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Histopathological effects induced in *Mytilus edulis* by *Mytilicola intestinalis* and the histochemistry of the copepod intestinal cells

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Localised metaplastic changes in the gut epithelial cells of *Mytilus edulis* were found to be associated with the presence of the parasitic copepod, *Mytilicola intestinalis*. These changes involved the replacement of the ciliated columnar cells by non-ciliated cuboidal cells. Experimental thermal stress induced a significant increase in the numbers of macrophages in the connective tissues of both infected and uninfected mussels, but there was no evidence of any synergistic effect in infected hosts. A number of lysosomal hydrolases are associated with the gut cells of the parasite where they may be involved in intracellular digestion. It is concluded that *Mytilicola intestinalis* has no significant effect on the basic cellular functions in *Mytilus edulis*.

Introduction

A number of studies in the early 1950's on the effects of the copepod *Mytilicola intestinalis* on *Mytilus edulis* resulted in the parasite being considered a serious pest of shellfish. Meyer and Mann (1950) came to the conclusion that the presence of between two and eight *Mytilicola* seriously affected the metabolic balance of the host which would result in the loss of "condition" postulated by Odlaug (1946) and Cole and Savage (1951). Sparks (1962) found that *Mytilicola orientalis* induced metaplasia in the gut epithelial cells of *Crassostrea gigas* and a similar condition was found in *Crassostrea glomerata* by Dinamani and Gordon (1974). More recently, Williams (1969) could find very little effect of the parasite on the gross biochemical composition of *Mytilus*, except possibly in the immediate postspawning period, while Dethlefsen (1975) found no evidence for a reduction in meat content of infected mussels.

In view of this dichotomy of opinion in published data it was thought useful to re-examine the effects of *M. intestinalis* on the histology, physiology and general condition of *M. edulis*. In the present study, mussels collected from three populations around Plymouth (Davey and Gee, 1976) have been examined for evidence of histopathological changes and cellular stress responses induced by the parasite. The effect of thermal stress on macrophage infiltration (Moore and Lowe, 1977) was also examined in both infected and uninfected animals in order to determine the presence or absence of a synergistic effect at the cellular level. Finally the gut of *Mytilicola* was examined histochemically for lysosomal hydrolases in order to gain some insight into the type of digestive processes involved in the nutrition of the parasite.

Materials and methods

Mytilus edulis (shell length 50–65 mm) were collected from the River Lynher, Cattewater Power Station cooling outlet and the River Erme in the general vicinity of Plymouth, England (Fig. 1) at intervals of approximately six weeks for a period of two years. Two of these populations are infested with the copepod *Mytilicola intestinalis* (Lynher and Cattewater) while the Erme group has a very low incidence of infection (Davey and Gee, 1976, Fig. 1).

For histopathological and histochemical examination, the visceral mass, rectum and surrounding mantle were fixed in Baker's formol calcium (+ 2·5% NaCl) at 4°C for 24 h. Material for histopathology was dehydrated in a graded alcohol series and embedded in wax for sectioning (2 and 7 µm), while specimens for enzyme histochemistry were transferred to gumsucrose (4°C) for 24 h and sectioned in a Bright's cryostat at 10 µm. For routine histology and cytology, wax sections were stained with hae-

