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Sykes, Paul Frederick, Ph.D.
University of California, San Diego, 1991
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UNIVERSITY OF CALIFORNIA, SAN DIEGO

PHYSIOLOGICAL-ECOLOGY AND CHEMICAL-ECOLOGY

OF

COPEPOD-DINOFLAGELLATE INTERACTIONS

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Marine Biology

by

Paul Frederick Sykes

Committee in Charge:

Dr. Mark E. Huntley, Chairman
Professor Farooq Azam
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1991
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Chairman

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1991
To Linda Sykes:
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* * *

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ABSTRACT OF THE DISSERTATION

Physiological-Ecology and Chemical-Ecology of Copepod-Dinoflagellate Interactions

by

Paul Frederick Sykes

Doctor of Philosophy in Marine Biology

University of California, San Diego, 1991

Dr. Mark E. Huntley, Chairman

Copepods are the most important metazoan grazers of phytoplankton in the sea. In order to more fully understand the flow of energy from phytoplankton through copepods (and beyond), it is necessary to know what factors modify their feeding. Contrary to the classical paradigm of passive, mechanical feeding by copepods, copepods are quite selective in their preferred prey. Some of this selectivity appears to be mediated by chemicals produced by their phytoplankton prey. Knowing the time required for selectivity to occur and the persistence of that selectivity should give us a better understanding of copepod sensory and decision-making capabilities.

Some members of the marine phytoplankton, particularly some dinoflagellates, produce toxic (or noxious) chemicals. The biosynthesis, biochemical modes of action, and structures of some of these chemicals are known, but virtually nothing is known about the natural function of these toxins. One of the likely functions is chemical defense against herbivores. Chemically-defended dinoflagellates may be selectively avoided, and
preferred cells selectively ingested. If true, then chemical defense may provide an ecological advantage over co-occurring undefended cells. Otherwise, all cells may be equally rejected or accepted, thus conferring no advantage on the defended cell.

The goal of my doctoral dissertation research was to investigate the physiological and behavioral aspects of the feeding biology of *Calanus pacificus* vis a vis the presence of noxious dinoflagellates. In addition, I studied the significance of chemical defense to the noxious dinoflagellate *Gonyaulax grindleyi*.
CHAPTER 1

INTRODUCTION
Copepod Feeding Biology and Physiological Ecology

Marine herbivorous copepods are the most important metazoan consumers of phytoplankton in the ocean (Vinogradov 1970). Because of this, copepods are an important link in the food web. Assimilated carbon and nitrogen are passed up the trophic ladder (Conover 1978), fecal pellets transport primary production to the benthos (or are at least partially utilized by the microbial food web), and the products of excretion and "sloppy feeding" may be utilized by microbes as well (Eppley et al. 1981). Because of this significant, central position in the food web, those parameters which modify grazing by copepods on phytoplankton are of great importance if we are to understand oceanic trophodynamics.

Cannon (1928) described the water currents produced by copepods placed in drops of seawater. He stated that circular water currents produced by the second antennae swept particles into the mouthparts where the setae acted like a sieve to catch the particles. Although his experimental design caused him to give an incorrect description of the water flow, it was the first concerted effort to investigate the mechanism by which copepods feed.

Until the late 1970s, most researchers of copepod feeding biology believed that copepods were passive, automatic, strictly mechanical filter feeders of any and all particles of a specific size class. Mullin (1963) showed that the filtration rate was inversely related to the cell concentration, duration of the experiment, and culture age. The explanation for these results was that at low cell concentrations the copepod beats its mouthparts faster to gain more food; at increased cell abundance the mouthparts do not need to work so hard to gain the same amount of cells; at some high cell concentration, the ingestion rate is maximal and the amount of water swept clear to gain this
maximal amount of food/time is minimal. With too long an experimental
duration copepods might become satiated and cease or slow their feeding rate.
Mullin (1963) suggested that with culture age, inhibitory chemicals may
affect the filtering rates of copepods.

Boyd (1976) coined the term "leaky sieve" to explain the mechanics of
grazing by copepods. He stated that some cells impinging on the setules of
the mouthparts could bend them enough to allow the cells to slip past and
that the more flexible cells such as unarmored flagellates should be able to
slip through the setules. In the model of Lam and Frost (1976), only cell size
and cell concentration determine the filtration rate of copepods. Nival and
Nival (1976) explained that size selection was a mechanical consequence of
variable retention efficiencies of the setae and setules of the second maxillae.
Frost (1977) described copepods as passive grazers without any behavioral
component to their feeding on particles.

Ample evidence exists, however, that copepods are exquisitely
equipped sensorally, physiologically, and behaviorally to feed selectively on
phytoplankton. Lowndes (1935), based on his visual observations of feeding
copepods, and analysis of fecal pellets and mouthpart anatomy, strongly
asserted that copepods were selective feeders. In a comprehensive review of
the literature, Marshall and Orr (1955) gave numerous indications of potential
selective feeding including one that may be relevant to my research here.
They stated that Calanus rarely eats the dinoflagellate Ceratium, which is a
relatively common "bloomer" off the Southern California coast (Allen 1941).
Mullin (1963) suggested that factors other than cell size or concentration
might be important and should be investigated. Urry (1965) demonstrated
that not all phytoplankton cells were good food for Pseudocalanus elongatus
and that size could not always account for this finding. He concluded that some of the cells he investigated might be toxic. In his book on suspension feeding, Jørgensen (1966) described direct seizing of large phytoplankton cells in addition to filter feeding (this presumes a sensory capability not allowed by Frost (1977)), selectivity for living versus dead cells, and a preference for diatoms.

The first demonstration of selective feeding based solely on the chemical nature of the food comes from the work by Poulet and Marsot (1978) who fed copepods nylon microcapsules containing either phytoplankton homogenates or seawater controls. The copepods significantly preferred the microcapsules containing homogenates, proving that copepods were capable of chemically mediated, active selection of food items. In a study of the selection capabilities of Acartia clausi, Donaghay and Small (1979) showed that the copepods would avoid polystyrene beads and eat diatoms even if the beads in the diatom/bead mixture were larger than the cells.

The authors also showed that the preferred size of the diatom selected depended upon the preconditioning environment of the copepods (i.e. prefed large or small diatoms). Similarly, Runge (1980) found that the clearance rates of Calanus pacificus females depended on their preconditioning. Starved copepods fed at higher rates than fed ones and filtering rates of copepods varied with the season (highest in June and July). Furthermore, he found that within the concentration range examined (23–231 µg C/l), the filtration rates increased with cell size regardless of the concentration.

Copepods may select preferred foods on the basis of their nitrogen or protein content. Houde and Roman (1987) found that copepods fed growing
and senescent diatoms, dinoflagellates, or coccolithophores preferred the growing diatoms (containing the highest organic nitrogen concentration). Cowles et al. (1988) demonstrated that Acartia tonsa preferred exponential phase Thalassiosira weisflogii over stationary phase T. weisflogii. They also determined that the preference for faster growing cells was strongly correlated to the nitrogen content of the cells.

While much of "food quality" may be related to the nutritional value of the cell, there are cells (particularly some dinoflagellates) that may have negative value to zooplankton predators due to toxins or noxious chemicals. That the presence of one cell could reduce the grazing by a copepod on another cell was demonstrated by Tomas and Deason (1981) who showed that two species of Acartia experienced reduced feeding rates on Dunaliella tertiolecta in the presence of Olisthodiscus luteus. Huntley et al. (1983) found that Calanus pacificus females discriminated between similarly sized (and shaped) cells and beads, and more importantly, that the ingestion rates of copepods on similarly sized cells could be different. Copepods ingested Gyrodinium dorsum faster than Scripsiella trochoidea=[Peridinium trochoideum] which was fed upon at approximately the same rate as beads.

Huntley et al. 1986 found that Calanus pacificus and Paracalanus parvus females fed poorly on certain dinoflagellates in comparison to other similarly sized (or smaller) cells. Protophormalex tamesensis=[Gonyaulax tamerensis], Gonyaulax grindleyi, Scripsiella trochoidea, and Ptychodiscus brevis were rejected by these copepods. Starvation of Calanus pacificus did not induce them to feed on the noxious cells at higher rates and animals maintained in G. grindleyi suffered a higher mortality rate than those maintained in a suspension of readily eaten cells (Gyrodinium resplendens).
The animals maintained in *G. grindleyi* also failed to produce eggs even though surrounded by carbon-rich food. Copepods presented with mixtures of accepted and rejected cells experienced reduced clearance rates on the accepted cells when the ratio of noxious/readily eaten cells increased. Filtrates of *G. grindleyi* culture medium inhibited feeding on preferred cells, indicating that the rejection of this species is chemically mediated. With the findings of the above authors, that copepods were, in fact, actively sensing their environment and capable of selective feeding, new questions concerning the mechanism of feeding and behavior arose that are still important today.

With the use of high-speed cinematography, the actual movements involved in particle capture and selection are now fairly well known. Alcaraz *et al.* (1980) described the technique of glueing copepods to dog hairs and subsequently showed that the mouthparts of copepods do not act as sieves so much as paddles; the authors also described the handling of particles by copepods. Other authors (Koehl and Strickler 1981, Paffenholzer *et al.* 1982, and Price *et al.* 1983) provided more detailed information on the same subject. They showed that, contrary to Cannon (1928), the flow of water about the mouthparts of a copepod was not circular, but linear; that copepods generally do not touch the particles, but rather, process parcels of water containing cells; that copepods can capture cells by using the endites of the mouthparts in a so-called "chopstick" fashion; and that copepods occasionally reject captured objects. Price and Paffenholzer (1984) and Vanderploeg and Paffenholzer (1985), using the marine copepod *Eucalanus elongatus* and the freshwater copepod *Diaptomus sicilis* respectively, observed both active and "passive" filtration modes depending on the size of the cells presented. In the active mode, large cells were sought out, captured and individually processed,
whereas in the passive mode the positioning of the mouthparts allowed the entrained cells to pass by the mouth and be eaten.

Active searching and processing of food items presupposes a suite of sensory apparatus capable of detecting and analyzing particulate matter. Poulet and Ouellet (1982) demonstrated that the copepods Eurytemora herdmani and Acartia hudsonica were most stimulated by the amino acid leucine, and other authors have shown that copepods have chemosensory apparatus on their antennae (Ong 1969, Friedman and Strickler 1975, and Gill 1986). Further evidence for the chemoreceptive abilities of copepods comes from Gill and Harris (1987) who observed the first maxillae of Calanus helogolandicus and Temora longicornis. The authors found that both copepods responded more positively to cells and extracts of diatoms than to cells and extracts of dinoflagellates. Furthermore, Calanus responded to Scrippsiella trochoidea as well as it did to filtered seawater, and Temora responded in a similar fashion to S. trochoidea and Protogonyaulax tamarenis, but demonstrated a lesser response than to seawater when presented with Gymnodinium aureoleum.

Along with chemoreception, mechanoreception appears to play a major role in the detection of potential food items. Gill (1986) located and elucidated the structure of mechanoreceptors in Temora longicornis and Léguer-Visser et al. (1986) used a mathematical model to show that mechanoreception should be an important factor in determining the location, size, and swimming speed of prey.

If, as indicated above, copepods are chemosensory, active grazers, are they capable of moving to "greener" pastures or avoiding those of poor quality? The evidence to this date is equivocal at best. Bird and Kitting
(1982) used a miniature water column in the laboratory to show that *Temora turbinata* would migrate with its dinoflagellate food. Additionally, Huntley *et al.* (1982) and Fiedler (1982) demonstrated in field studies that copepods avoided monospecific blooms of the dinoflagellates *Gymnodinium flavum* and *G. splendens*, respectively. This last report is curious in that *G. splendens* is readily eaten by *Calanus pacificus* (Huntley *et al.* 1986), and supports rapid development rates and high survival in *Calanus* nauplii (Huntley *et al.*, 1987). Furthermore, *C. pacificus* will not avoid a proven noxious dinoflagellate, *Gonyaulax grindleyi* (Huntley *et al.* 1986). Uye (1986) demonstrated, using a simulated red-tide, that *Chattonella antiqua* was readily grazed by copepods.

Copepod feeding biology can be summarized by stating that marine herbivorous copepods are capable of sensing and responding to chemical and mechanical stimuli in their environment. They actively capture and select particles based on mechanical and chemical stimuli (often without regard to cell size), and do not appear to feed in the thickest parts of phytoplankton blooms (for unknown reasons).

How does *Calanus pacificus* reject noxious dinoflagellates, such as *Gonyaulax grindleyi*? Are these copepods immediately selective, or do they have to eat some cells first? Does *C. pacificus* learn to not ingest *G. grindleyi*, or are the copepods incapacitated? How long do these effects last? How does the long-term presence of *G. grindleyi* affect the survival and fecundity of *Calanus pacificus*?

In the following chapters, I will present the results of direct visual observations of *Calanus pacificus* feeding on several toxic or noxious dinoflagellates. From these observations, as well as the results of more classical feeding experiments, I will describe the behavioral and physiological
responses of *C. pacificus* to a variety of dinoflagellates.

**Chemical Ecology and Chemical Defense**

Much is known about interactions of terrestrial herbivores with plant secondary metabolites, which function primarily to defend against grazing. What has been learned in that field has had a major impact on theories of optimal foraging, animal behavior, and co-evolution. It has led to the development of entirely new areas of research such as optimal defense theory and to an explosive development in ecological biochemistry.

Marine and terrestrial environments are so fundamentally different that many of the generalities which apply to defense mechanisms of terrestrial plants may not hold true for marine plants. Unicellular marine plants are challenged by predators and environmental circumstances that are peculiar to the marine environment.

Secondary metabolites from some marine algae are well known both structurally and functionally. The following examples illustrate the wide variety of functions that secondary metabolites may have.

*Prymnesium parvum*, a eukaryotic chrysophyte common in fish ponds, induces a broad spectrum of effects on target organisms, primarily fish (Shilo 1981). Extracts and purified toxins show cytotoxic, hemolytic, and neurotoxic effects, and blooms of this alga are responsible for fish kills in ponds and bays. The toxins are produced and released into the medium in high quantity only if the phosphate concentration is low. Because of their structure, and the fact that very specific environmental conditions are needed to cause toxin production, "prymnesins" are believed to be membrane precursors that are oversynthesized and released under the right conditions. As a result, the toxins of *P. parvum* are not believed to provide any ecological
advantage to the alga.

*Prorocentrum minimum* produces the antibiotic β-diketone in culture at the cessation of growth (Trick *et al.* 1981 and Trick *et al.* 1984). The authors stated that the compound is not a photodestruction product of carotenoids. Andersen *et al.* (1980), however, determined the structure of the molecule (1-(2,6,6-trimethyl-4-hydroxycyclohexenyl)-1,3-butanedione), and further determined that its structure is consistent with a carotenoid (or specifically, zeaxanthin) origin. The norcarotenoids (carotenoids with a methyl or CH$_2$ group missing) were implicated in the inhibition of the bacteria *Chromobacterium lividum* and *Arthrobacter* sp. in the laboratory. The compounds were noncompetitive inhibitors of glucose uptake and respiration in these bacteria (Reichardt 1981). It appears, then, that some marine phytoplankters produce compounds, most being breakdown products of primary metabolites, that inhibit the growth of bacteria. The importance of this is that phytoplankton cells are capable of growing without bacteria attaching to them and fouling their surfaces. As most of the inhibitory compounds are diluted as they disperse away from the phytoplankter, the bacteria can grow near phytoplankton and benefit from leaking organic compounds (eg. amino acids). The phytoplankton benefit by not being fouled by bacteria, and, hypothetically, by encouraging the growth of bacteria at a distance, which in turn can remineralize phosphorous and other elements needed by the phytoplankton (Azam and Ammerman 1984). This hypothesis mainly applies to oligotrophic waters.

