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Host defence mechanisms in marine invertebrate larvae

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Invertebrate immunity has been extensively investigated in adult animals from most phyla, however, very little is known of defence mechanisms in larval invertebrates. Existing information has established that motile phagocytic cells are present in the veliger larvae of bivalves and that the pluteus larvae of echinoderms possess antibacterial substances. Antimicrobial and cytotoxic activities have also been found in the eggs of opisthobranch molluscs, but have yet to be described in the larvae. Chemical defences, which may have antimicrobial or cytotoxic capabilities in addition to anti-predatory purposes, have been recorded from zoanthid, echinoderm and tunicate larvae. Many other aspects of immunity remain to be investigated in invertebrate larvae, and additional methods need to be developed for easier recognition and quantification of the larval immune response. The marine mussel, *Mytilus edulis*, is a species of particular importance, from both economic and ecological viewpoints. A disaggregation technique has been tested by the authors on *M. edulis* larvae and used to show that the larval cells possess certain enzymes associated with adult mussel haemocytes and are capable of phagocytosis.

Key words: Invertebrate larvae, immunity, cytotoxicity, phagocytosis, antimicrobial activity.

Introduction

A wide range of defence mechanisms has been reviewed in invertebrate phyla (e.g. Ratcliffe *et al.*, 1985; Smith, 1991). These include processes such as phagocytosis or encapsulation, and the production of soluble components which may be antimicrobial or cytotoxic. The blood cells of invertebrates are the primary effector cells in host defence and their involvement in numerous immune processes has frequently been demonstrated. However, very little is known of the origin of invertebrate blood cells in some groups, or of immune reactions in early stages of invertebrate life cycles. Information on such topics is particularly sparse with respect to host defence mechanisms in marine invertebrate larvae. This is probably due in part to their strictly seasonal availability, and the difficulties inherent in working with larvae, which cannot be bled in the same way as adult invertebrates.

Generally, marine invertebrate larvae are either planktotrophic or lecithotrophic. Planktotrophic larvae may spend several months in the water column before metamorphosis and it is therefore likely that they need some basic measure of immunocompetence to survive. Lecithotrophic larvae may spend only a few hours in the water column before metamorphosis and are possibly

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at less risk from pathogens. Observations of induced bacterial infection in larval bivalves have shown disease manifestation and progression between 1 and 4 days post-inoculation (Elston & Leibovitz, 1980), by which time many lecithotrophic larvae would have metamorphosed into their adult form. To date, immune functions have been recorded only in planktotrophic larvae (e.g. Stabili *et al.*, 1994), although lecithotrophic larvae have yet to be studied with respect to host defence mechanisms.

In this article, the existing information on defence mechanisms in marine invertebrate larvae is reviewed and new data are presented on immunity in the planktotrophic larvae of the mussel, *Mytilus edulis*.

PHAGOCYTOSIS

The process of phagocytosis is of primary importance in the immune response of all organisms, and blood cells from adult animals of all phyla are capable of phagocytic activity. The first observations of phagocytic cells in marine larvae were made by Yonge (1926), working on the European flat oyster, *Ostrea edulis*. After oyster larvae had been incubated in suspensions of iron saccharate, cells in the connective tissue surrounding the digestive diverticula were full of the iron suspension. Yonge referred to these cells as phagocytes, and hypothesised that digestion in both larval and adult oysters involved the transfer of absorbed material to motile phagocytic cells for transport around the body. Erdmann (1935) also observed 'free, mesenchymal cells' in *O. edulis* larvae, which are likely to be the phagocytes referred to by Yonge (1926), but made no mention of any phagocytic activity.

Subsequent work on oysters referred to migratory phagocytic cells, which were reported to degrade obsolete larval organs during settlement and metamorphosis. Prytherch (1934) observed the breakdown of the eyespots in larvae of the American oyster, *Crassostrea virginica*, describing the separation of the constituent cells and their migration to the circulatory system, commenting that they bore a close resemblance to the 'leucocytes of the spat and adult oyster'. Cole (1938) working on the pediveliger larvae of *O. edulis*, recorded the invasion of phagocytes into the areas occupied by the velum and foot and observed the uptake of recognisable larval material into these cells. The phagocytes were also numerous in the connective tissue under the eyespots, participating in their invasion and destruction at metamorphosis. Cole (1938) quoted a personal communication from Prytherch, in which the latter hypothesised that the eyespots acted mainly as haemopoietic tissue from which blood cells migrated into the circulation at metamorphosis. However, Cole (1938) suggested that migration of phagocytes around the body while containing the remnants of pigmented cells may have given a misleading impression of blood cell generation from the eyespots.