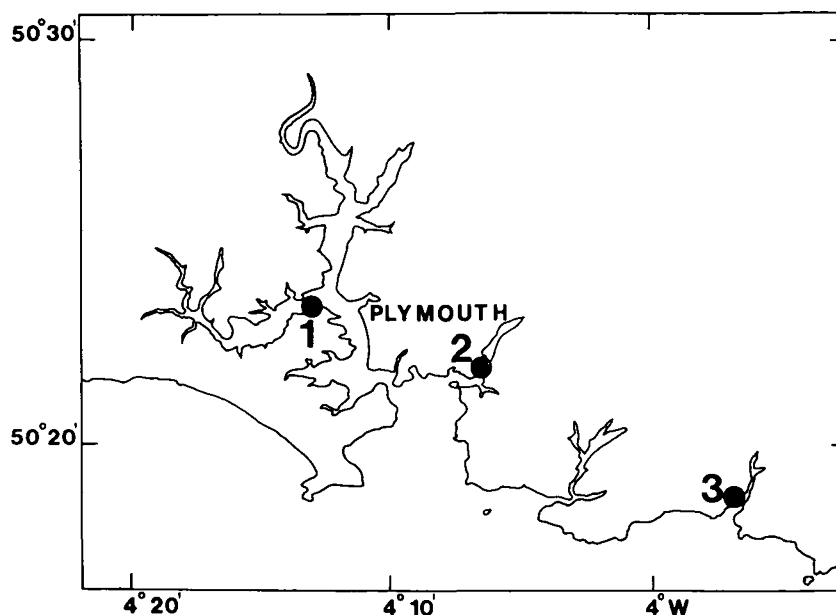

Figure 1. Map of the coastline in the vicinity of Plymouth showing the three sampling sites for *Mytilus edulis* used in this investigation: 1. River Lyhner; 2. Cattewater power station; 3. River Erme.

matoxylin and eosin, the Feulgen reaction for DNA, methyl green-pyronin Y for RNA (with 0·8% ribonuclease control), periodic acid-Schiff (PAS) (with amylase control for glycogen), alcian blue/PAS for acid and neutral mucopolysaccharides and Schmorl reaction for lipofuscin (Bancroft, 1967).

Cryostat sections were used to determine the histochemical distributions and staining intensities of lipids and certain hydrolytic enzymes. Lipids were demonstrated using a saturated solution of oil red-0 in 60% tri-ethyl phosphate (Bancroft, 1967). The Nile blue sulphate method was used to distinguish neutral and acidic lipid (Adams, 1965). Naphthol AS-BI methods were used to demonstrate acid phosphatase (Pearse, 1968), β-glucuronidase (Pearse, 1972) and N-acetyl-β-glucosaminidase (Pearse, 1972).

The simultaneous coupling reactions were carried out at pH 5·0 with fast red violet LB or fast garnet GBC. Incubation was for 40 min at 37°C for acid phosphatase and β-glucuronidase and 30 min at 37°C for glucosaminidase with the appropriate controls. Indoxyl esterase was demonstrated by the 6-bromo-indoxyl acetate method (Pearse, 1972). The leucyl-4-methoxynaphthylamide method (Pearse, 1972) was used to demonstrate naphthylamidase.

Artificial infection of mussels with *Mytilicola* copepodites was carried out by placing individual uninfected mussels (from Mothecombe Bay) in plastic tanks (20 × 13 × 8 cm) containing 1 litre of filtered sea water to which approximately 50 newly metamorphosed copepodites had been added. Each tank was aerated, continuously supplied with a culture of the diatom *Phaeodatcylum tricornutum*, covered with black polythene and left for 24 h. Infected and uninfected mussels were maintained at 10°C and 22°C and all four groups were sampled after 12, 32 and 81 days.

The densities of macrophages in the connective tissue (Moore and Lowe, 1977) were determined in cryostat sections (10 µm) stained for β-glucuronidase. A Wild M20 microscope with attached viewing head was used for macrophage counts, with five counts per section of an individual mussel. The area counted was 200 × 200 µm; counts of macrophages carried out on serial sections of single animals gave consistently similar values (not significant on analysis of variance).

