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Evidence of a Chemical Defence Mechanism in the Echiuran Worm *Bonellia viridis* Rolando (Echiura: Bonelliidae)

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Evidence for the existence of chemical defence in the echiuran *Bonellia viridis* is provided by investigating the palatability of *Bonellia* tissues to the shrimp *Palaemon elegans*, the teleosts *Oblada melanura* and *Mugil labeo*, and the anthozoan *Anemonia sulcata*. *Bonellia* tissues are shown to be highly distasteful to the shrimp. The results with the teleosts and the anthozoan are less clear-cut though *Bonellia* extracts are shown to be distasteful also to these species.

INTRODUCTION

Bonellia viridis Rolando, 1822 is a soft-bodied worm with no visible means of defence. These worms normally live as commensals in the rock burrows of the shrimp *Upogebia deltaura* (Schembri and Jaccarini, 1978) but are also found moving freely beneath loose boulders (Rolando, 1822; Schembri and Jaccarini, 1977, 1978). During feeding, the proboscis is protruded from the burrows to graze over the surrounding substratum, and at full extension, the proboscis may have a length of up to 1.5 m (Jaccarini and Schembri, 1977a, 1977b). The animal is thus often exposed to predators. Fish stimulated to feeding frenzy by the breaking open of sea urchins in the vicinity of *Bonellia* individuals were observed not to nibble at or even investigate the proboscides of feeding worms, and the speed of retraction of the proboscides, when

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touched, is of the order of 1 m s^{-1} (Jaccarini and Schembri, 1977a) suggesting that in *Bonellia* there is no fast withdrawal response as is shown in some tubicolous polychaetes.

The above observations suggest that *Bonellia* has some means of chemical defence. Previous authors reached similar conclusions. Baltzer (1924) found that mixtures containing 1 part dried pulverized *Bonellia* proboscis to 30,000 parts seawater were toxic to a wide variety of animals including marine and freshwater protozoa, echinoid embryos and the annelid *Tubifex*. Fragments of the worm were found to be toxic to the cladoceran *Daphnia* and to amphibian tadpoles when ingested by the organisms. Michel (1931) found that the crabs *Pisa tetraodon*, *Maia verrucosa*, *Carcinus maenas*, *Portunus arcuatus*, *P. corrugatus*, *P. holsatus* and *Pilumnus hirtellus* as well as the cephalopod *Sepia officinalis* all refused to feed on the body wall of *Bonellia*, but the crab *Dromia* fed readily.

This paper reports on palatability experiments and behavioural assays of palatability on tissues of *B. viridis* in an attempt to confirm and quantify the general observations of previous authors.

MATERIAL AND METHODS

The animals used in these experiments, the shrimp *Palaemon elegans* Rathke, the teleosts *Oblada melanura* (L.) (juveniles) and *Mugil labeo* Cuv., and the anthozoan *Anemonia sulcata* (Pennant), were all collected from Marsaxlokk Bay, Malta. The animals were allowed to acclimatize to laboratory conditions for a period of 1–2 weeks in the case of *Palaemon* and *Anemonia*, and 8–24 weeks in the case of *Oblada* and *Mugil*, before experiments were started.

To determine the rate of consumption of food in *P. elegans*, individuals of different sizes were placed in aquaria holding 8 l of seawater each at a density of 5 shrimps per aquarium. Two hours before feeding the shrimps were removed, gently blotted dry between sheets of tissue paper and weighed in tared beakers of seawater. Preliminary experiments indicated that the weight of the shrimps did not vary significantly over the two-hour period and that the weighing procedure did not affect the behaviour of the shrimps. Fresh healthy specimens of the mussel *Mytilus galloprovincialis* were broken open, the mantle tissue removed, blotted dry on filter paper, divided into small pieces and weighed. The mussel flesh was then placed in the aquaria containing the shrimps and left for a period of two hours after which all remaining fragments were collected, blotted dry and weighed again. The shrimps were fed at the same time each day and were not given any other food between tests. Only one test per day was carried out. The shrimps were observed in either ordinary light or in dim red light.