The phagocytosis of bacteria by larval cells was first reported by Elston & Leibovitz (1980), in *C. virginica*. During an investigation of pathogenic vibriosis in oyster larvae, they observed motile phagocytes containing bacterial fragments in the visceral cavity, and recorded the extrusion of redundant phagocytes through the velum. Phagocytosis was evident in all ages of larvae tested and occurred early in the disease process, although the phagocytes were

eventually overwhelmed by the bacteria. Other free-living cells in the visceral cavity were found to react to the bacteria by increasing their amount of smooth and rough endoplasmic reticulum, which may be linked to elevated levels of secretory products in response to disease. Subsequent to this, Elston (1980*a* & *b*), published the only ultrastructural descriptions to date of larval blood cells, referred to as coelomocytes. Elston (1980*a*) gave a full account of larval ultrastructure in the prodissoconch I & II stages of *C. virginica*, and identified two free-living cell types. One was classified as phagocytic and the other as non-phagocytic, the latter containing significant amounts of smooth endoplasmic reticulum (SER). Both cell types were observed in the visceral cavity, and the channels of the nascent circulatory system.

A more detailed description of larval coelomocytes was given in Elston (1980*b*). At the light microscope level, the phagocytes were observed to ingest India ink particles and to contain eosinophilic cytoplasmic granules. Electron microscopy showed that the phagocytes contained membrane-bound vacuoles, mitochondria, and dense, granular cytoplasm. The content of the vacuoles varied considerably, with some empty and others containing discernible elements of cellular material or foreign matter. The non-phagocytic cells were referred to as SER cells, and were most numerous in the region of the umbones and the thickened mantle area near the valve hinge. In comparison with the phagocytic cells, the SER cells showed lower frequencies of mitochondria and Golgi complexes. Elston (1980*b*) also referred to other coelomocytes, which did not appear to belong to either the phagocytic or SER cell types. These cells were described as having a high nuclear:cytoplasmic ratio, dense, granular cytoplasm and they frequently had prominent cytoplasmic processes. It is possible that these cells represent a precursor or an intermediate stage, between the two coelomocyte types.

ANTIMICROBIAL ACTIVITY

Antimicrobial activity in marine larvae was first demonstrated by Canicatti (1990), using lysates from the eggs and pluteus larvae of the sea urchin, *Paracentrotus lividus*. Lysozyme activity was assessed by incubating larval lysate in wells of an agar plate previously inoculated with the bacterium *Micrococcus luteus*. The development of a clear zone surrounding the wells in the agar indicated lysis of the bacterial cell walls and provided a means of quantification. The lysate from both eggs and larvae produced a zone of around 5 mm diameter.

This work was continued by Stabili *et al.* (1994), also working on *P. lividus*, who extended the techniques to test the effects of lysates against several species of bacteria. Only larval stages were used, namely the 4-, 6- and 8-armed pluteus larvae. Lysozyme-like activity was assessed as described by Canicatti (1990), although the results were compared with those produced by standard concentrations of hen egg-white lysozyme. Stabili *et al.* (1994) found that a dose-response curve was evident when measuring the lysis zone with increasing protein concentration of the larval lysate. When comparing the different larval stages at a defined protein concentration (0·2 mg ml^{-1}), lysate from the 6-armed pluteus larvae showed the greatest level of activity. Antibacterial

activity against *Vibrio alginolyticus, V. vulnificus, Vibrio* sp., *Salmonella* sp. and *Escherichia coli* was assessed by incubating the bacteria with larval lysates from the 4-armed stage and subsequently counting the number of emerging bacterial colonies on an agar plate. The shellfish pathogen, *V. alginolyticus*, was found to be the most sensitive to the lysates, with approximately 70% inhibition of growth evident compared to the control. The results were similar to those from the lysozyme assay, in that the percentage of inhibition was related to increasing protein concentration, and the 6-armed larval stage showed the highest level of antibacterial activity. Stabili *et al.* (1994) suggested that the decrease in lysozyme-like and antibacterial activity between the 6- and 8-armed larval stages may reflect the development of additional defence mechanisms in the later larval stages.

