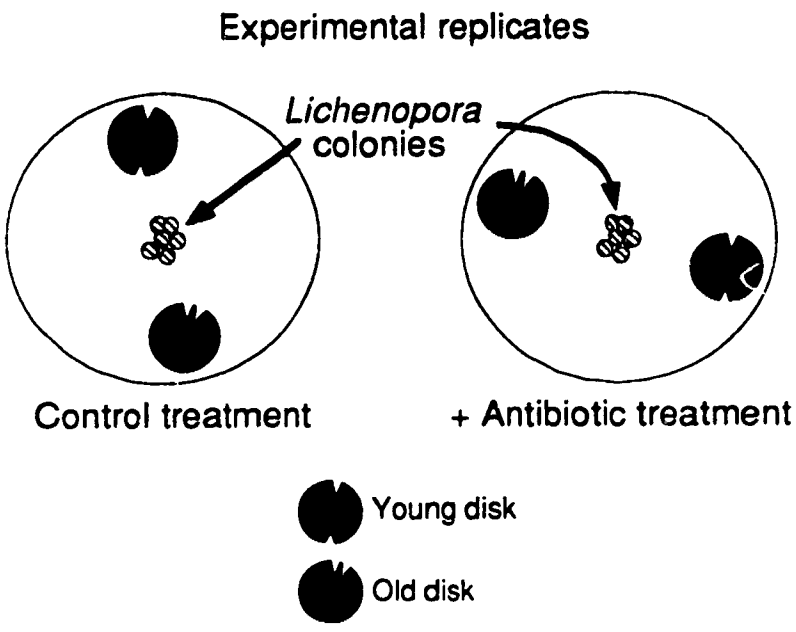

Experimental replicates
Lichenopora colonies
Control treatment
+ Antibiotic treatment
Young disk
Old disk

Figure IV-6: Diagram of one "otoco" or making artificial substrata that were used in a larval settlement experiment. Aqueous extracts of young and old kelp tissue (see extraction details in text) were mixed with 4% agar in FSW, and this mixture poured into molds constructed of 15 ml polystyrene centrifuge tubes. Once the mixtures had congealed, the solidified extract-agar cylinders were gently removed from their molds and sliced into equal sized disks. Scoring lines from the centrifuge tubes were transferred onto the extract-agar cylinders and provided marks for slicing. To make one experimental block, one disk from each cylinder was adhered (with a drop of 4% agar) onto opposite ends of a glass microscope slide, and the slide transferred to a glass dish with FSW and adult bryozoans.

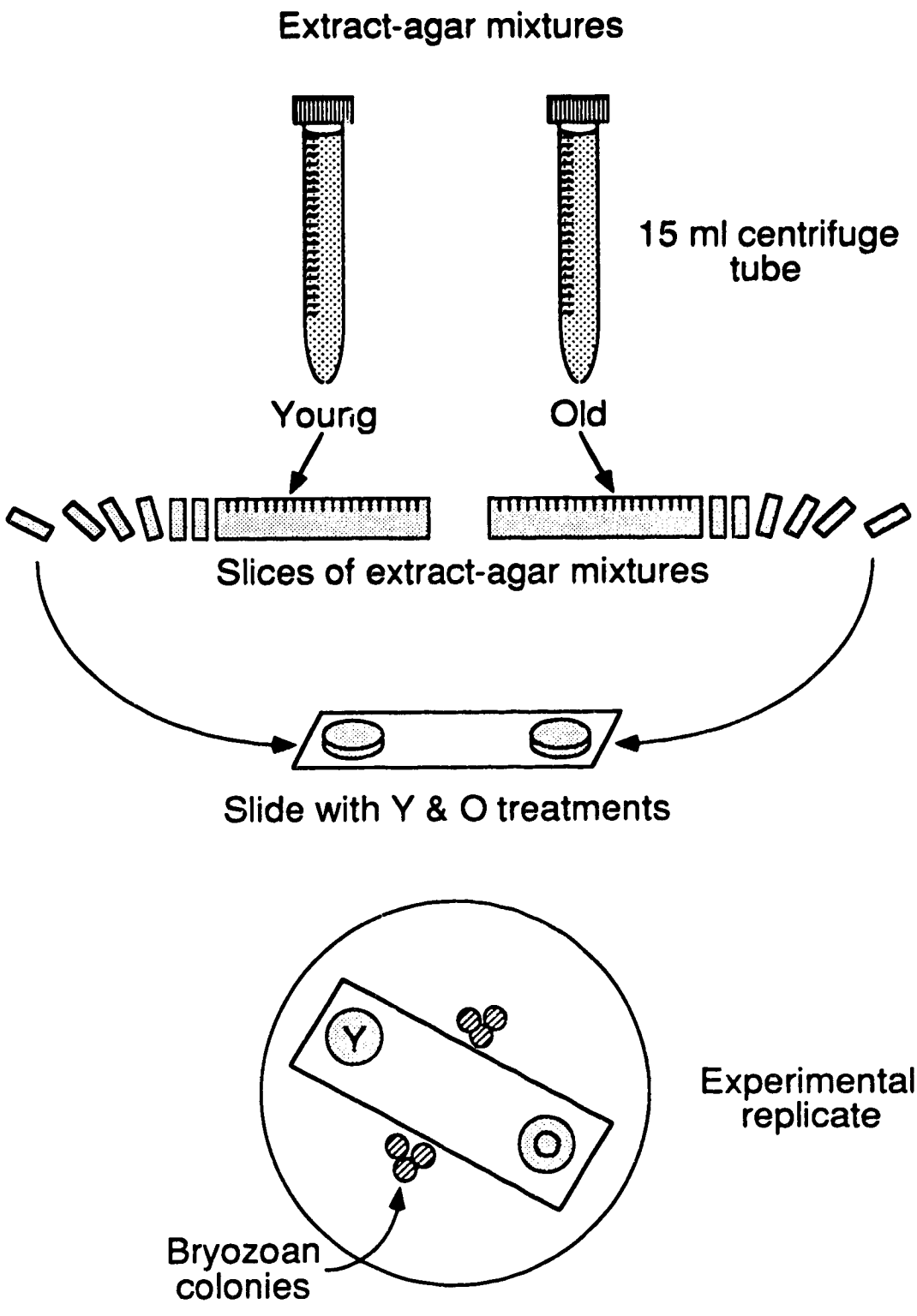

Extract-agar mixtures
15 ml centrifuge tube
Young
Old
Slices of extract-agar mixtures
Slide with Y & O treatments
Y
O
Experimental replicate
Bryozoan colonies

Figure IV-7: Settlement of *Lichenopora novae-zelandiae* larvae onto two choice artificial substrata made with homogenates of young and old blade tissue from the kelp *Agarum fimbriatum*. Plotted are means and standard errors of numbers of bryozoan ancestrulae (log transformed), with the values above histogram bars indicating means. A randomized block ANOVA showed that young "flavored" extract was preferred over old extract.

Settlement of *Lichenopora novae-zelandiae*
26 May - 1 June 1993

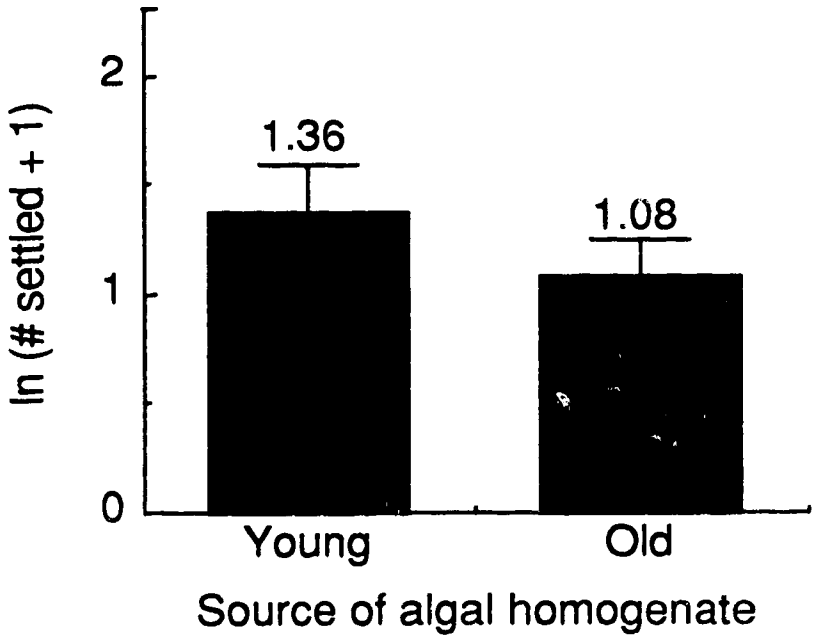
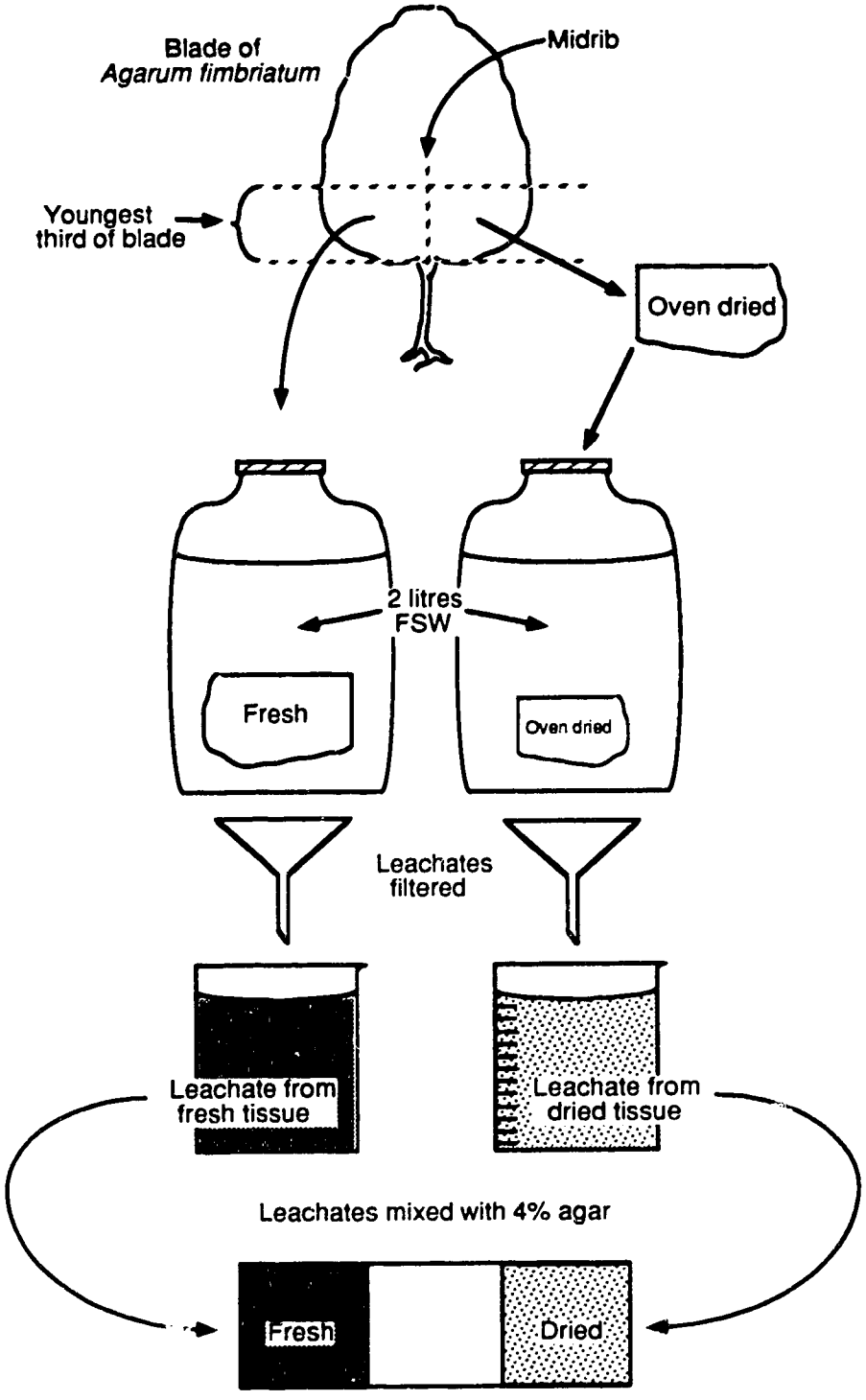


Figure IV-8: Diagram of protocol used to obtain leachates from young blade tissue of the kelp *Agarum fimbriatum*. The youngest third of the blade (nearest the intercalary meristem) was divided in halves along the midrib, and each half soaked in 2 l of FSW for 3 days. One half was oven dried at 80° C for 24 hours before soaking. The FSW in which algae were soaking were filtered to remove particulate matter, and mixed with equal volumes of 4% agar in FSW. Before the agar mixtures congealed, they were used to coat opposite ends of microscope slides by dipping slides into the warm mixtures. Densely- and sparsely-stippled patterns on the leachate-agar mixtures represent fresh and oven-dried treatments, respectively. When the mixtures congealed on slides, they were offered as choice substrata to bryozoan larvae in a laboratory settlement experiment (see text).



Ends of microscope slide dipped in leachate-agar solutions

Figure IV-9: Settlement of *Lichenopora novae-zelandiae* larvae onto artificial substrata made with leachates from fresh and oven-dried young blade tissue of the kelp *Agarum fimbriatum*. Plotted are means (also shown as values above histogram bars) and standard errors of numbers of bryozoan ancestrulae present on each of the two choice substrata after 4 days. A randomized block ANOVA showed that there was no difference in bryozoan settlement between the two choice substrata.

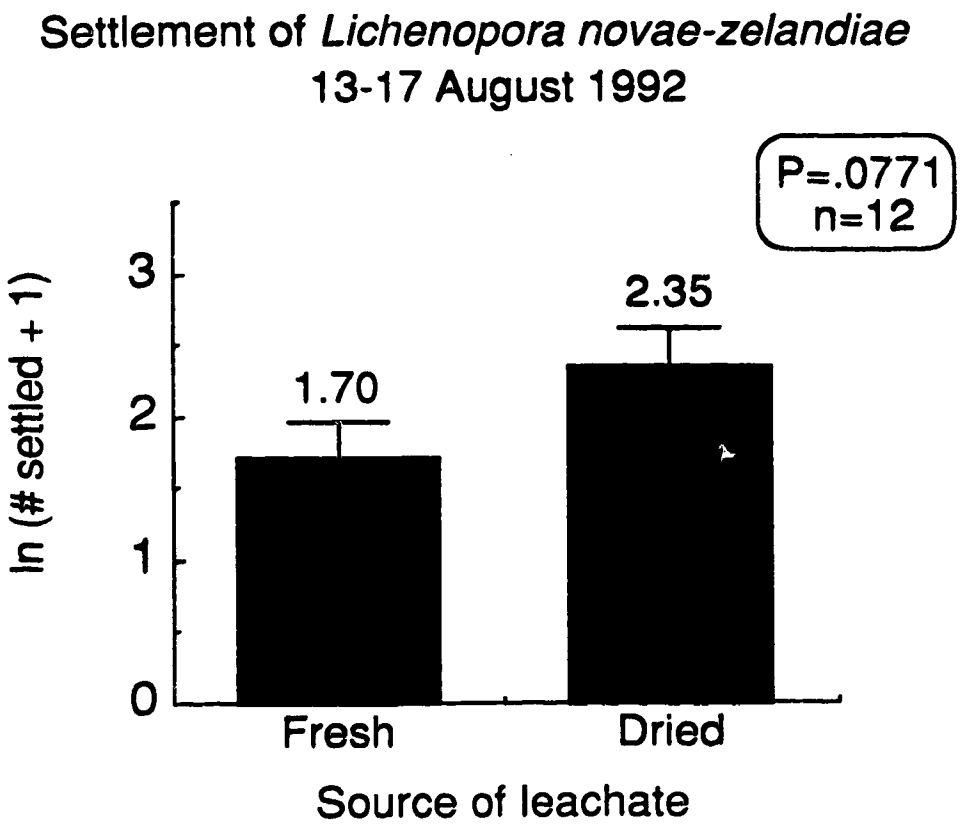
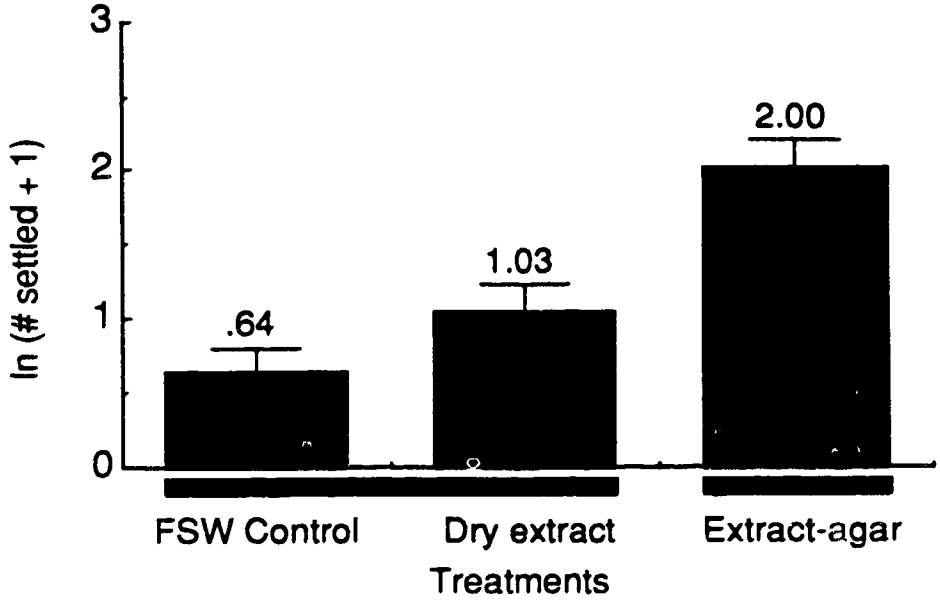

Settlement of *Lichenopora novae-zelandiae*
13-17 August 1992
P=.0771
n=12
ln (# settled + 1)
3
2
1
0
1.70
2.35
Fresh
Dried
Source of leachate

Figure IV-10: (a) Settlement of *Lichenopora novae-zelandiae* larvae onto artificial substrata made with aqueous homogenate from young blade tissue of the kelp *Agarum fimbriatum*. The 3 choice substrata are: FSW + agar, kelp extract, and kelp extract + agar (see text for further explanation of treatments). Plotted are means and standard errors of numbers of ancestrulae on each of three choice substrata, with values above histogram bars indicating means. A randomized block ANOVA showed that larval settlement differed among the three treatments, and a Student-Newman-Keuls multiple comparisons test (results summarized by horizontal bars beneath histogram) found settlement on the extract-agar mixture to be higher than that on the other two choices. (b) Diagram of one experimental replicate, showing one of 10 random arrangements of treatments within blocks. The three treatments, as indicated by open, sparsely hatched, and densely hatched circles, were adhered to glass microscope slides, which were then placed in glass dishes with FSW and adult bryozoan colonies.