Results

Histopathology of infected mussels

The epithelial cells lining the intestine and rectum exhibited metaplasia in the infected mussels, the normal ciliated columnar cells (Fig. 2;1) being replaced by a non-ciliated cuboidal cell type (Fig. 2;2). There was a corresponding change in nuclear structure

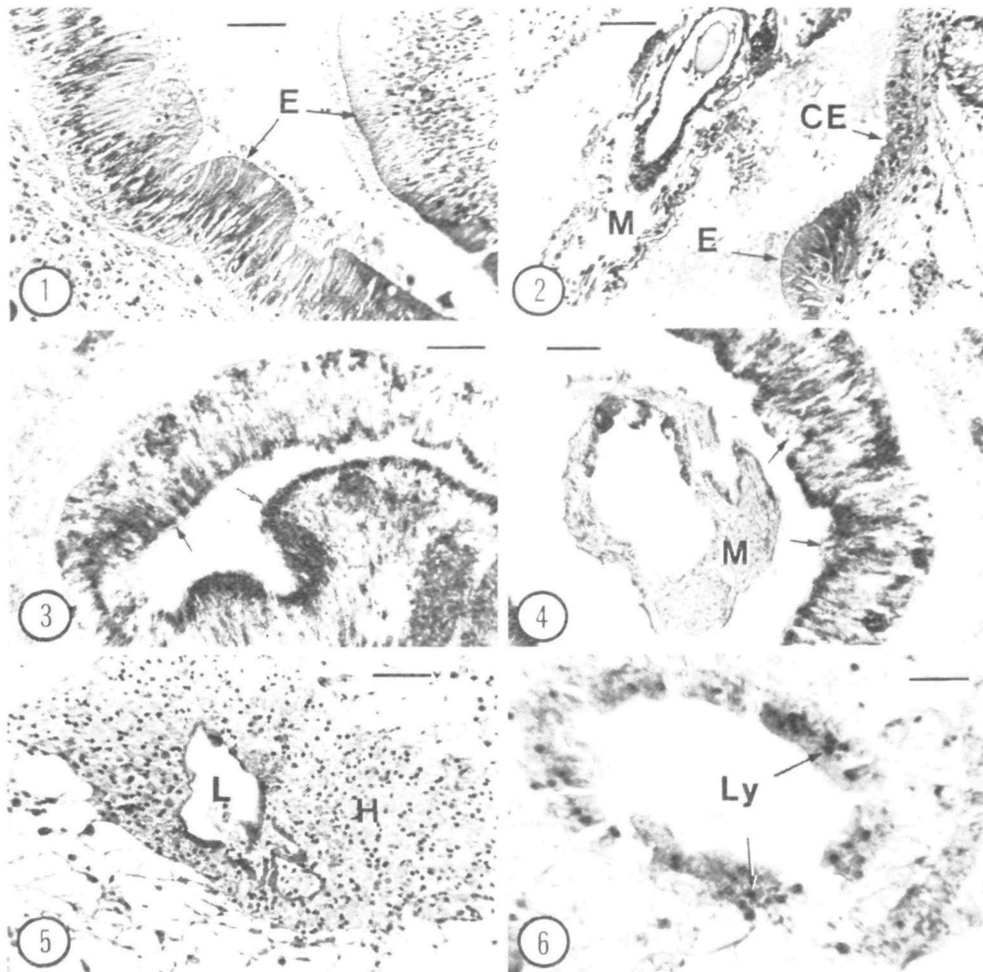

Figure 2.