*Pandorina morum* is a volvocoid, colonial, marine, green alga, with an allelopathic compound(s). Much is known about the toxicity, the mode of action of the toxin(s), and its/their possible function, but the structure of the
compound(s) has not been elucidated. In a study to determine the allelopathic capabilities of different volvocoid green algae (using culture filtrates), Harris (1971a) found that Volvox globator was the most sensitive to allelopathic compounds and that P. morum produced compounds that were most inhibitory to growth. In other experiments, Harris (1971b) found that oxygen evolution in V. globator was reduced by 65% after 1 hour exposure to P. morum culture filtrate and 91% after 12 hours exposure. In this study respiration rate was unaffected. Harris and Caldwell (1974) found that an extract of the alga produced a 49% reduction in respiration of V. globator after 10 min exposure. Furthermore, they determined that the extract inhibited the light reaction of photosynthesis in Volvox and isolated spinach chloroplasts. The mode of action of the extract is to uncouple electron flow in photosystem II. It also effectively uncouples electron flow in the electron transport chain of white potato tuber mitochondria (Patterson et al. 1979).

While these few examples are well understood, little is known about antiherbivore compounds from marine phytoplankton. Most of the research in this area has been done by those working with benthic macroalgae.

The number of marine benthic macroalgae for which compounds with antiherbivore activity is known is too large to completely relate here, but the marine macroalgae are a source of a myriad of novel secondary compounds as the following examples should illustrate.

Tropical green algae, and some of their temperate relatives contain potent antifeedants. McConnell et al. (1982) found that the sea urchin Lytechinus variegatus was inhibited by "cymopol", a monoterpenel bromohydroquinone from the algae Cymopolia barbata and by "caulerpenyne", an oxygenated sesquiterpenoid from Caulerpa prolifera. The sea urchin was
not inhibited by other Caulerpa species. Paul and Fenical (1983) isolated "halimedatrial", a diterpenoid trialdehyde of extreme bioactivity. This compound inhibits the growth of marine bacteria and fungi, stops sea urchin egg development, interrupts sea urchin sperm motility, kills fish at doses of 1 \( \mu g/ml \), and is highly inhibitory to feeding by fish. Paul and Fenical (1986) reported that, of the forty tropical members of the order Caulerpales they investigated, nearly all contained toxic compounds (linear diterpenoids with bisenol acetate functional groups). They found that the degree of chemical defense was strongly correlated to regional herbivore pressure.

Some species of marine brown algae are also chemically defended. Geiselman et al. (1981) demonstrated that the polyphenols (molecular weights of \(<30,000\) to \(>300,000\)) from the algae Fucus vesiculosus and Ascophyllum nodosum inhibited feeding by the marine snail Littorina littorea. The polyphenolic metabolites of Alaria marginata also inhibit predation by snails (Steinberg 1984). Furthermore, the compounds are concentrated in the sporophylls (reproductive parts), thus concentrating the antiherbivore properties in the most "valuable" regions of the plant.

The red algae are perhaps the most chemically rich group of marine macroalgae. Toxins produced by this group include 1, 3, 7, and 15 carbon haloacetogenins, linear and cyclic terpenoids, many with a high degree of halogenation (unknown in terrestrial plants), and many other classes of secondary metabolites (Fenical 1978; Faulkner 1984). Laurencia and Asparagopsis are two strongly chemically defended red algae. Among the many compounds that Laurencia obtusa produces, "elatol", a bromochamigrene sesquiterpene, was found to be lethal to damselfish at concentrations of 5–8 \( \mu g/ml \) (Norris and Fenical 1982). Asparagopsis
*taxiformis* is apparently avoided by all fish - not surprising considering it contains "bromoform", an exceedingly toxic substance (Norris and Fenical 1982).

What chemical evidence exists for the presence of antiherbivore-active compounds in phytoplankton? Among the freshwater phytoplankton, the cyanobacterium, *Microcystis aeruginosa*, has been shown to contain a variety of toxins with differing effects on daphnids (Nizan *et al.* 1986). While the exact nature of the antidaphnid toxins are still unknown, they are different than the previously isolated fractions that are known to kill mice. Lampert (1981) found that *Daphnia pulec*aria was severely affected by *M. aeruginosa*; 100% of the animals were killed in two days whereas only 50% of the animals were killed in 5-6 days by starvation.

*Chlamydomonas reinhardtii* contains fatty acids which may reduce the growth of other algae and which may have lethal effects on mixed copepod assemblages at doses as low as 10 ppm (Spruell 1984).

Among the marine phytoplankton, some cells are known to have negative effects on the feeding rates and/or survival of copepods. Clones of *Gonyaulax tamerensis* induce greatly different clearance rates in copepods (Ives 1985), suggesting that the antifeedant property must be a chemical. Whole cells and filtrates of cultures of *Gonyaulax grindleyi* caused a reduction in the clearance rate of *Calanus pacificus*; cell homogenate has no effect (Huntley *et al.* 1986). Under constant exposure, the copepods suffer high mortality and zero fecundity. First-feeding nauplii larvae of *Calanus pacificus* had reduced clearance rates and high mortalities in the presence of *Amphidinium carteri, Glenodinium sp., Gonyaulax grindleyi,* and *Pychodiscus brevis.* These reduced clearance rates were clearly not correlated to size,
carbon content, shape (all cells were approximately spherical), or texture (presence or absence of thecae) (Huntley et al., 1987).

Chemical defense, therefore, is to be expected in the planktonic environment. What is the nature of chemical defense in marine dinoflagellates, and to what extent does chemical defense afford these cell an adaptive advantage? In addition to the studies of the responses of Calanus pacificus to noxious dinoflagellates (Chapters 2 and 3), I have studied the effects of cell age and the presence or absence of bacteria on the noxious quality of Gonyaulax grindleyi. Furthermore, I will present data on the significance of chemical defense for G. grindleyi and co-occurring dinoflagellates (Chapter 4).
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CHAPTER 2

ACUTE PHYSIOLOGICAL REACTIONS OF Calanus pacificus

TO SELECTED DINOFLAGELLATES:

DIRECT OBSERVATIONS
Acute physiological reactions of *Calanus pacificus* to selected dinoflagellates: direct observations

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Abstract

*Calanus pacificus* (Copepoda: Calanoida) females were collected off the California (USA) coast from November 1984–April 1985. A video system was used to observe and record the behavior of restrained individual females presented with a variety of dinoflagellate prey. Two species, *Gonyaulax grindlayi* and *Pychodiscus brevis*, elicited acute physiological reactions. In 40°C of the trials (*n* = 10), copepods fed *G. grindlayi* regurgitated after 45 to 120 min and, in nearly all cases, did not maintain full guts. Copepods in the presence of *P. brevis* exhibited rapid heart rate and loss of motor control. *Scispspellia trochoidea* elicited an intermediate response by *C. pacificus*. The copepods occasionally displayed mouthpart twitching or failure to maintain gut fullness. *Olschadesis latea* elicited no unusual behavior in an intermediate temporal range (sec-hours), although the mouthpart movements appeared different than in copepods fed *G. recondita* (used as control). Placing the copepods in *G. recondita* suspension restored normal feeding behavior in all cases.

Introduction

Free-living, herbivorous, planktonic copepods are discriminant feeders. The first evidence for selective feeding by copepods was presented by Poulet and Marot (1978), who showed that copepods ingested seawater-filled microcapsules at a much lower rate than phytoplankton homogenate-filled microcapsules. Since then many studies have shown that copepods are discriminant feeders (Donaghy and Smill, 1979; Fernández, 1979; Bartram, 1980; Huntley, 1982; Huntley et al., 1983, 1986). All the above researchers used conventional grazing experiments which integrate feeding behavior over a period of hours to days.

In the latest study, Huntley et al. (1986) showed that *Calanus pacificus* females rejected (greatly reduced filtration rates) five of thirteen dinoflagellate species tested. One of them, *Gonyaulax grindlayi* (previously *Proterocentrum reticulatum*), is much larger (45 μm diam) than *Gyrodinium recondita* (30 μm diam), which was readily eaten. Starvation did not increase the ingestion rate, and copepods maintained for nearly one month in *Gonyaulax grindlayi* suspension suffered significantly greater mortality and loss of egg production even though the copepods were offered a high concentration of carbon-rich cells. Grazing experiments with filtrates of *G. grindlayi* showed that the suppression of feeding by *C. pacificus* was chemically mediated. We became interested in observing the behavioral or physiological mechanisms which caused these results. Our question now is: What are the direct consequences of eating undesirable/toxic cells?

Using high-speed cinematography, Alcaraz et al. (1980), Koehl and Steckler (1981), and Price and Paffenholzer (1984), have illustrated the selective capabilities of copepods. Copepods handle and manipulate particles, rejecting some. The behaviors these researchers described were manifested for milliseconds to a few seconds.

Our study shows that direct visual observations can be used to answer questions about the long-term physiological state of copepods fed “undesirable” food. We find that copepods elicit behaviors in response to their food in an intermediate temporal range (seconds to hours) that has not been explored previously.

Materials and methods

We collected copepods approximately 2 to 5 km west of the Scripps Pier (32°N; 117°W) from 1 November 1984 to 18 April 1985 with 0.5 or 1 m zooplankton nets (205 μm mesh) having protected cod-ends (Reeve, 1981). Copepods were immediately transferred to 8-liter buckets containing seawater. The buckets were covered and brought back to the laboratory within 30 to 60 min.

*Calanus pacificus* females were sorted under a dissecting microscope using a “wide-mouth” Pasteur pipette, and
were placed into filtered seawater (FSW) within several hours of being brought to the laboratory. Individuals to be used the next day were starved overnight under dim light at 19°C. Copepods not to be used for several days were fed a dense suspension of *Gyrodinium resplicens* and then starved for 12 to 72 h before use.

We selected active, healthy (no obvious damage) *Calanus pacificus* females for our experiments and placed them on a fine-mesh platform stage that had a FSW-soaked tissue underneath to keep the ventral side of the copepod moist. We glued a fine glass wire to the dorsal side of the copepod's cephalothorax with a minimal amount (~1 mm) of nearly dry (gummy consistency) cyanoacrylate glue. Copepods survived very well on glass wires if fully capable of moving their mouthparts. They feed on *Gyrodinium resplicens* and can lay viable eggs. One copepod lived for several weeks on its glass wire, approximating its natural lifespan (Huntley and Brooks, 1982). After gluing, the copepods were allowed to rest for periods ranging from 1 to 24 h (if not previously starved) before running the video experiments.

Our video system is a Sony 1800 camera (Sony Corporation of America) mounted on a Wild M-3 dissecting microscope (Fig. 1). Images were taped on a Sony 4800 tape deck or routed through the deck to a monitor for closed-circuit viewing. Lighting was provided by a “Fiber-Lite” optical fiber illuminator (Delan-Jenner Industries Inc.). In order to view the copepod from the side and at the same time have the copepod in an upright feeding position and – perhaps more importantly – to keep the fluid flow past the copepod parallel to the long axis of the chamber, we viewed the copepod through a mirror held at 45° to the axis of the microscope.

Copepods were held by their glass wires in a 3 ml spectrophotometer cuvette or a 100 ml chamber containing either FSW, *Gyrodinium resplicens* or one of the test cells (*Gonvaulax grinulata*, *Psychodiscus brevis*, *Scraptiella trochoidea*, or *Olistodiscus luteus*). After allowing about 15 min for the copepod to adapt to its environment, we observed its feeding behavior and monitored fecal pellet production, heart rate, and mouthpart movements (MPM).

Timing of fecal pellet production was determined to within approximately 30 s by simply noting the time when a fecal pellet was passed. Heart rate was measured as beats per minute (bpm) by counting beats and dividing by the stopwatch. MPM and interval durations were measured with a stopwatch randomly selected portions of tape. We were unable to measure MPM or interval durations near the time required to press a stopwatch button twice (~0.15 s), so we ignored them. Very brief MPM and short interval times were mainly observed when *Calanus pacificus* females were presented with *Psychodiscus brevis* and *Scraptiella trochoidea*; thus, the largest errors in the reported MPM and interval times are somewhat underestimated.

**Results**

Table 1 shows our collection data and experimental conditions for the video observations reported in this paper. In nearly all cases, *Calanus pacificus* fed *Gyrodinium resplicens* rapidly filled their guts and maintained full guts throughout the observation period (Table 1). They generally had a slow and intermittent heartbeat (about 240 bpm), although periodic bursts of activity could cause short periods of constant heart beating. These “normal” behaviors were consistent with our previous experience with *C. pacificus* females feeding on *G. resplicens* in conventional graze experiments (Huntley et al., 1986). To test long-term gutfulness of restrained *C. pacificus* females, we placed a copepod in a 100 ml chamber with a suspension of *G. resplicens* (above the level to allow maximum ingestion) and two copepods in a similar chamber with an equally high concentration of *Gonvaulax grinulata* and observed them then 18 h later. The individual in the *Gyrodinium resplicens* suspension had a full gut and the two in the *Gonvaulax grinulata* had empty guts. These results indicated that the feeding behavior and physiological status of *C. pacificus* feeding on *Gyrodinium resplicens* was a good standard with which to compare the effects of other test cells.

As Frost (1977) demonstrated, we found that *Calanus pacificus* females ingested beads more readily when living cells, *Gyrodinium resplicens* in our experiment, were present.

Compared with its “normal” reaction to *Gyrodinium resplicens*, the reaction of *Calanus pacificus* to *Gonvaulax grinulata* and *Psychodiscus brevis* was radically different (Table 1). In the ten observations we made of *C. pacificus* feeding on *G. grinulata*, the copepods regurgitated their guts' contents after 45 to 120 min in four cases, made strong reverse peristaltic contractions (“retching”) but did not regurgitate in two cases, and ate few cells in one case (maybe an injured copepod – no *Gyrodinium resplicens*...
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<td>9.8 ± 0.6 (6)</td>
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*Coprod from previous in P. brevis suspension
Very brief observation
Coprod feeding rapidly
Motor twitching, coprod previously in S. rexohaeus suspension
Only a few beads ingested
Beads ingested after G. resplendens added to chamber
Very few cells ingested
Observation in immediate sequence
Ehren mouthparts movement, but no twitching

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comparison using this individual, No. 7). In the remaining three cases, as well as in six of the previously mentioned cases, the copepods failed to maintain full guts and, in many cases, ceased feeding entirely.