(a) Settlement of *Lichenopora novae-zelandiae*
13-17 August 1992



(b) Experimental Replicate

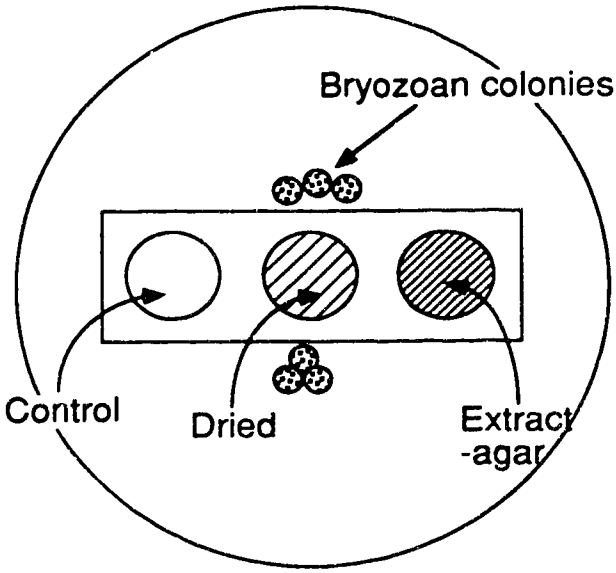
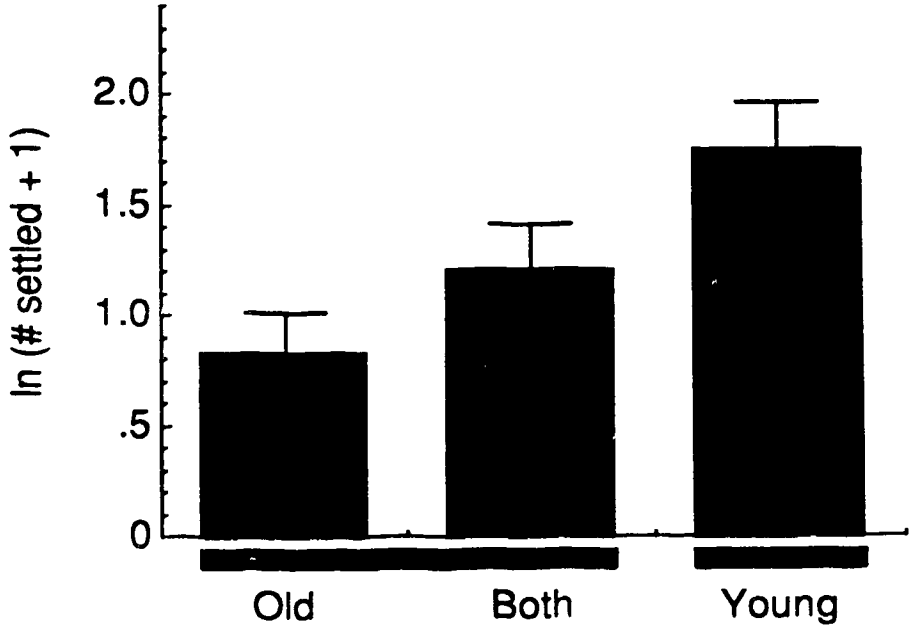


Figure IV-11: (a) Settlement of *Lichenopora novae-zelandiae* larvae onto artificial substrata made with aqueous extracts of blade tissue from the kelp *Agarum fimbriatum*. The three choice substrata were all made by mixing kelp extracts with 4% agar solutions in a 1:1 ratio, and the three extract treatments were young blade extract, old blade extract, and 1:1 ratio of young:old extracts. Plotted are means and standard errors of numbers of bryozoan ancestrulae on each of three choice substrata, with values above histogram bars indicating means. A randomized block ANOVA showed that larval settlement differed among the three treatments, and a Student-Newman-Keuls multiple comparisons test (results summarized by horizontal bars beneath histogram) showed that settlement on the young choice was significantly higher than that on the other two choices. (b) Diagram of one experimental replicate showing the three choice substrata on glass coverslips (with white, gray, and black squares representing old, both, and young treatments, respectively) which have been randomly arranged on a microscope slide. A total of 20 slides were placed in glass dishes containing FSW and adult bryozoans.

(a) Settlement of *Lichenopora novae-zelandiae*
2-5 June 1993



(b) Experimental replicate

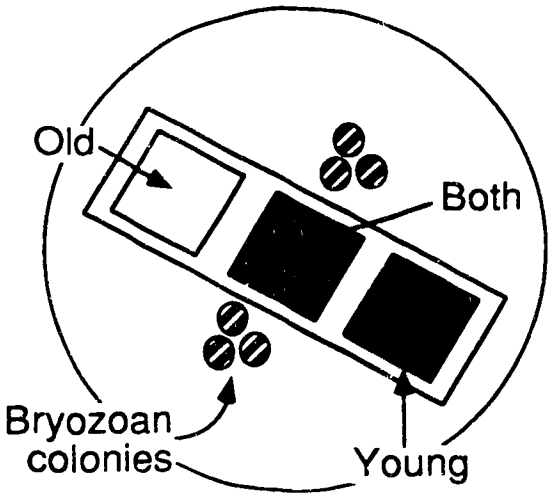
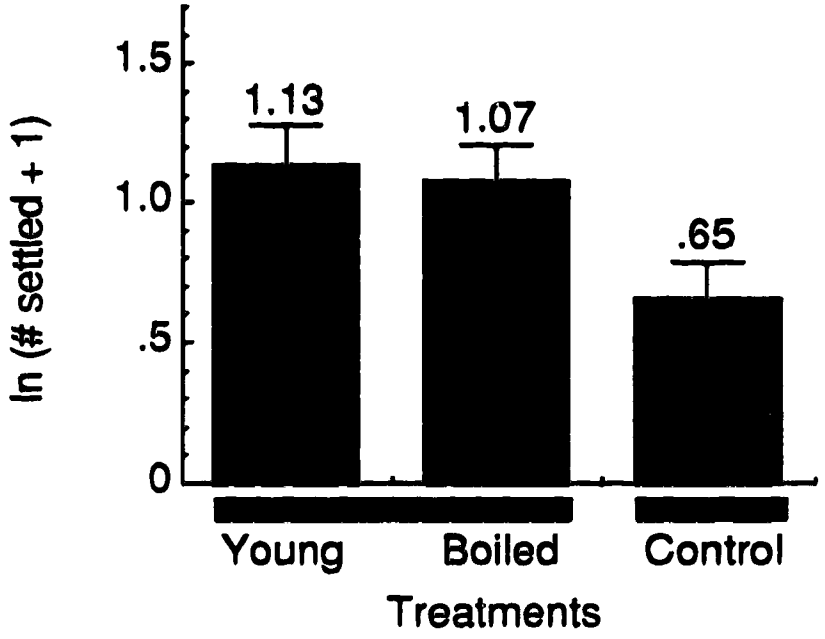
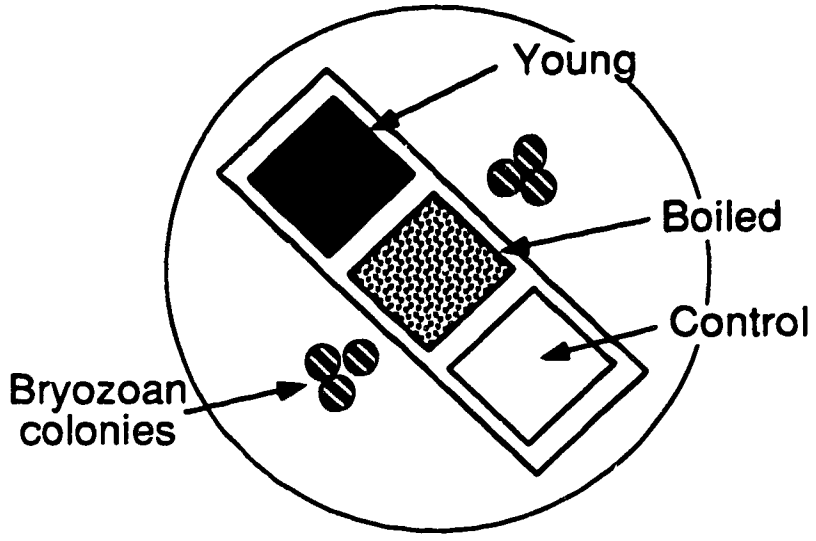


Figure IV-12: (a) Settlement of *Lichenopora novae-zelandiae* larvae onto three choice substrata made with algal extract from young tissue of the kelp *Agarum fimbriatum* and a 4% agar solution. In the "Young" treatment, aqueous extract was mixed directly with agar. Extract in the "Boiled" treatment was heated to the point of boiling before mixing with agar, and the control substituted FSW for extract. Plotted are means and standard errors of numbers of bryozoan ancestrulae on each of three choice substrata, with values above histogram bars indicating means. A randomized block ANOVA showed that larval settlement differed among the three treatments, and a Student-Newman-Keuls multiple comparisons test (results summarized by horizontal bars beneath histogram) showed that settlement on the young and boiled choices were significantly higher than that on the control substratum. (b) Diagram of one experimental replicate showing the three choice substrata on glass coverslips (black, dotted, and open squares representing young, boiled, and control treatments, respectively) which have been randomly arranged on a microscope slide. A total of 20 slides were placed in glass dishes containing FSW and adult bryozoans.

(a) Settlement of *Lichenopora novae-zelandiae*
19-22 July 1993



(b) Experimental replicate



Chapter V

Differential allocation of polyphenolic chemical defenses in the subtidal kelp *Agarum fimbriatum* Harv. (Phaeophyta: Laminariales)

Introduction

Like most terrestrial plants, marine algae contain secondary metabolites (Scheuer 1978-1983, Fenical 1982), chemicals manufactured and sequestered by plants for no apparent reason relating to primary metabolic function. In terrestrial plants, these secondary compounds function as chemical signals to, for example, deter grazing by herbivores, attract pollinators, suppress growth in competing plant species, and prevent infection and disease (reviewed in Whittaker & Feeny 1971, Rhoades & Cates 1976, Rhoades 1979, Spencer 1988, Rosenthal & Berenbaum 1992). Secondary chemicals in marine algae have also been shown to have important ecological function, particularly as anti-herbivore defenses, (Hay & Fenical 1988, Hay 1992, Hay & Steinberg 1992, Paul 1992, Steinberg 1992).

In temperate brown algae, the most common secondary metabolites are polyphenolic compounds, which display the tannin-like property of binding to proteins (Ragan & Glombitza 1986, Steinberg 1992). Brown algal polyphenolics are all polymers of the monomer phloroglucinol, (i.e., 1,3,5 - trihydroxybenzene), and hence are called phlorotannins, to distinguish them from terrestrial tannins (Ragan & Glombitza 1986), which have a different chemical structure (Zucker 1983). Unlike certain plant secondary

metabolites that act as toxins on potential herbivores, polyphenolics and other digestibility-reducing compounds function differently: they can bind to plant proteins, rendering the tissue indigestible, or they can bind to proteolytic enzymes in an herbivore's gut, essentially destroying digestive enzyme function (Rhoades & Cates 1976, Reese 1979). Numerous studies have confirmed that brown algal polyphenolics deter grazing (Taniguchi et al. 1991), and that herbivore preference for the algae is inversely proportional to the polyphenolic content of the algal tissue (Geiselman & McConnell 1981, Anderson & Velimirov 1982, Steinberg 1984a, b, 1985, 1988, Van Alstyne 1988, Denton & Chapman 1991, Winter & Estes 1992). In other words, polyphenolic function is dose dependent, and plants with higher levels of polyphenolics in their tissues are less likely to be grazed.

Among temperate brown algae in the northeast Pacific, the subtidal kelp *Agarum fimbriatum* Harv. (Phaeophyta: Laminariales) is one of the least preferred algae to generalist herbivores such as gastropods (Durante & Chia 1991, see Chapter II) and urchins (Vadas 1968, 1977). Polyphenolic content in *A. fimbriatum* is high relative to that in other kelps, and it is thought to vary intraspecifically as much as or more than it does interspecifically (Steinberg 1984b). There is some evidence that juvenile blades of *A. fimbriatum* have higher polyphenolic content than adult blades collected from the same site at the same time (Steinberg 1984b), but that study did not indicate exactly where on the plants samples for the polyphenolic analyses were taken. The same study also found that structures anchoring *A. fimbriatum* to the bottom, stipe and holdfast, are more heavily defended with polyphenolics than blade tissue.

In this study, I examined intraspecific variation in polyphenolic content in *Agarum fimbriatum*, with particular attention to how it varies

along a gradient of algal age. Like other members of the Laminariales, *A. fimbriatum* has an intercalary meristem, located between the stipe and the blade, but because it is a single blade species, this meristem is the only location where new tissue is generated. Should the meristem be lost entirely, it is unlikely that the plant could regenerate another blade. Also, reproductive sori in this kelp appear as patches of spore-bearing tissue on the vegetative blade, so plants whose blades are removed or seriously damaged during a grazing event would not likely reproduce that year. Hence, the intercalary meristem is critical to the survival of the kelp, and should receive a disproportionately high allocation of polyphenolic defenses. In addition, seasonal patterns in the allocation of polyphenolics within plants was examined by comparing blades censused from the same site over thirteen months.

Although polyphenolic compounds are polar and thus soluble in water (Ragan & Glombitza 1986), little is known about how, if at all, they are transported from *Agarum fimbriatum* cells to locations where other organisms may detect them. To determine whether polyphenolics escape from algal tissues into the surrounding seawater, I measured polyphenolic content in aqueous extracts of *A. fimbriatum* blade tissue, and in pieces of intact tissue subjected to a period of leaching.

Materials and Methods

Collection and maintenance of algae

Blades of *Agarum fimbriatum* were collected haphazardly using SCUBA from 5-20 m depth at Dixon Island [48° 51.24' N; 125° 7' W] or Helby Island [48° 50.86' N; 125° 10.18' W] in Barkley Sound, on Vancouver Island, British Columbia. The field sites were located 2.4 km (Dixon) and 3.3 km (Helby) from the Bamfield Marine Station, and transportation from the field to laboratory holding facilities took no longer than 1 h. Constrained by the large size of *A. fimbriatum*, usually only five plants were collected per sampling, but when tissue subsamples or juvenile plants were collected, then sample sizes were larger. During transport, whole algae were held in cooled seawater aquaria aboard the research vessel, and then transferred to laboratory holding tanks with running seawater. For some collections, subsamples were taken from whole algae aboard the research vessel, held on ice during transport to the laboratory, and then transferred to a -80°C freezer for storage.

