# *In vitro* cultivation and developmental cycle in culture of a parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters

P. L. APPLETON *and* K. VICKERMAN*

*Division of Environmental and Evolutionary Biology, University of Glasgow, Glasgow G12 8QQ*

SUMMARY

Dinoflagellates are common and often important parasites of aquatic organisms, but their developmental cycles are poorly known and have not been established in *in vitro* culture. The parasitic dinoflagellate (*Hematodinium* sp.) associated with mortality of the Norway lobster (*Nephrops norvegicus*) in British waters has been cultivated *in vitro* in 10% foetal calf serum in a balanced *Nephrops* saline. In culture the parasite undergoes a characteristic cycle of development. Circulating sporoblasts from the host's haemolymph *in vitro* generate 2 kinds of flagellated uninucleate dinospore, macrospores and microspores, either of which will, after 5 weeks in fresh medium, germinate to produce multinucleate unattached filamentous trophonts. These trophonts multiply by fragmentation and growth and may be serially subcultured in this form, at 2 week intervals, indefinitely. If not subcultured, the filamentous trophonts give rise to colonies of radiating filaments ('gorgonlocks') which subsequently attach to the substratum to form flattened web-like 'arachnoid' multinucleate trophonts. Arachnoid trophonts become arachnoid sporonts when they synthesize trichocysts and flagellar hairs and may give rise to secondary arachnoid sporonts or to dinospores which initiate a new cycle.

Key words: dinoflagellate, Syndinea, *Hematodinium* sp., *Nephrops norvegicus*, *in vitro* culture, developmental cycle.

## INTRODUCTION

The Norway lobster or langoustine (*Nephrops norvegicus*) forms the basis of one of Europe's most important fisheries. Off the west coast of Scotland and in the Irish Sea, seasonal outbreaks of disease are currently seriously harming stocks. The diseased animal is characterized by increasing lethargy and an increased body opacity as the crustacean's blood cells are replaced by up to 8 times their number of uni- or multi-nucleate cells of a syndinean dinoflagellate of the genus *Hematodinium* Chatton & Poisson 1931 (Field *et al*. 1992). Epizootics of *Hematodinium* spp. have severely affected other commercial crustacean fisheries, notably the Alaskan tanner crab (*Chionoecetes bairdi*) industry (Meyers *et al*. 1987) and the velvet swimming crab (*Necora puber*) fishery of southern Brittany (Wilhelm & Miahle, 1996), in recent years.

Parasitic dinoflagellates are common in the marine environment, but little is known about their transmission, development in the host and pathogenicity (reviewed by Cachon & Cachon, 1987; Shields, 1994). Parasitic forms are found in both the major subdivisions of the Dinoflagellata, the Dinokaryota and the Syndinea (Fensome *et al*. 1993). The entirely parasitic Syndinea differ from the Dinokaryota (well known as free-living autotrophic components of marine and freshwater plankton, and notorious for giving rise to toxic blooms) in that they have relatively few chromosomes which lack the unique 'screw-carpet' arrangement of DNA fibrils prominent in transmission electron micrographs of dinokaryote nuclei. Dinokaryote chromosomes lack both histones I–IV and typical nucleosomal organization; syndinean chromosomes may have more conventional eukaryote organization (Hollande, 1974; Ris & Kubai, 1974). Histological studies on infected hosts, moreover, suggest that the syndineans have elaborate development cycles in which most of the stages lack flagella and look nothing like free-living dinoflagellates. Our present knowledge of these life-cycles is fragmentary, however, and little experimental work has been conducted on these organisms, largely due to lack of a suitable culture system.

In order to understand the complex life-cycles of the syndineans we attempted to cultivate the *Nephrops* parasite *in vitro*. Serial cultivation has now been practised for 5 years using 10% foetal calf serum in *Nephrops* saline with added antibiotics. At 8 °C the parasites undergo a series of developmental changes, which probably correspond to those taking place in

* Corresponding author: Division of Environmental and Evolutionary Biology, Graham Kerr Building, University of Glasgow, Glasgow G12 8QQ, UK. Tel: +141 330 4433. Fax: +141 330 5973. E-mail: k.vickerman@bio.gla.ac.uk

Table 1. Origin of the isolates maintained in *in vitro* culture

(Isolates were examined every day; time from isolation to sporogenesis, the spore type produced and the time from sporogenesis to germination into trophonts were recorded.)