*Calanus pacificus* females showed acute reactions to the presence of high concentrations (800 to 1,000 μg C l⁻¹) of *Psychrodictus brevis*. This was not unexpected, because *P. brevis* is known to produce two neurotoxins (Steidinger, 1983). After as little as 30 min, the mouthpart movements (MPM) of the copepod became erratic (i.e., the maxilliped swung through a much larger arc than when feeding on any other cell). This was accompanied by nervous twitching of the mouthparts and/or the distal setae and an accelerated (400± bpm) and constant heartbeat (Table 1).

When the copepod was transferred to a *Gryhdinum resplendens* suspension, it invariably "calmed down"; the twitching and erratic MPM stopped in about 15 min. The racing heartbeat sometimes took much longer to slow down to the more frequently seen slow and intermittent one.

Three copepods fed *Scaphoeca trochoidea* on four occasions reacted in a manner which was intermediate to the responses caused by *Gonystellax grindleyi* and *Psychrodictus brevis*. In the first observation (Copepod No. 14), gut fullness was maintained, the heart rate was slow and intermittent, but the copepod displayed exaggerated MPM. In the second observation, the same individual failed to maintain a full gut, and suffered from minor mouthpart twitching. The copepod (No. 15) in the third observation failed to maintain a full gut; all other behavior appeared normal. In contrast, the copepod (No. 17) in the fourth observation failed to maintain a full gut, had a rapid constant heartbeat, and made strong twitching movements in its gut (Table 1).

*Olyrtohdicus lutus* was fed to a *Calanus pacificus* female on one occasion with results similar to those observed when fed *Gryhdinum resplendens*. The MPM looked more like those seen when copepods were fed *Psychrodictus brevis*, except that we observed no twitching. The details of this different behavior cannot be adequately described through the use of video observations, but might be observable via high-speed cinematography. The copepod packed its gut and produced fecal pellets with no signs of distress.

We observed that, regardless of the food offered, fecal pellet production was highly predictable (Fig. 2). Once a copepod had filled its gut and passed a few fecal pellets it continued to produce them at a rate of about 10 per hour. In four cases, the observed fecal pellet production rate was about one-half of that in the other cases. In two of these the copepod had been used in an experiment the previous day, when the fecal pellet production rate was higher (about 10 per hour). Perhaps the lowered rate was an indication of decreasing copepod "health". Even when the copepod cleared its gut due to regurgitation or cessation of feeding (i.e., after ingesting *Gonyaulax grindleyi*), hind-gut contractions and production of mucous fecal membranes continued for some time. Our observations usually continued for no more than 30 min after the gut had been cleared.

![Fig. 2. *Calanus pacificus*. Fecal-pellet production by females fed *Gryhdinum resplendens* (Gr), *Gonyaulax grindleyi* (Gg), *Scaphoeca trochoidea* (St) and *Olyrtohdicus lutus* (Ol). Fecal-pellet (FP) production was nearly always about 10 per hour (10.2±2.6 (SE) FP h⁻¹; n=4) regardless of food offered. In some cases, fecal-pellet production was roughly half (4.7±1.2 (SE) FP h⁻¹; n=3) of the pool of four observations. No. of time measurements = no. of time measurements (total no. of fecal pellets - 1)

![Fig. 3. *Calanus pacificus*. Mouthpart movement (MPM) durations and durations of intervals between mouthpart movements in females fed *Gryhdinum resplendens* (Gr), *Gonyaulax grindleyi* (Gg), *Psychrodictus brevis* (Pb) and *Scaphoeca trochoidea* (St). Most noticeable difference between MPM durations in different cell solutions is that standard deviation of MPM duration is greater for copepods in *Psychrodictus brevis* than for other tests; arrows indicate that error bars are off scale. FSW: filtered seawater.
used by a "microbial-loop" food web (Fuhrman and Azam, 1982; Azam et al., 1983). Furthermore, copepods that regurgitate, or otherwise fail to maintain full guts, will not produce as many fecal pellets, reducing the flow of energy to deeper waters and the benthos. The degree to which regurgitation plays a role in the energy budget of copepods may be minor, but has yet to be determined.

_Calanus pacificus_ females presented with _Psychodiscus brevis_ showed a different acute reaction. Heart rate increased to over 400 bp/min and became constant. Mouthpart movements became erratic, accompanied by twiching. Some of the movements such as the broader arc of MPM may be due to the copepod's attempt to feed on small cells. Neurotoxins may be used by _P. brevis_ to reduce predation and allow the cells to form "red-tide" blooms (Huntley et al., 1986).

Huntley and Ciminiello (unpublished data) have shown that non-feeding _Calanus pacificus_ nauplii twitch constantly when maintained in the presence of _Psychodiscus brevis_, and suffer 100% mortality after three days (beginning of the first feeding stage).

The copepod _Oithonidus luescens_ (another small cell, 19 μm) packed its gut as it does when fed _Gyrodinium resplendens_. This result is interesting; considering the findings of others that _O. athidius_ is an unsatisfactory food for a variety of organisms (Fretter and Montgomery, 1968; Blaxter, 1969; Choiyaputna and Hirayama, 1978; Verity and Stoecker, 1982) including two species of the copepod _Acartiia_ (Tomas and Deason, 1981). The copepod's mouthparts were moving as with _Psychodiscus brevis_ (without the twiching) when the copepod was presented with _O. luescens_, but we were not able to analyze the movements further. We suspect, however, that the difference in MPM may be due to the copepod's attempt to eat a small cell. High-speed cinematography might clear up this question (Price and Paffenhofer, 1986). Additionally, conventional grazing experiments will help determine whether _O. luescens_ is an unsatisfactory or toxic cell for _Calanus pacificus_.

All visible consequences of eating toxic cells (_Gonyaulax grimaldi_ and _Psychodiscus brevis_) disappeared shortly after the copepod was returned to FSW or a suspension of _Gyrodinium resplendens_.

Most of the data presented here points out the importance of long-term visual observations of copepod feeding behavior. Physiological or behavioral events that take place over relatively long periods of time (maintenance of gut fullness, fecal pellet production, mouthpart twichings) or rapidly occurring, unpredictable events (regurgitation) might not be monitored easily or economically by high-speed cinematography. Conventional grazing experiments either do not detect the events or integrate the data without allowing analysis of them. We believe that the recording of visual observations of restrained copepods provides a useful tool for analyzing some of the otherwise unpredictable and long-term physiological and behavioral mechanisms of feeding. This technique should allow a better understanding of the complex behavior of many marine zooplankters.
Acknowledgments. We gratefully thank Mr. V. Marin, Mr. S. Rohan, Mr. C. J. Toni, and Mr. D. Gross for assistance in collecting and sorting copepods and for many productive discussions. Thanks go to Mr. D. Lee for helping us set up the video system. We also thank Mr. F. Crowe and his staff for their artistic talents. Many thanks are due Dr. D. Anderson and an anonymous reviewer who critiqued our manuscript. Support for this work was provided in part by the National Science Foundation, Grant No. OCE-8215903 and in part by NOAA, National Sea Grant College Program, Department of Commerce, under Grant No. NA85AA-D-SG140, Project No. R/F-106.

Literature cited


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P.F. Sykes and M.E. Huntley: Reactions of a copepod to dinoflagellates
This reprint of published material is the result of experiments designed and performed by the primary author. Dr. Mark E. Huntley provided the initial idea of observing copepods through the video system, the laboratory space and equipment, and invaluable assistance in editing the manuscript.
CHAPTER 3

IT MUST HAVE BEEN SOMETHING I ATE:

REACTIONS OF *Calanus pacificus*

TO THE NOXIOUS DINOF Lagellate

*Gonyaulax grindleyi*
ABSTRACT

*Calanus pacificus* is a selective particle grazer, but what are the dynamics of selectivity *vis a vis* chemically noxious dinoflagellates; does *C. pacificus* immediately select against noxious cells, or must it learn to avoid them? In addition, how long will this selectivity last? This study was undertaken to elucidate the mechanisms of and timescales for selectivity in *Calanus pacificus*. The results of the following study demonstrate that *C. pacificus* females in the presence of the noxious cell *Gonyaulax grindleyi* suffered a mortality rate similar to that of copepods in filtered seawater. When naive *C. pacificus* were presented with *G. grindleyi*, the copepods ingested these noxious cells at rates comparable to those of copepods presented similar concentrations of a preferred dinoflagellate. *C. pacificus* showed high variability in ingestion rates regardless of the species of cell presented. Copepods ingested *Gonyaulax grindleyi* at rates from 0.42 - 120 ng C min⁻¹, corresponding to clearance rates of 14.6 - 38 ml copepod⁻¹ h⁻¹. However, *G. grindleyi* was eaten at these "normal" rates for only for a short time (ca. 5 min). The copepods then appeared incapacitated, eating neither *G. grindleyi* nor preferred dinoflagellates at the initial conditioning rate. The duration of incapacitation (roughly 30 min - 12 h) appears to be dose dependent and may have other components as well. *C. pacificus* regained the ability to eat a preferred dinoflagellate before regaining the ability or "willingness" to eat *G. grindleyi*. Associative learning from single-trial conditioning persisted for approximately 12 h. The copepods appeared to be completely recovered, eating *G. grindleyi* at the initial rate, within 10 - 24 h. These results indicate that, as far as chemically defended dinoflagellates and *C. pacificus* are concerned, past feeding history on a timescale of 24 h should
be considered when designing feeding experiments. In experiments designed to elucidate the selective powers of *C. pacificus*, copepods were unable to completely distinguish between *G. grindleyi* and *Gyrodinium dorsum* (the preferred cell in the mixture). Because *C. pacificus* ingested both noxious and preferred dinoflagellates, these copepods made associations between some characteristic(s) of the cells in the mixture, and the negative experience of having eaten *G. grindleyi*. Consequently, copepods with experience in the algal mixtures ingested fewer of the preferred dinoflagellates that had been in the mixture than control copepods with no negative feeding experiences. These results indicate that learning and memory may be involved in associational defense that may play a role in determining the distribution of phytoplankton species when chemically-defended cells are present.
INTRODUCTION

Some herbivorous, marine copepods are strongly selective feeders, capable of distinguishing between cells and abiotic particles (Donaghay and Small 1979; Huntley 1983), live and dead cells (Paffenhöfer and Van-Sant 1985), rapidly growing and non-growing cells (Houde and Roman 1987; Cowles et al. 1988), autotrophic and heterotrophic organisms (Stoecker and Sanders 1985), and chemically noxious and innocuous cells (Huntley et al. 1986). Some mechanisms of selection (Huntley et al. 1986, Sykes and Huntley 1987 [chapter 2]) and sensory capabilities are also known (Ong 1969; Friedman and Strickler 1975; Koehl and Strickler 1981; Gill 1986; Légier-Visser et al. 1986; Gill and Harris 1987).

One of the more elusive questions in the study of marine copepods concerns the extent to which feeding history affects present feeding behavior (including selection). If significant, then would the persistence of a changed behavior be due to either acclimation or associative learning? Nothing is known about associative learning and memory in marine zooplankton although learning from gustatory experience has been suggested (Kranse 1973) and acclimation and adaptation are known to occur (Price and Paffenhöfer 1984).

The ability of copepods to learn from feeding experiences may enhance their ability to select edible foods from a melange of food items. A knowledge of the learning capability and memory of copepods may help us to explain some of the variability in feeding rates observed in these copepods, and may help us understand the dynamics of dinoflagellate–zooplankton interactions.

How do copepods react to a chemically defended alga (in this study Gonyaulax grindleyi), and how are they affected by this cell? To answer
these major questions, I asked the following sub-questions:

1) **Is *Calanus pacificus* poisoned by *Gonyaulax grindleyi* or does it starve in the presence of this cell?** Huntley *et al.* (1986) demonstrated that *C. pacificus* has a higher mortality in a suspension of *G. grindleyi* than in *Gyrodinium dorsum*. Uye and Takamatsu (1990) demonstrated that *Acartia omorii* and *Pseudodiaptomus marinus* suffer high mortalities in the presence of *Olisthodiscus luteus*, and the dinoflagellates *Gymnodinium nagasakiense, Heterosigma akashiwo, Chattonella marina*, and *Fibrocapsa japonica*. This appeared to be mainly due to starvation, although intoxication was attributable to at least some of the algal species. White (1980, 1981) has shown that copepods and other zooplankton can accumulate paralytic shellfish poisons (PSP) with no apparent harm to themselves. He did not, however, investigate the sub-lethal effects, if any, which the PSP containing cells might have had on the zooplankton, and did not investigate the feeding responses of zooplankton to the PSP bearing cells.

In freshwater systems, lethal and sublethal effects of chemically defended algae on herbivores are well documented. Lampert (1981) has shown that the cladoceran *Daphnia pulicaria* was poisoned by *Microcystis aeruginosa*. Nizan *et al.* (1986) also demonstrated that some cladocerans were poisoned by this cell while others simply stopped eating. The authors stated this was due to different suites of toxins in the cells. Fulton and Paerl (1987) determined that different species of copepods, cladocerans, and rotifers were differentially susceptible to *M. aeruginosa* and one species of rotifer appeared to be resistant to the toxic effects of this cell. Are similar situations to be found in the marine environment?

2) **How rapidly is *Calanus pacificus* affected by *Gonyaulax grindleyi*?**
Preliminary observations of *C. pacificus* feeding on *G. grindleyi* (Sykes and Huntley 1987, [chapter 2]) revealed that under their experimental conditions, starved copepods would often eat this noxious cell rapidly for a while before regurgitating and/or subsequently virtually ceasing to feed. DeMott (1988) has suggested that hungry copepods are less selective than fed ones, but Huntley *et al.* (1986) demonstrated that starvation did not increase the clearance rate by *C. pacificus* feeding on *G. grindleyi*; their experiments were relatively long-term (~3 h), so perhaps the timescales we need to investigate are much shorter. Determining the time-course of selectivity will allow us to understand more about information processing in marine copepods and perhaps allow us to better understand their impact on algal populations.

Because my preliminary observations (Sykes and Huntley 1987, [Chapter 2]) led me to believe that *C. pacificus* eats rapidly for approximately 1 h before any significant reduction in ingestion rate, the question of mechanism of rejection became obvious.

3) *Is C. pacificus* incapacitated by *Gonyaulax grindleyi* or does it learn to avoid it?

4) In either case, how long do these effects last?

The role past history plays in copepod feeding has been reviewed recently by Huntley (1988). Of the 12 papers dealing with factors affecting feeding history (light, food concentration, food size, and quality), in only one paper (McAllister 1970) was the persistence of an effect of conditioning mentioned. He found that starvation for a period of 12–24 hours led to enhanced feeding rates that persisted for 10–60 hours.

The persistence of a change in behavior following a period of conditioning has been loosely called "learning" by Krasne (1973). A more
rigorous definition of learning and memory, associative learning (reviewed by Carew and Sahley 1986), requires that the change in behavior is not due to habituation or sensitization (or incapacitation or adaptation), but, rather, to an association with some identifiable characteristic of the conditioning substance (eg. look, feel, taste) with the negative experience (eg. natural: endogenous toxins, or experimental: negative reinforcement) of having eaten the conditioning substance.

5) **Does Calanus pacificus make associations between preferred cells in mixtures with Gonyaulax grindleyi and the negative experience of having eaten these noxious cells?** If so, this could explain the mechanism for the reduced ingestion of preferred dinoflagellates (Huntley et al. 1986).