Measurements of polyphenolic content

I measured polyphenolic content along gradients of algal age in vegetative, reproductive, and structural tissue of *Agarum fimbriatum*. For each measurement, ~ 50 - 500 mg (blotted wet weight) of algal tissue were homogenized in a ground glass tissue homogenizer with 15 ml of 70% aqueous methanol (including wash volume). At the same time tissues for extraction were sampled from whole plants, adjacent pieces of tissue were removed and dried for 24 h @ 80° C, and their dry masses used to calculate the wet:dry ratio of each tissue sample. Homogenates were

allowed to extract for 24 h in a dark refrigerator (ca 5 ° C), after which they were filtered through a Whatman GF/A glass fiber filter. Polyphenolic content in the fitrates was determined using the Folin-Denis assay (Folin & Denis 1915), following the protocol in Swain & Hillis (1959) and reagent recipes in AOAC (1990). Absorbance was read at 725 nm, and polyphenolic concentrations were determined using a phloroglucinol standard. To reduce cuvette and spectrophotometer error, Folin-Denis assays were replicated 2-3x and averaged. To be consistent with reports in the literature, I reported all polyphenolic data as % of dry mass.

Specific collections and sampling protocols

Censuses for seasonal variation

On each of five sampling dates from January 1992 to February 1993, five blades of *Agarum fimbriatum* were collected from Dixon Island, and the blades divided into three equal sized sections, representing young, medium, and old relative age categories. A sample of vegetative blade tissue was cut from the middle of each section (Fig. V-1a), and assayed for polyphenolic content.

Effects of tissue damage on polyphenolic content

As polyphenolic defenses in fucoid brown algae can be induced by herbivore damage (Van Alstyne 1988), I tested whether polyphenolic content in whole *Agarum fimbriatum* changes in response to blade tissue removal. For each of five plants collected on two occasions, 8 d apart, I sampled young, medium, and old blade tissue (as in Fig. V-1a). Polyphenolic content in the tissue samples was compared between sampling dates using a paired Student's *t*-test.

Polyphenolic content along blades of young sporophytes

Steinberg (1984b) reported that, at least in the northeastern Pacific, juvenile blades of *Agarum fimbriatum* have higher polyphenolic content than adult blades collected at the same time and location. My preliminary evidence suggested, however, that polyphenolic content decreases with increasing algal age in blades of *A. fimbriatum*, regardless of plant size. Thus, to determine the scale of polyphenolic variation in blades of this alga, I sampled blades of young *A. fimbriatum* at 5 cm increments along the long axis of the alga (Fig. V-1b, c), beginning at the intercalary meristem. In November 1991, I collected four young sporophytes of *A. fimbriatum* from Dixon Island, ranging from 20-35 cm in blade length, and measured polyphenolic content at 5 cm increments from the meristem (Fig. V-1b). In September of 1992, to examine variation in plants of approximately the same size, I collected eight young sporophytes that were all ca 15 cm in blade length, and measured polyphenolic content in each of the three, 5 cm bands of blade tissue (Fig. V-1c).

Polyphenolic content in older sporophytes

For older (i.e., representative of the population mean) blades of *Agarum fimbriatum* haphazardly collected from Dixon Island in July and September 1992, and from Helby Island in June 1992, I measured polyphenolic content in anchoring tissues, (i.e., holdfast and stipe), vegetative tissues from the blade and stipe, and, where present, reproductive sori (Fig. V-1d).

Allocation of polyphenolic defenses to vegetative vs. reproductive tissue

To determine whether polyphenolic compounds are allocated differentially to vegetative and reproductive tissue in blades of *Agarum*

fimbriatum, I collected samples of blades from 14 plants at Dixon Island in April 1992. Tissue subsamples from reproductive and vegetative portions of each blade were then assayed for polyphenolic content, and those values compared between tissue types.

Polyphenolic concentration in aqueous extracts of young and old blade tissue

Although the quantitative analysis of polyphenolic content in algal tissues requires that tissues be extracted using methanol as a solvent, these compounds are reported to be soluble in water (Ragan & Glombitza 1986). To test whether polyphenolics are present in aqueous extracts of algal tissue, I made a 10% (w/v) homogenate of young and old vegetative blade tissue from *Agarum fimbriatum*. Homogenates were filtered (to 0.45 μm) 1 h later and immediately frozen in a -80° C freezer, where they were stored until assayed for polyphenolic content.

Leaching of polyphenolic compounds from cut algal tissues

To determine whether polyphenolic compounds leach from blade tissues of *Agarum fimbriatum* when those tissues are cut, I set up an experiment comparing polyphenolic content in fresh algal tissue, tissue that had been allowed to leach in 25 ml filtered seawater for 72 h, and in the 25 ml filtered seawater in which algal tissues were held. For each of five *A. fimbriatum* collected from Dixon Island in June 1993, three patches of tissue (ca 50 cm^2) were removed from the youngest third of the blade. Each patch was further sampled by excising six disks of tissue (19.65 mm in diameter) using a cork borer. Two disks were extracted immediately and assayed for polyphenolic content, two disks were placed in vials with 25 ml filtered seawater for leaching, and the remaining two disks were oven dried for 24 h at 75° C to obtain the wet/dry ratio for tissue in that patch.

Statistical analyses

Because sample sizes for most analyses were small, all data were rank-transformed prior to applying parametric analyses. Using Student's *t*-tests, and randomized block design, repeated measures, or nested ANOVAs, I tested the effects of tissue location and type on polyphenolic content among plants (i.e., blocks). For analyses with significant effects, I also applied *a posteriori* Student-Newman-Keuls (S-N-K) multiple comparisons tests. Non-significant interaction terms were deleted from the models, as were treatment x block interactions. All analyses were conducted at a significance level of 0.05.

Results

Seasonal and spatial patterns of polyphenolic content

Blades of *Agarum fimbriatum* collected during five censuses from January 1992 to February 1993 showed a distinct peak in polyphenolic content in April relative to the other three months (Table V-1). Overall, relative age of blade tissue was not a significant factor affecting polyphenolic content ANOVA ($P = .0614$, Table V-1). When individual randomized block design ANOVAs were conducted on data from each of the five censuses, however, the relative age of blade tissue was a significant factor affecting polyphenolic content in blades collected during February (Fig. V-2). Results of S-N-K multiple comparisons procedures showed that the significance of the ANOVAs for February censuses was due to elevated polyphenolic content in young blade tissue, with medium and old age categories being the same. Because in four of the five censuses, mean

polyphenolic content was higher in young blade tissue than in medium or old tissue, these initial census data suggested that plant tissue age may be an important determinant of polyphenolic levels in this kelp species, and hence I addressed this question further by examining allocation of polyphenolic defenses on a finer scale.

Effects of tissue damage on polyphenolic content

Polyphenolic content along blades of *Agarum fimbriatum* collected from Dixon Island in November 1991 was significantly different among three relative age categories (Table V-2), with the youngest category near the intercalary meristem having the highest polyphenolic content (Fig. V-3). Repeated measurements of polyphenolic content in the same plants 8 d later were significantly lower than the initial measurements (Table V-2), but the spatial patterns remained the same. Polyphenolic content was highest in young blade tissue, and declined in medium and old blade tissue (Fig. V-3). For both sets of measurements, S-N-K multiple comparisons procedures identified young blade tissue as having the highest levels of polyphenolics. In the initial measurements, medium and old blade tissue were not significantly different, but in the repeated measurements, medium blade tissue had a significantly higher polyphenolic content than old tissue (Fig. V-3).

Polyphenolic content in young sporophytes

Young *Agarum fimbriatum* ranging from 15 to 35 cm in blade length also exhibited declining polyphenolic content with increasing algal age. Young sporophytes collected in November 1991 (Fig. V-4) and September 1992 (Fig. V-5) from Dixon Island had the highest mean polyphenolic

content in the 5 cm increment nearest to the intercalary meristem. Randomized block design ANOVAs comparing 5 cm bands of blade tissue for both collections showed that tissue location significantly affected polyphenolic content (Table V-3). Because of low sample size (n=4) and high between-plant variation in the November 1991 collection, an *a posteriori* S-N-K multiple comparisons test identified only two groups at $\alpha = .05$ (Fig. V-4), but the mean polyphenolic content was highest in the 0-5 cm category and this declined gradually towards the distal, older end of the blades. In the smaller blades (ca 15 cm in length) collected in September 1992, an S-N-K test identified three groups with different polyphenolic content, and they are, in order from highest to lowest: (0-5 cm > 5-10 cm > 10-15 cm) (Fig. V-5).

Polyphenolic content in older sporophytes

In older blades of *Agarum fimbriatum* collected from Dixon Island in July (Figs. V-6, V-7) and September (Figs. V-8, V-9) 1992, and from Helby Island in June 1992 (Figs. V-10, V-11), polyphenolic content varied significantly among the tissues tested (Table V-4). Blade lengths (mean ± standard error) for these collections were: 136 ± 9.8 cm, 108 ± 10.3 cm, and 80.6 ± 7.1 cm for July at Dixon, September at Dixon, and June at Helby, respectively.

For plants collected at Dixon in July, highest polyphenolic content was found in structural tissues anchoring the plant to the substratum (e.g., holdfast and stipe), and in blade tissue within 10 cm of the intercalary meristem (S-N-K test, Figs. V-6, V-7). The remainder of the tissues fell into two overlapping groups identified by the S-N-K test, both with significantly lower polyphenolic content than the first group (Fig. V-7).

For plants collected in September at Dixon Island, anchoring structures and tissue from a patch of reproductive sorus were found to contain the highest polyphenolic content (S-N-K test, Figs. V-8, V-9). Blade tissue within 10 cm of the meristem, and, interestingly, tissue from the oldest 10 cm of the blade were included with the group having the second highest polyphenolic content (Fig. V-9). In addition, three other overlapping groups with lower polyphenolic content were identified with the S-N-K test (Fig. V-9).

In the plants collected from Helby Island in June 1992, tissue from proximal (i.e., near the holdfast) and distal (i.e., near the meristem) regions of the stipe had the highest polyphenolic content (S-N-K test, Figs. V-10, V-11). The group with the second highest polyphenolic content included tissues from the holdfast, reproductive sori, and the vegetative blade and midrib within 10 cm of the meristem (Fig. V-11). Two additional overlapping groups with lower polyphenolic content included the remainder of the tissues (Fig. V-11).

Polyphenolic content of vegetative blades and reproductive sori

In samples of blade tissue collected from 14 *Agarum fimbriatum* at Dixon Island in April 1992, polyphenolic content was significantly higher in reproductive sori than in adjacent vegetative blade tissue (paired *t*-test, Fig. V-12). Although I did not record where along the long axis of the plant samples were taken, reproductive and vegetative tissue pairs were adjacent, and thus removed from blade tissue of the same age.

Polyphenolic concentration in aqueous extracts of young and old blade tissue

Aqueous extracts made from a 10% (w/v) solution of blade tissue in distilled deionized water contained measurable levels of polyphenolic compounds. Extracts made with the youngest third of blade tissue contained a higher concentration of polyphenolics than equivalent extracts made with the oldest third of blade tissue (Student's *t*-test, Fig. V-13). Although this experiment demonstrated the presence of polyphenolics in aqueous extracts of kelp tissue, it did not distinguish whether those compounds were released from broken cells, or had diffused from intact cells into the surrounding water solvent.

Leaching of polyphenolic compounds from excised disks of kelp tissue

Polyphenolic content in disks of tissue excised from the youngest third of *Agarum fimbriatum* blades was the same as that in disks from adjacent tissue that were held in vials of filtered seawater for 72 hours, (Table V-5, Fig. V-14), but levels of polyphenolics measured in the surrounding seawater at the end of the 72 hour experimental period were at least one third as high as those in the kelp disks (Fig. V-14). These data suggest that the disks, while being held in vials for the leaching experiment, continued to produce more polyphenolic compounds, thus releasing detectable amounts of polyphenolics into the surrounding seawater without losing a significant amount of tissue polyphenolic content. Although polyphenolic content in this leachate was significantly lower than that in the kelp tissue, it appears that polyphenolics are diffusing into the surrounding seawater either from cut surfaces, or across cell membranes.

Discussion

In this study of polyphenolic variation in the kelp *Agarum fimbriatum*, I found that polyphenolic chemical defenses were allocated differentially among reproductive, vegetative, and structural tissue types. Reproductive sori contained higher levels of polyphenolics than adjacent vegetative blade tissue, and structural tissues generally had higher polyphenolic content than blade and midrib tissues located more than 10 cm from the intercalary meristem. Tissues near the intercalary meristem had a higher polyphenolic content than those further from the meristem. For most plants examined, the concentration of polyphenolics near the meristem resulted in a declining gradient in polyphenolic content from young to old blade tissue. In young (i.e., juvenile, *sensu* Steinberg 1984b), sporophytes, this declining chemical gradient with increasingly older blade tissue was evident even on a scale of centimeters.

Differential allocation or inducible response?

Theory predicts that chemical defenses in plants should be allocated to particular tissues in direct proportion to their vulnerability (McKey 1974); i.e., meristematic tissue, reproductive structures, and other tissues critical for maintaining fitness should be more heavily defended than non-critical, replaceable tissues. This prediction must be clarified, however, depending on the structure and physiological capabilities of the plant. Whether plants store defensive chemicals in all vulnerable tissues or in a central location from which they are translocated to sites where needed must depend on the ratio of the rate of tissue removal by herbivores and the rate of translocation. If defensive chemicals reach the site of wounding after most

of the grazing damage has occurred, then there would be little selection for an inducible response. Some terrestrial plants, when under attack by herbivores, can immediately shunt chemical defenses from storage sites to the sites of wounding (reviewed in Rhoades 1985, Karban & Meyers 1989), eliminating to some degree the necessity for site-specific storage of secondary metabolites.

Inducible responses may be lacking or limited in marine algae, which unlike vascular plants, generally lack the veinous architecture through which defensive substances can be rapidly translocated. Kelps are, however, capable of translocating substances in sieve elements along the stipe (Nicholson & Briggs 1972) at a rate of up to 78 cm/hr (Parker 1965, 1966), so they may similarly move polyphenolics among tissues. Another simple form of vascularization is present in fucoid brown algae, and has been proposed to explain how an inducible chemical defense mechanism can operate in a so-called non-vascular plant (Van Alstyne 1988). Within two weeks of experimental wounding, the intertidal brown alga *Fucus distichus* (Fucales) can increase its polyphenolic content by approximately 20%, which is sufficient to reduce grazing by littorinid gastropods (Van Alstyne 1988). Although this induced reponse in wounded plants occurs over a longer time period than analogous responses in terrestrial plants, the amount of tissue that gastropods are likely to consume during that period is considerably smaller. Thus, before the littorinid grazers have removed enough tissue to kill the plant, defensive compounds will have reached the site of wounding and successfully halted grazing. Evidence presented here suggests that inducible production or translocation of polyphenolic compounds probably does not occur in *Agarum fimbriatum*. Blades from which disks of tissue were removed on two occasions showed

a slight decline in polyphenolic content after the first sampling, rather than an increase. The amount of tissue removed for each sample was no more than ~3 g, however, so perhaps a larger amount of tissue needs to be removed to induce further production of chemical defenses.

For algae, having primitive vascularization, individual plant size may be an important factor in determining whether inducible chemical defense is selective, given physiological constraints on translocation and ecological constraints on grazing rates. In the largest kelps (e.g., *Nereocystis luetkeana*) with thallus size often in excess of 30 m, chemical translocation may be too slow to support an inducible response, particularly as the major herbivores in kelp communities are urchins (reviewed in Lawrence 1975, Hawkins & Hartnoll 1983, Dayton 1985), which can probably remove enough kelp tissue in an hour to kill the plant. The red urchin *Strongylocentrotus franciscanus* can consume up to 20 g algal tissue in 36 hours (Vadas 1968), and if that tissue were removed from the kelp's anchoring structures or meristem, the grazing damage would be lethal. For kelps, there may be stronger selection for protecting vulnerable tissues with localized concentrations of defensive compounds. In the intertidal kelps *Alaria marginata* (Steinberg 1984a) and *A. nana* (Pfister 1992), reproductive blades (sori) have higher polyphenolic content than vegetative blades, and for *A. marginata*, they are, consequently, consumed less by herbivores. The evidence presented here for *Agarum fimbriatum* supports the hypothesis that irreplaceable tissues, being more vulnerable to attack by herbivores, receive a higher allocation of chemical defense than replaceable tissues.

Correlation of polyphenolic content with nutrient state: the devil's advocate position

Because there appears to be some seasonal phenotypic plasticity in polyphenolic content in brown algae (Ilvessalo & Tuomi 1989, this study), polyphenolics may simply serve as a carbon sink that is filled under conditions of nitrogen limitation or high photosynthesis (Tuomi et al. 1989). Algal tissues undergoing rapid growth (e.g., meristems and sites of wounding) would perhaps have a localized deficiency in nitrogen, and hence a surplus of carbon. That regions with enhanced polyphenolic content are subsequently rejected by herbivores has prompted the recognition of polyphenolics as antiherbivore defenses. For a chemical defense to have evolved in response to grazing pressure, however, it should have significant negative effects on the herbivore's fitness and population dynamics (see Fowler & Lawton 1985, Tallamy & Krischik 1989).