The same procedure was used for the experiments on the rate of consumption of *Bonellia* tissues. In all experiments two control aquaria without shrimps were set up. Weighed pieces of the tissue under test were placed in the control aquaria and left for the two hour feeding period then removed, blotted and weighed again. The difference in weights gave a measure of the dissolution rate of the tissue in the water, and the mean dissolution rate so determined was subtracted from each value obtained from the shrimp experiments to correct for the loss in weight due to dissolution of the test tissue in the water.

TABLE I

Composition of mixtures of *Bonellia viridis* proboscis homogenate and *Mytilus galloprovincialis* homogenate employed in palatability experiments. Each homogenate mixture was blended with 50 ml of 20% hot gelatine solution

Mixture	*Mytilus* tissue homogenate weight in g	*Bonellia* proboscis homogenate weight in g
I	0	5
II	1	4
III	2	3
IV	3	2
V	4	1
VI	5	0
VII	0	0

The palatability of various *Bonellia* tissue/*Mytilus* meat mixtures was assayed using all four experimental animals. *Oblada* and *Mugil* were maintained in large (50 × 90 × 90 cm) aquaria at densities of 6 fish per aquarium in the case of *O. melanura* and 3 fish per aquarium in the case of *M. labeo*. For observation, a 60 W light was positioned 30 cm above the water surface with the observer behind the light to prevent the fish reacting to the presence of the observer. *A. sulcata* were placed in 18 × 37 × 16 cm aquaria and observed in ordinary light.

All aquaria were supplied with a constant flow of aerated seawater at a temperature of 20°C and salinity 36.5‰.

To establish a baseline for the assay, the behaviour of the four test organisms to palatable (*Mytilus* mantle tissue) and unpalatable (*Mytilus* byssus threads) food was analysed before presenting them with the *Bonellia*/*Mytilus* mixtures.

The test mixtures were prepared by homogenizing separately *Bonellia* proboscides and *Mytilus* mantle tissue and combining the two homogenates in various proportions to a total weight of 5 g. Each homogenate mixture was then added to 50 cm^3 of a hot 20% gelatine solution, mixed thoroughly and allowed to solidify. The composition of the mixtures used is given in Table I.

For the palatability assays on *P. elegans*, the various *Bonellia/Mytilus/* gelatine mixtures were cut into cubes of side 8 mm and two cubes of different composition placed at opposite ends of the aquaria and left for exactly 10 minutes during which the behaviour of the shrimps was observed closely. For the assays on *O. melanura* and *M. labeo* single cubes of side 2 mm were dropped one at a time into the water and the behaviour of the fish observed. For the assays on *A. sulcata* a cube of side 10 mm of the test mixture was placed on the marginal tentacles of each individual and the behaviour of the anemone observed. Only one test per day on each test organism was carried out.

RESULTS

Feeding rates of *P. elegans*

The normal rate of consumption of *Mytilus* tissue by *P. elegans* was determined over a continuous period of 7 days. These results are shown in Table II. These experiments gave an overall mean of 0.0560 (s.e. 0.0107) g wet weight *Mytilus* tissue/g wet weight shrimp/hour as the normal rate of consumption under the experimental conditions employed.

TABLE II

Consumption rates of *Mytilus* tissue by the shrimp *Palaemon elegans* over a 7-day period with a two hour feeding period each day. Figures are g wet weight *Mytilus* tissue/g wet weight shrimp/hour and are means of 5 replicate experiments (25 shrimps). Standard errors in brackets

Day 1	2	3	4	5	6	7
0.0406 (0.0221)	0.0508 (0.0047)	0.0648 (0.0223)	0.0528 (0.0079)	0.0741 (0.0311)	0.0543 (0.0169)	0.0545 (0.0228)

In one set of experiments, shrimps were starved for a two day period, after which they were given (i) *B. viridis* proboscis tissue, and (ii) *B. viridis* body wall tissue, for a two-hour feeding period. The rates of consumption of these two tissues recorded in these experiments are given in Table III. In another set of experiments, starved shrimps were presented with both *Mytilus* mantle tissue and *Bonellia* proboscis tissue simultaneously for a two hour feeding period. The rates of consumption of these two tissues are given in Table IV.