In this chapter, I report on short-term and long-term experiments where videotaped observations were made to determine the timescales for learning and memory. In addition, I will demonstrate that Calanus pacificus is capable of associative learning from a single feeding experience. Furthermore, I will report evidence that C. pacificus experiences negative gustatory consequences of eating G. grindleyi in mixtures of cells containing readily eaten foods (eg. Gyrodinium resplendens and Gyrodinium dorsum), that they associate the negative feeding experience with some characteristic of the preferred cell in the mixture, and that they subsequently reduce their feeding on that cell for at least 12 hours.
METHODS

Zooplankton collection

*C. pacificus* females were caught from the Spring of 1988 to early Summer of 1989 approximately 2 to 5 km west of Scripps Institution of Oceanography Pier (32°50'N; 117°10'W) with a 0.5 m zooplankton net (505 μm mesh) terminating with a glass 4 l cod-end. The zooplankton catch was immediately diluted into a 46.2 l cooler containing a seawater rinsed plastic garbage bag. Live female *C. pacificus* were sorted under a dissecting microscope and either used immediately for the experiments or fed *Gyrodinium dorsum*, *Gyrodinium resplendens*, or *Gymnodinium splendens* until needed for experiments.

Algal cultures

All dinoflagellates used in the following experiments were cultured in *Gonyaulax polyedra* Medium (GPM; Loeblich 1975) at approximately 25° C, in constant light with a range of intensities from ~ 0.3 - 1.0 X10^6 Q s^-1 cm^2. Exponential phase cells were used in all experiments.

Survival Experiment

Huntley *et al.* (1986) demonstrated that copepods in the continued presence of *Gonyaulax grindleyi* suffered higher mortality than copepods fed *Gyrodinium dorsum*. I repeated this experiment as before with the added control of copepods in filtered seawater (FSW) to determine whether mortality was due to starvation or intoxication.

Time series Experiments

To determine how rapidly *Calanus pacificus* demonstrated reduced ingestion rates on noxious dinoflagellates, I measured the loss of chlorophyll *a* from a series of cell suspensions in a time series experiment. Individual 280
ml bottles were filled with a 100–300 μg C l⁻¹ suspension of either *Gonyaulax grindleyi* or *Gyrodinium resplendens*. The time was marked when 10 or 15 copepods were added to each jar (except those jars designated as controls). Every 10 or 20 minutes, I removed a bottle with *G. grindleyi* and *G. resplendens*, strained out the copepods on 330 μm mesh screens and measured the chlorophyll *a* that remained in the bottle using the fluorescence technique of Holm-Hansen and Riemann (1978) and equations of Parsons (1984). In this experiment and in others where fluorescence techniques were used, the fluorometer used was a Turner model 10-005.

**Video Technique Test**

In order to investigate the effects of glueing on copepods, so I devised the following experiment to address both of these problems. From a net sample, I sorted 25 copepods, placed them in individual 200 ml dishes with 100 ml of a 100 cells ml⁻¹ suspension of *Gymnodinium splendens* for four hours at a time. Because egestion rate is related to ingestion rate (Ayukai 1987), I counted and measured the fecal pellets produced by the copepods. I repeated these measurements three times, in the morning, afternoon, and evening. I then glued each copepod to a glass wire (Sykes and Huntley 1987, [Chapter 2]) and suspended it within a 200 ml dish with the same concentration of the same food for the same length of time. Each copepod was glued within 40 seconds. After counting and measuring the fecal pellets the second time (again, in the morning, afternoon, and evening), I compared the pre-glued egestion volume with the post-glued volume. Animals whose post-glued egestion rate was consistently much less than the pre-glued egestion rate were considered to have been damaged by glueing. Animals whose pre- and post-glued egestion rates were equal to or greater
than their pre-glued rates were regarded as undamaged by glueing.

**Video methods**

I examined the response of *Calanus pacificus* to exposure to *Gonyaulax grindleyi* and readily eaten cells to determine the time scales for incapacitation (or learning) and recovery. Animals were glued to acetone-washed hairs (Alcaraz et al. 1980, Cowles and Strickler 1983) mounted on glass rods or wooden sticks in the same manner as they were glued to glass wires in Sykes and Huntley (1987, Chapter 2). To insure that the copepods I observed were in good health, I used a shortened version of the above mentioned test. I placed individual glued copepods into 200 ml crystallization dishes with the preferred food that I would use for a control. After several hours (or overnight), I counted the number of fecal pellets produced by each copepod and only used those copepods which had produced the largest numbers of full pellets. I viewed the copepods as before (Sykes and Huntley 1987, [chapter 2]) except that the copepods were in a horizontal position and they were in 200 ml crystallization dishes. In both types of experiments the ingestion rates were determined by directly counting ingested cells from the taped observations.

**Short and Long-term Time series**

The fluorescence time series tests mentioned above indicated that *Calanus pacificus* stopped eating *Gonyaulax grindleyi* in 20 minutes or less, so I used the video system to determine the short-term timescales of rejection and to determine how long the copepods were affected by eating this noxious cell. Five copepods were selected. These copepods were fed *G. grindleyi* and/or *Gyrodinium dorsum* for 20 minutes. Every 5 minutes the copepod being observed was placed in a new dish with a fresh suspension of cells. A
similar approach was taken for the long-term timescale tests except that the copepods were tested for 5 minutes and then placed in FSW between tests for periods as long as 11 hours.

**Associative Learning and Memory**

Animals were observed feeding on the noxious dinoflagellate *Gonyaulax grindleyi* for ~5-10 min. After this initial conditioning, the copepods were placed in FSW for periods ranging from 1 h to 21 h. The copepods were then observed in suspensions of either the readily eaten food, *Gyrodinium resplendens*, or *G. grindleyi* to determine when the copepods became incapacitated, when learning was manifest, and to ascertain the memory of the copepods.

I observed the tapes and recorded the total time of each observation and the duration each copepod moved its mouthparts (data not used in this report). I then observed the tapes again to count the cells each copepod ate during the observation period. For days in which I have several observations for one recovery time, I plotted the final ingestion rate as a function of the initial ingestion rate and compared those copepods tested in *Gonyaulax grindleyi* against those tested in *Gyrodinium resplendens*. I used a 1-tail, completely randomized t-test (Edgington 1980) to compare the recovery ratios (final ingestion rate over initial ingestion rate) of copepods tested in *G. resplendens* and *G. grindleyi*. I computed significance using a computer program written by Dr. Richard Bray of California State University, Long Beach. Some copepods were "conditioned" and "tested" in *Gyrodinium resplendens* to determine the effects of handling.

**Association Experiments with Congeners**

In order to elucidate the dynamics of selectivity in *Calanus pacificus*, I
used another associative learning test. In this test, copepods were given a mixture of a *Gyrodinium dorsum* and *Gonyaulax grindleyi* food for varying amounts of time. After the conditioning period, the copepods were allowed to recover in the preferred food alone and then the copepods were tested on the original preferred food or *Gyrodinium resplendens*, the other highly edible congener the copepods had not had before. If the copepods had associated a negative gustatory experience (*i.e.* regurgitation due to the ingestion of *G. grindleyi*) with the ingestion of the preferred food in the mixture, then the filtration rate on the original edible food was expected to be reduced relative to its control while the filtration rate on the new preferred food was not expected to be lower than its control. If, however, the copepods were simply incapacitated, then both groups of copepods from the mixture should have had lower filtration rates than their respective controls.

I sorted *C. pacificus* females into two 4 l bottles. One bottle contained 300 µg C l⁻¹ each of *Gonyaulax grindleyi* and *Gyrodinium dorsum* while the control bottle contained 600 µg C l⁻¹ of *G. dorsum*. I conditioned the copepods for periods of 30 min (Fig. 3.1). After conditioning, the copepods were allowed to recover for periods of 0, 2, 6, and 12 h in 600 µg C l⁻¹ *G. dorsum*. The recovery period was immediately followed by the association test.

I placed 10 copepods each from the mixture group and from the control group into each of four replicate 280 ml jars containing filtered seawater and 100 µg C l⁻¹ *G. dorsum*. Another set of jars contained *G. resplendens*. Four jars of each cell suspension (without copepods) were sampled for the initial (*i.e.* control) value of chlorophyll a. The jars were allowed to stand for one hour and then the contents of the jars were strained.
through 330 μm mesh screens to retain the copepods. The cells were then filtered onto glass-fiber filters for fluorescence determinations as mentioned before. Four jars, each with cell suspensions of either cell, but without copepods served as controls (Fig. 3.1). I used the formulae of Marin et al. (1986) to calculate the clearance rates. Because the variances were not homogeneous in some of the experiments (F-test; p >0.05), I used the completely randomized t-test on the individual data points. I performed one double experiment, using G. dorsum as the initial preferred food in the first half of the experiment, and G. resplendens as the initial preferred food in the second half of the experiment. The initial conditioning period was 24 hours in this experiment, and the recovery period was 0 hours. The purpose of this experiment was to determine if C. pacificus preferred one preferred dinoflagellate over the other, and would react to these cells differently after exposure to the noxious cell, G. grindleyi.
Figure 3.1. Flow chart for associative learning experiment using congeners. *Calanus pacificus* females were sorted into two groups. The first group was conditioned with a mixture of *Gonyaulax grindleyi* and *Gyrodinium dorsum* both at a concentration of 300 $\mu$g C l$^{-1}$. The control group was conditioned in a 600 $\mu$g C l$^{-1}$ suspension of *G. dorsum*. After conditioning (30 min), the copepods were allowed to recover in suspensions of *G. dorsum*. In order to test whether or not the copepods had associated a negative feeding with some characteristic of the preferred food *G. dorsum*, a clearance rate test was performed on the test and control copepods. Each group of copepods was tested with the preferred food *G. dorsum* as well as a preferred food with which the copepods had had no previous experience. Significance of differences between test and control group in both cell was detected by a 1 tail randomized t test. Associative learning and memory was indicated by a significant reduction of clearance rate by the test group in *G. dorsum* and a nonsignificant difference between the test and control group in *G. resplendens*. 
ASSOCIATIVE LEARNING

Gonyaulax grindleyi
Gyrodinium dorsum
Gyrodinium resplendens

EXPERIENCED
G. grindleyi
G. dorsum

CONDITIONING

NAIVE CONTROL
G. dorsum

RECOVERY

TEST

1
2
3
4

INCAPACITATION: $1 < 2$ & $3 < 4$

ASSOCIATIVE LEARNING: $1 < 2$ & $3 = 4$
RESULTS

Survival experiment

The group of copepods fed *G. grindleyi* produced relatively few fecal pellets in approximately 24 hours, and the bottle still contained a great quantity of cells, whereas the group fed *Gyrodinium dorsum* produced a large quantity of fecal pellets, and the copepods ingested nearly every cell in the same time period. By the end of the experiment (day 27), 79.5% of the copepods fed *G. dorsum* were still alive as compared with 1% and 12.2% for the *G. grindleyi* and filtered seawater group, respectively. Because the onset of rapid mortality and the median death times for the *G. grindleyi* and filtered seawater groups were about the same (roughly 15 and 16 days respectively for onset of rapid mortality and 19 and 20 days respectively for median death), I conclude that the copepods starved, and were not poisoned by *G. grindleyi*.
Figure 3.2. Survival of *Calanus pacificus* as a function of food quality. Open circles represent the group fed *Gyrodinium dorsum*. The copepods in filtered seawater are represented by "X"s, and the *Gonyaulax grindleyi* group is represented by the asterisks (*).
Time series Experiment

Copepods fed *Gonyaulax grindleyi* demonstrated strongly reduced ingestion in less than 10 minutes. Animals fed *Gyrodinium resplendens* substantially reduced the concentration of cells in both experiments while not obviously ingesting *G. grindleyi* in one experiment and only slightly reducing its concentration in another (Fig. 3.3).
Figure 3.3. Time series experiments. Reduction of Gonyaulax grindleyi and Gyrodinium resplendens over time. In both experiments, Gyrodinium resplendens (O) was dramatically reduced whereas Gonyaulax grindleyi (*) was not.
Pre-Video Test

Free copepods produced a minimum of 0 fecal pellets to a maximum of 3.73 fecal pellets h$^{-1}$ with a maximum egestion rate of $\sim 6 \times 10^6 \ \mu$m$^3$ h$^{-1}$. Of the copepods not damaged by glueing and not showing obvious signs of damage, the egestion rates ranged from 0 to $\sim 4.5 \times 10^6 \ \mu$m$^3$ h$^{-1}$ indicating that glueing did not interfere with the individual variability of the sample. Glueing copepods to supports does not consistently reduce their egestion (and by inference, ingestion) rates (Table 3.1 and Fig. 3.4). Most copepods egested roughly equal volumes from the pre-glued to post-glued conditions, particularly at night. Some of the copepods showed few signs of having eaten anything.
Table 3.1. Egestion rates (X 10^6 μm³ h⁻¹) of Calanus pacificus fed Gymnodinium splendens. Egestion rates were measured for unrestrained (Free) copepods in the morning (AM), early afternoon (PM), and evening (NT). The following day, the same copepods were then glued to glass wires and egestion rates were measured as before. The data are ordered by the NT, Free data in ascending order, and the Glued/Free Ratio:NT column shows the extent to which each of these copepods was affected by gluing. Ratios of one or greater indicate that the animal was not harmed by gluing. Ratios below one indicate that the copepods were either inhibited by gluing, were in some other way inhibited. Copepods in the separate group at the bottom were not used in Figure 3.4.

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1. One A1 not showing.
3. Animal appeared dead by end of experiment.
4. Animal looked nearly dead by end of experiment.
Figure 3.4. Pre-video test of glueing procedure. Egestion rates ($\times 10^6 \, \mu m^3 h^{-1}$) of copepods glued to glass wires were compared with the egestion rates of the same copepod when swimming freely. 25 copepods were tested in the morning (a), afternoon (b), and night (c). The results for 14 copepods are plotted. The solid line indicates a slope of 1.
Video Experiments

The 83 copepods I examined with video displayed tremendous individual variability in terms of their initial ingestion rates. This variability did not appear to be influenced by the species of cell presented (Table 3-2).

Table 3.2. Initial ingestion rates (ng C copepod\(^{-1}\) min\(^{-1}\)) of *Calanus pacificus* on three species of dinoflagellates. Ingestion rates were calculated from direct counts of ingested cells. Restrained copepods were observed for approximately 5 min. Number of observations, n; range of ingestion rates; and the mean and standard deviation are displayed.

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Short-Term Time series

Figure 3.5a demonstrates that the copepod fed *Gyrodinium dorsum* fed well over the total 20 min period, but copepods that fed well on *Gonyaulax grindleyi* for the first 5 min (Fig. 3.5b&d) ate any cell less well subsequently. These results indicate that the copepods may become incapacitated in as little as 5 minutes if they eat a sufficient amount of the cell. Note that copepod #1 (Fig. 3.5c) ate all cells proffered, and appeared not to have eaten a sufficient amount of *G. grindleyi* to become negatively affected. Animal #5 (Fig. 3.5e) was fed *G. dorsum* first and showed some reduction in the ingestion of subsequent cells after 5 min suggesting that some reduction in the ingestion rates may be due to satiation.