In addition, both antibacterial and antifungal activity have been found in eggs of the sea hare, *Aplysia kurodai*. Kisugi *et al.* (1989) have described the mode of action of an antibacterial glycoprotein, designated aplysianin E, against a range of bacteria. The growth of both Gram-positive and Gram-negative bacteria was inhibited and the factor responsible was heat-labile, and sensitive to extreme pH values. Aplysianin E was found to be bacteriostatic but not bactericidal. The synthesis of DNA and RNA by *E. coli* was stopped completely within 4 h of exposure to aplysianin E, suppressing the incorporation of thymidine and uridine.

Recent work by Iijima *et al.* (1995) has also reported that aplysianin E inhibits growth of the yeast species *Saccharomyces cerevisiae, Schizosaccharomyces pombe* and *Candida albicans*, showing significant decreases in colony number with increasing concentrations of the protein. Aplysianin E appears to have similarities with the glycoprotein dolabellanin A (Iijima *et al.*, 1995), which is found in adults of the sea hare species, *Dolobella auricularia*, suggesting that antimicrobial glycoproteins may be important factors for host defence throughout the life history of these molluscs. Generally, opisthobranch molluscs of the genus *Aplysia* hatch from eggs into planktotrophic veligers (Thomson, 1976). It is likely, but so far unrecorded, that antimicrobial factors in the eggs are also present in the larvae.

CYTOTOXIC ACTIVITY

To date, the only cytotoxic activity recorded in the early stages of an invertebrate life cycle relates to aplysianin E, which has been shown to lyse murine and human tumour cells (Yamazaki *et al.*, 1984, 1985). Inhibition of thymidine and uridine incorporation was observed, and occurred in a 10–100 ng ml^{-1} range of protein concentrations. Normal murine spleen cells were lysed only at concentrations equal to or greater than 10 μg ml^{-1}, and erythrocytes were not susceptible at all. Aplysianin E-induced tumour cell lysis was inhibited by *N*-acetyl neuraminic acid, and it was therefore suggested that the recognition of carbohydrate moieties may be a component in the cytotoxic process (Kisugi *et al.*, 1987).

CHEMICAL DEFENCES

Chemical defences are widespread in sessile adult marine invertebrates and are effected by toxic secondary metabolites such as alkaloids or terpenoids

(Hashimoto, 1979). Potential roles of these chemical defences include protection against invasion, settlement by other invertebrates and predation (Dyrynda, 1985). The majority of these compounds have been found to have antimicrobial or cytotoxic properties, and it is likely that they serve in more than one defensive capacity. Several examples of these have been found in immature invertebrate stages. The anticarcinogenic toxin, palytoxin, has been found in high concentrations in the eggs of zoanthids in the genus, *Palythoa* (Hashimoto, 1979). Saponins, which are cytotoxic steroid or triterpenoid compounds and are common in echinoderms, have been found in eggs and larvae of the starfish, *Acanthaster planci* (Lucas *et al.*, 1979). Examples from ascidians include the cytotoxic didemnins, some of which have been found in the larvae of the ascidian, *Tridemnum solidum* (Lindquist *et al.*, 1992). It seems likely that these compounds may provide an important line of defence for marine invertebrate larvae.

DEFENSIVE MECHANISMS IN LARVAE OF THE MUSSEL, *MYTILUS EDULIS*

The larvae of *M. edulis* are particularly important, not only for the economic value of the adults, but from the ecological viewpoint. An average female mussel can produce up to 4 million eggs in a single season, making *M. edulis* the dominant lamellibranch larva in the zooplankton, with peak abundance of up to 25 thousand larvae per cubic metre (Fish & Johnson, 1937). In addition, the species is extremely widespread, can be induced to spawn under laboratory conditions and can be used as a model for bivalve species generally. Only limited information is available on the coelomocytes of larval bivalves and to date, no information exists on any elements of immunity in larval mussels. Knowledge of immune function in larval bivalves generally is of particular importance as the larvae are susceptible to bacterial infection, which can result in the significant loss of stock in hatcheries (Guillard, 1959; Tubiash *et al.*, 1965).

