Evidence of a defensive role for limatulone, a novel triterpene from the intertidal limpet *Collisella limatula*

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**ABSTRACT:** *Collisella limatula*, a limpet found abundantly in the rocky intertidal from Oregon to Baja California, has recently been found to contain a novel triterpene, limatulone. The present paper describes laboratory experiments and field observations that strongly suggest that limatulone acts as a potent chemical defense against some intertidal predators. Intertidal fish and crabs rejected pieces of the foot of *C. limatula*, but readily ate the foot tissue of co-occurring gastropods. In contrast, individuals of *C. limatula* were eaten by seastars, an octopus and sea gulls. Fish antifeedant activity was restricted to a single compound, limatulone, which was present in the foot of the limpet at a mean concentration of 3.5 ppt dry weight. Food pellets containing limatulone at concentrations of ≥ 0.5 ppt dry weight induced regurgitation in the intertidal fish *Gibbonsia elegans*, a known limpet predator. Concentrations of limatulone in *C. limatula* varied from one geographic location to the next and from one month to the next. Reasons for these variations are not understood. Surveys of the 5 most common limpets in an undisturbed rocky intertidal site revealed that *C. limatula* was the most abundant limpet inside tidepools and on intertidal boulders. Limatulone may act to defend foraging limpets from predatory fishes in tidepools. From the boulder field habitat, shell damage incurred by limpets from wave-borne rocks and debris may expose foot tissue prior to shell regeneration, during which time the chemical defense may release *C. limatula* from predatory constraints imposed on other intertidal limpets.

**INTRODUCTION**

Most gastropod molluscs bear a calcified shell which protects the animal from predation and physical injury. Some opisthobranch gastropods, especially nudibranchs, are believed to have lost their shells with a concomitant elaboration of various chemical defense strategies (Thompson 1960, Faulkner & Ghiselin 1983). These sea slugs are, however, restricted in their distribution to subtidal and cryptic intertidal habitats where physical stresses are reduced.


Recent evidence has suggested that some pulmonate limpets augment the physical defense provided by a shell with a chemical defense against predation. Branch & Cherry (1985) found that irritated individuals of *Siphonaria capensis* produce copious amounts of a milky mucus which thwarts predatory gastropods, seastars, fishes and birds. Rice (1985) reported that *Trimusculus reticulatus*, when agitated, secretes a milky-white mucus which immobilizes the tube feet of attacking seastars and inhibits predation by them. Polypropionate metabolites, some of which are pharmacologically active, have been isolated from 9 species of *Siphonaria* investigated to date: *S.
denticulata (Hochlowski et al. 1983), S. diemenensis (Hochlowski & Faulkner 1983), S. pectinata (Biskupiak & Ireland 1983), S. atra, S. zealindica, S. normalis, S. laciniosa (Hochlowski et al. 1984), S. australis (Hochlowski & Faulkner 1984) and S. lessoni (Capon & Faulkner 1984), but the ecological consequences of the presence of these compounds in any of these pulmonates has yet to be demonstrated.

Collisella (= Acmaea) limatula, is a prosobranch limpet found abundantly in the rocky intertidal from Oregon to Baja California (Morris et al. 1980). Albizati et al. (1985) have recently demonstrated that C. limatula contains a novel triterpene, limatulone, the chemical structure of which is shown in Fig. 1.

The present paper is the result of an investigation into the possible ecological importance of limatulone.

MATERIALS AND METHODS

Biological assays; field experiments. Three or more individuals of each of 3 species of limpets (Collisella limatula, C. scabra and Lottia gigantea) were freshly collected, dissected, and the foot of each diced into \( \sim 3 \times 3 \times 3 \) mm pieces. Foot pieces were randomly offered to groups of 4 to 10 individuals of each of 2 species of tidepool fishes (juvenile Girella nigricans and Clinocottus analis) found in 2 tidepools near Dike Rock, approximately 1 km north of Scripps Pier, La Jolla, California (Fig. 2). Food offerings were recorded as having been eaten or, after repeated inspection, abandoned at the bottom of the tidepool.