Long-Term Time series

As shown in the short-term time series, the copepod fed *Gyrodinium dorsum* throughout the experiment ate at high rates at all times (Fig. 3.6a). The copepod fed *Gonyaulax grindleyi* initially and *Gyrodinium dorsum*...
subsequently (Fig. 3.6b) demonstrated incapacitation for 10 h followed by recovery by 21 h. The copepod fed *G. grindleyi* throughout the experiment (Fig. 3.6c) appeared incapacitated for 8 h followed by recovery by ~10 h which subsequently incapacitated the copepod for at least another 11 h. Animal #32 (Fig. 3.6d) appeared incapacitated for at least 5 h. By 21 h the copepod had recovered in *G. dorsum*, and probably had recovered completely. The maximum persistence of the effects of *G. grindleyi* appears to be of the order of one day.
Figure 3.5. Short-term video time series. Open bars represent ingestion rates on *Gyrodinium dorsum* (Gd) while shaded bars represent ingestion rates on *Gonyaulax grindleyi* (Gg). Ingestion rates were calculated from direct counts of ingested cells over the 5 min observation period. Feeding regimes were as follows:  

a)Gd,Gd,Gd; b)Gg,Gd,Gd,Gd; c)Gg,Gd,Gg,Gd; d)Gg,Gd,Gg,Gd; e)Gd,Gg,Gd,Gg.
Figure 3.6. Long-term video time series. Individual restrained *Calanus pacificus* females were observed for 5 min at a time beginning at the times indicated below each bar. The open and shaded bars as well as the initials represent the same algal species. Feeding regimes were as follows:
a)Gd,Gd,Gd,Gd,Gd; b)Gg,Gd,Gd,Gd,Gd,Gg; c)Gg,Gg,Gg,Gg,Gg,Gd;
d)Gg,Gg,Gd,Gd.
Single-Trial Associative Learning Experiments

Table 3 shows the raw data for the video experiments. Over the course of the 5 - 10 min initial feeding period, these copepods attained ingestion rates of 0.42-120.2 ng C min\(^{-1}\), corresponding to clearance rates in the range of 14.6-38 ml copepod\(^{-1}\) hr\(^{-1}\).
Table 3.3. Single - Trial associative learning and memory in *Calanus pacificus*. Date of observation, copepod number (#), Initial ingestion rate (ii) in ng C min⁻¹, time between initial and final observations, final ingestion rate (ff), and the "recovery ratio" (If/ii) are given. Recovery ratios of 1.00 or more indicate that the copepod had eaten the test cell (If) at the same rate as the conditioning cell (ii). "Rg" and "Rt" indicate respectively that the copepod had either regurgitated or demonstrated strong reverse paristalsis ("retching") during the second observation (test) period. The first group of copepods were conditioned and tested in *Gyrodinium resplendens* (Gr vs Gr). The second group of copepods were conditioned in *Gonyaulax grindleyi* and tested in *G. resplendens* (Gg vs Gr). The third group of copepods were conditioned and tested in *G. grindleyi* (Gg vs Gg).

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**Gr vs Gr**

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Copepods generally ate *Gonyaulax grindleyi* rapidly with few signs of distress although some copepods did demonstrate strong reverse paristalsis within seconds of eating this cell for the first time. No copepod, however, regurgitated *G. grindleyi* during the initial feeding period. Of the copepods tested with *Gyrodinium resplendens*, recovery ratios of < 1.0 (16 out of 35 observations) indicate that the copepods were incapacitated to some degree (Table 1). For animals tested in *G. grindleyi*, recovery ratios in this group of 1.0 or greater indicate that the copepods had fully recovered. Recovery ratios less than 1.0 (24 out of 34 observations) indicate that (for the given recovery period) the copepod was either incapacitated or (if the Gg vs Gr copepods for the given date, initial dose and recovery period were recovered) had learned to avoid *G. grindleyi*. Figure 3.7 documents that copepods tested in *G. resplendens* after 4 h (3/8/89) and 6 h (2/27/89) had recovered the ability to eat preferred dinoflagellates, whereas the copepods tested in *G. grindleyi* would not (or could not) eat these noxious cells. One animal regurgitated *G. resplendens* 5 h (6/30/88) after eating only a small amount of *G. grindleyi*. Figure 3.7 shows that *Calanus pacificus* females exposed to either cell at 10h (3/8/89) were still incapacitated. Copepods tested 3/24/89 after 12 h were completely recovered. These results indicate that *C. pacificus* may have a memory for single-trial experiences as long as 6 to 12 h, or ~0.014 of its adult life-span of about 5 weeks (Huntley and Brooks 1982).
Figure 3.7. Selected single-trial associative learning video observations. All copepods were conditioned in *Gonyaulax grindleyi* for 5 min. Animals tested in *G. grindleyi* (*) were usually below the "recovery line" (the solid line with a slope of 1) at 4, 6, and 10 h, indicating that the copepods were not eating that cell at the initial rate. Animals tested in *Gyrodinium resplendens* (●) generally were above the "recovery line" except at 10 h. Graphs a and b, where *G. grindleyi*-tested copepods are below the line and *G. resplendens*-tested copepods are above the line indicate associative learning. Graph c, at 10 h, indicates incapacitation (below the recovery line), and the copepods in graph d appear fully recovered (above the recovery line).
Association Experiments with Congeners

Figure 3.8 shows results which were similar to those from the video experiments. Significant learning periods started after 0 hours recovery indicating that the copepods in the mixture ate enough of the noxious cell to cause a negative experience within the 30 min conditioning period without a long period of incapacitation. Copepods with a 12 h recovery period still demonstrated the ability to distinguish between the preferred cell that had been in the mixture and the preferred cell that the copepods were naive to. Figure 3.9 demonstrates that *Calanus pacificus* had no significant preference for either *Gyrodinium dorsum* or *Gyrodinium resplendens*. Copepods conditioned with and tested on *G. dorsum* ingested these cells at the same rate as copepods conditioned with and tested on *G. resplendens* ($p=0.571$, 2 tail randomization test). Copepods condition with *G. dorsum* and tested on *G. resplendens* had clearance rates not significantly different than copepods conditioned with *G. resplendens* and tested on *G. dorsum* ($p=0.228$, 2 tail randomization test). The extent to which learning and memory is manifest in *C. pacificus* may differ depending on which preferred cell is present in the mixture. The left half of figure 3.9 shows that the copepods conditioned with *G. dorsum* in the mixture were not incapacitated by the time of the test (just a few minutes). Copepods conditioned with *G. resplendens* in the mixture (right half of figure 3.9) were still incapacitated when tested.
Figure 3.8. Associative learning and memory of *Calanus pacificus* when tested with preferred congeneric algal species. Experimental copepods were conditioned in a mixed suspension of *Gonyaulax grindleyi* and *Gyrodinium dorsum*. Control copepods were conditioned only in the preferred cell, *G. dorsum*. In each graph, the left pair of replicates represent copepods tested in *Gyrodinium dorsum*, the preferred cell that was present in the conditioning suspension. The second pair of replicates were tested in a suspension of *Gyrodinium resplendens*, a preferred cell the copepods had had no experience in. Associative learning is indicated in all experiments. Clearance rate (F) is in units of ml copepod$^{-1}$ h$^{-1}$. Negative filtration rates indicate that the "no copepod control" from which clearance rates were calculated contained slightly less chlorophyll than the tested sample.
Figure 3.9 Associative learning and memory of *Calanus pacificus* when tested with preferred congeneric algal species. Experimental copepods were conditioned in a mixed suspension of *Gonyaulax grindleyi* and *Gyrodinium dorsum* or *G. grindleyi* and *Gyrodinium resplendens*. Control copepods were conditioned only in the preferred cell, *G. dorsum* or *G. resplendens*. In the graph, the left two pairs of replicates represent copepods conditioned in *G. dorsum*. The first pair were tested in *G. dorsum* and show a significant difference between them (p=0.014). The second pair were tested in *G. resplendens*, and do not show a significant difference between them (p=0.300). This indicates that *C. pacificus* is demonstrating learned behavior. The right two pairs of replicates were conditioned in a suspension of *Gyrodinium resplendens*. Incapacitation is indicated with this group because a significant difference (p=0.014) between "mix" and "control" was found in the pair tested on *G. resplendens* and the pair tested on *G. dorsum*. No significant differences between the clearance rates of like controls (shown in open circles [p=0.571] and asterisks [p=0.228]). Clearance rate (F) is in units of ml copepod⁻¹ h⁻¹.
DISCUSSION

*Calanus pacificus* is definitely a selective organism in terms of food quality. But what is the time course of this selectivity? Does *Calanus* "smell" or "taste" its food before ingesting it, or does it eat any living cell and then learn from negative experience? I have presented evidence that *Calanus* follows the later scenario as far as *Gonyaulax grindleyi* is concerned.

In the constant presence of *Gonyaulax grindleyi*, these copepods will starve rather than consume a significant quantity of this noxious cell. *Calanus pacificus* may ingest *G. grindleyi* rapidly for 5 to 10 minutes (or until a sufficient dosage of the cell is accumulated). The copepod then appears incapacitated as it eats much less *G. grindleyi* or the preferred dinoflagellates. Because copepods having had the same initial dosage of *G. grindleyi* generally tend to recover the ability to eat a preferred cell before recovering the ability to eat *G. grindleyi*, we can infer that *C. pacificus* is capable of learning to discriminate between cells when the cells are presented one at a time.

Associative learning is known in a variety of freshwater, marine, and terrestrial invertebrates (Carew and Sahley 1986), but appears to be unknown in planktonic invertebrates. To my knowledge, this represents the first report of associative learning and memory in a planktonic organism.

*Calanus pacificus* cannot perfectly distinguish between cells in a mixture as shown by reduced feeding on a preferred cell (Fig. 3.8). Note, however, that the lowered rates are still higher than observed for copepods presented *Gonyaulax grindleyi* after previous conditioning on that cell. What must take place, then, is that the naive, hungry copepod eats any and all live cells of an appropriate size at a high initial rate (Demott 1988 and Cowles et
al. 1988). If a noxious cell is in the mixture, and the copepod eats a sufficient quantity of that cell, the copepod will become incapacitated. After initial recovery, the copepod can distinguish between-preferred cells that were in the mixture and congeners that were presented later. This supports the hypothesis that copepods may have to ingest some cells before they become strongly selective. This is probably due to learning and not due to satiation induced selectivity.

I propose a simple model to describe learning and memory in *Calanus pacificus*. Copepods fed preferred foods for a short initial duration will invariably eat the cell at different rates (Fig. 3.4). This is most likely due to the past nutritional history of the animals (Huntley 1988). Some of the variability is undoubtedly due to net damage and other handling. If the copepods are then fed the same preferred cell at some later time, the copepods should eat at approximately the same rates as they did initially. If the copepods are initially conditioned on *Gonyaulax grindleyi*, for some time afterward only those animals whose initial ingestion rates were low may recover while the other copepods would show lowered ingestion rates regardless of the cell used to test the final ingestion rate. This indicates that the copepods are incapacitated. Depending on the initial dose of *G. grindleyi* ingested, copepods will begin to recover. Copepods ingesting less of the noxious cell will recover more rapidly than copepods whose initial ingestion rates on the noxious cell were higher. Copepods tested on *Gyrodinium resplendens* (or other preferred cell) at a later time should be expected to have higher ingestion rates on this cell than copepods with the same initial ingestion rate but tested on *G. grindleyi*. So in general, a copepod who has learned to not eat the noxious cell will have recovered the ability to eat *G.*
*resplendens*, but not *G. grindleyi*. The difference in the time required for a *G. grindleyi*-tested copepod and a *G. resplendens*-tested copepod to recover (assuming similar initial ingestion rates on *G. grindleyi*) is the memory of the learned behavior.

How might associative learning and memory affect the survival of copepods and the distribution of algae in a natural setting? Clearly, a copepod capable of avoiding a noxious cell will eat less of that cell if it is encountered again within the timespan of the copepod's memory. Less time will be spent in an incapacitated state and more edible cells may be consumed.

Under some conditions, this may allow a slower growing noxious cell to continue to grow while undefended cells are removed from a region of high grazing pressure. Somewhat mitigating this effect is the fact that *Calanus pacificus* eats preferred cells that were present in the mixture with *Gonyaulax grindleyi* at a somewhat reduced rate (also observed by Huntley *et al.* (1986) and Fulton and Paerl (1987) for freshwater organisms feeding on *Microcystis aeruginosa*) due to associative learning indicating that undefended cells may derive an associational defensive advantage from the presence of noxious cells (Pfister and Hay 1988).
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CHAPTER 4

THE CHEMICAL ECOLOGY OF Gonyaulax grindleyi:

THE SIGNIFICANCE OF CHEMICAL DEFENSE
ABSTRACT

The chemical ecology of *Gonyaulax grindleyi* was studied to determine the function and value of its noxious compound(s) as a defense against grazing by *Calanus pacificus*. These studies revealed that exponentially growing *G. grindleyi* was more noxious to *C. pacificus* than stationary phase cells, and that bacteria did not play a role in the production of this noxious quality. *G. grindleyi* usually grew more slowly than *Gyrodinium resplendens*, a readily eaten dinoflagellate, though under some circumstances their growth rates were the same. Neither species was allelopathically inhibited by the other, and *G. grindleyi* experienced a higher net growth rate than *G. resplendens* only when the noxious cell was in high enough concentration to allow copepods to receive a sufficient dosage of noxious compounds. Under most conditions, the rapidly growing, undefended cell was associationally defended to some degree by the presence of *G. grindleyi*. Furthermore, *G. grindleyi* was made more palatable in the presence of the preferred cell. Chemical defense in *G. grindleyi* probably has a greater value in maintaining an established population under heavy grazing pressure than in facilitating bloom formation.
INTRODUCTION

Unicellular, planktonic algae, containing toxic compounds, are well known. Because of their role in mortalities in fisheries, and in human illness and deaths, the toxigenicity, pharmacology, biosynthesis, and chemical structures of phytoplankton toxins, particularly of the Dinoflagellata, have been the focus of many studies culminating in at least three major symposia (LoCicero 1975, Taylor and Seliger 1979, and Anderson et al. 1985). While the much may be known about the compounds from acutely toxic phytoplankton, very little is known about the natural function of these compounds, or of compounds with less acute pharmacologies.

In the late 19th century, the suggestion that certain plants could reduce herbivory through the use of chemical defenses was first offered by Stahl (1888). Evidence that chemical defenses play a major role in the terrestrial environment is abundant and thorough (Harborne 1972, Wallace and Mansell 1976, Rosenthal and Janzen 1979). While chemical defenses are well known in the terrestrial environment, they are less well studied in the marine environment, mainly for a lack of intense investigation.