TABLE III

Consumption rates of (i) *Bonellia* proboscis tissue and (ii) *Bonellia* body wall tissue by the shrimp *Palaemon elegans* during a two hour feeding period. Figures are g wet weight *Bonellia* tissue/g wet weight shrimp/hour and are means of 6 replicate experiments (30 shrimps)

	$\bar{x}$	s.e.
Bonellia proboscis tissue	−0.0070	0.0127
Bonellia body wall tissue	0.0027	0.0054

TABLE IV

Consumption rate of (i) *Bonellia* proboscis tissue and (ii) *Mytilus* mantle tissue by the shrimp *Palaemon elegans* during a two hour feeding period when presented with both foods simultaneously. Figures are g wet weight food tissue/g wet weight shrimp/hour and are means of 6 replicate experiments (30 shrimps)

	$\bar{x}$	s.e.
Bonellia proboscis tissue	0.0092	0.0139
Mytilus mantle tissue	0.0451	0.0117

Palatability assays on *P. elegans*

Behaviour of the shrimps when presented with palatable and unpalatable food

When hungry, the shrimps patrol the bottom of the aquarium, exploring the substratum and picking up faecal pellets and other sediment with the chelae of the first pereiopods and conveying them to the mouth. On being presented with palatable food (*Mytilus* tissue) all the shrimps swim up towards the sinking morsel and attempt to feed. However, it is always the largest individual which takes possession of the food and prevents the other shrimps from feeding by pushing them off with the antennae and pereiopods. The shrimp in possession of the food sometimes also walks around the food morsel and chases away any individual which comes too close, occasionally engaging in "fencing" matches with the second pereiopods if the intruder persists in attempting to approach the food. When several individuals crowd round the food, the shrimp "defending" the food takes hold of it and swims away with it to one of the corners of the aquarium.

Any shrimp which manages to take hold of the food already held by another individual jerks at it violently tearing off small fragments and immediately swims to a corner of the aquarium and proceeds to feed. The smallest individuals do not attempt to steal food in this way but pick up and feed on any small pieces dropped by the larger animals.

When the shrimp in possession of the food is satiated, it drops the food and swims away, whereupon the next largest individual takes possession of the food. This is repeated till all the shrimps have fed.

When presented with unpalatable food (*Mytilus* byssus threads) the shrimps do not rush towards the sinking particles but ignore them. Occasionally a shrimp comes upon the threads and conveys them to the mouth, but soon discards them.

Behaviour of the shrimps when presented with Bonellia/Mytilus *mantle homogenate mixtures* For these experiments two cubes of different mixtures of *Bonellia/Mytilus* mantle tissues (Table I) were presented simultaneously for a 10 minute period. The number of times "Ingestion" and "Fencing" behaviour was shown during this period was recorded. One period of "Ingestion" was taken to last from the time the food was picked up and transferred to the mouth and pieces chewed off and swallowed, till the food was dropped by the shrimp. Food could be seen to pass into the stomach through the transparent cuticle of the light adapted animals. "Fencing" was taken as an encounter between the shrimp in the possession of the food and one of the other shrimps (see above). The results of these experiments are presented in Table V.

Palatability assays on *O. melanura, M. labeo* and *A. sulcata*

Behaviour of the animals when presented with palatable and unpalatable food *O. melanura* juveniles normally patrol the aquarium in shoals in midwater. As soon as a food fragment hits the water, all the fish rush towards it. The first fish to reach it takes it in its mouth and swims away, pursued by the other members of the shoal. Small fragments of palatable food are swallowed immediately; larger particles are alternately spat out of the mouth and immediately sucked in again, the fish biting off and swallowing a small piece each time the food is taken in the mouth. This is repeated till all the food is consumed or till another fish manages to steal the food particle spat out.

Unpalatable food is also taken into the mouth, but is spat out and allowed to sink to the bottom after a few seconds. No food is taken from the bottom even if highly palatable.