Biological assays; laboratory experiments. Three or more individuals of each of 3 species of limpets (Collisella limatula, C. digitalis and Lottia gigantea) and 1 species of snail (Tegula funebralis) were diced as before and the pieces randomly offered to 2 species of tidepool fishes, 10 individuals of Gibbonsia elegans and 3 individuals of Hypsoblennius gilberti, housed in individual compartments in laboratory aquaria. Food offerings were presented to fish in a pipette at the top of the compartment; normal consumption of a food offering was followed by normal respiratory movements, while rejection of a food offering involved flaring of the gill covers and subsequent regurgitation of the ingested piece.

Experiments with the hermit crab Pagurus samuelis entailed placing a \( \sim 4 \times 4 \times 4 \) mm piece of foot tissue from each of the 2 limpets Collisella limatula and Lottia gigantea together in the middle of a 250 ml finger bowl along with a single crab (20 crabs used in assay). The type of foot tissue initially held in the claws of the crab, and the type held 10 min thereafter was
recorded (foot tissue of *C. limatula* is pale yellow, that of *L. gigantea* is white).

Whole, similarly-sized, overturned individuals of *Collisella limatula* and *Lottia gigantea* were randomly added to aquaria containing individual intertidal sea-stars, 2 each of *Pisaster giganteus* and *Astrometis sertulifera*, and the results recorded after 24 h. Whole limpets were similarly offered to an octopus (*Octopus* sp.). Whole foot pieces of *C. limatula* and *L. gigantea* were randomly offered to 2 captive California sea gulls (*Larus californicus*) that had been starved for 12 h preceding the assay; their responses to the food offerings were recorded.

Extracts of freeze-dried *Collisella limatula* (see below) were used in feeding assays performed on the spotted kelpfish *Gibbonsia elegans*. Extracts were dissolved in their respective solvents and injected into freeze-dried krill pellets (*Euphausia* sp., San Francisco Bay Brand Ocean Plankton, approximately 4 mg each) so as to saturate each pellet at 100 ppt pellet dry weight, thereafter the pellets were dried under nitrogen. Experimental pellets and control pellets treated with solvent alone (paired) were randomly fed to fish, with the responses recorded as previously described. The assay was repeated with purified limatulone at 5.0, 1.0, 0.5 and 0.1 ppt dry weight of pellet.

Where applicable, the non-parametric G-test of goodness of fit (hermit crab assay) or the G-test of independence (all others) was employed to ascertain significance of the difference between frequency distributions (Sokal & Rohlf 1981).

**Extraction of limpets and isolation of limatulone.** Freeze-dried *Collisella limatula* (about 400) were extracted sequentially in hexane, diethyl ether, ethyl acetate and methanol, and the extracts assayed for inhibition of fish feeding. The combined hexane and diethyl ether soluble extracts were fractionated by flash chromatography (Still et al. 1978) on silica gel (E. Merck, 230 to 400 mesh) with eluants of increasing polarity from 100% hexane to 100% diethyl ether. The active component, limatulone (Fig. 1), was eluted in the 100% diethyl ether fraction (95 to 99% purity). Final purification of limatulone was achieved by high-performance liquid chromatography on Partisil with 60% hexane: 40% ethyl acetate as eluant. The structural determination of limatulone has been previously described (Albizati et al. 1985). In the present study, analysis for limatulone relied on thin-layer chromatography against a purified standard together with ^1H nuclear magnetic resonance spectroscopy.

The localization of limatulone in the limpet was determined in the following manner: pedal mucus was transferred from the foot of each of 100 limpets onto filter paper. The limpets and filter paper were freeze-dried and the limpets dissected into 3 parts (shell, viscera and foot). Shells, visceral masses, foot tissue and the pedal mucus on filter paper were each separately extracted and analyzed for limatulone.