1. A section (2 µm) through the intestine of uninfected *Mytilus edulis* showing the normal appearance of the ciliated columnar intestinal epithelium (E). Papanicolaou method. Scale = 50 µm.
2. A section (2 µm) through the intestine of *Mytilus edulis* infected with *Mytilicola intestinalis* (M). In this example of an unusually severe host response, the intestinal epithelial cells (E) in a localised region of the gut have been replaced by non-ciliated cuboidal cells (CE). There is no evidence of any haemocytic response associated with the intestine. Papanicolaou method. Scale = 50 µm.
3. A cryostat section (10 µm) through the intestine of uninfected *Mytilus edulis* showing dark staining reaction product (arrowed) for N-acetyl-β-glucosaminidase, associated with lysosomes in the apical region of epithelial cells. Naphthol AS-BI glucosaminide method. Scale = 50 µm.
4. A section as in Figure 2; 3 through mussels infected with *Mytilicola* (M). There is little evidence of metaplastic change although there has been slight erosion (arrowed) probably due to the appendages of the parasite. There is no change in localisation of glucosaminidase activity. Naphthol AS-BI glucosaminide method. Scale = 50 µm.
5. A section (2 µm) through the interstitial connective tissue of the digestive gland region of *Mytilus* showing the remnants of an encapsulated larval *Mytilicola* (L). The encapsulation reaction consists of haemocytes (H) and is localised to the immediate vicinity of the larva. Papanicolaou method. Scale = 50 µm.
6. A cryostat section (10 µm) through the mid-gut of *Mytilicola* showing β-glucuronidase reactivity associated with lysosomes (Ly) in the gut epithelial cells. Naphthol AS-BI glucuronide method. Scale = 20 µm.

from the elongated nuclei of the columnar cells with a diffuse chromatin pattern to the round or oval denser nuclei of the cuboidal cells. In a few instances of high infection there were indications of a more sustained metaplastic response (Fig.2;1 and 2).

The presence of a large parasite or several smaller ones induced slight erosion of the gut epithelial cells. This erosion appeared to be associated with the appendages of the parasite. Necrotic cells associated with this erosion showed a slight increase in β-glucuronidase staining activity, possibly indicating autolysis. Cuboidal and slightly eroded cells did not show any change from that of columnar cells in either intensity or granule-associated apical localisation of staining for acid phosphatase, β-glucuronidase or N-acetyl-β-glucosaminidase (Fig. 2;1, 3 and 4). No changes could be detected in the staining intensity or distribution of the mucosubstances coating the luminal surface of the intestinal epithelial cells. There was no evidence of increased haemocytic infiltration of the basal connective tissue of the gut in infected animals from the Lynher river. However, both infected and uninfected animals from the power station outlet showed distinct haemocytic infiltration, although this was probably due to thermal stress. No fibrosis was observed in the basal connective tissue of any infected mussels.

Occasional groups of haemocytes were associated with the chitinous cuticle of copepods in the gut of infected animals. In a few instances, large multinucleate cells were also associated with these sites. However, both haemocytes and multinucleate cells were also found in the gut lumen of uninfected mussels.

There were no significant differences in the levels of glycogen in the Leydig or digestive cells of infected and uninfected mussels. Levels of granulocytes and macrophages associated with mantle connective tissues were also unaffected. Experimental infections with larval copepodites showed no evidence of cellular damage associated with the larvae in either the intestine or digestive tubules. In a small number of instances copepodites had apparently penetrated the epithelium of the digestive tubule and become lodged in the connective tissue. Where this had occurred, there was a localised haemocytic encapsulation surrounding the larvae (Fig. 2;5) which were observed in varying states of necrosis.

There were no changes in levels of amylase-labile PAS staining in either digestive cells or Leydig cells, after 12, 32 or 81 day infections. No significant differences were observed in the numbers of β-glucuronidase-positive macrophages in the connective tissues of uninfected or infected animals maintained at 10°C after 12 or 81 days. However, uninfected and infected mussels maintained at 22°C for 12 days showed significant increases (uninfected – 163%, $P < 0{\cdot}05$; infected – 189%, $P < 0{\cdot}01$) in β-glucuronidase-positive macrophages in the connective tissues over uninfected animals maintained at 10°C. These increases were apparently due to the increase in temperature as no differences could be detected between infected and uninfected groups at the two temperatures. After 81 days, no significant differences could be detected in numbers of macrophages between the four groups of animals, indicating that the animals at the higher temperature had attained a steady state after the initial response.