Research on benthic algae has provided most of what is known about chemical defenses in marine algae, and this body of knowledge is increasing rapidly. Many species of tropical green algae are known to contain antiherbivore toxins (Paul and Fenical 1986). Geiselman et al. (1981) and Steinberg (1984) demonstrated that polyphenolic compounds from brown algae inhibited grazers. A wide variety of toxic, antiherbivory compounds are also known from red algae (Fenical 1978, Norris and Fenical 1982, Faulkner 1984). Our knowledge of chemical defenses in the planktonic environment is growing, but still lags behind the research on benthic algae.
Huntley et al. (1986) demonstrated that *Gonyaulax grindleyi* was strongly rejected by *Paracalanus parvus* and *Calanus pacificus*, and that *C. pacificus* suffered higher mortality in the presence of *G. grindleyi* than more preferred dinoflagellates. Huntley et al. (1986), Huntley et al. (1987), and Sykes and Huntley (1987,[Chapter 2]) elucidated some of the lethal and sublethal effects of this cell on *C. pacificus*. In chapter 3 of this thesis, I demonstrated that *C. pacificus* is not poisoned, but rather, starves in the presence of *G. grindleyi*, and that most of these dinoflagellate cells remained in the jar after each 24 h period with the copepods.

To better understand the chemical ecology of *Gonyaulax grindleyi*, I asked the following questions: 1) What effect does cell age have on the noxious quality of *G. grindleyi*? 2) What role, if any, do bacteria play in producing this noxious quality? 3) Under conditions of copepod grazing, will *G. grindleyi* experience a greater net growth rate than an undefended, more rapidly growing dinoflagellate? 4) Does the presence of a chemically noxious cell affect the edibility of an undefended, preferred dinoflagellate? 5) Does the presence of a preferred dinoflagellate make *G. grindleyi* more edible?
METHODS

Algal Culture and Zooplankton Collection

Algal cultures were maintained and zooplankton collected as per the methodology described in chapter 3.

Noxious quality vs Cell Age

Cowles et al. (1988) elegantly demonstrated that *Acartia tonsa* ingests exponentially growing *Thallasiosira weisflogii* more rapidly than stationary phase or senescent phase cells. Studies of phytoplankton toxicity (Hall 1982, Singh et al. 1982, White and Maranda 1978, Boyer et al. 1985, Prakash 1967) have shown that toxicity is usually higher in exponential phase algal cells than in stationary phase cells.

To determine which of these two conflicting phenomena would dominate the edibility of *Gonyaulax grilldeyi* to *Calanus pacificus*, I conducted the following experiment. Following the methods for grazing experiments (Huntley et al. 1986), I measured the clearance rates of an exponentially growing culture of *G. grilldeyi*, a stationary phase culture of the same, and an exponential phase culture of *Gymnodinium splendens*. To summarize the experimental conditions, 280 ml jars were filled with the experimental suspensions, 4 replicates each. Ten *C. pacificus* females were added to each jar. Jars containing each experimental suspension, without copepods, were used as controls. The bottles were rotated on a grazing wheel at 1 rpm for 3 h under dim light at 19° C. Each suspension was sampled prior to the experiment, and each bottle was sampled at the end of the experiment. The suspensions were preserved in basic Lugol's solution. Using the Utermöhl method (Lund 1958), I counted 3 subsamples from each sample and calculated mean cell abundances for each jar. Clearance rates were
determined with the formulae of Marin et al. (1986), and I performed a randomized one tail t test (Edgington 1980) to measure significance as described in chapter 3.

Noxious quality vs the presence of bacteria

Bacteria are believed to form associations with marine algae (Azam and Ammerman 1984), and are known to exist endosymbiotically in some algal species of phytoplankton (Martinez et al. 1983, Dodema and Veer 1983, Chesnick and Cox 1986). Endosymbiotic bacteria have been indicated as the primary source of toxic compounds in a coelenterate (Guyot and Van Preat 1985). Other studies have shown that bacteria were not involved with the formation of toxins (Singh et al. 1982, Durand-Clement 1986). Is Gonyaulax grindleyi noxious to Calanus pacificus due to associated bacteria, or are the noxious compounds created endogenously?

Are bacteria closely associated with Gonyaulax grindleyi? If they are, then the likelihood of them being involved in the production of noxious compounds would be higher. I examined Gonyaulax grindleyi under SEM to see if the cells were covered with bacteria. I strained a 4 day old culture of G. grindleyi through a coarse Nitex mesh (~100 μm) and fixed the cells in 1 - 2% formalin (total concentration). I then filtered the cells onto a 0.8 μm Nuclepore filter, briefly rinsed with a few ml of deionized water, and placed the filter onto a coverslip. When the filter was dry (Venrick, personal communication), I sputter-coated the filter with gold/paladium on a Technics "Hummer" sputter-coater and viewed the specimen under a Cambridge "Stereoscan 360" SEM.

The noxious characteristics of Gonyaulax grindleyi were directly examined with the use of axenic cultures. I created an axenic culture of G.
grindleyi by first straining an early exponential phase culture of *G. grindleyi* through a ~100 μm Nitex mesh to remove large particles which could harbor bacteria. I added these cells to a GPM (Loeblich 1975) containing 10 μg/ml Ampicillin (Amp). Ampicillin was chosen after screening a variety of antibiotic impregnated disks against the bacteria in the *G. grindleyi* culture. For approximately 5 days I added more GPM and Amp. I tested for the presence of bacteria by inoculating a tube containing a complex seawater bacterial medium and incubating it overnight with shaking.

The grazing experiment to test the edibility of axenic versus xenic *Gonyaulax grindleyi* was conducted and analyzed in the same way as the "cell age" experiment.

**Filtration rate versus % seawater**

In order to test the hypothesis that *Gonyaulax grindleyi* has an ecological advantage in the presence of a copepod grazer, I grew cells in an algal growth medium with and without copepods (chemical defense experiments described below). The growth medium (GPM) contains 25% distilled water and *Calanus* is known to be injured by lowered salinity (Marshall 1973). To determine the percentage of distilled water *Calanus pacificus* could tolerate in the growth medium, I tested the clearance rate of *C. pacificus* on *G. splendens* in 50%, 80%, 90% and 100% seawater. The experiment was conducted and analyzed in the same fashion as the cell age experiment and axenic *G. grindleyi* experiments. The results of this experiment were used to determine the best modification of GPM which would allow the copepods to feed well and yet allow good cell growth.

**Preliminary Chemical Defense Experiments**

In these experiments I asked the following questions: Does *Gyrodinium*

Each of these experiments consisted of four bottles containing GPM with < 5% distilled water (SW-GPM). The first bottle contained a preferred dinoflagellate, Gyrodinium dorsum or Gyrodinium resplendens, without a copepod, the second contained a suspension of G. grindleyi and the preferred cell. Initial, total particulate nitrogen levels were equal to the single-species suspensions. The third bottle contained the mixed-cell suspension with a Calanus pacificus female, and the fourth bottle contained G. grindleyi without a copepod.

The first experiment in this series differed in that Gyrodinium dorsum was used instead of Gyrodinium resplendens and the experiment was conducted in 2.8 l Fernbach flasks with 2 l of SW-GPM. All of the other experiments were conducted with G. resplendens as the preferred cell, and with 4 l bottles containing 3 l SW-GPM. The initial concentrations in the first experiment were 25 μg N l⁻¹ for G. dorsum and 19 μg N l⁻¹ for Gonyaulax grindleyi. In experiments 2-4 the initial concentrations were 60 μg N l⁻¹, G. resplendens, and 21 μg N l⁻¹, G. grindleyi. In the last experiment these concentrations were approximately doubled.

I prefed the copepods the preferred dinoflagellate so that I could pick out the healthiest ones as determined by gut fullness, and these were added after the cells had begun to grow. The bottles were placed in an incubator at 18-20° C. A 14:10 h light:dark light cycle was maintained at 0.3-0.4 x 10¹⁶ Q (s cm⁻²)⁻¹. The experiments lasted ~14-38 days.
I counted samples up to 10 ml in volume by the Útermohl technique (Lund et al. 1958) using a Zeiss inverted microscope. From cell counts, μg N 1⁻¹ were calculated from the known nitrogen contents of our laboratory cultures (Huntley et al. unpublished data). Linear regression analysis was performed on log transformed data to determine the slopes of the various growth curves. After analysis of variance on each group of regression lines to determine if significant differences in the slopes existed, I tested pairs of slopes (1 tail F test α=0.05) to test the above mentioned hypotheses (Zar 1974).

Chemical Defense: Algal Concentration ratios and Associational defense

Results from the preliminary experiments revealed that little or no allelopathy existed between Gonyaulax grindleyi and Gyrodinium resplendens. Thus, I set up this series of experiments to answer the question: Is G. resplendens defended from predation by the existence of G. grindleyi in the suspension? Additionally, I changed the initial ratios of G. grindleyi to G. resplendens to determine the effect the starting concentration ratios might have on G. grindleyi's ability to avoid predation.

In this series of experiments, the first bottle contained a suspension of G. resplendens and a copepod. The second and third bottles contained suspensions of both cells, the second bottle containing a copepod. The experiment was run four times with the ratio of Gonyaulax grindleyi to G. resplendens varied. The ratios were 10:90, 30:70, 50:50, and 90:10. A fifth experiment was attempted with a ratio of 70:30, but the medium contained too much precipitate, and the copepods could not eat. The initial, total dinoflagellate nitrogen in the mixed suspensions was approximately 23 μg N 1⁻¹
The experimental conditions, sampling methods, and statistical analysis were conducted in the same fashion as the previous experiments.

**Chemical Defense Experiment: "Associational Liability"**

I set up the following experiment to determine whether or not *Gonyaulax grindleyi* was made more edible in the presence of *Gyrodinium resplendens*. This experiment was set up in the same fashion as the last one except that an extra bottle was used, containing a suspension of *G. grindleyi* and grazers. For this experiment, I placed 4 copepods in each of the test bottles because the copepods did not appear to be eating as rapidly as in earlier experiments as determined by egestion rates previous to the experiment.

The experimental conditions, sampling methods, and statistical analysis were carried out in the same fashion as the other experiments.
RESULTS

Noxious quality vs Age

Regardless of cell age, *Calanus pacificus* strongly rejected *Gonyaulax grindleyi* when compared with *Gymnodinium splendens* (Figure 4.1). However, exponential phase *G. grindleyi* was significantly more noxious than the stationary phase cells (α=0.05, p=0.014). As a result, I used exponential phase cultures in all of the following experiments.

Noxious quality vs the presence of Bacteria

*Gonyaulax grindleyi* is a highly reticulated cell, with numerous pores. The cells are ~45–50 μm dia., and showed no evidence of strongly attached bacteria (Figure 4.2).

Figure 4.3 shows that no significant differences (α=0.05, p=0.143) in clearance rates were found between xenic and axenic *Gonyaulax grindleyi* cultures.

Clearance rate vs Salinity

*Calanus pacificus* was strongly affected by reduced salinity. A 10% dilution of filtered seawater resulted in a reduction of the clearance rate by ~50%. I also found a significant difference between 80% and 50% seawater (Figure 4.4). As a compromise between *Calanus* clearance rates and algal growth rates, I used GPM (Loeblich 1975) with a minimum seawater strength of 95%. This seawater strength does not appear to have inhibited the copepods because in the following experiments, clearance rates on *Gonyaulax grindleyi* were as high as 40 ml animal⁻¹ h⁻¹.
Figure 4.1. Clearance rates (ml animal$^{-1}$ h$^{-1}$) on *Gonyaulax grindleyi* by *Calanus pacificus* relative to cell age. 1-tail randomized t test revealed significant reduction of the noxious quality of stationary phase *G. grindleyi*. Neither exponential nor stationary phase *G. grindleyi* were eaten as readily as exponential phase *Gymnodinium splendens*. 
Figure 4.1

G. splendens

G. grindleyi

P = 0.014

stationary

exponential
Figure 4.2. Scanning electron micrograph of *Gonyaulax grindleyi* showing a lack of superficial bacteria; a) whole cell, scale bar=20 μm; b) 6X zoom of insert showing reticulated surface and pores.
Figure 4.3. Clearance rates (ml animal$^{-1}$ h$^{-1}$) on Gonyaulax grindleyi by Calanus pacificus relative to the presence or absence of bacteria in the culture. 1-tail randomized t test revealed no significant difference between axenic G. grindleyi and xenic G. grindleyi. Both cultures were obviously more noxious than Gymnodinium splendens.
Figure 4.3

G. splendens

G. grindleyi

\[ P = 0.143 \]

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Figure 4.4. Clearance rates (ml animal$^{-1}$ h$^{-1}$) on *Gymnodinium splendens* by *Calanus pacificus* relative seawater strength. 1-tail randomized t tests revealed significant reductions of clearance rates between 100% and 90% seawater strength, and between 80% and 50% seawater strength.
Preliminary Chemical Defense Experiments

In all cases, analysis of covariance revealed that significant differences existed within the experiments, making multiple comparisons legitimate. Tables 4.1 and 4.2 display the results of the experiments and data analysis.

In all cases, *Gyrodinium resplendens* grew faster than *Gonyaulax grindleyi*. *G. resplendens* was not inhibited by *G. grindleyi*, and in 2 cases *G. grindleyi* was inhibited by *G. resplendens*. As expected, *G. resplendens* was readily eaten by *Calanus pacificus*. *G. grindleyi* was also eaten by the copepods. *Gonyaulax grindleyi* experienced a higher net growth rate in 2 experiments (a and b).

Table 4.1. Preliminary Chemical Defense Experiments. Slopes are given for log-transformed biomass (µg N l⁻¹) data. In each of these experiments, four 4l bottles were used. The first contained the preferred dinoflagellate (*Gyrodinium dorum* in experiment "a" and *Gyrodinium resplendens* in the rest). The second was a control containing a mixed (Mx) suspension of the preferred cell and *Gonyaulax grindleyi*. The third contained the mixed suspension of cells and a *Calanus pacificus* female (C). The last bottle contained a suspension of *G. grindleyi*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
<th>e</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preferred: Alone</td>
<td>0.017</td>
<td>0.042</td>
<td>0.047</td>
<td>0.053</td>
<td>0.051</td>
</tr>
<tr>
<td>Preferred: Mx</td>
<td>0.088</td>
<td>0.051</td>
<td>0.060</td>
<td>0.058</td>
<td>0.058</td>
</tr>
<tr>
<td>Preferred: Mx:C</td>
<td>-0.060</td>
<td>-0.028</td>
<td>0.016</td>
<td>0.046</td>
<td>0.044</td>
</tr>
<tr>
<td><em>G. grindleyi</em>: Alone</td>
<td>0.029</td>
<td>0.028</td>
<td>0.028</td>
<td>0.022</td>
<td>0.038</td>
</tr>
<tr>
<td><em>G. grindleyi</em>: Mx</td>
<td>0.040</td>
<td>0.027</td>
<td>0.016</td>
<td>0.031</td>
<td>0.033</td>
</tr>
<tr>
<td><em>G. grindleyi</em>: Mx:C</td>
<td>-0.009</td>
<td>-0.001</td>
<td>0.018</td>
<td>0.021</td>
<td>0.024</td>
</tr>
</tbody>
</table>
Table 4.2. Results of 1 tail F test comparisons between slopes. Significant results (Ho rejected, \( p < 0.05 \)) are indicated with (*) ; insignificant results are also indicated (ns).