In an attempt to isolate limatulone from the algal diet of the limpet, rocks covered with enrusting red algae of the genera *Hildenbrandia*, *Peyssonelia*, *Li-thothamnion* and *Lithophyllum*, algae upon which *C. limatula* are known to feed (Eaton 1968, J. R. Pawlik unpubl. obs.), were extracted and analyzed for limatulone.

**Bi-monthly determination of limatulone concentrations.** The levels of limatulone in limpet foot tissue were quantified for *Collisella limatula* collected from Dike Rock (1.1 km north of Scripps Pier, La Jolla, California), Sunset Cliffs (Ladera Street, Point Loma, San Diego, California) and San Nicolas Island (N. W. side, Fig. 2) from June 1984, through October 1985. For each collection, 90 to 100 limpets were frozen, freeze-dried and dissected into 3 parts (shell, visceral mass and foot). The parts were weighed, individual shell lengths measured, shells checked for evidence of repair and limatulone quantitatively isolated from the foot tissue.

**Field surveys.** Field transects were carried out in the lower intertidal at San Nicolas Island, where the intertidal environment is free of human interference. Two adjacent sites on the N. W. side of the island were surveyed (Fig. 2). They consisted of (1) an intertidal boulder field, approximately 100 m in length, composed of large (1 to 3 m) boulders interspersed with sand and cobbles, and (2) a rock shelf outcrop, approximately 50 m in length, pitted with large (2 to 4 m diameter, irregular) tidepools.

At the boulder site, 8 similarly shaped boulders with approximately equal surface areas were randomly selected and the number of each species of limpet counted, dividing the count into limpets on the seaward side (mostly vertical plane facing the water), landward side (mostly vertical plane facing the land), and top (mostly horizontal plane) of each boulder. At the tidepool site, 4 pools were haphazardly chosen, a grid was established and 5 random 0.25 x 0.25 m quadrat samples were taken. The number of each limpet species was counted within each quadrat sample.

**RESULTS**

**Biological assays**

The results of biological assays on whole and dissected limpets are presented in Table 1. Adult sculpins *Clinocottus analis* and juvenile opaleye *Girella nigricans* promptly ate small pieces of the foot of *Collisella scabra* and *Lottia gigantea*, but refused pieces of the
Table 1. *Collisella limatula*. Feeding responses of intertidal predators to *C. limatula* and alternative sympatric gastropods. *T* = tidepool assay, *L* = laboratory assay; *P* = foot pieces assayed, *W* = whole limpet assayed, *F* = whole foot assayed. Except for hermit crab assay, number in replicates column is the number of randomized, paired comparisons of the response of assay predators to the foot of *C. limatula* versus the foot of the listed alternative gastropods. *E/C* is the number of *C. limatula* foot offerings eaten/number of alternative gastropod foot offerings eaten. For hermit crab assay, the number in the replicates column is the number of times the choice experiment was run; *E/C* is the number of crabs holding *C. limatula*/alternative gastropod foot pieces after 10 min.