M. labeo normally swim just beneath the water surface in loose groups. Palatable food is taken into the mouth and swallowed immediately it hits the

TABLE V

Results of comparative palatability tests on various *Bonellia* proboscis tissue/*Mytilus* mantle tissue/gelatine mixtures on the shrimp *Palaemon elegans*. VII represents pure gelatine cubes. VI represents a *Mytilus* tissue homogenate/gelatine mixture. V–II represent gelatine/*Mytilus*/*Bonellia* tissue homogenates with increasing proportions of *Bonellia* homogenate. I represents a *Bonellia* homogenate/gelatine mixture (see Table I). Figures represent the number of times the shrimps showed "Ingestion" behaviour (Ing.) and "Fencing" behaviour (Fen.) during each 10 min test period. For fuller explanation see text

		Tank													
	Food	A		B		C		D		E		F		Totals	
Test	mixture	Ing	Fen	Ing	Fen	Ing	Fen	Ing	Fen	Ing	Fen	Ing	Fen	Ing	Fen
1	VII	3	0	1	0	0	0	1	0	1	1	4	5	10	6
	VI	3	3	5	1	3	3	6	1	5	6	8	7	30	21
2	VI	6	3	1	2	4	2	4	1	4	6	3	3	22	17
	V	5	4	1	2	1	3	0	0	4	3	3	5	14	17
3	V	3	2	4	5	3	4	1	1	4	5	2	2	17	19
	IV	3	1	4	5	1	1	4	5	1	1	2	2	15	15
4	IV	2	2	2	3	3	2	1	0	1	1	4	3	13	11
	III	1	1	1	1	1	1	3	0	0	0	1	1	7	4
5	III	2	1	1	2	0	0	1	0	2	2	2	1	8	6
	II	0	1	0	1	0	0	0	0	1	1	1	1	2	4
6	II	0	0	1	0	0	0	1	0	1	1	0	1	3	2
	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0

water. Unpalatable food is taken into the mouth but is ejected after a few seconds and allowed to sink to the bottom. Occasionally, however, the fish take the rejected food into the mouth again once or twice before finally allowing it to sink. No food is taken from the bottom even if highly palatable.

When food particles are placed on the oral tentacles of *A. sulcata* these tentacles immediately retract, pulling the food onto the oral disc. The oral aperture then dilates till it can accommodate the food whereupon the tentacles push the food into the gastrovascular cavity. If the food is palatable the oral aperture closes and the animals assume a characteristic bloated appearance. Unpalatable food is, however, pushed out of the gastrovascular cavity and the tentacles then push it over the edge of the oral disc. The tentacles do not attach to rejected food is they again make contact.

Behaviour of the animals when presented with Bonellia/Mytilus *mantle homogenate mixtures* In each experiment individual cubes of the food mixture under test (Table I) were given to the test animals. With the fish the test was scored positive if the food was swallowed by any member of the shoal and negative if the food was rejected by all the individuals in the shoal and allowed to sink to the bottom. With the anemone a test was scored positive if the food was ingested and negative if the food was rejected.

TABLE VI

Palatability indices (ratio of number of positive tests to total number of tests) for various *Mytilus* tissue/*Bonellia* tissue/gelatine mixtures (I–VII) tested on two species of teleosts and one anthozoan species. See Table I for the composition of the mixtures. The total number of tests done with each mixture is given in parentheses

Test animals	Food mixtures (see Table I) I	II	III	IV	V	VI	VII
Oblada melanura	0.33	0.00	0.00	0.46	—	1.00	1.00
	(12)	(10)	(10)	(13)	—	(13)	(7)
Mugil labeo	0.80	0.20	0.88	0.00	—	1.00	1.00
	(10)	(5)	(8)	(2)	—	(10)	(5)
Anemonia sulcata	0.00	0.75	0.50	0.50	0.83	0.60	0.25
	(4)	(8)	(4)	(4)	(6)	(5)	(4)

The results of these experiments are presented as palatability indices in Table VI. The palatability index of each food mixture was defined as the ratio of the number of positive tests to the total number of tests, and ranges from 0 for totally unpalatable food to 1 for palatable food. The use of palatability indices was necessary as not every food mixture was tested an equal number of times on the different assay organisms and, hence the results were not directly comparable.