<table>
<thead>
<tr>
<th>Hypothesis</th>
<th>Experiments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ho1. <strong>G. resplendens</strong> growth rate ( \leq ) <strong>G. grindleyi</strong></td>
<td>a b c d e</td>
</tr>
<tr>
<td>Ho2. <strong>G. resplendens</strong> not inhibited by <strong>G. grindleyi</strong></td>
<td>ns ns ns ns ns</td>
</tr>
<tr>
<td>Ho3. <strong>G. grindleyi</strong> not inhibited by <strong>G. resplendens</strong></td>
<td>ns ns * ns *</td>
</tr>
<tr>
<td>Ho4. <strong>G. resplendens</strong> not eaten</td>
<td>* * * * *</td>
</tr>
<tr>
<td>Ho5. <strong>G. grindleyi</strong> not eaten</td>
<td>* * * * *</td>
</tr>
<tr>
<td>Ho6. <strong>G. grindleyi</strong> net growth rate ( \leq ) <strong>G. resplendens</strong></td>
<td>* * ns ns ns</td>
</tr>
</tbody>
</table>

Chemical defense: Algal Concentration Ratios and Associational Defense

As with the previous experiments, the results of analysis of covariance allowed the use of multiple comparisons. The results of these experiments are displayed in Tables 4.3 and 4.4.

**Gyrodinium resplendens** grew faster than **Gonyaulax grindleyi** in two of the four cases. The other times the growth rates were not significantly different. In all cases, **G. resplendens** was eaten. **G. grindleyi** was eaten in all cases but one. In this case, the concentration of **G. grindleyi** was so low that accurate detection of feeding from cell counts was difficult (experiment a). **G. grindleyi** experienced a higher net growth rate than **G. resplendens** in the presence of **Calanus pacificus** in experiment d, where the **G. grindleyi:****G. resplendens** ratio (in terms of cellular nitrogen) was 10.

The experimental design of these experiments allowed me to determine whether the undefended cell, **Gyrodinium resplendens**, gained some protection from herbivory in the presence of the defended cell, **Gonyaulax grindleyi**. In all but 1 case **G. resplendens** obtained associational defense from **Calanus pacificus** in mixed suspensions.
Table 4.3. Algal Ratios and Associational Defense. Slopes are for log-transformed biomass (μg N 1⁻¹) data. Three 4l bottles were used in each of these experiments. The first bottle contained a mixed suspension of *Gyrodinium resplendens* and *Gonyaulax grindleyi*. The second bottle contained the same suspension and a *Calanus pacificus* female (C). The third bottle contained a suspension of *G. resplendens* and a copepod. The ratio of *G. grindleyi/G. resplendens* was varied in this series of tests. The ratios for these experiments were, respectively: 0.1, 0.43, 1, & 10.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. resplendens</em>:Mx</td>
<td>0.030</td>
<td>0.025</td>
<td>0.033</td>
<td>0.0151</td>
</tr>
<tr>
<td><em>G. resplendens</em>:Mx:C</td>
<td>0.037</td>
<td>-0.023</td>
<td>0.005</td>
<td>-0.019</td>
</tr>
<tr>
<td><em>G. resplendens</em>:Alone:C</td>
<td>-0.009</td>
<td>-0.013</td>
<td>-0.103</td>
<td>-0.081</td>
</tr>
<tr>
<td><em>G. grindleyi</em>:Mx</td>
<td>0.008</td>
<td>0.026</td>
<td>0.021</td>
<td>0.016</td>
</tr>
<tr>
<td><em>G. grindleyi</em>:Mx:C</td>
<td>0.004</td>
<td>-0.031</td>
<td>-0.023</td>
<td>0.009</td>
</tr>
</tbody>
</table>

Table 4.4. Results of 1-tail F tests between slopes. Significant results (Ho rejected, p < 0.05) are indicated with (*); insignificant results are also indicated (ns).

<table>
<thead>
<tr>
<th>Experiment</th>
<th>a</th>
<th>b</th>
<th>c</th>
<th>d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ratio <em>G. grindleyi/G. resplendens</em></td>
<td>0.1</td>
<td>0.43</td>
<td>1</td>
<td>10</td>
</tr>
<tr>
<td>Ho1. <em>G. resplendens</em> growth rate ≤ <em>G. grindleyi</em></td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td>ns</td>
</tr>
<tr>
<td>Ho2. <em>G. resplendens</em> not eaten</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ho3. <em>G. grindleyi</em> not eaten</td>
<td>ns</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Ho4. <em>G. grindleyi</em> net growth rate ≤ <em>G. resplendens</em></td>
<td>ns</td>
<td>ns</td>
<td>ns</td>
<td>*</td>
</tr>
<tr>
<td>Ho5. <em>G. resplendens</em> not defended by <em>G. grindleyi</em></td>
<td>*</td>
<td>ns</td>
<td>*</td>
<td>*</td>
</tr>
</tbody>
</table>
Chemical Defense: "Associational Liability"

Results of the "Associational Liability" experiment (Tables 4.5 and 4.6) show that *Calanus pacificus* ate *G. grindleyi* more rapidly when it was in a mixed suspension with *Gyrodiunium resplendens* than when presented alone. As with most of the other experiments, 1 tail F tests between the slopes indicated that *G. resplendens* grew at a faster rate than *Gonyaulax grindleyi*, and that both of these dinoflagellates were eaten. When presented in a mixed suspension to copepods, *G. grindleyi* did not grow any faster than *G. resplendens*, demonstrating that chemical defense, in this case, did not confer a competitive advantage. However, as shown before, *G. resplendens* in the mixed suspension was eaten less rapidly than this cell when fed to copepods alone.
Table 4.5. Slopes for chemical defense experiment including test for "associational liability". This experiment consisted of four 4l bottles. The first contained a mixture of *Gonyaulax grindleyi* and *Gyrodinium resplendens*. The second contained the same mixture with female *Calanus pacificus* grazers. The third bottle contained a suspension of *G. resplendens* with a copepod(s), and the fourth bottle contained a suspension of *G. grindleyi* with a copepods. Four copepods were placed in each test bottle.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Slope</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. resplendens</em>:Mx</td>
<td>0.033</td>
</tr>
<tr>
<td><em>G. resplendens</em>:Mx:Copepod</td>
<td>-0.003</td>
</tr>
<tr>
<td><em>G. resplendens</em>:Alone:Copepod</td>
<td>-0.04</td>
</tr>
<tr>
<td><em>G. grindleyi</em>:Mx</td>
<td>0.007</td>
</tr>
<tr>
<td><em>G. grindleyi</em>:Mx:Copepod</td>
<td>-0.018</td>
</tr>
<tr>
<td><em>G. grindleyi</em>:Alone:Copepod</td>
<td>-0.004</td>
</tr>
</tbody>
</table>

Table 4.6. Results of 1 tail F tests between slopes. Significant results (Ho rejected, p < 0.05) are indicated with (*); insignificant results are also indicated (ns).

Ho1. *G. resplendens* growth rate ≤ *G. grindleyi*  
Ho2. *G. resplendens* not eaten  
Ho3. *G. grindleyi* not eaten  
Ho4. *G. grindleyi* has advantage over *G. resplendens*  
Ho5. *G. resplendens* not defended by *G. grindleyi*  
Ho6. *G. grindleyi* not made more edible by *G. resplendens*
DISCUSSION

While copepods may find log phase phytoplankton to be more edible than stationary phase cells (Cowles et al. 1988), they react differently to Gonyaulax grindleyi. This cell, known to be rejected by Calanus pacificus (Huntley et al. 1986, Sykes and Huntley 1987 [Chapter 2], Huntley et al. 1987), follows the pattern of many toxic phytoplankters, being more noxious in exponential phase and, so, less readily eaten than stationary phase cells. This finding suggests that the compound(s) involved have some use to the growing cell, and are not produced as a result of some mechanism triggered during conditions of nutrient limitation as found in Prymnesium parvum (Shilo 1981).

The noxious compound(s) is produced endogenously. Calanus pacificus strongly rejects axenic Gonyaulax grindleyi, and SEM of nonaxenic G. grindleyi failed to demonstrate attached superficial bacteria. The compound may affect bacteria, however, because only one visually distinct morph was found when I observed the bacteria under epifluorescence and inoculated them onto nutrient agar plates whereas many forms were found in the readily eaten cultures (unpublished observations).

The evidence from previous studies (Huntley et al. 1986, Sykes and Huntley 1987 [Chapter 2], Huntley et al. 1987), as well as that given in the previous chapter suggests that the compound(s) in Gonyaulax grindleyi is an antiherbivore defense. Allelopathy is an unlikely function because neither Gyrodinium resplendens nor Gyrodinium dorsum was inhibited by G. grindleyi. Obviously, other algal cells may be inhibited, but the major evidence points to a defense against herbivores.
Gyrodinium resplendens grew at a greater rate than Gonyaulax grindleyi, in 8 of 10 experiments. This appears to be the general case as G. grindleyi and other rejected cells grew at significantly lower rates than preferred cells (Huntley et al. 1986). When Calanus pacificus is present, both cells in the mixture may be eaten, and occasionally G. grindleyi will experience a net growth rate greater than G. resplendens or G. dorsum. Under some conditions, then, G. grindleyi may be able to gain a strong refuge in heavily grazed environments. However, it appears that chemical defense may not generally confer upon G. grindleyi a competitive advantage in the form of a net growth rate higher than that of an undefended cell. Chemical defense may, however, allow established populations of this noxious cell to sustain heavy attack by grazers and may actually exclude them. Thus, a refuge for chemically undefended cells may exist by way of associational defense (Pfister and Hay 1988).

My final experiment also demonstrated that Gonyaulax grindleyi is made more edible by Gyrodinium resplendens and that G. resplendens is made less edible by the presence of G. grindleyi. This agrees with my evidence from chapter 3 that in algal mixtures Calanus pacificus is not perfectly capable of selecting edible cells from a suspension containing noxious cells.

How will the chemical defenses of Gonyaulax grindleyi affect the abundance and distribution of this cell in nature? From this study and others in this thesis, it appears possible that in a mixed community, G. grindleyi will be eaten with little or no effect on the grazers if the abundance of G. grindleyi is low. At higher cell densities, and particularly when G. grindleyi is a dominant member, this cell, and the whole assemblage to some degree, will be defended. Under some conditions G. grindleyi may experience higher
net growth rate than more readily eaten, rapidly growing cells. In conclusion, the chemical defenses of *G. grindleyi* should provide the strongest refuge from grazing when high levels of grazing pressure are applied to an established, rapidly growing population of this cell.
REFERENCES


CHAPTER 5

SUMMARY

PHYSIOLOGICAL-ECOLOGY AND CHEMICAL-ECOLOGY

OF

COPEPOD-DINOFLAGELLATE INTERACTIONS
ABSTRACT

The goal of my doctoral dissertation research was to investigate the physiological and behavioral aspects of the feeding biology of *Calanus pacificus* vis a vis the presence of noxious dinoflagellates. In addition, I studied the significance of chemical defense to the noxious dinoflagellate *Gonyaulax grindleyi*.

This research has led to several important discoveries in copepod feeding biology, behavior and in dinoflagellate chemical-ecology. These discoveries will give us a better understanding of copepod sensory and decision-making capabilities, and provide insight into dinoflagellate-copepod interactions. These findings show that *Calanus pacificus* is not only a selective feeder, but is an organism whose selectivity is highly plastic. The plasticity in selectivity is due to past feeding experiences, and involves associative learning when the copepod has ingested the noxious dinoflagellate *Gonyaulax grindleyi*. *C. pacificus* is capable of distinguishing between cells it has had experience with and cells it has had no experience with before. Occasionally *C. pacificus* is capable of selecting preferred cells out of mixed suspensions containing *G. grindleyi*.

At high concentrations, *Gonyaulax grindleyi* has several profound effects on *Calanus pacificus*, causing regurgitation, reduced fecundity, and starvation in the copepods. However, my studies show that chemical defense assists the defended cell only when it is in relatively high abundance, indicating that chemical defense probably plays a role in supporting existing blooms, and not in forming them.
Due to the critical position of herbivorous copepods in marine metazoan trophodynamics, the mechanisms of copepod feeding biology have long been a focal-point of study and debate (Huntley 1988 for a review). For the most part, researchers in the field recognize that marine herbivorous copepods are selective feeders. Indeed, these copepods are capable of discriminating on the basis of size (e.g. Frost 1972); nutritive value: live cells vs inanimate objects (e.g. Donaghay and Small 1979), live vs dead cells (e.g. Paffenhofer and Van-Sant 1985), high nitrogen vs low nitrogen (e.g. Houde and Roman 1987, Cowles et al. 1988), dinoflagellates vs tintinnids (e.g. Stoecker and Sanders 1985); and quality, noxious vs undefended dinoflagellates, (e.g. Huntley et al. 1986).

A major thrust of my thesis has been an investigation of the mechanism of selection against noxious dinoflagellates, Gonyaulax grindleyi in particular. In this chapter, I will discuss my findings in relation to what is known about the sensory capabilities of Calanus pacificus, and compare this copepod with other invertebrates. I will also offer a modification to Huntley's (1988) "new perspective" in which I will elaborate on Calanus' ability to learn, remember, and make decisions.

Many marine algae are known to produce secondary metabolites. Dinoflagellates are well known to produce very powerful toxins, but the ecological significance of such toxins is still relatively unknown. The possible functions for these metabolites could be antibacterial, as found in the dinoflagellate Prorocentrum minimum (Trick et al. 1981, Trick et al. 1984), allelopathic, as found in the green alga Pandorina morum (Harris 1971a,b, Harris and Caldwell 1974), or antipredatory, as found in the freshwater...
cyanobacterium *Microcystis aeruginosa* (Nizan et al. 1986), and as suggested by Ives (1985) for *Gonyaulax tamerensis*.

*Gonyaulax grindleyi* definitely produces some kind of noxious compound of unknown structure (Huntley et al. 1986), but it is not known to be a strongly toxic organism. The function of the compound(s) is clearly antipredatory (Huntley et al. 1986, Sykes and Huntley 1987 [Chapter 2], and Huntley et al. 1987a), but to what extent does chemical defense provide an ecological advantage for the defended cell? Could chemical defense be responsible for "Red Tides"?

In this chapter, I will also discuss the ecological ramifications of chemical defense in *Gonyaulax grindleyi* and similarly defended planktonic algae.

**Copepod feeding biology and sensory capabilities: Calanus pacificus vs Gonyaulax grindleyi**

Huntley et al. (1986) demonstrated that *Calanus pacificus* rejected *Gonyaulax grindleyi*, and that this copepod suffers high mortality when presented with this cell alone. High mortality in the presence of *G. grindleyi* is due to starvation as opposed to intoxication (Chapter 3). Closer examination (Chapters 2 and 3) of the mechanisms of rejection of *G. grindleyi* by *Calanus pacificus* revealed that this copepod was not immediately selective against *G. grindleyi*, but ingested it at high rates (up to 48 ml h\(^{-1}\)) for a short time. Thus, contrary to our present belief, *C. pacificus*, and other copepods, may in fact be rather cavalier in this selection of food once the copepod has determined that the prey is alive.

The duration of and intervals between mouthpart movements were similar to those made by copepods feeding on preferred cells (Sykes and
Huntley 1987 (Chapter 2)). Detailed frequency analysis of mouthpart movements made by Gill and Harris (1987) demonstrated that the mouthpart beat-frequency of *Calanus helgolandicus* and *Temora longicornis* was affected by noxious dinoflagellates. They demonstrated that mouthpart beat-frequency increased relative to seawater controls when they fed *Gonyaulax tamaensis* to *Calanus helgolandicus*. However, mouthpart beat-frequency decreased for *C. helgolandicus* in the presence of *Scrippsiella trochoidea* and *Gonyaulax aureolum*. Similarly, *T. longicornis* experienced reduced mouthpart activity in the presence of *G. tamaensis* and *G. aureolum*.