Given the potential covariation between polyphenolics and nutrients in brown algae, one could also argue that herbivore preference for low-phenolic algae is caused not by higher phenolics in other choices, but by the carbon/nitrogen balance in the preferred alga's tissues. Nitogen content, as a measure of plant nutritional quality, is thought to be an important factor affecting herbivore food choices (Mattson 1980, but see Nicotri 1980, Steinberg 1985). Pfister (1992) found that although reproductive sporophylls of *Alaria nana* contained higher levels of polyphenolic content than vegetative frond tissue, they also had 15% higher carbon content. Urchins consuming tissue from *Agarum* spp. have been shown to have reduced assimilation efficiencies compared to those consuming higher preference algae, such as *Nereocystis luetkeana* (Vadas 1968). Is this reduction due to the presence of protein-binding polyphenolics, or to a

nitrogen deficiency? In a study on nutrient uptake dynamics on *Agarum fimbriatum*, however, reproductive sorus tissue was found to uptake nitrate and ammonium at a significantly lower rate than vegetative tissue from the same blades (Durante & Hurd, unpublished data). More information is needed to determine whether the uptake rates are lower in sorus tissue because the tissue doesn't grow appreciably, or because the tissue needs a higher carbon:nitrogen ratio in order to manufacture polyphenolic defenses.

Recent studies by Steinberg (1985, 1988) have revealed that polyphenolics are more important than nitrogen content in determining food preference among brown algae. Grazing by the herbivorous gastropod *Tegula funebralis* was negatively correlated with algal phenolic content, and postively correlated with nitrogen content, but a partial correlation analysis revealed that, when phenolic effects were controlled for, the relationship between nitrogen and feeding was no longer significant (Steinberg 1985). In another series of experiments using bioassays for *in vitro* testing of several types of phenolic compounds, Steinberg (1988) presented strong evidence that polyphenolics alone effectively reduce grazing. When phenolics were added to artificial food items constructed with kelp extract and agar, feeding by *Tegula* spp. and *Strongylocentrotus purpuratus* was reduced in a dose-dependent manner (Steinberg 1985).

Ecological implications of polyphenolic allocation in *Agarum fimbriatum*

Aside from the accumulation of polyphenolic compounds in their tissues, *Agarum fimbriatum* have little protection from grazing by urchins or gastropods. Vegetative blade and reproductive sporophyll tissues are thin and easily ripped (personal observation), and would seem to be easy

prey even for herbivores with small, inefficient feeding structures. Along blades of *A. fimbriatum*, as in other kelps, there is an age gradient from young, newly formed tissue at the meristem to old and senescing tissue at the distal end. Old blade tissue sloughs from the distal end regularly, and is replaced by new growth. Removal of older blade tissue by herbivores is unlikely to cause serious harm to the plant, but damage to the meristem may prevent any future growth. The concentration of polyphenolic compounds near the meristem would reduce the probability of damage to that vulnerable tissue. Sporophyll tissue, bearing zoospores (i.e., the haploid dispersal stage in kelps), is critical for reproduction, and hence maintaining plant fitness. This tissue receives a disproportionately high allocation of polyphenolic compounds as well, and thus is protected from damage or removal by herbivores. Finally, the holdfast and stipe, tissues anchoring the plant to the substratum, are well defended with polyphenolics. Should an individual plant be dislodged from the substratum, its chances of surviving to reproduce are minimal. Because marine algae absorb nutrients from the surrounding seawater rather than through roots, kelp tissue can theoretically survive and grow when detached. A detached piece of algae will be transported, however, by the currents in coastal waters to locations where its survival is unlikely. If the drift alga sinks into deeper water, the light levels will be insufficient for photosynthesis, and if it is washed ashore, desiccation stress, particularly for a subtidally adapted plant, will kill the tissue. As the holdfast and stipe, which contain very high concentrations of polyphenolics, are the oldest tissues in this plant, it is unlikely that the decline in polyphenolics along increasingly older blade tissue is simply due to leaching or degradation of these defensive compounds.

Consistent with observations of UV-absorbing substances in coastal waters (Sieburth 1968), results presented here show that polyphenolics do leach from *Agarum fimbriatum* tissues into the surrounding seawater. Phlorotannins are known to deter fouling and to have toxic effects on a variety of marine organisms (Sieburth & Conover 1965, Sieburth 1968). If polyphenolics regularly leach from blade tissue of *A. fimbriatum* in the field, then epiphytic organisms may be deterred from using this alga as a host substratum. As the other chapters in this thesis show, however, stenolaemate bryozoans preferentially settle on *A. fimbriatum*, and appear to settle on blade tissue near the meristem, which has the highest polyphenolic content. Because blades of *A. fimbriatum* appear free of epiphytes other than stenolaemate bryozoans, the specialist epiphytes may have evolved resistance to polyphenolic toxicity, while other species are effectively deterred from settling on this kelp.

Implications for future research

Patterns in polyphenolic distribution documented here reveal an important consideration for future research on brown algal polyphenolics. It is no longer acceptable to make inferences about allocation of polyphenolic defenses based on single measurements that supposedly span large areas of algal tissue. On a scale of centimeters, polyphenolic content can change nearly an order of magnitude, so it is important to sample tissues on a small scale, particularly for studies on larger plants such as kelps.

Although inducible chemical defense was not the main focus of this study, results of the one experiment measuring polyphenolic content after removal of tissue contradicted those of Van Alstyne (1988). Future studies

of this phenomenon in brown algae could benefit from determining which tissues and how much of them must be removed to induce increased production of chemical defenses. Also, direct evidence for polyphenolic translocation can only be obtained either by measuring polyphenolic levels in fluid removed from sieve elements (where translocation occurs in kelps), or by specifically staining tissues for polyphenolics either *in vivo* or in section.

Because of the potential interaction between polyphenolics and the carbon:nitrogen ratio in kelp tissues, further understanding of the selective pressures determining polyphenolic content may be gained from experiments in which nutrient states of algae are manipulated. A lack of change in polyphenolic content under conditions of severe nitrogen depletion may indicate that polyphenolic allocation is under genetic control.

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Figure V-1: Protocols for sampling tissue from the kelp *Agarum fimbriatum*. Scale bars to the right of blade diagrams = 5 cm. Black squares on diagrams indicate locations where tissue samples were removed. (a) Blades collected as part of a year-round census were divided into three equal-length sections, and a sample of tissue removed from the center of each section. Young sporophytes that were ca 35 cm (b) and ca 15 cm (c) in blade length were divided into 5 cm sections along the long axis of the plant. (d) Plants collected for a more detailed investigation of phenolic patterns were sampled at the holdfast, proximal and distal sections of the stipe, vegetative blade, midrib, and where present, the reproductive sorus (shaded region).

Measurement of polyphenolic content in *Agarum fimbriatum*
Sampling protocols

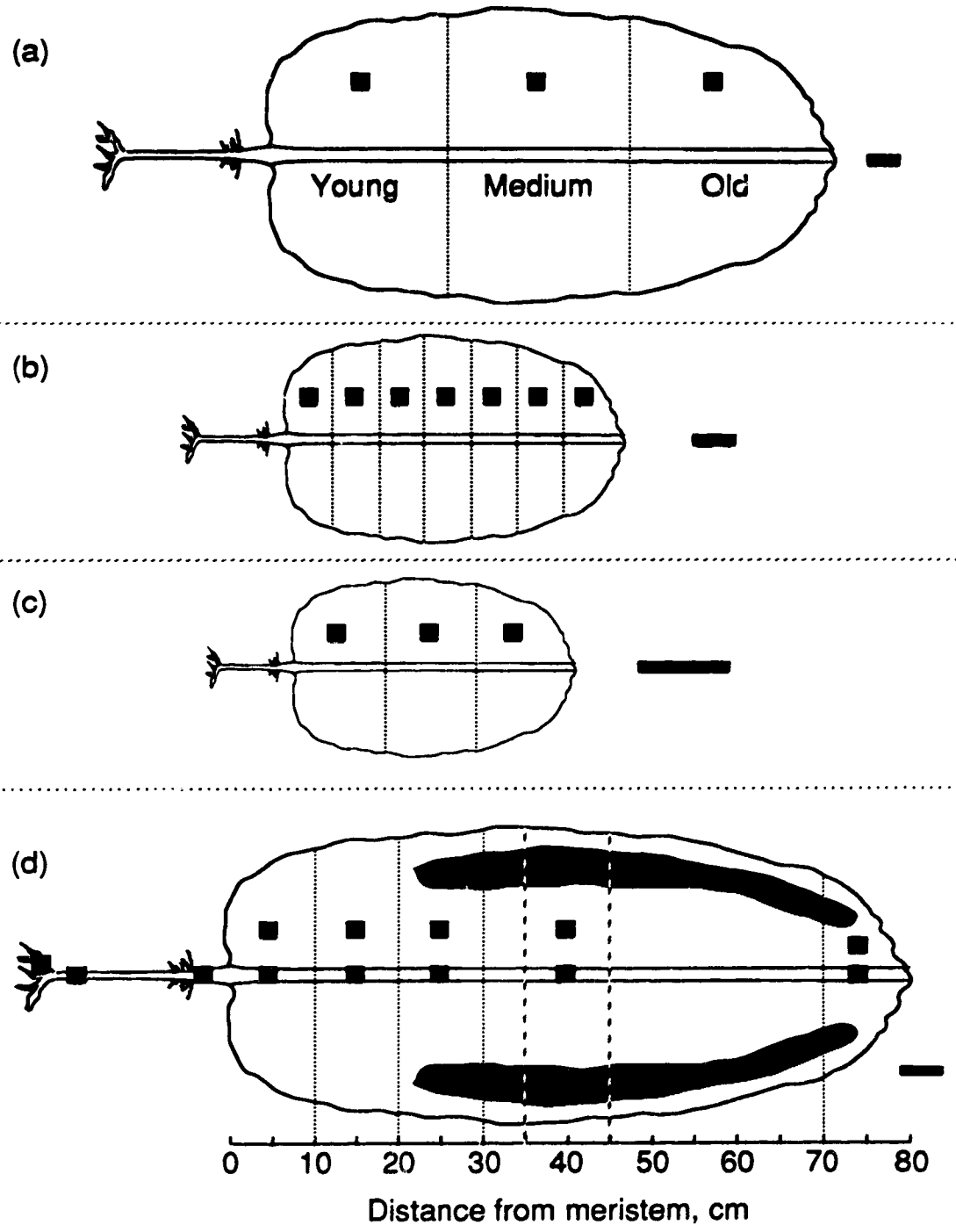

Figure V-2: Polyphenolic content in young, medium, and old age blade tissue from plants of *Agarum fimbriatum*, collected on five occasions at Dixon Island from January 1992 to February 1993. The means and standard errors of five plants are plotted for each of five censuses, and the diagram of the algal blade at the bottom indicates location of tissue age sections. Analyses of variance (ANOVAs) conducted on rank-transformed data showed that polyphenolic content, expressed as the % of algal dry mass, varied significantly with tissue age in plants collected during February 1992 and 1993, but not in the other plants tested. P-values from the ANOVAs are indicated to the right of histograms, and horizontal bars beneath histograms indicate separate groups identified by Student-Newman-Keuls (S-N-K) multiple comparisons procedures. In plants from the two February collections, young blade tissue had a higher polyphenolic content than the medium or old tissue. In all but the January 1992 census, young blade tissue had the highest mean polyphenolic content of the three tissue age categories, but significant differences were only recorded for two censuses.

Agarum fimbriatum collected from Dixon Island
Polyphenolic content of young, medium, & old blade tissue

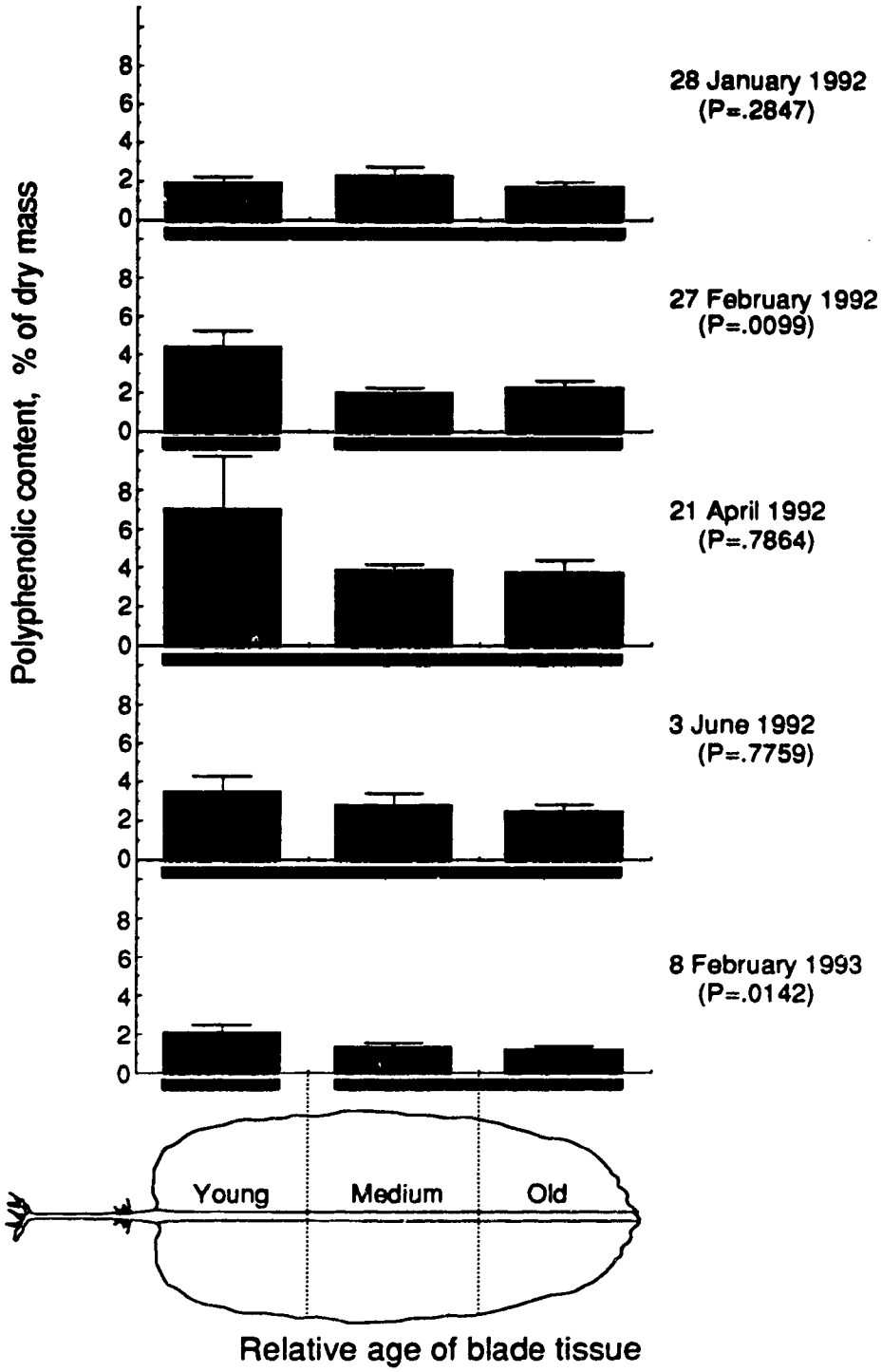

Figure V-3: Effects of tissue sampling on polyphenolic content in blades of *Agarum fimbriatum*. Data plotted are the means and standard errors of polyphenolic content in young, medium, and old age blade tissue from five plants, collected from Dixon Island in November 1991. Tissue samples were removed for phenolic assays on 6 November (solid bars), and again on 14 November (hatched bars). A repeated measures ANOVA on rank-transformed data showed that tissue age and the repeated measure significantly affected polyphenolic content (see Table V-2). Horizontal bars beneath the histogram indicate groups identified by S-N-K multiple comparisons tests.