<table>
<thead>
<tr>
<th>Potential predator</th>
<th>Common name</th>
<th>Alternative gastropod tested</th>
<th>Experimental conditions</th>
<th>Replicates</th>
<th>E/C</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Girella nigricans</em></td>
<td>Opaleye</td>
<td><em>Lottia gigantea</em></td>
<td><em>T/P</em></td>
<td>15</td>
<td>0/15*</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Collisella scabra</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Clinocottus analis</em></td>
<td>Woolly sculpin</td>
<td><em>L. gigantea</em></td>
<td><em>T/P</em></td>
<td>7</td>
<td>0/7</td>
</tr>
<tr>
<td><em>Gibbonsia elegans</em></td>
<td>Spotted kelpfish</td>
<td><em>C. scabra</em></td>
<td><em>L/P</em></td>
<td>21</td>
<td>0/21*</td>
</tr>
<tr>
<td><em>Hypsoblennius gilberti</em></td>
<td>Notch brow blenny</td>
<td><em>Tegula funebralis</em></td>
<td><em>L/P</em></td>
<td>18</td>
<td>3/18*</td>
</tr>
<tr>
<td><em>Pagurus samuelis</em></td>
<td>Hermit crab</td>
<td><em>L. gigantea</em></td>
<td><em>L/P</em></td>
<td>56</td>
<td>10/46*</td>
</tr>
<tr>
<td><em>Pisaster giganteus</em></td>
<td>Knobby seastar</td>
<td><em>L. gigantea</em></td>
<td><em>L/W</em></td>
<td>4</td>
<td>4/4</td>
</tr>
<tr>
<td><em>Astrometis sertulifera</em></td>
<td>Soft seastar</td>
<td><em>L. gigantea</em></td>
<td><em>L/W</em></td>
<td>4</td>
<td>4/4</td>
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<tr>
<td><em>Octopus sp.</em></td>
<td>Octopus</td>
<td><em>L. gigantea</em></td>
<td><em>L/W</em></td>
<td>3</td>
<td>3/3</td>
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<tr>
<td><em>Larus californicus</em></td>
<td>Sea gull</td>
<td><em>T. funebralis</em></td>
<td><em>L/F</em></td>
<td>6</td>
<td>6/6</td>
</tr>
</tbody>
</table>

* p < 0.05, G-test

foot of *C. limatula*. In laboratory experiments, the common tidepool fishes *Gibbonsia elegans* and *Hypsoblennius gilberti*, both known limpet predators (Mitchell 1953, Stephens et al. 1970), rejected the pedal tissue of *C. limatula*, although pieces of the foot of co-occurring gastropods were eaten. Both species of fish readily ate pieces and whole portions of the visceral mass dissected from *C. limatula*.

In 56 choice experiments with the hermit crab *Pagurus samuelis*, pieces of the foot of *Collisella limatula* were held by 24 crabs and pieces of the alternative limpet were held by 32 crabs at the onset of the experiment; after 10 min, 10 crabs held *C. limatula* and 46 held the alternative limpet. The final frequency distribution was significantly different from both a random distribution and from the distribution at the onset of the experiment (G-test, *p* < 0.005).

Intertidal seastars *Astrometis sertulifera* and *Pisaster giganteus* readily consumed whole, overturned limpets, including *Collisella limatula*. Moreover, the foot tissue of both *C. limatula* and *Lottia gigantea* was eaten by an octopus and by captive California sea gulls *Larus californicus*.

Only the hexane and diethyl ether soluble extracts of whole *Collisella limatula* inhibited fish feeding. Separation of the active component of these extracts yielded limatulone, which induced regurgitation in *Gibbonsia elegans* at concentrations as low as 0.5 ppt dry weight of food pellet (Table 2).

Table 2. *Gibbonsia elegans*. Feeding responses of an intertidal predatory fish to food pellets containing limatulone. *N* = the number of randomized, paired comparisons of control and treated pellets. *L/C* is the number of limatulone-treated pellets eaten/number of control pellets eaten.

<table>
<thead>
<tr>
<th>Limatulone concentration* ppm</th>
<th>N</th>
<th>L/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.0</td>
<td>10</td>
<td>0/10**</td>
</tr>
<tr>
<td>1.0</td>
<td>10</td>
<td>0/10**</td>
</tr>
<tr>
<td>0.5</td>
<td>10</td>
<td>0/10**</td>
</tr>
<tr>
<td>0.1</td>
<td>10</td>
<td>9/10</td>
</tr>
</tbody>
</table>

* *p* < 0.05, G-test

**Extraction of limpets and isolation of limatulone**

Limatulone was found in the foot of *Collisella limatula*, but not in the shell, visceral mass, or pedal mucus. The mean concentration of limatulone was 3.5 ppt dry weight of the foot (*N* = 18 collections of 90 to 100 limpets, *SD* = 0.99 ppt, range = 1.9 to 5.2 ppt). Limatulone was not found in extracts of rocks covered with encrusting red algae of the genera *Hildenbrandia*, *Peyssonelia*, *Lithothamnion* and *Lithophyllum*. 
Bimonthly determination of limatulone concentration