Although histology at both light and electron microscope levels may be of great use for studying larval host defences, these techniques are slow to perform and are not suited for quantification of immune reactions in live cells. For the investigation of larval immunology in *M. edulis*, a method was developed in which live larval cells could easily be manipulated and used in immune functional assays (Dyrynda, unpublished). The technique was based on that of Odintsova & Khomenko (1991), which was originally designed for the establishment of primary invertebrate cell cultures and involved the disaggregation of scallop larvae into their constituent cells. In brief, the method involved an initial, 20 min incubation of *M. edulis* larvae with calcium-magnesium free saline (CMFS), followed by a second 20 min incubation in CMFS and trypsin. The disaggregated cells were then washed in Tris-buffered saline and either resuspended in the buffer for functional assays with living cells, or in fixative for cytochemical techniques. Using this method, it would not be possible to identify specifically the developing haemocytes in a mixture of cells, however, the existence of defensive capabilities could be demonstrated.

Initially, basic characterisation in terms of the morphology and cytochemistry of the disaggregated larval cells was attempted. This involved compiling

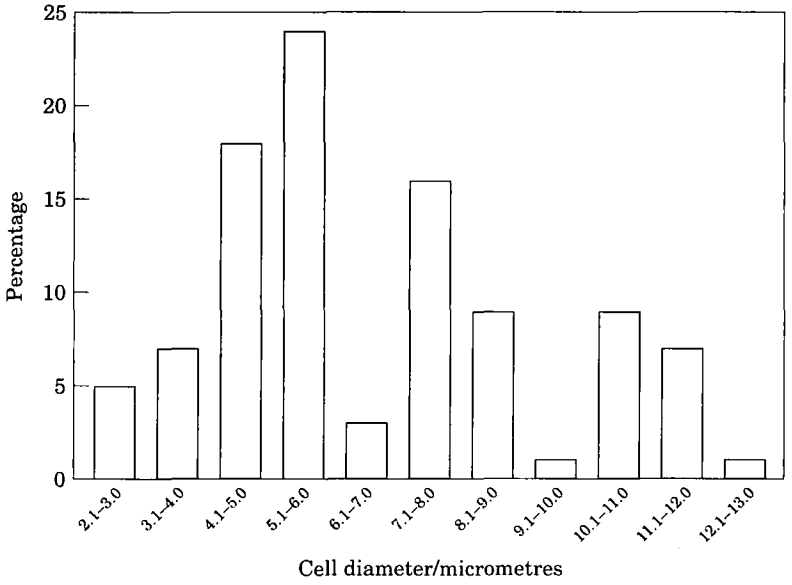

Fig. 1. Frequency distribution of size classes of disaggregated trochophore cells.

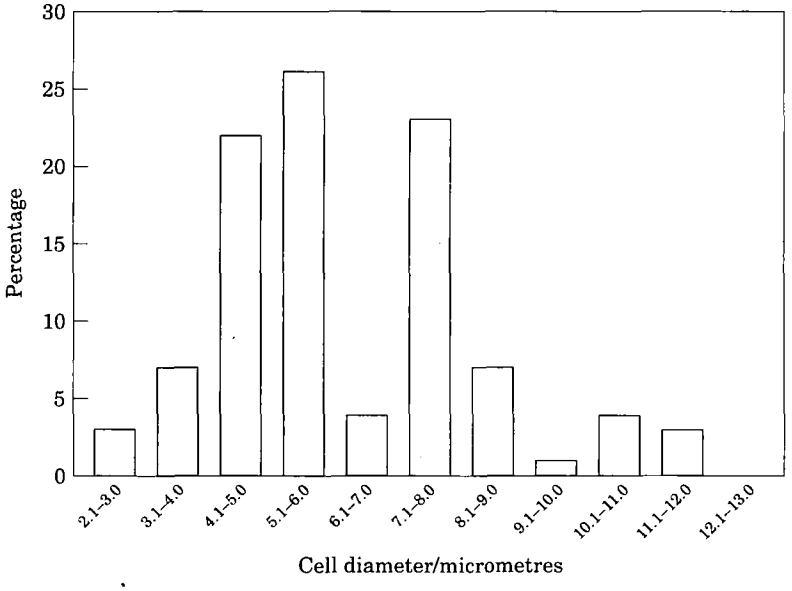

Fig. 2. Frequency distribution of size classes of disaggregated veliger cells.

frequency distributions of disaggregated trochophore and veliger cells (Figs 1 & 2), differential staining and tests for the presence of common enzymes associated with adult mussel haemocytes. These enzymes included peroxidase, phenoloxidase, acid phosphatase, arylsulphatase, β-glucuronidase, N-acetyl-β-hexosaminidase and non-specific esterase, and were tested for using the methods described in Holden *et al.* (1994). Subsequently, live disaggregated