High ingestion rates in my studies, however, were transitory and *Calanus pacificus* then suffered lowered ingestion rates, and often regurgitated (Chapter 2 and 3). The selective behavior of *Calanus pacificus* then changed dramatically. To the best of my knowledge, this thesis contains the first report of associative learning in a planktonic organism (Chapter 3). *C. pacificus* with experience in the noxious cell *Gonyaulax grindleyi* began to ingest preferred dinoflagellates at pre-experience rates before ingesting *G. grindleyi* at naive rates. This is an example of associative learning with adverse conditioning. In the case of *G. grindleyi* and *C. pacificus*, the adersive stimulus is provided by the dinoflagellate itself. In associative learning experiments with congeneric species of preferred dinoflagellates, *C. pacificus* demonstrated the ability to distinguish between *Gyrodinium dorsum* and *Gyrodinium resplendens*.

Huntley (1988) discussed the importance of past feeding history on ingestion rates and decried the fact that so little was actually known about it. The previous paradigm as developed over the last 25 years was based on the proposition that all forcing functions (light, temperature, food abundance,
food size, food quality, feeding history, and body size) act in the present, and have some minor affect on feeding history. Huntley (1988) proposed that that while the forcing functions may act instantaneously, the integration of these factors in past feeding history may have the greater effect on feeding rates.

My studies have shown that due to the capacity of Calanus pacificus to learn and remember short-term (single-trial, 5min) feeding experiences, at least 12 h of past feeding history may override most ingestion rate forcing functions, particularly when the copepod has experienced noxious dinoflagellate prey. I propose the following conceptual model for decision-making in Calanus pacificus diagramed below (Figure 5.1). In this model, a particle is first detected by mechanoreceptors (Légier-Visser et al. 1986), and rejected instantaneously as being out of the present correct size range (modulated by past experience), or accepted for further analysis. Mechanoreception appears to be more sensitive to the presence of a cell than chemoreception because cells are detected at distances (Strickler 1982, Koehl 1983, Price et al. 1983) that exceed the likely detectable deformed active spaces of exuded chemical cues (Andrews 1983, Strickler 1982, Friedman and Strickler 1975). In my own experiments (Chapter 3), experienced Calanus pacificus captured Gonyaulax grindleyei cells, and then flicked them away. This corroborates the hypothesis that the copepod first senses the physical presence of a particle before it detects a chemical scent. Chemoreception (Andrews 1983) may occur simultaneously, but the decision to reject a noxious cell usually follows its capture.

In the next step, the "primary" quality of the cell is determined by chemoreceptors (alive vs dead or inanimate (Donaghay and Small 1979,
Paffenhöfer and Van-Sant 1985), autotroph vs heterotroph (Stoeker and Sanders 1985), and high nitrogen vs low nitrogen (Houde and Roman 1987, Cowles et al. 1988, and Butler et al. 1989)). The interpretation of "quality" may be modified by past history as well. For example, Price and Paffenhöfer (1984) found that Eucalanus pileatus ingested larger diatoms at higher rates if the copepods had had recent previous experience ingesting these cells. If the cell is not rejected, it is then captured and its taste (due to secondary metabolites) or "secondary" quality is determined.

This is the step that is most dramatically affected by recent past history. If the copepod is naive, then most large, live, nitrogen-rich cells will be ingested at a high rate, regardless of any noxious, secondary metabolites the cell may contain (Sykes and Huntley 1987 [Chapter 2] and Chapter 3). If the copepod has had recent experience with a noxious cell, then it may be rejected even though the animal may ingest other, nontoxic cells. If nontoxic cells are ingested with no ill-effects to the copepod, the experience will positively affect the previous selection processes for that cell (Price and Paffenhöfer 1984). If, on the other hand, the naive copepod ingests a sufficient quantity of noxious cells (eg. Gonyaulax grindleyi), the copepod will suffer reduced ingestion rates, and regurgitate (Sykes and Huntley 1987 [Chapter 2] and Chapter 3). For some period after regurgitating, C. pacificus is incapacitated, ingesting few, if any, cells of any species. The duration of this incapacitation should be a function of the dose of the noxious compound received minus the rate at which it is destroyed or excreted. C. pacificus associates negative experiences (i.e. regurgitation) with past feeding bouts; therefore, this copepod will now avoid ingesting these noxious dinoflagellates. Note that C. pacificus will also reject preferred cells
if that cell was present in a mixture with *Gonyaulax grindleyi*. This suggests that copepods cannot determine which cell caused the negative experience, but associates that experience with any recently ingested cell (Chapters 3 and 4).

The positive and negative experiences, as well as a memory of the feeding conditions are retained for some period, and will modulate the decision-making steps of the copepod. I propose that the ingestion rate equation of a copepod be organised into two components, and be simplistically written as \( I = H I_p \) where "H" stands for the sum of all past feeding history, and \( I_p \) stands for the effects of all forcing functions acting in the present to determine the ingestion rate of the copepod.

Feeding history is the summation of all feeding experiences and feeding conditions. The formula for long-term feeding history should be written as follows: \( H = M_1 + M_2 + M_3 \ldots M_n \) where "M" is the effect of a past feeding experience.

A simple dimensional analysis of memory is presented in Table 5.1. Memory of feeding experience on noxious food items will be a function of the initial mass of the offensive compound ingested, "m", the mass of the copepod, "W", the conditioning period, "t", and the length of time since the initial conditioning (the "recovery period"), "T".
Table 5.1. Dimensional analysis of the effect of memory on copepod feeding rates. In this case, *Calanus pacificus* is feeding on the noxious dinoflagellate *Gonyaulax grindleyi*. All other forcing functions affecting ingestion rates are ignored.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Units</th>
<th>Proportionality with Memory</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass of Compound</td>
<td>m</td>
<td>g</td>
<td>M m</td>
<td>$6.5 \times 10^{-11} - 1.2 \times 10^{-8}$ g$^a$</td>
</tr>
<tr>
<td>Copepod Mass</td>
<td>W</td>
<td>g</td>
<td>M W$^{-1}$</td>
<td>$0.15 - 0.30 \times 10^{-8}$ g$^b$</td>
</tr>
<tr>
<td>Conditioning Period</td>
<td>t</td>
<td>h</td>
<td>M t</td>
<td>0.08-0.2 h$^c$</td>
</tr>
<tr>
<td>Recovery Period</td>
<td>T</td>
<td>h</td>
<td>M T$^{-1}$</td>
<td>4 -24 h$^d$</td>
</tr>
</tbody>
</table>

a. Approximated as 1% of the *Gonyaulax grindleyi* carbon ingested by *Calanus pacificus* during videotaped experiments (Chapter 3).
c. Chapter 3
d. Chapter 3

From the relationships of the parameters with memory, we may begin to understand the effect that memory will have on ingestion rates. Memory can be represented as the following function: \( M = (m)(t)(W^{-1})(T^{-1}) \). Using the estimated ranges of each parameter in Table 5.1, we find that memory may exert an influence over ingestion rate of four orders of magnitude. Clearly, this may explain much of the variability found in the copepod feeding biology literature (Huntley 1988).

The selection of a particular size class, abundance, species, temperature regime, or nutritional status over another due to recent past experience is probably caused by simple learned behavior such as sensitization and desensitization. A memory function for these forcing functions may be radically different from the one I have just described. Selection against noxious cells, or preferred cells in a mixture with noxious ones is due to associative learning. In the case of a copepod with recent feeding experience
with a noxious dinoflagellate, part of the short-term memory function may contain natural decay functions ($m_o e^{-kT}$) to explain the loss of the noxious compound from the copepod, and perhaps to describe the loss of associative learning. In this equation, $m_o$ stands for the initial dose of the noxious compound ingested, $T$ is the time from the initial encounter, and $k$ stands for a decay constant. Short-term memory as portrayed here must be expanded to become an integration of memories of all beneficial and harmful feeding experiences.

Forcing functions affecting ingestion rates in the present have been exhaustively studied by others (Huntley 1988). Light, temperature, cell size, quality (Huntley 1988), and cell abundance should affect short-term memory, long-term history, as well as instantaneous ingestion rate. The extent to which these forcing functions will contribute to these different components is still unknown.

Short-term memory of aversive feeding experiences suggests that while herbivorous copepods may have extremely sophisticated mechanosensory and chemosensory capabilities (Gill 1986, Gill and Harris 1987, Koehl and Strickler 1981, and Friedman and Strickler 1975), they can only use them to their fullest extent when phytoplankton prey are diverse, and noxious cells are present in the assemblage. If the copepod is in conditions of rapidly changing food assemblages, the copepod will be able to select preferred foods from the available food items.

Invertebrate learning and memory have been well studied (see the following for reviews Carew and Sahley 1986 and Corning, Dyal, and Willows 1973a, 1973b, and 1975). Relatively simple mollusks such as terrestrial and marine slugs are known to reject preferred foods after a negative
reinforcement has been administered. *Aplysia faciata* was trained to avoid preferred foods by enclosing the algae in a mesh bag making taste possible, but denying ingestion of the food. Memory of this training lasted 24 hours (Suiswein and Schwarz 1983 and Susswein *et al.* 1986). Thompson (1917) demonstrated associative learning to a positive stimulus. The freshwater snail *Physa* retained its memory for approximately 96 d after 250 paired trials. Gelperin (1975) showed that *Limax* had learned to avoid previously preferred foods after a single trial. Using the same mollusc, Sahley *et al.* found that single-trial associations were retained for 3 d.

Dahl (1884) demonstrated associative learning in the jumping spider *Attus arcuatus*. This spider avoids certain bees and beetles, and will avoid edible insects if the spider had attacked a turpentine coated conspecific. This spider continued to make the association for a few hours after the initial experience. In other works on spiders, Drees (1952) found that the spider *Salticus* would avoid ants after attacking them a few times. Similar to my findings, the spiders became unresponsive to preferred prey as well for a short time indicating a general malaise. Eventually, the spider would once again become responsive to preferred prey, but would avoid ants.

Benthic crustaceans also are known to demonstrate learned behavior. The hermit crab, *Pagurus striatus*, associated a colored light with being tapped between the eyes. After a training period of 12 days, the learned behavior gradually declined after a 20 day period (Mikhailof 1922). In this, and most of the previous examples, the unresponsive behavior was manifest only after repeated trials. In my studies, a 5 minute conditioning period was sufficient for *Calanus pacificus* to learn to avoid (significantly lowered ingestion rate) *Gonyaulax grindleyi* for periods exceeding 12 hours.
Thus, it appears that, like other invertebrates, herbivorous marine copepods such as *Calanus pacificus* are capable of relatively sophisticated decision-making based on surprisingly rapid associative learning. Perhaps this is not so surprising in light of the chemically complex array of potential food items copepods must select from.

**Chemical Ecology**

During the course of my thesis, I concentrated on the noxious effects of *Gonyaulax grindleyi*, a large (~45 µm dia.), thecate dinoflagellate. This dinoflagellate was most noxious to *Calanus pacificus* when dividing exponentially, and the noxious quality did not appear to be derived from exosymbiotic bacteria (Chapter 4). I did not investigate the possible presence of endosymbiotic bacteria, but this would be good idea for further investigation in light of the findings of Kodama *et al.* (1989) who demonstrated strong evidence for endosymbiotic, saxitoxin-producing, bacteria in *Protogonyaulax tamerensis*.

The noxious chemical(s) in dinoflagellates could have several functions: antibacterial, allelopathic, or antipredatory. Most likely, *Gonyaulax grindleyi*, whose noxious compounds are as yet undescribed, uses these noxious compounds as antipredatory agents. That *G. grindleyi* negatively affects *Calanus pacificus* is now well documented (Huntley *et al.* 1986, Huntley *et al.* 1987, Sykes and Huntley 1987 [Chapter 2], and Chapter 3). Copepods starve in the continued presence of this cell, often regurgitate *G. grindleyi* if it is ingested, and learn to avoid ingesting this cell with a memory that lasts for 12 or more hours. The ecological significance of chemical defense is still somewhat unclear.
Huntley et al. (1986) suggested that chemical defense and selective feeding may play a role in the formation of dinoflagellate blooms. Kim et al. (1989) indirectly supported this hypothesis in their investigation of the effects of 5 species of cladocerans on the populations of 2 species of Prorocentrum and a species of Ceratium. The authors determined that the cladocerans could not appreciably graze the forming bloom. This may be due to the low grazing rates of cladocerans, but could also be due to the probable noxious quality of these dinoflagellates. Copepods are capable of significant grazing of phytoplankton (potentially 90% of the daily primary production) to the extent that they may control bloom formation (Bathmann et al. 1990). Uye (1986) and Uye and Takamatsu (1990) have shown that some red-tide organisms were readily ingested by copepods while others appeared to be chemically defended. Perhaps chemical defense allows certain dinoflagellates to form blooms in the presence of grazing copepods.

My results suggest that chemical defense may indeed play a role, but probably not so much in the formation of the bloom. The strongest theory of bloom formation by dinoflagellates is that blooms are caused by the germination of cysts under appropriate conditions (Stiedinger 1983 for a review) rather than being formed as a result of chemical defense of the dinoflagellate while under strong grazing pressure.

When a chemically defended cell is at low concentrations in a natural assemblage, the copepods will most likely not be affected by its presence, and the chemically defended cell will be eaten along with the preferred prey items. Only occasionally did G. grindleyi demonstrate a higher net growth rate than the preferred cell due to chemical defense and selective feeding (Chapter 4). In nearly all of these experiments, the preferred cell acquired
an associational defense from the presence of the noxious cell. This makes sense in view of the results of my associative learning experiments (Chapter 3) where experienced *Calanus pacificus* ingested preferred cells that had been in a mixed suspension with *G. grindleyi* at a lower rate than another species of preferred dinoflagellate. Additionally, *G. grindleyi* was more readily ingested when in the presence of preferred cells. Thus, any advantage provided by chemical defense is shared, to some extent, by all of the members of the assemblage.

Chemical defense (if similar to that of *Gonyaulax grindleyi*) will definitely allow the persistence of an established population of chemically defended dinoflagellates should herbivorous copepods increase in the community. Indeed, the establishment of a defended cell such as *G. grindleyi* may serve to inhibit the increase of herbivores in the system.
Figure 5.1. Conceptual model for decision-making by *Calanus pacificus* as it and other factors will effect ingestion rate. In this model, *C. pacificus* analyses the quality of cells before and after ingesting them. The decisions made from these analyses are highly plastic being affected by previous experience. Note that past feeding history has been split into short-term "Memory" and long-term "History". Memory includes simple learned behavior such as sensitization and desensitization, and complex learned behaviors such as associative learning. Memory probably acts over time periods of minutes to several days. History is the long-term integration of all past feeding experiences (positive and negative) and feeding conditions. Symbols: + and - symbols indicate the positive or negative effects on short-term memory or decision-making, Y = Yes, N = No.
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