The concentration of limatulone was greater in *Colisella limatula* from San Nicolas Island than in limpets from the 2 collection sites near San Diego for contemporaneous collections from the 3 sites (Fig. 3a). Levels of limatulone in the foot tissue of limpets from all 3 sites showed similar fluctuations over the course of the sampling period; most notably, a drop in limatulone concentration for the April 1985 collections. The same concentration of limatulone was found in the foot of limpets from both the boulder field and tidepool habitats of San Nicolas Island based on 2 separate collections: one from February, the other from July, 1985. Recovery of limatulone from the foot of *C. limatula* with the outlined extraction procedure proved to be highly reproducible in a limited number of replicates. With the possible exception of *C. limatula* from the Sunset Cliffs collection site, the fluctuations in limatulone concentration did not appear to follow trends in mean shell size or weight, or mean freeze-dried viscera or foot weight (Fig. 3b to e).
At the San Nicolas Island site, shell heights of *Collisella limatula* from the boulder field were significantly greater than those of their conspecifics in tidepools (Fig. 4). The most commonly observed type of shell damage was 'skirt' formation ([Fig. 5c, d]), with a concentric, marginal growth of more recently accreted shell material around an apical 'cap'. Of 1751 specimens of *C. limatula* collected during the present study, 30% showed skirt formation, with obvious variation between collection sites: 34.0% of 288 limpets collected from the San Nicolas Island boulder field had suffered concentric marginal damage, whereas only 4.4% of 137 limpets from the tidepools showed similar damage.

*Collisella limatula* from the Sunset Cliffs site were collected from a high-energy boulder field similar to that found on San Nicolas Island: 39.0% of 934 limpets collected there exhibited skirt formation. The Dike Rock population of *C. limatula* inhabited boulders on the shoreward side of a rock reef that bore the brunt of wave energy: 14.5% of 392 limpets collected from that site showed evidence of margin regeneration.

**Field surveys**

*Collisella limatula* was the most abundant limpet on boulders, with nearly twice as many individuals as the next most abundant limpet, *C. scabra* (Fig. 6a). *C. limatula* was found predominantly on the sides of the boulders, especially on the seaward side, with *C. scabra*, *C. digitalis* and *Lottia gigantea* predominating on the top surface. At the tidepool site, *C. limatula* was found almost exclusively submerged in tidepools, whereas the other limpets sampled were found almost exclusively outside tidepools (Fig. 6b). *C. scabra* was the most abundant limpet at the tidepool site, followed closely by *C. limatula*.

**DISCUSSION**

In laboratory assays, limatulone is a highly potent inhibitor of fish feeding – nearly 10 times more effective on a dry weight basis at inducing regurgitation than polygodial, a defensive compound isolated from nudibranchs (Cimino et al. 1982, Okuda et al. 1983). Although effective against fish and crab predators, limatulone had no apparent effect on seastars, octopuses or sea gulls. This would imply that *Collisella limatula* may be vulnerable to these predators. However, *C. limatula* has a characteristic behavioral avoidance response upon contact with, or in the presence of, predatory seastars (Bullock 1953, Feder 1963, Phillips 1975, 1976), and Wells (1980) concluded that *C. limatula* avoided octopus predation by remaining inactive and inconspicuous when awash during daylight hours. Black oyster catchers *Haematopus* spp. prey on limpets (Hartwick 1976, Branch & Cherry 1985), and sea gulls may have some impact (Lindberg & Chu 1982), although the overall importance of seabird predation on intertidal gastropods is thought to be minor in comparison to that caused by marine organisms (Bertness et al. 1981 and references therein).