Polyphenolic content of blade tissue from *Agarum fimbriatum*
Effects of sampling damage

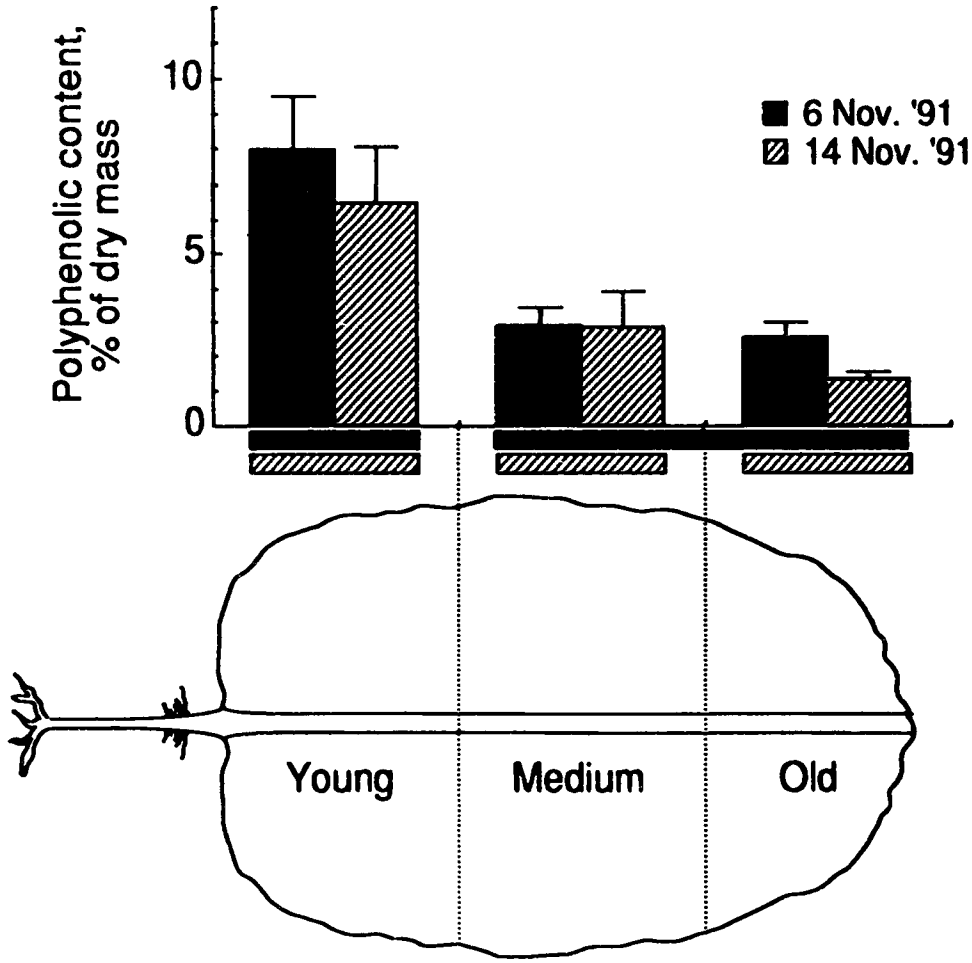

Figure V-4: Polyphenolic content, expressed as the % of algal dry mass, measured at 5 cm intervals along blades of young *Agarum fimbriatum* sporophytes, collected from Dixon Island in November 1991. Data shown are means ± standard errors of four blades. Blade intervals where tissue samples were removed for analysis are shown on diagram beneath histogram. A randomized block design ANOVA on rank-transformed data showed a significant difference in polyphenolic content among the 5 cm blade sections (see Table V-3), and a multiple comparisons test (S-N-K) identified two groups, shown by the horizontal bars beneath the histogram.

Polyphenolic content of blade tissue from young *Agarum fimbriatum* sporophytes

13 November 1991: Dixon Island

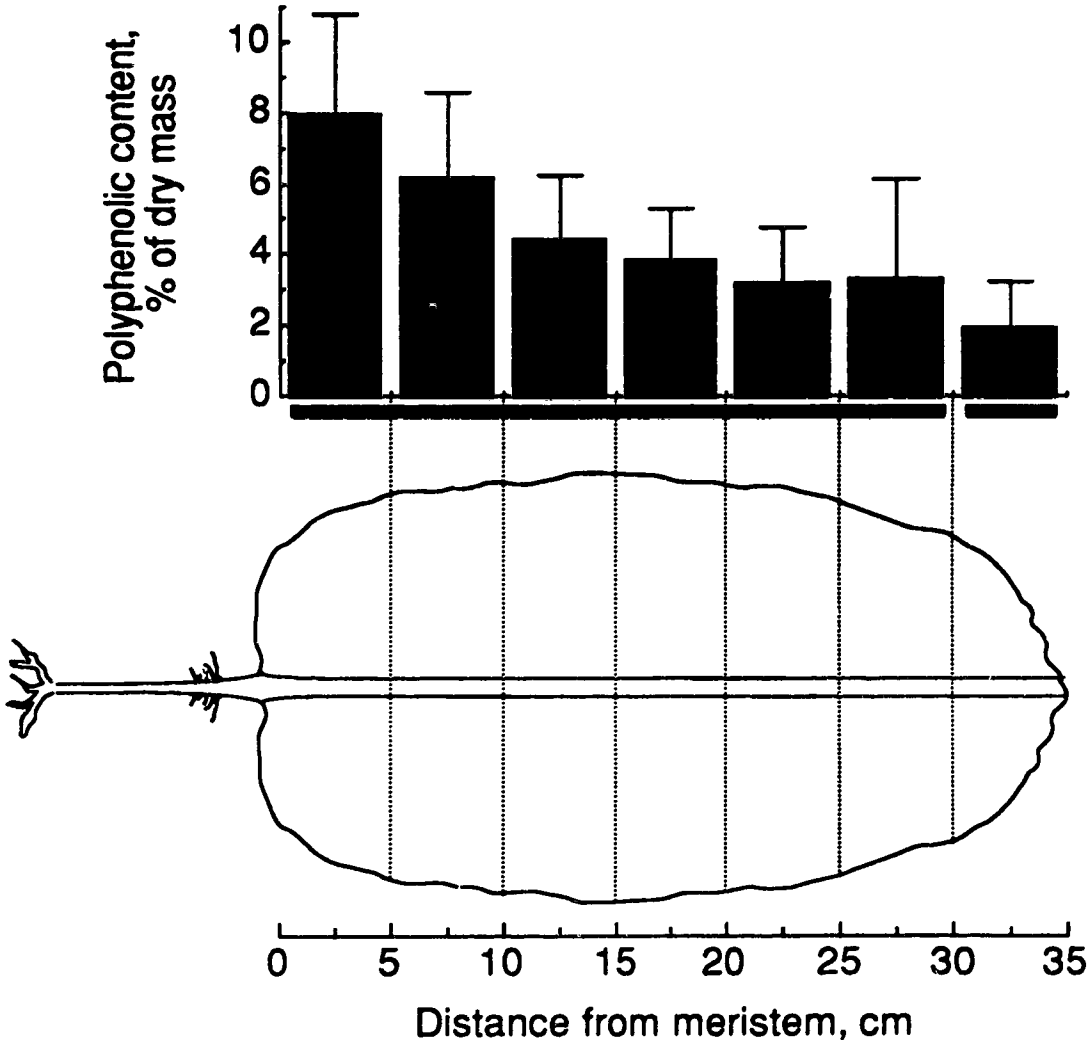

Figure V-5: Polyphenolic content in juvenile sporophytes of *Agarum fimbriatum* collected from Dixon Island in September 1992. Blades (n=8) were divided into three sections, representing 5 cm increments along the long axis of the blade (see diagram beneath histogram). Data plotted are means and standard errors of polyphenolic content, expressed as the % of algal dry mass. A randomized block design ANOVA showed that polyphenolic content differed significantly among the three blade sections (see Table V-3). An *a posteriori* S-N-K multiple comparisons procedure showed that polyphenolic content in the 0-5 cm section > in the 5-10 cm section > in the 10-15 cm section, (groups indicated by horizontal bars beneath histogram).

Polyphenolic content of blade tissue from juvenile *Agarum fimbriatum* sporophytes

17-18 September 1992

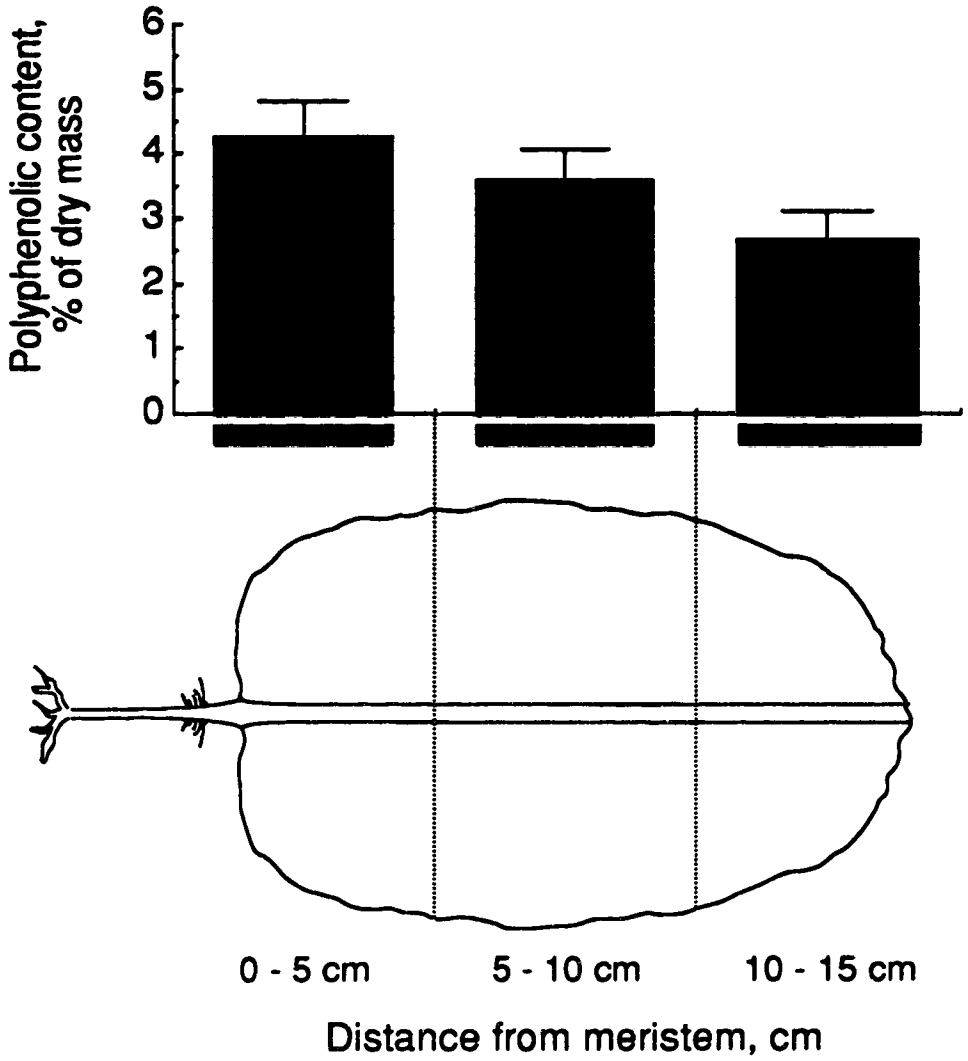

Figure V-6: Polyphenolic content in tissues of *Agarum fimbriatum* collected from Dixon Island in July 1992. Arrows beneath each histogram bar point to the location on the diagram where the tissue was sampled for polyphenolic analysis. In order from left to right, histogram bars represent the following tissues: holdfast, stipe near holdfast, stipe near meristem, blade within 10 cm of meristem, midrib within 10 cm of meristem, youngest third of blade, youngest third of midrib, middle third of blade, middle third of midrib, oldest third of blade, oldest third of midrib, oldest 10 cm of blade, and oldest 10 cm of midrib. Data plotted are the means and standard errors of polyphenolic content measured for five plants. A randomized block design ANOVA showed that polyphenolic content differed significantly among tissue types (see Table V-4), and an *a posteriori* S-N-K multiple comparisons test identified three groups, the overlapping pattern of which is depicted in Fig. V-7.

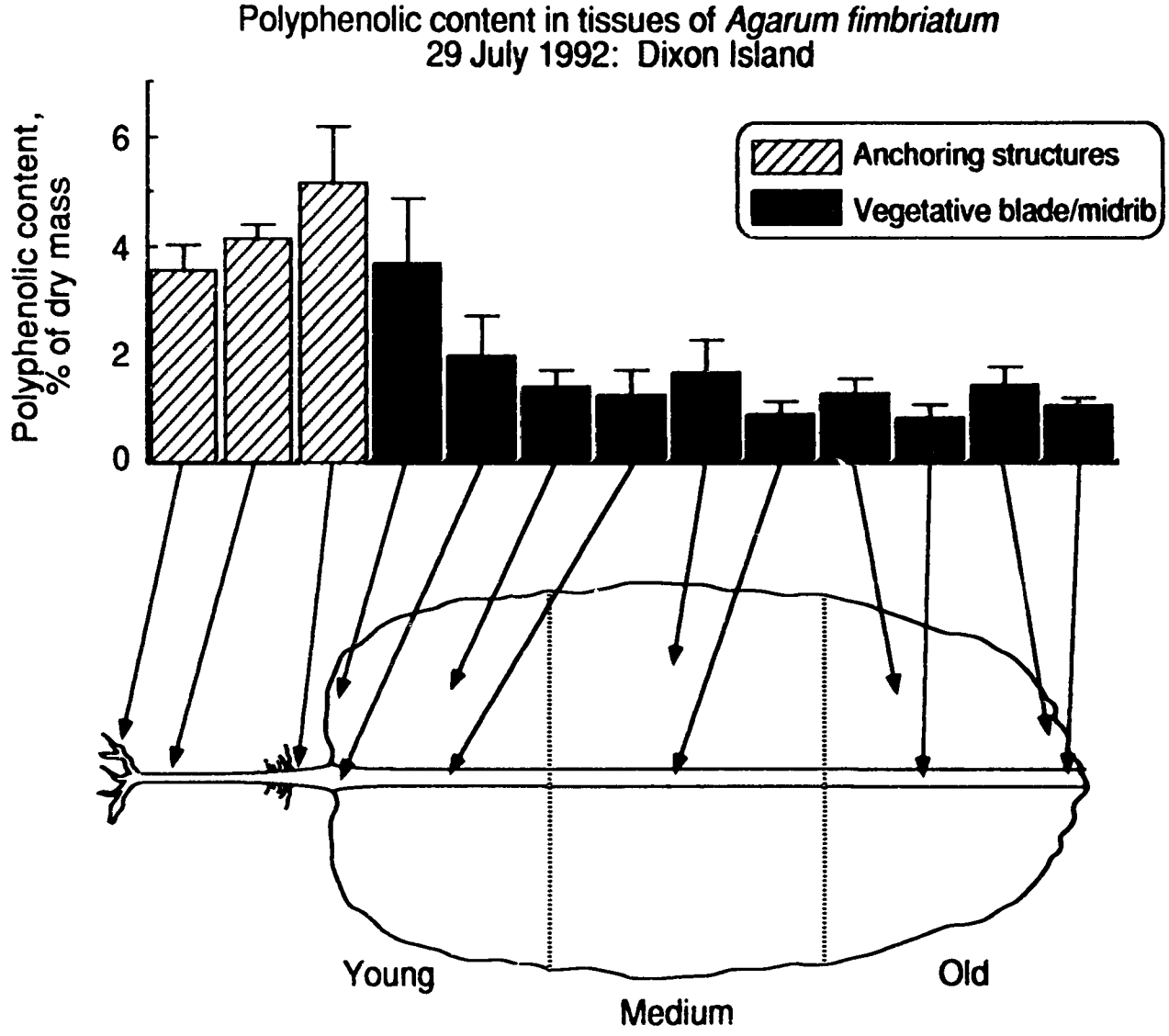
Polyphenolic content in tissues of *Agarum fimbriatum*
29 July 1992: Dixon Island
Polyphenolic content, % of dry mass
6
4
2
0
Anchoring structures
Vegetative blade/midrib
Young
Medium
Old

Figure V-7: Results of a Student-Newman-Keuls (S-N-K) multiple comparisons procedure testing for differences in polyphenolic content among tissues of *Agarum fimbriatum* collected from Dixon Island in July 1992. From top to bottom, tissues are listed in order of decreasing polyphenolic content, and the vertical black bars to the right of the tissue list indicate groups identified by the S-N-K test at the significance level α = .05.