Table 1. Cytochemistry of disaggregated cells from larval *M. edulis*

	Trochophore	Veliger (5 days old)	Veliger (11 days old)
Wright's stain	Basophilic	Basophilic	Basophilic
Peroxidase	–	–	–
Phenoloxidase	+	++	++
Hexosaminidase	–	–	–
β-glucuronidase	–	–	–
Arylsulphatase	+++	+++	+++
Acid phosphatase	–	–	–
Esterase	–	–	–

+ =weak reaction, ++ =moderate reaction, +++ =strong reaction.

cells were used to investigate immune functions. These included phagocytosis, using fluorescent-labelled *E. coli* in a similar protocol to that developed by Rohloff *et al.* (1994).

Cytochemistry

The cytocentrifugation of disaggregated, fixed cells proved to be an effective method for preparing cell suspensions for various staining techniques. The diameter of the cells ranged from 3–13 μm (Figs 1 & 2), which was slightly smaller than that of adult *M. edulis* haemocytes, quoted at 4–24 μm (Renwrantz, 1990). The average size of cells from the trochophore and veliger stages was found to be approximately 6 μm, with no significant difference evident between the trochophore and veliger stages. Length-frequency distributions based on cell diameters revealed a predominantly bimodal distribution (Figs 1 & 2), with peaks occurring in the 4–6 and 7–8 μm bands. It is possible that these bands could be separated by isopycnic centrifugation and that differences in terms of cytochemistry or function could be established.

The results of the cytochemical staining are summarised in Table 1. The basophilic nature of these cells contrasts with other observations in the larvae of *C. virginica* and *O. edulis*, in which some phagocytic coelomocytes, mantle and byssal gland cells were observed to be eosinophilic (Cole, 1938; Elston, 1980*a* & *b*). Possibly the cells of *M. edulis* larvae become eosinophilic at a later stage, or this is a fundamental species difference. There is no indication from the available information on haemocytes from adult mussels and oysters that eosinophilic cells have more prominence in one species compared to another.

Phenoloxidase is commonly found in blood cells from vertebrates (Schmidt, 1988) and invertebrates (Smith & Söderhäll, 1991) and is present in haemocytes of the adult mussel (Coles & Pipe, 1994). The enzyme has also been previously observed in sections of the pediveliger of *O. edulis*, in cells from the inner mantle fold (Cranfield, 1974). Phenoloxidase was detected in all the disaggregated larval cells from *M. edulis*, showing a stronger reaction in the veligers. Phenoloxidase can be regarded as a marker enzyme for the prophenoloxidase (proPO) system, which has been described in detail in arthropods (Ratcliffe,

1991), but appears to be of less significance in the immune systems of molluscs. It is possible that the proPO system exists in molluscs, but is primarily involved with aspects of immunity such as opsonisation rather than melanin production. Alternatively, phenoloxidase may be functioning mainly in a non-defensive capacity, as melanin (an end product of the proPO system) is present in byssal threads and involved in shell repair. The presence of phenoloxidase in *M. edulis* larvae, and the stronger reaction in the veliger stage, may be due to a maturing immune response, or may reflect the role of the enzyme in shell development and maintenance.

Lysosomal enzymes number around 40 acid hydrolases, many of which have been found in invertebrate haemocytes (e.g. Janoff & Hawrylko, 1963; Cheng, 1976; Ballarin *et al.*, 1993). All the larval cells in both trochophores and veligers of *M. edulis* were found to be strongly positive for arylsulphatase. This enzyme catalyses the hydrolysis of aryl sulphate esters, is widely distributed in both vertebrate (Bainton *et al.*, 1976) and invertebrate (Pipe, 1990; Ballarin *et al.*, 1993) blood cells and has been implicated in the cytotoxic activities of human natural killer cells (Zucker-Franklin *et al.*, 1983). The presence of arylsulphatase in larval *M. edulis* cells and in developing embryos of the sea urchin, *Hemicentrotus pulcherrimus* (Sasaki *et al.*, 1987) suggests that these larval cells have a degradative, defensive ability. It is possible that in the early stages of the life history only arylsulphatase is present and that other degradative enzymes occur later.