DISCUSSION

The shrimp *P. elegans* refuses to feed on the tissues of *B. viridis* even when starved as can be seen by comparing the consumption rates of *Mytilus* meat (Table II) with the consumption rates of *B. viridis* proboscis and body wall tissues by the shrimp (Table III). Statistical comparison of the raw data from which Tables II and III were compiled showed that the rates of consumption of both proboscis and body wall tissues were significantly different from the rates of consumption of *Mytilus* tissue at $P = 0.05$ (Mann–Whitney U-test, Siegel, 1956). Similarly if starved shrimps are offered a choice between *Mytilus* meat and *Bonellia* proboscis tissue, very little *Bonellia* tissue is consumed (Table IV) and again the Mann–Whitney U-test shows that the two rates of consumption are significantly different at $P = 0.05$.

The behaviour of *P. elegans* when presented with mixtures containing varying amounts of *Mytilus* and *Bonellia* tissues in a gelatine base (Table I) again shows that as the proportion of *Bonellia* tissue to *Mytilus* meat increases, the mixture becomes increasingly more unpalatable to the shrimps. Thus, the number of times the shrimps chewed off and swallowed bits of the mixture in a 10 minute test period decreased as the concentration of *Bonellia* tissue increased (Table V). Mixture I, which is made up of *Bonellia* tissue and gelatine only, was ignored completely. With highly palatable food, the dominant shrimp takes possession and defends the food against attempts to steal it by the subordinate individuals. As expected, as the proportion of *Bonellia* tissue in the mixture increases, the number of fights over possession of the food during the 10 minute test period decreases (Table V), with mixture I again being completely ignored. Gelatine by itself (mixture VII) is not very palatable to the shrimps (Table V).

The data from the palatability tests on *O. melanura*, *M. labeo* and *A. sulcata* is more difficult to interpret. There is no consistent trend in ranking of the test mixtures in order of decreasing palatability by the three test organisms. However, Friedman's χ_r^2 test (Siegel, 1956) showed that there is no significant difference in the mean levels of the three species in their ranking of the food mixtures tested. *O. melanura* ranks mixtures VI and VII as the most palatable, and mixtures II and III the least so, while *M. labeo* ranks mixtures VI and VII as the most and IV as the least palatable. *A. sulcata* ranks mixture I as the least palatable, but then surprisingly ranks mixture II as the second most palatable. Gelatine alone (mixture VII) and gelatine plus *Mytilus* tissue only (mixture VI) are ranked as the most palatable food by the two fish and as having medium palatability by *A. sulcata*. The presence in all the mixtures of gelatine, which appears to be highly palatable to the fish and somewhat palatable to the anemone might be masking to some extent the reactions to the *Mytilus* and *Bonellia* tissue homogenates. However, if all samples containing *Bonellia* extract tested on the three species are grouped together and compared with samples having no *Bonellia* extract, a statistically significant difference (at the 5% level) is found in the palatability of the mixtures to the three species, the mixtures containing *Bonellia* extract being less palatable (Wilcoxon's two-sample rank test, Snedecor and Cochran, 1967). In connection with this it is significant that none of the test organisms accepted pieces of fresh *Bonellia* proboscis even when previously starved, but rejected them immediately.

The results presented here confirm the existence of a chemical defence mechanism in *Bonellia* as has been suggested by our observations of the worms *in situ*, and by previous workers (Baltzer, 1924; Michel, 1931). The nature of the active compound or compounds in the tissues of *Bonellia* is unknown but we suggest that it is the pigment bonellin or a close relative. Bonellin solutions have been shown to be toxic to a wide variety of vertebrates and invertebrates and to be physiologically active in other ways (Baltzer, 1924; Lederer, 1939; Ruggieri and Nigrelli, 1962; Bridges, 1963; Nigrelli *et al.*, 1967; Schembri, 1977; Agius, 1978; Agius *et al.*, 1979). It is significant in this respect that when irritated, the worm extrudes large quantities of green-tinted mucus. A possible mechanism for the mode of action of bonellin has been postulated by Schembri (1977) and by Agius *et al.* (1979).

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