Limatulone was found only in the foot of *Collisella limatula*, not in the pedal mucus, viscera or shell. Some intertidal pulmonates possess defensive mucoid secretions, including onchids (Ireland & Faulkner 1978) and siphonariid limpets (Branch & Cherry 1985, Rice 1985, D. J. Faulkner unpubl. obs.). *C. limatula*, a prosobranch, does not exude limatulone into its pedal mucus, yet the avoidance response of potential pre-
dators is similarly immediate and dramatic. Tidepool fishes were never observed to make repeated attacks on overturned *C. limatula* (as they do with other limpet species), and attacks that were made resulted in no apparent harm to the limpet. Limatulone may be released directly from the foot as the limpet is attacked. *C. limatula* appears to avoid the expense of secreting a chemical defense along with its pedal mucus, a substance that is produced in copious amounts during gastropod locomotion (Calow 1974, Denny 1980).

The absence of limatulone in the visceral mass of *Collisella limatula* suggests that the compound is not directly derived from the diet, corroborated by the absence of limatulone in extracts of rocks covered with the encrusting red algae preferred by these limpets (Eaton 1968). It seems most likely that limatulone is synthesized by *C. limatula*, probably from related but inactive dietary precursors.

There are many potential explanations for the variation in the concentration of limatulone in the foot of *Collisella limatula* collected from San Nicolas Island versus those collected near San Diego, and for seasonal variation at all 3 study sites. If algal precursors are necessary for the synthesis of limatulone, the algae may show variations in their local or temporal abundance or chemical constitution. The levels of limatulone were the same for both limpets collected from within tidepools and on boulders at San Nicolas Island, even though the abundance of encrusting red algae was much greater within the pools. Other dietary algae may be important in precursor production.
The non-feeding planktonic larvae of *Collisella limatula* are competent to settle and metamorphose after 4 to 5 d, but can remain healthy and settle many weeks thereafter (D. R. Lindberg pers. comm.). It seems unlikely that genetic variation between San Nicolas Island and San Diego populations could explain the difference in the concentration of limatulione between limpets at the 2 sites. Predation pressure could be higher at the San Nicolas Island site, resulting in differential survival of limpets with higher levels of limatulione in their foot tissues.

Temporal variation may be a function of the reproductive state of the limpets. This does not, however, seem to be reflected in the mean visceral weight over time. Seapy (1966) found that at least 50% of the population of *Collisella limatula* he studied was ripe for spawning throughout much of the year, and spawning occurred during fall, winter and spring. Seasonal fluctuations in the levels of limatulione in *C. limatula* merit further study.

The field survey reveals that *Collisella limatula* was the most abundant limpet in 2 very different intertidal habitats: inside tidepools and on exposed boulders. Limpets inside tide pools were continuously submerged and were surrounded by an abundance of encrusting red algae. The shells from *C. limatula* in tidepools were flatter than the shells of conspecifics living on boulders (Fig. 4 and discussion below), and rarely showed evidence of repaired damage. *C. limatula*, along with the other limpets surveyed, were inactive when not submerged or awash during daylight hours, as similarly observed by Eaton (1968) and Wells (1980). Contrary to Wells (1980), however, *C. limatula* that were continuously submerged in tidepools actively foraged during daylight hours; in contrast, the small number of *C. digitalis* found in the pools appeared inactive. Several southern California tidepool fishes are known to feed on acmaeid limpets (Mitchell 1953, Johnston 1954, Stephens et al. 1970). In particular, the kelpfish *Gibbonsia elegans* was found to be the most abundant of 22 species of tidepool fishes in a survey by Mitchell (1953). It seems likely that, within the tidepool habitat, limatulione may protect *C. limatula* from predation by both fishes and crabs,
allowing utilization of a food source unavailable to the other limpet species.

 Apart from emersion between tides, the boulder field habitat contrasted with the tidepool habitat in several respects. The sand and cobble-scoured boulders were free of macro-algae; limpets feed on microscopic algae which inhibits fish predation while these limpets actively forage in tidepools. In addition, non-fatal shell breakage, combined with the chemical defense, would permit survival of *C. limatula* on the sides of intertidal boulders where the incidence of waveborne rock damage is particularly high.

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LITERATURE CITED


Chapin, D. (1968). Some observations of predation of Acmaea species by the crab Pachygrapsus crassipes. Veliger 11 (Supp.): 67–69


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