Polyphenolic distribution in *Agarum fimbriatum*
Dixon Island, Jul/92: S-N-K results, $\alpha = .05$

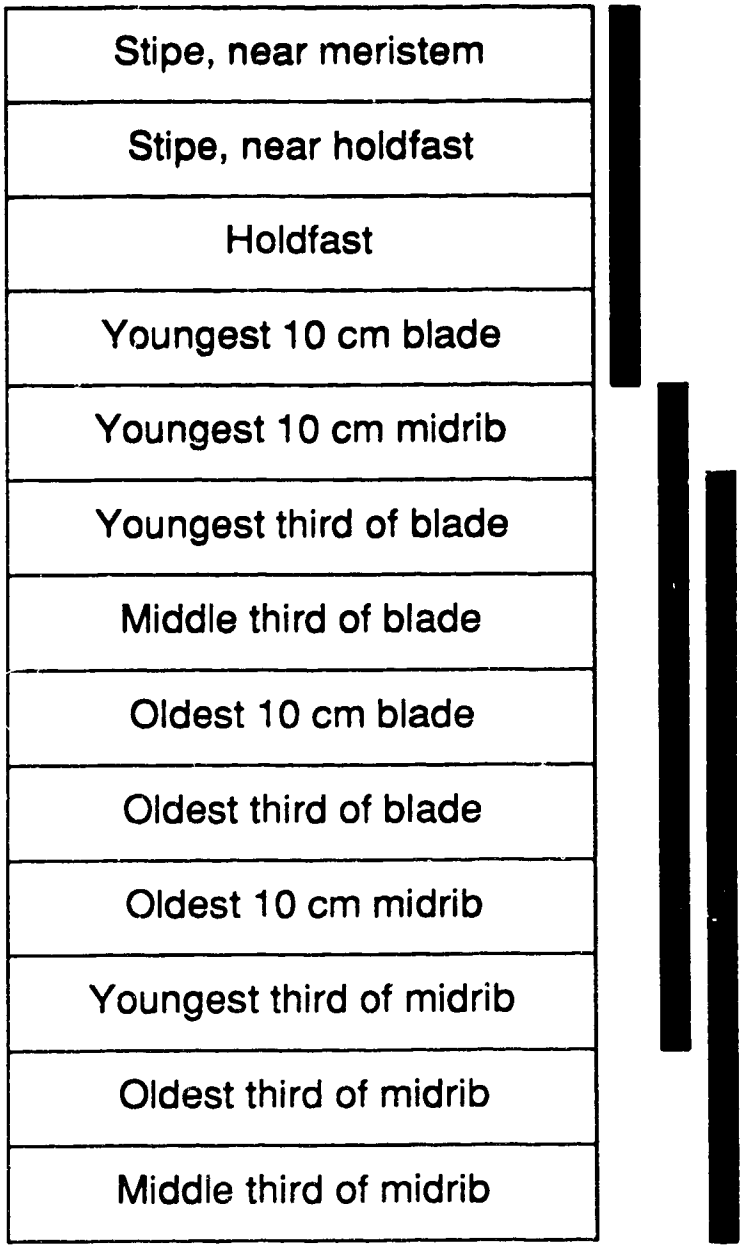

Figure V-8: Polyphenolic content in tissues of *Agarum fimbriatum* collected from Dixon Island in September 1992. Arrows beneath each histogram bar point to locations on the diagram where tissues were sampled for polyphenolic analysis. From left to right, histogram bars represent mean ± standard error of polyphenolic content in tissue sampled from: holdfast, stipe near holdfast, stipe near meristem, youngest 10 cm blade, youngest 10 cm midrib, youngest third of blade, youngest third of midrib, youngest third of sorus (shaded region), middle third of blade, middle third of midrib, oldest third of blade, oldest third of midrib, oldest 10 cm of blade, oldest 10 cm of midrib. A randomized block design ANOVA showed that polyphenolic content differed significantly among tissues sampled (see Table V-4). Results of an *a posteriori* S-N-K multiple comparisons test identified five groups, but with a sufficiently complex pattern of overlap that they could not be depicted clearly on this figure (see Fig. V-9).

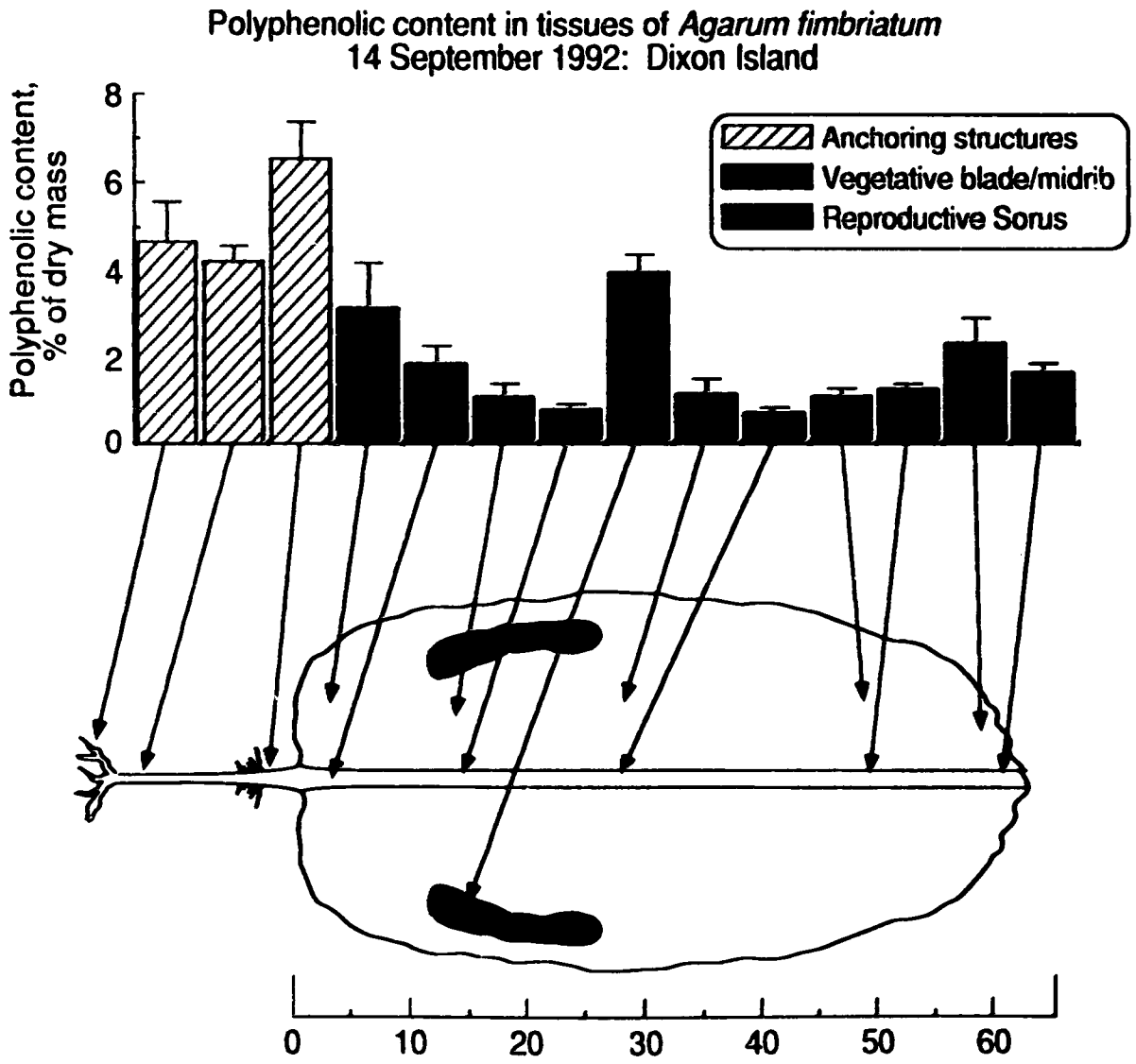
Polyphenolic content in tissues of *Agarum fimbriatum*
14 September 1992: Dixon Island
Polyphenolic content, % of dry mass
8
6
4
2
0
Anchoring structures
Vegetative blade/midrib
Reproductive Sorus
0
10
20
30
40
50
60

Figure V-9: Results of a Student-Newman-Keuls (S-N-K) multiple comparisons procedure testing for differences in polyphenolic content among tissues of *Agarum fimbriatum* collected from Dixon Island in September 1992. From top to bottom, tissues are listed in order of decreasing polyphenolic content, and the vertical black bars to the right of the tissue list indicate groups identified by the S-N-K test at the significance level $\alpha = .05$.

Polyphenolic distribution in *Agarum fimbriatum*
Dixon Island, Sep/92: S-N-K results, $\alpha = .05$

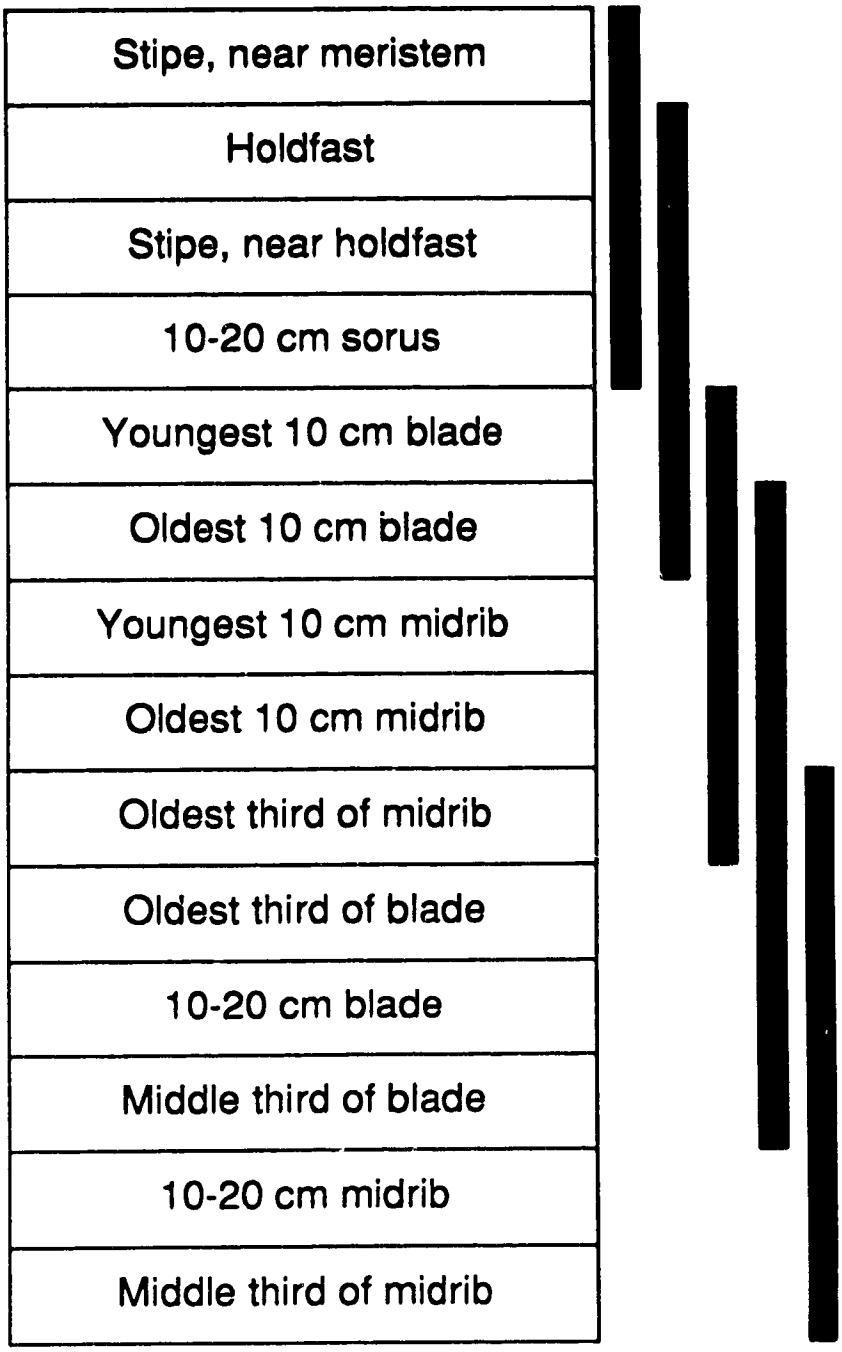

Figure V-10: Polyphenolic content in tissues of *Agarum fimbriatum* collected from Helby Island in June 1992. Arrows beneath each histogram bar point to locations on the diagram where tissues were sampled for polyphenolic analysis. From left to right, histogram bars represent means ± standard errors of polyphenolic content in tissue sampled from: holdfast, stipe near holdfast, stipe near meristem, youngest 10 cm blade, youngest 10 cm midrib, 10-20 cm blade, 10-20 cm midrib, 20-30 cm blade, 20-30 cm midrib, 20-30 cm sorus (shaded region), middle 10 cm blade, middle 10 cm midrib, middle 10 cm sorus, oldest 10 cm blade, oldest 10 cm midrib, oldest 10 cm sorus. Histogram bars with a hatched pattern represent anchoring structures, solid bars represent vegetative tissue from blades or midribs, and shaded bars represent reproductive sorus tissue. A randomized block design ANOVA showed that polyphenolic content differed significantly among tissues sampled (see Table V-4). Results of an *a posteriori* S-N-K multiple comparisons test identified five groups, but with a sufficiently complex pattern of overlap that they could not be depicted clearly on this figure (see Fig. V-11).

Polyphenolic content in tissues of *Agarum fimbriatum*
22 June 1992: Helby Island

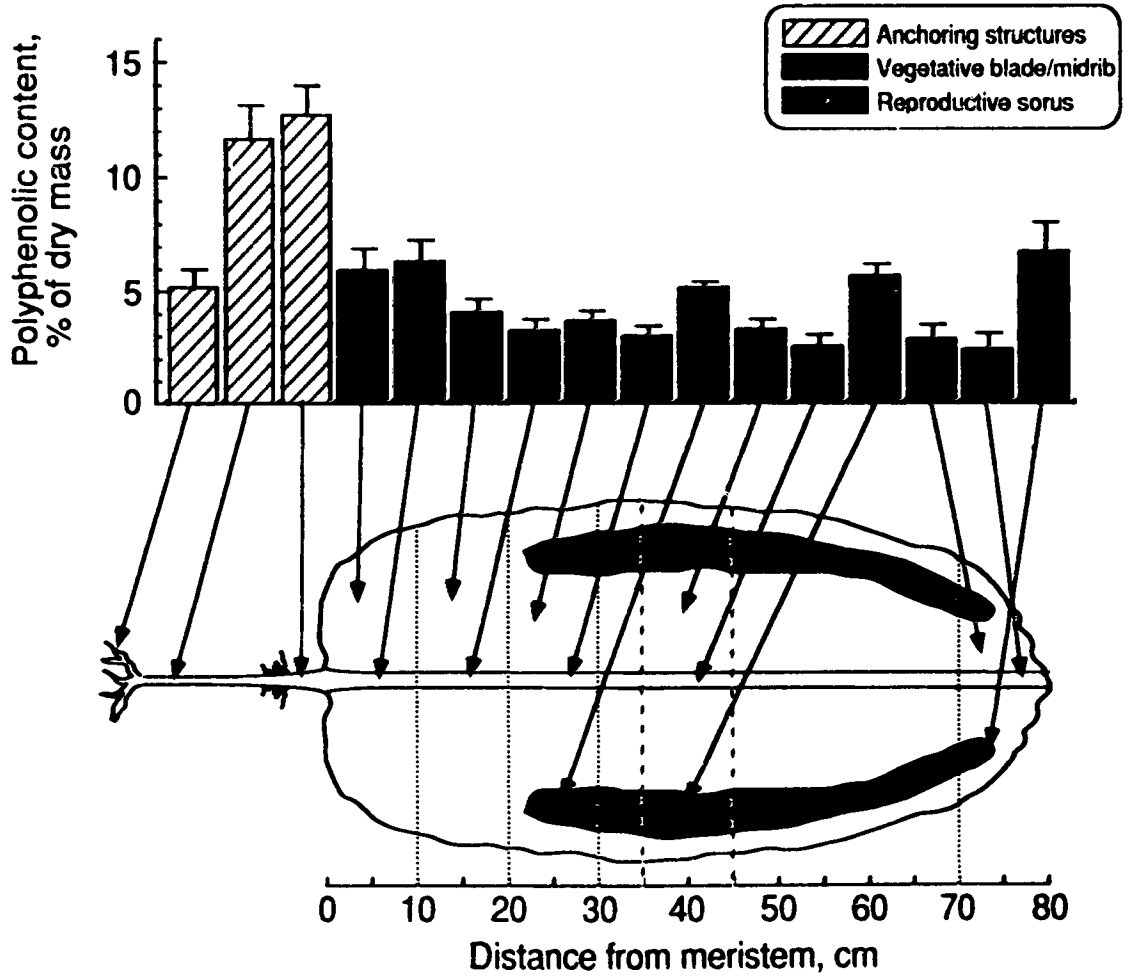

Figure V-11: Results of a Student-Newman-Keuls (S-N-K) multiple comparisons procedure testing for differences in polyphenolic content among tissues of *Agarum fimbriatum* collected from Helby Island in June 1992. From top to bottom, tissues are listed in order of decreasing polyphenolic content, and the vertical black bars to the right of the tissue list indicate groups identified by the S-N-K test at the significance level α = .05.