Cytology and histochemistry of *Mytilicola* gut cells

The intestinal cells of *Mytilicola*, sectioned *in situ*, form a cuboidal epithelium (Fig. 2;6) with strongly basophilic cytoplasm and frequent brown or green coloured spherical inclusions. Microvilli occurred at the luminal border of the intestinal cells, although there was no evidence of any mucosubstance associated with this region. The luminal contents were similar in appearance to the gut contents of *Mytilus*, consisting of remnants of algal cells.

The cytochemical distributions of five hydrolytic enzymes have been examined in the intestinal cells, namely indoxyl esterase, naphthylamidase, acid phosphatase, β-glucuronidase (Fig. 2;6) and glucosaminidase. Slight activity for indoxyl esterase and naphthylamidase was localised in the cytoplasm and did not appear to be associated with any granular components. However, reactivity for the other three enzymes was associated with cytoplasmic inclusions as well as smaller granules. The staining reactions for acid phosphatase, glucosaminidase and particularly β-glucuronidase were also associated with the general cytoplasmic matrix of the intestinal cells. The staining intensity of the azo-dye reactions was strongest for acid phosphatase and weakest for glucosaminidase.

Some of the intestinal cells, in the posterior region of the midgut, had large apical vacuoles which frequently contained smaller inclusions. These large vacuoles showed a strong staining reaction for β-glucuronidase. Acid phosphatase and glucosaminidase activities were also localised at this site.

Discussion

The results indicate that the histopathological effects induced in *Mytilus* by *Mytilicola* are, in general, limited to areas of the host gut where there is erosion or irritation of the columnar epithelium by the

appendages of the copepod. It is interesting to note that although fibrosis of underlying connective tissues occurred in *C. gigas* (Sparks, 1962), this condition was not encountered in our infected mussels The absence of any significant haemocytic response is in general agreement with the observations of Sparks (1962) although Dinamani and Gordon (1974) found some instances of haemocytic infiltration in the basal connective tissues of *C. glomerata.* As the metaplastic regions of the host gut epithelium are localised, and in view of the known mobility of the copepod (Hockley, 1951), it seems probable that repair of the damaged areas is rapid.

The increased numbers of macrophages (Moore and Lowe, 1977) following experimental thermal shock ($\Delta C = 12°C$) and the increased haemocytic infiltration in animals from the power station outlet, which are subjected to marked diurnal temperature fluctuations (Bayne, Widdows and Worrall, 1977), obviously constitute a cellular stress response. Experimental animals subjected to a constant temperature differential returned to the control levels of macrophage counts after the initial response. Because these responses are the same in both infected and uninfected mussels, it seems unlikely that there is a synergistic effect between the parasite and thermal stress.

Cole and Savage (1951) noted that *M. intestinalis* had an adverse effect on the condition of *Mytilus* but Dethlefsen (1975) could find no such evidence in mussels from the Waddensea. Gee, Maddock and Davey (1977), working with the same mussel populations as in the present study, found that the "condition" of mussels was not affected by *Mytilicola* except, possibly, during one or two months when the mean number of parasites per host reached about 30 in sublittoral mussels. In the present study there is little evidence to support the view that infections with *Mytilicola* constitute a stress condition in *Mytilus edulis.* Campbell (1970) was of the opinion that it was the larval stages of the parasite which caused most damage when they occupied sites in the digestive tubules of the host. Again, no evidence for this is apparent from the present investigation; those few copepodites which penetrated the tubule epithelium and lodged in the connective tissues were effectively encapsulated and killed.

The localisation of some lysosomal-type hydrolases in granular and vacuolar components of the copepod gut cells is probably indicative of intracellular lysosomal digestion. These types of enzymes are known to be associated with lysosomal function in the digestive glands of many molluscs (Sumner, 1969; Moore and Halton, 1973; Moore, 1976) and the crab *Scylla serrata* (Monin and Rangneker, 1974). The prominence of the reactions for carbohydrases as opposed to the proteolytic naphthylaminidase would suggest that the diet of the copepod is primarily herbivorous, presumably feeding on the gut contents of the host and not on host tissue.

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