Acid phosphatase, hexosaminidase, β-glucuronidase and esterase were not detected in larval *M. edulis* cells, but have previously been detected in haemocytes from *M. edulis* (Moore & Lowe, 1977; Pipe, 1990) and *M. californianus* (Bayne *et al.*, 1979). It is possible that these other enzymes may be present in larval cells, but some degree of challenge may be necessary to increase their levels to a detectable degree. Levels of lysosomal enzymes in both vertebrate and invertebrate blood cells have been found to increase with bacterial challenge (Cohn & Fedorko, 1969; Cooper-Willis, 1979; Cheng, 1992).

PHAGOCYTOSIS

Although phagocytosis has been recorded in oyster larvae (Yonge, 1926; Cole, 1938; Elston, 1980*a* & *b*; Elston & Leibovitz, 1980), no published record of this exists for mussel larvae. A phagocytosis assay utilising fluorescein isothiocyanate (FITC) labelled *E. coli* was used to demonstrate phagocytic capability in larval *M. edulis* cells. Following larval disaggregation in CMFS, the cells were washed in Tris-buffered saline and incubated for 30 min with the bacteria, using an approximate ratio of 1:50 respectively. Subsequently, the cells were washed and fixed, with cell monolayers prepared by cytocentrifugation. The percentage of phagocytic cells and the number of ingested bacteria per cell were recorded. The levels of phagocytosis observed in disaggregated cells showed very little difference between the trochophore and veliger cells, in either the percentage of phagocytic cells or the mean number of bacteria ingested per cell, and were very much lower than those observed in adult mussel haemocytes (Table 2).

The percentage of phagocytic haemocytes varies considerably in adult molluscs and values between 5% and 100% have been reported under various

Table 2. Phagocytosis of FITC-labelled *E. coli* by disaggregated larval cells from *M. edulis*

	% Phagocytic cells	Mean no. bacteria/cell
Adult *Mytilus* haemocytes	80·2 ± 9·7	5·4 ± 2·1
Trochophore cells	4·3 ± 1·4	3·0 ± 0·8
Veliger cells	4·6 ± 1·9	1·9 ± 0·9

Larval results represent mean from 3 pools of disaggregated cells, ± standard deviation.

Table 3. Summary of defence mechanisms recorded in *M. edulis*

Activity	Adults	Larvae	Reference
Degradative enzymes	+	+	Pipe, 1990: Dyrynda, unpublished data
Phagocytosis	+	+	Noël *et al.*, 1993: Dyrynda, unpublished data
Cytotoxic reactions	+	ND	Leippe & Renwrantz, 1988
ROM generation[1]	+	+	Pipe, 1992: Dyrynda, unpublished data
NO generation[2]	+	ND	Ottaviani *et al.*, 1993
LK-like activity[3]	+	ND	Hughes *et al.*, 1990
Antimicrobial factors	+	ND	Nottage & Birkbeck, 1990
Agglutinins	+	ND	Renwrantz & Stahmer, 1983

[1]ROM-reactive oxygen metabolites, [2]NO-nitric oxide, [3]LK-lymphokine
ND-not determined.

conditions (Renwrantz & Stahmer, 1983; Tripp, 1992). Larval *M. edulis* cells are capable of phagocytosis, however the apparently low percentage of phagocytic larval cells when compared with adult mussel haemocytes is likely to be due to the presence of all larval cell types in the assay and influenced further by the disaggregation process itself. Phagocytosis in adult invertebrate haemocytes has been shown to be enhanced by opsonic factors in haemolymph. Renwrantz & Stahmer (1983) working on *M. edulis*, recorded significant increases in phagocytosis after the test particles were incubated with whole haemolymph from the mussel, or with purified agglutinin from *M. edulis*, or in saline solution plus calcium. It is possible that disaggregated larval cells may need the presence of natural opsonin for optimal phagocytosis, or that additional calcium ions should be added to the buffer.

In conclusion, some elements of the immune system in adult *M. edulis* are also present in the trochophore and veliger larvae of this species (Table 3). The enzymes phenoloxidase and arylsulphatase have been found in larval cells, which have also been observed to phagocytose *E. coli*. The rates of phagocytosis recorded to date are low, however, it is likely that under different conditions an increase in the percentage of phagocytic cells may be seen. The generation of reactive oxygen metabolites has been recorded in both trochophore and veliger cells of *M. edulis* (Dyrynda, unpublished data).

Further work is being undertaken to quantify other aspects of the immune response, as well as establishing possible sites of haemopoiesis in larval and juvenile mussels by the use of monoclonal antibodies to adult mussel haemocytes.

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