Polyphenolic distribution in *Agarum fimbriatum*
Helby Island, Jun/92: S-N-K results, $\alpha = .05$

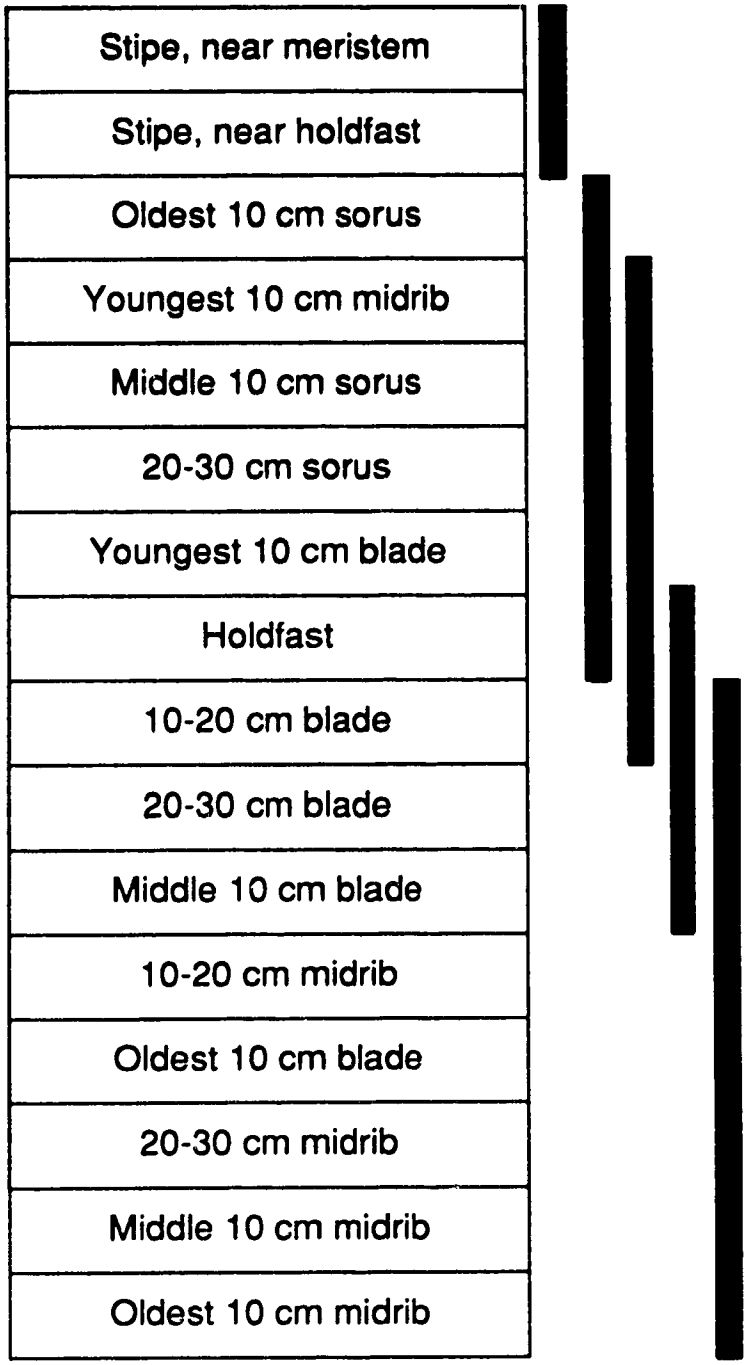

Figure V-12: Polyphenolic content in vegetative and reproductive tissues from blades of *Agarum fimbriatum* collected from Dixon Island in April 1992. Shaded patches on the diagram represent reproductive sori (heavier arrows); open area is vegetative blade tissue (lighter arrows). Data plotted are the means ± standard errors of polyphenolic content, expressed as the % of algal dry mass, from 14 plants. A paired Student's *t*-test applied to rank-transformed data showed that polyphenolic content was significantly higher in reproductive sori than in adjacent vegetative blade tissue, (df = 13, paired $t = 7.1006$, $P = .0001$)

Polyphenolic content of reproductive vs. vegetative tissue from *Agarum fimbriatum*

23 April 1992: Dixon Island

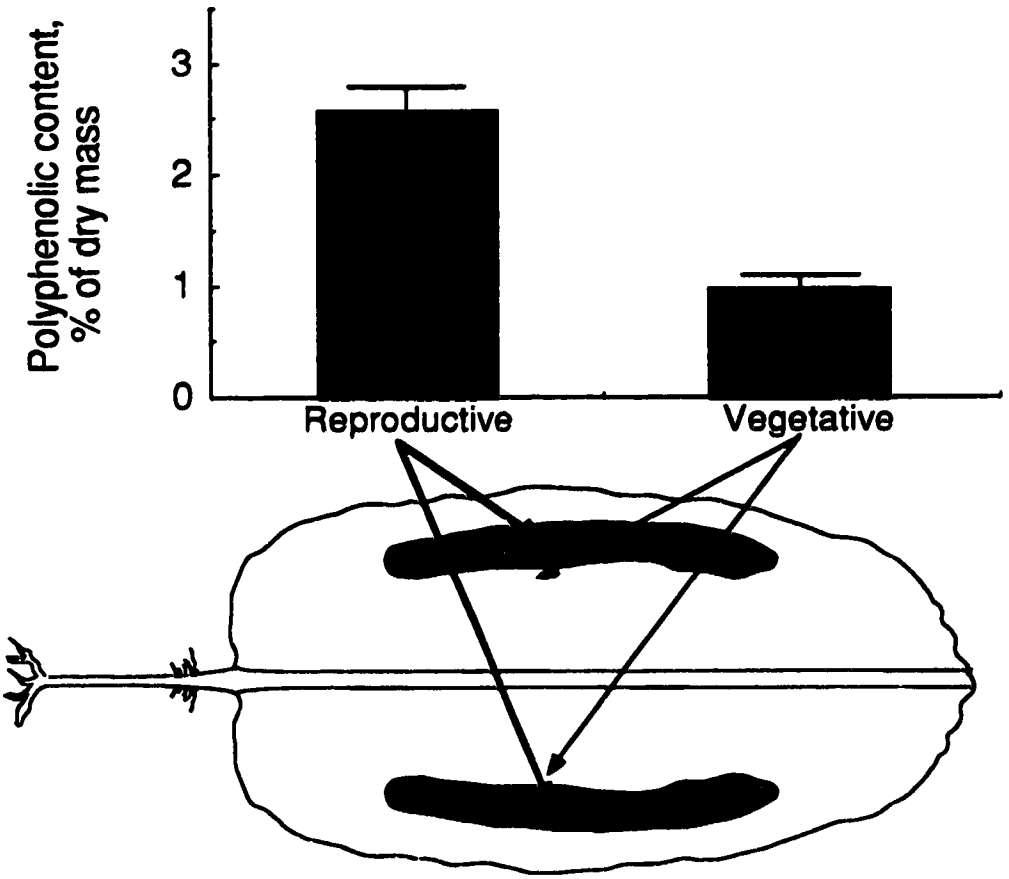

Figure V-13: Concentration (in mg/ml) of polyphenolic compounds in aqueous extracts made with vegetative tissue from the youngest and oldest thirds of *Agarum fimbriatum* blades (see location of tissue on diagram). Data plotted are means ± standard errors of five extract samples, although the errors are so small (3.9×10^{-4} and 3.3×10^{-4} for young and old extracts, respectively) that the error bars are not visible on the graph. An unpaired Student's *t*-test on rank-transformed data showed that extract of young blade tissue had a higher polyphenolic concentration than extract of old blade tissue (df = 8, t = 5.4233, P = .0006).

Polyphenolic concentration of aqueous extracts from young and old *Agarum fimbriatum* tissue

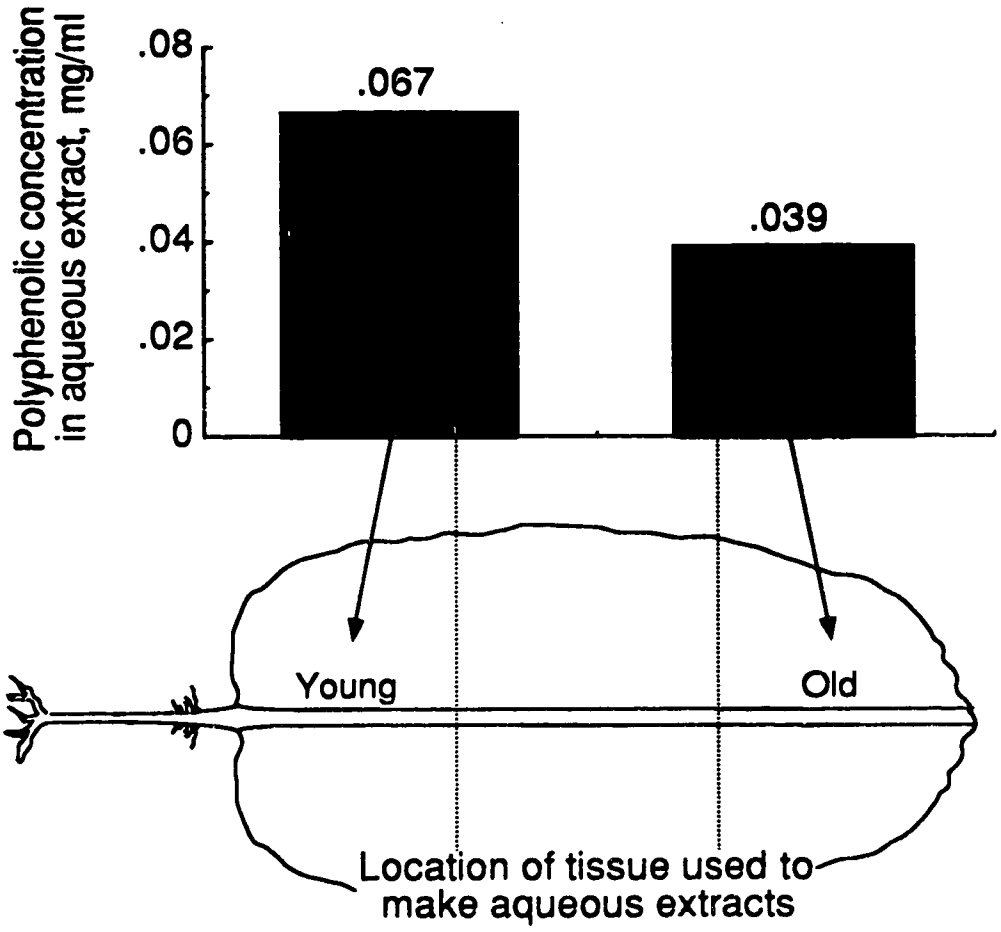

Figure V-14: Effect of leaching on polyphenolic content in young blade tissue of *Agarum fimbriatum* collected from Dixon Island in June 1993. "Fresh" treated tissue was extracted immediately following collection. "Leached" treated tissue was held for 72 h in 25 ml filtered seawater prior to extraction. "Leachate" was the seawater in which the "leached" treated tissue was held for 72 h. Data plotted are the means ± standard errors of 15 replicates. A nested ANOVA on rank-transformed data showed that polyphenolic content significantly differed among the treatments (see Table V-5). An *a posteriori* S-N-K multiple comparisons test (results depicted as horizontal bars beneath histogram) showed that polyphenolic content in freshly extracted and leached tissue did not differ significantly, but they both had significantly higher polyphenolic content than the leachate.

Effect of leaching on polyphenolic content in blade tissue from *Agarum fimbriatum*

22 June 1993: Dixon Island

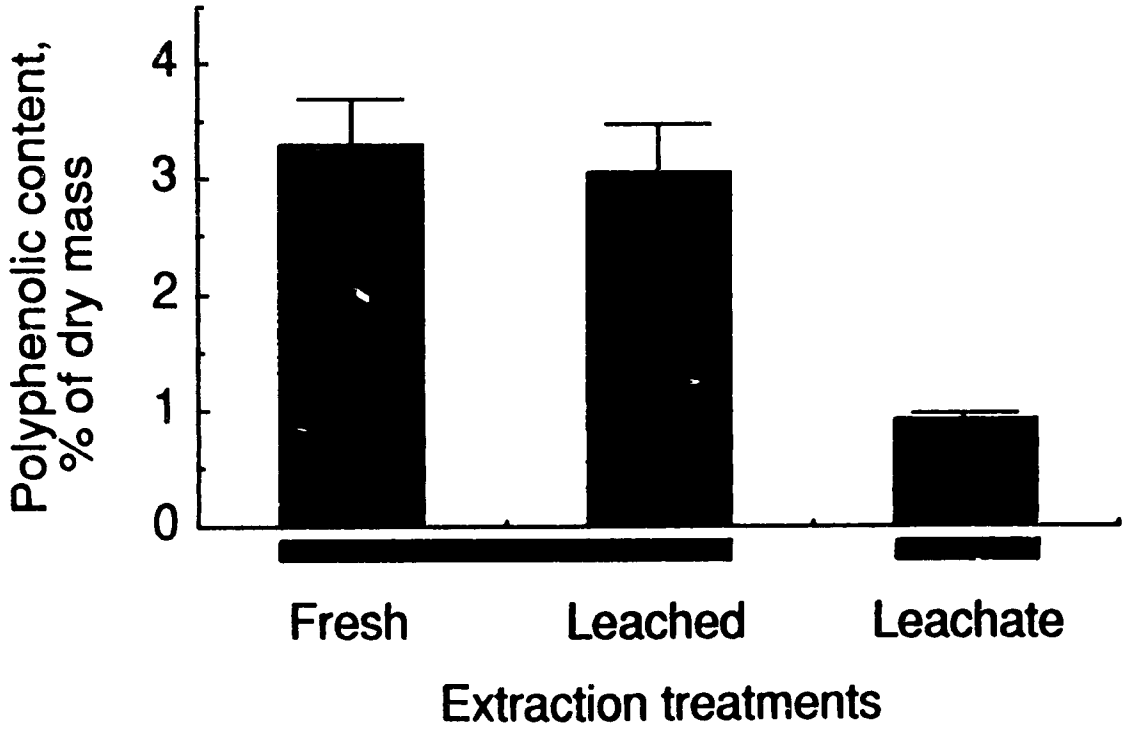

Table V-1: Results of a two-factor, randomized block design ANOVA testing the effects of month and relative tissue age on polyphenolic content in blades of *Agarum fimbriatum* collected at Dixon Island from January 1992 to February 1993 (see Fig. V-2). The month x age interaction term was non-significant and deleted from the model. Also shown are results from an *a posteriori* S-N-K multiple comparisons procedure testing pairwise comparisons between all months. Months contained within the same rectangle are not significantly different at $\alpha = .05$. Data were rank-transformed prior to analysis.

Source	df	MS	F	P
Month	3	3335.644	10.184	.0001
Tissue Age	2	954.120	2.913	.0614
Plant (block)	4	486.267		
Residual	65	327.535		

April	June	February	January

Table V-2: Results of repeated measures (RM) analysis of variance (ANOVA) testing the effects of sampling damage and week of measurement (the repeated measure) on polyphenolic content in young, medium, and old age blade tissue of *Agarum fimbriatum* collected from Dixon Island in November 1991 (see Fig. V-3). The ANOVA was conducted on rank-transformed data, and non-signficant interaction terms were removed from the model.

Source	df	MS	F	P
Age	2	594.700	29.569	.0001
Week (RM)	1	132.300	6.578	.0177
Plant (block)	4	120.833		
Residual	22	20.112		

Table V-3: Results of randomized block design ANOVAs testing the effect of tissue age section on polyphenolic content in young sporophytes of *Agarum fimbriatum*. Plants collected in November 1991 ranged from 20-35 cm in blade length (see Fig. V-4), and those collected in September 1992 were all ca 15 cm in blade length (see Fig. V-5). For both collections, polyphenolic content was measured at 5 cm intervals along the blade beginning at the intercalary meristem. Data were rank transformed prior to analysis.

Date	Source	df	MS	F	P
Nov/91	Age	6	30.643	3.069	.0426
	Plant (block)	3	218.787		
	Residual	13	9.985		
Sep/92	Age	2	118.625	23.924	.0001
	Plant (block)	7	120.476		
	Residual	14	4.958		

Table V-4: Results of randomized block design ANOVAs testing the effect of tissue location on polyphenolic content in *Agarum fimbriatum*, collected from Dixon Island in July (see Fig. V-6) and September 1992 (see Fig. V-8), and from Helby Island in June 1992 (see Fig. V-10). Data were rank transformed prior to analysis.

Date	Source	df	MS	F	P
July 1992	Tissue	12	910.919	15.733	.0001
(Dixon Island)	Plant (block)	4	1269.370		
	Residual	43	57.898		
September 1992	Tissue	13	806.692	14.110	.0001
(Dixon Island)	Plant (block)	3	637.762		
	Residual	39	57.172		
June 1992	Tissue	15	1809.452	17.948	.0001
(Helby Island)	Plant (block)	4	1617.930		
	Residual	58	100.814		

Table V-5: Results of a nested ANOVA testing the effects of leaching treatments and replicate patches within blocks on polyphenolic content (see Fig. V-14). Data were rank-transformed prior to analysis.

Source	df	MS	F	P
Treatment	2	2418.467	49.657	.0001
Patch within Plant	2	370.147	7.600	.0016
Plant (i.e., block)	1	113.344		
Residual	39	48.703		

Chapter VI

General Summary

Research presented in this thesis has addressed the ecological relationship between the subtidal kelp *Agarum fimbriatum* Harv. and the stenolaemate bryozoans using this kelp as a living substratum in Barkley Sound, Vancouver Island, British Columbia. In protected shallow subtidal regions around the Deer Group Archipelago, colonies of the bryozoan *Lichenopora novae-zelandiae* (Cl. Stenolaemata: O. Cyclostomata: F. Lichenoporidae) primarily colonized *A. fimbriatum*, with one exception. During the summer of 1992, several colonies of *L. novae-zelandiae* were observed on fronds of *Macrocystis integrifolia* collected from a site in close proximity (ca 100 m) to a fuel dock and marine railway (at Ostrom's Machine Shop in Bamfield Inlet: 48° 49.45'N; 125° 8.15'W). As my only observation of *L. novae-zelandiae* growing on a substratum other than *A. fimbriatum* occurred in a habitat exposed to contamination from engine oil, gasoline, diesel, copper, tributyl tin, and sewage, it appears that, in the Barkley Sound region, this bryozoan grows in a specific epiphytic relationship with *A. fimbriatum*. Several species of *Tubulipora* (F. Tubuliporidae) were also common epiphytes on *A. fimbriatum*, although they were found occasionally on other kelps and on non-living substrata such as empty gastropod shells.

Three lines of evidence suggested that interactions between *Agarum fimbriatum* and its bryozoan epiphytes were more complex and ecologically interesting than those between most sessile invertebrates and their substrata. First, among subtidal kelps in the northeast Pacific, *A.*

fimbriatum is reported to be one of the least preferred food items for herbivorous sea urchins (Vadas 1968, 1977), and the rejection of this algal food by generalist herbivores has been attributed to its unusually high levels of polyphenolic chemical defenses (Steinberg 1984b). Second, *A. fimbriatum* is a perennial kelp with a single, and thus highly vulnerable, intercalary meristem, and theoretical predictions that plants allocate defensive chemicals to tissues most vulnerable for maintaining fitness (McKey 1974) have been upheld repeatedly for brown algae (Steinberg 1984a, Pfister 1992). Third, my initial observations of bryozoan distributions along blades of *A. fimbriatum* revealed that adult colonies were found towards the distal end of the blades, on older tissue, whereas juvenile colonies and newly settled ancestrulae were present mostly on younger tissue, towards the intercalary meristem. Thus, the major questions addressed in this thesis were: (1) Do the larvae of bryozoans that grow epiphytically on *A. fimbriatum* actively choose to settle on this kelp species, or is differential mortality responsible for non-random distributions of bryozoans?, (2) Does larval preference also explain spatial patterns of bryozoan distribution along blades of *A. fimbriatum*?, (3) Do epiphytic bryozoans select *A. fimbriatum* because it provides a refuge from herbivory?, and (4) Is there within-thallus variation in polyphenolic defenses in *A. fimbriatum*, and if so, can this variation explain bryozoan larval choice or colony distributions?

For three consecutive years, I sampled *Agarum fimbriatum* from Dixon Island and other sites in Barkley Sound, and found that both bryozoan abundance and kelp growth seemed to peak in late spring to early summer. During the winter, bryozoan epiphytes on *A. fimbriatum* were absent or rare, but were present in high densities every summer during the

study. Blades of *A. fimbriatum* were relatively short during the winter, presumably because their growth rate slowed (due to low light levels) while tissue continued to senesce and erode from the distal end. Although colonies of *Tubulipora* spp. were occasionally found growing on fronds of the co-occurring kelp *Eisenia arborea* and on non-living substrata, *Lichenopora novae-zelandiae* were never found on substrata other than *A. fimbriatum* at the sites studied. In laboratory experiments, larvae of both bryozoan genera exhibited preferential settlement on blade tissue from *A. fimbriatum* when offered as a choice with analogous tissue from other kelp species. Also, when offered the choice of young and old blade tissue from the same kelp species, larvae preferred younger tissue.

What properties of young *Agarum fimbriatum* blade tissue attract bryozoan larvae? From an evolutionary perspective, epiphytic organisms settling on the youngest part of their host plant will maximize their potential lifespan on that plant. If, on the other hand, bryozoan larvae settled on older kelp tissue, that tissue could reach the point of senescence and distal erosion before the adult bryozoans have reproduced: their settlement choice being a failure in terms of evolutionary fitness. Feeding experiments with the generalist herbivorous gastropod *Tegula pulligo* were consistent with previous work, showing that *A. fimbriatum* is a low-preference food item (Vadas 1968, 1977). Bryozoans settling on this kelp might ensure an associational refuge from herbivory-related costs; indeed, the presence of *Lichenopora novae-zelandiae* colonies on both young and old blade tissue reduced grazing rates by *T. pulligo*. Proximate reasons why bryozoan larvae exhibit strong preference for their host alga may be related to proximate reasons why herbivores are deterred from grazing on this kelp species. Not only is *A. fimbriatum* protected against herbivory

with an unusually strong arsenal of polyphenolic compounds (Steinberg 1984b), but research in this thesis also demonstrated that polyphenolics generally exhibit a gradient along the kelp blades with the highest concentration in young tissue near the meristem. That bryozoan larvae prefer settling not only on a polyphenolic rich alga, but also on the region of the blades where polyphenolics are in highest concentration suggests that they might be induced to settle by polyphenolic compounds themselves.

At least two puzzling questions arise with this hypothesis, however, beginning with why bryozoan larvae (particularly those of the specialist epiphyte *Lichenopora novae-zelandiae*) do not settle in lower abundance on other kelps, which contain polyphenolics in lower concentrations than those found in *Agarum fimbriatum*. In the experiment offering larvae of *Tubulipora tuba* a choice of young and old blade tissue from three kelp species, larvae chose young tissue of each species over the corresponding old choice. If age-specific gradients in polyphenolic content also occur in kelps other than *A. fimbriatum*, then the tendency for *T. tuba* larvae to settle on younger blade tissue of all three species may be explained by larval attraction to polyphenolic compounds. Also, as certain tissues of *A. fimbriatum* may have higher polyphenolic content than blade tissue near the meristem (e.g., reproductive sorus, holdfast, and stipe), if bryozoan larvae are attracted by polyphenolics alone, why does their settlement appear restricted to blade tissue? Although, in settlement choice experiments, bryozoan larvae settled in higher abundance on young *A. fimbriatum* tissue, in some cases they did settle in lower numbers on the other choices. Why then were these alternate substrata apparently not colonized by *L. novae-zelandiae* in the field?

Although their holdfasts may co-occur with those of *Agarum fimbriatum*, both *Macrocystis integrifolia* and *Nereocystis luetkeana* are long plants, sometimes extending tens of meters up from their holdfasts to surface waters. Fronds of these large kelps are supported by gas-filled floats, and thus are held near the surface by the associated buoyant tissue. As the larvae of many invertebrates are sensitive to ultraviolet radiation, bryozoan larvae settling on kelp blades near the surface may not survive to metamorphosis. Because larvae of stenolaemate bryozoans are lecithotrophic (i.e., non-feeding), they may be incapable of swimming the long distance between the *A. fimbriatum* bed and the surface (≥ 10 m), or they may encounter a sufficiently large number of acceptable settlement sites within the *A. fimbriatum* bed that they need not seek substrata elsewhere.

Bryozoan larvae released from colonies on *Agarum fimbriatum* must occasionally get transported by currents to locations where settlement on alternate substrata is their only option. In these cases, even if the larvae successfully metamorphosed and were able to initiate colony growth, the chances of them growing to sexual maturity, especially on fronds of fast-growing ephemeral algae like *Macrocystis integrifolia* and *Nereocystis luetkeana*, are minimal. Also, almost any alternate algal substrata would have a higher risk of herbivory than *A. fimbriatum*, and may have chemically incompatible substances present on or leaching from the blade surface. For example, in several experiments I conducted using blade tissue from *M. integrifolia*, I observed a large amount of mucous exudate oozing from the blade surface. Although kelp mucus is likely non-toxic to bryozoans, its physical properties create a viscous microenvironment where the small ciliated larvae may become immobilized. From my

personal observations, *A. fimbriatum* releases considerably less mucus than other co-occurring kelps, and its relatively mucus-free surface may facilitate bryozoan settlement.

Even if polyphenolic compounds are wholly or partially responsible for attracting the bryozoan epiphytes of *Agarum fimbriatum* to their host plant, qualitative (i.e., chemical structure) differences in polyphenolics may exist between and within plants, and bryozoan larvae may be induced to settle only by polyphenolics with a particular chemical structure. Because of their highly polar structure, polyphenolics are difficult molecules to isolate and purify, and this barrier has constrained many ecological studies of brown algal polyphenolics (see discussion in Van Alstyne 1988). As the Folin-Denis assay used in this thesis is reported to perform similarly with a variety of brown algal polyphenolics (Steinberg 1988), values for polyphenolic content between algal species or between tissue types of one species may be similar even if polyphenolic structure differs. Polyphenolics found in high concentrations in anchoring structures and reproductive tissues may be structurally different from those in blade tissue, and even subtle differences in chemical structure may be sufficient to attract or repel bryozoan larvae.

Alternatively, whether polyphenolics from different tissues of *Agarum fimbriatum* are even available for detection by larvae of its bryozoan epiphytes may determine if larval settlement induction occurs. In this study, polyphenolics were extracted from algal tissues by homogenizing samples in aqueous methanol. Thus, polyphenolic content, as quantified by the Folin-Denis Assay, was a measure of the total polyphenolics in each tissue sample, but this measure may not necessarily be representative of the polyphenolic levels detectable to settling bryozoan

larvae. For example, although holdfast and stipe tissue regularly contained high concentrations of polyphenolics, they may be neither released from those tissues, nor detectable to larvae from the algal surface. My experiment with polyphenolic leaching showed that, at least from young blade tissue of *A. fimbriatum*, polyphenolics are released into the surrounding seawater. Although this experiment was conducted with disks of tissue excised from whole blades, and hence the release of polyphenolics may have occurred at the cut surface, all tissue disks used for bryozoan settlement experiments were allowed to heal following excision, and larvae nonetheless were able to discriminate phenolic-rich and phenolic-poor tissue choices.

In consideration of the devil's advocate position, might there be another water soluble chemical that not only distinguishes *Agarum fimbriatum* from other macroalgae, but also exhibits an age-specific gradient along blades of *A. fimbriatum*? Certain alginates, polysaccharides found in kelps, are known to be more prevalent in the cell walls of young plant cells, and less so in those of mature cells (Percival 1979). Alginates are not easily extracted from brown algal tissue (Dring 1982), however, so it is unlikely that they would readily leach from kelp tissues into the surrounding seawater where they would be detectable by bryozoan larvae. Even if alginates were detectable at the surface of the kelp blade, they would not likely be present in dissolved form in aqueous extracts of kelp tissue. Because experiments presented here demonstrated bryozoan larval attraction to water soluble components of *A. fimbriatum*, the bioactive compounds inducing larval settlement would have to be soluble in water. Dissolved nutrients and waste products of plant metabolism readily pass through the kelp-seawater interface, and they may be released in

concentrations that differ along a gradient of algal age. Specifically, one would expect higher metabolic activity to be found near the meristem, where the kelp tissue is growing. These dissolved substances would not distinguish *A. fimbriatum* from other kelps, however, so they are unlikely candidates for chemical cues attracting bryozoan larvae. Also, unless bryozoans could obtain some nutritional value from cell wall constituents or nutrients, there would be no selective pressure for their larvae to be attracted to these substances. Cell walls would have to be digested extracellularly in order to extract nutritive value, and nitrogen-based nutrients are the waste products of animal metabolism, and would be more likely to repel than attract bryozoan larvae. Water soluble polyphenolics are not only concentrated in tissues most preferred by bryozoan larvae, but their anti-herbivore properties also provide an adaptive reason for a bryozoan to settle on *A. fimbriatum* tissue.

Polyphenolic compounds in brown algae are manufactured from a phenylalanine precursor, which tends to accumulate in algal tissues when plant growth is constrained by low nutrient levels (Ilvessalo & Tuomi 1989). If accumulating phenylalanine is not used for protein synthesis during growth, then it may be diverted towards phenylpropanoid (i.e., phenolic) synthesis (Ilvessalo & Tuomi 1989). Thus, it seems logical that, in rapidly growing algal tissues such as the meristem, phenylalanine would not likely accumulate in excess. My data on temporal and spatial variation in polyphenolic content in *Agarum fimbriatum* contradict this prediction, however, because polyphenolics were in highest concentration during times of the year when nutrients are abundant (i.e., winter) and in plant tissues with high growth rates. This observed variation seems to indicate that polyphenolic production in *A. fimbriatum* is not merely the

accumulation of metabolic by-products, but rather may be the evolutionary result of selection for anti-herbivore chemical defense. The gradual decline in polyphenolic content with increasing algal age along vegetative blades perhaps could be explained by localized phenolic production at the meristem, followed by gradual degradation or leaching of the compounds from successively older blade tissue. This scenario does not explain, however, elevated polyphenolic content in sporophyll patches, which are produced on mature blade tissue, or in anchoring structures, which are the oldest structures on the plant.

Aside from the obvious correlations between bryozoan distribution, bryozoan larval preference, and polyphenolic gradients, the most compelling evidence that polyphenolics in *Agarum fimbriatum* may be directing the settlement of its epiphytic bryozoans is that (1) water soluble compounds extracted from young blade tissue induced larval settlement, and (2) aqueous extract from young blade tissue had a higher polyphenolic content than similarly prepared extract from old blade tissue. These results support the hypothesis that bryozoan larvae are attracted to young blade tissue of *A. fimbriatum*, not because of its location, texture, or bacterial film, but because they are attracted to water soluble polyphenolics emanating from, or available at the surface of their preferred tissue.

Implications for future work

In order to rule out the possibility that other compounds in *Agarum fimbriatum* exhibiting within-thallus distributions similar to those of polyphenolics might influence bryozoan larval choice, it would be necessary to use more sophisticated chemical techniques, including: (1) isolation and purification of polyphenolic constituents from different algal

tissues, (2) examination of chemical diversity in sympatric related species, (3) larval bioassays with purified chemical products from the host plant and from other algae, and (4) confirmation of the chemical induction response using immunohistological or neurophysiological techniques. Such experimental protocols, similar to those used in identifying the biomedical potential of marine natural products with (see Faulkner 1979), are not only lengthy and expensive, but are also fraught with difficulty and prone to failure at some point along the process. The most efficient use of scientific expertise would be to undertake collaborative ventures between ecologists who identify and characterize chemical relationships between species, and chemists and biochemists who can offer precise information about the structure and probable biosynthetic pathways of the ecologically active chemicals.

A number of studies have identified specific plant-herbivore relationships in which herbivores either consume (Paul & Van Alstyne 1988), or both consume and live upon a chemically defended algal species (Hay et al. 1989, 1990a, b). In some cases, herbivores sequester chemical defenses from their algal food, becoming chemically defended themselves (Paul & Van Alstyne 1988), and in others, simply being associated with a defended plant is sufficient to protect the herbivore from predation (Hay et al. 1989, 1990a, b). Research presented in this thesis is the first to suggest that a non-herbivorous (i.e., not feeding on macroalgae) sessile suspension feeding organism is attracted to, and receives associational protection from a chemically defended plant. Settlement, survival, growth, and reproduction of animal epiphytes on marine plants may be more closely related to biological and chemical properties of their plant substrata than to gradients in physical factors. Sessile invertebrates, particularly those

without true circulatory and respiratory systems, must exchange gases and dissolved substances with their surrounding medium, and if half of that medium is a living plant, then it is not unreasonable to assume that materials may be exchanged between plants and animal epiphytes. Bryozoans growing on marine algae have been shown to uptake dissolved organic carbon from their underlying plant substratum (De Burgh & Fankboner 1978), and kelp tissue has been shown to uptake bryozoan-derived nitrogen (Hurd et al. 1994) and CO_2 (Muñoz et al. 1991). Nothing is known about whether secondary metabolites are exchanged between marine algae and their invertebrate epiphytes, despite a large amount of published information about seaweed-epiphyte interactions (reviewed in Seed & O'Connor 1981, Seed 1986). There is great potential for exploring not only physiological exchange between partners in epiphytic associations, but also the ecological and evolutionary consequences of host plant choice, particularly when the plants are defended against herbivory with secondary metabolites.

Although there have been some excellent, comprehensive studies conducted on the biogeography (Estes & Steinberg 1988, Steinberg 1989, Van Alstyne & Paul 1990, Targett et al. 1992,), efficacy (Steinberg 1985, 1988, Steinberg & van Altena 1992), and differential allocation (Steinberg 1984a, Pfister 1992) of polyphenolic compounds in brown algae, there still remain critical unanswered questions about how and why polyphenolics are synthesized. The controversy that polyphenolics may be a carbon sink fo. nitrogen-limited algae rather than a response to grazing pressure (Ilvessalo & Tuomi 1989, Tuomi et al. 1989) must be addressed further if we are to understand their role in the ecology and evolution of marine brown algae.

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