| Isolate number | Date of isolation | Location | Infection status of lobster* | Origin of isolate | Time from isolation to sporogenesis (days) | Dinospore type | Time from sporogenesis to germination (days) |
|---|---|---|---|---|---|---|---|
| 1 | 30/5/92 | Clyde Sea Area | IV | Haemolymph | 15 | Macro | 37 |
| 2 | 5/2/93 | Clyde Sea Area | III | Haemolymph | 46 | Macro | 37 |
| 3 | 5/2/93 | Clyde Sea Area | IV | Haemolymph | 5 | Macro | 36 |
| 4 | 11/3/94 | Clyde Sea Area | III/IV | Ovary | 20 | Macro | 36 |
| 5 | 11/3/94 | Clyde Sea Area | III/IV | Midgut | 20 | Macro | 39 |
| 6 | 16/2/95 | Clyde Sea Area | Uncertain | Haemolymph | 155 | Macro | 53 |
| 7 | 16/2/95 | Clyde Sea Area | II† | Haemolymph | 16 | Macro | 27 |
| 8 | 13/4/95 | Irish Sea | I | Haemolymph | 32 | Macro | 62 |
| 9 | 5/2/93 | Clyde Sea Area | II† | Haemolymph | 32 | Micro | 38 |
| 10 | 11/3/94 | Clyde Sea Area | I/II† | Haemolymph | 31 | Micro | 36 |
| 11 | 11/4/95 | Irish Sea | II | Haemolymph | 24 | Micro | 18 |
| 12 | 13/4/95 | Irish Sea | III | Haemolymph | 22 | Micro | 19 |
| 13 | 13/4/95 | Irish Sea | III/IV | Haemolymph | 22 | Micro | 19 |

* See Field & Appleton, 1995.
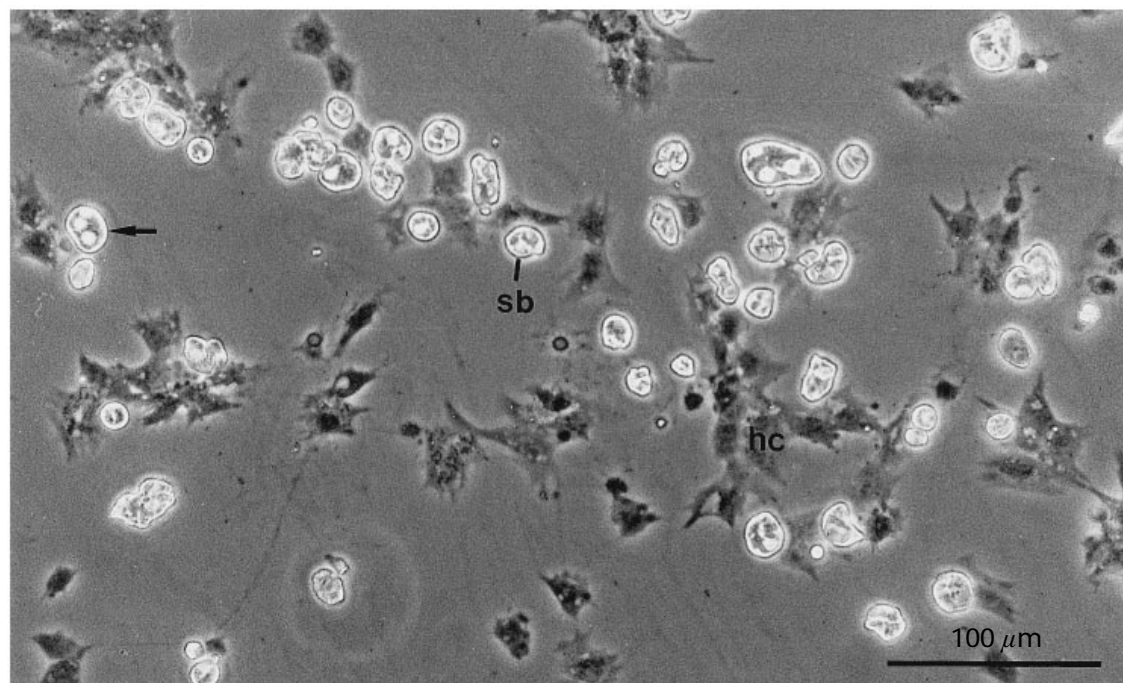† Isolate taken post-mortem.



Fig. 1. Fresh haemolymph preparation from infected *Nephrops*. Host haemocytes (hc) have settled onto the substratum and spread while the dinoflagellate sporoblasts (sb) remain subspherical. Arrow indicates a trinucleate parasite. Phase contrast.

the infected host, and culminate in the production of the flagellated dinospores which are of 2 types, macrospores and microspores. The principal aim of this paper is to characterize morphologically and behaviourally the developmental stages produced *in vitro*, and where possible to relate these to *in vivo* stages. The availability of a simple culture system for *Hematodinium* adds another subject for the study of the control of complex parasite life-cycles to the parasitologist's repertoire.

## MATERIALS AND METHODS

### Initiation of cultures

Haemolymph samples were taken from adult *Nephrops norvegicus* collected by trawl from the Clyde Sea Area and diagnosed as infected with the parasite by pleopod and body colour examination (Field *et al.* 1992; Field & Appleton, 1995). Haemolymph was withdrawn aseptically from the base of a fifth pereiopod or from the venter of the terminal abdominal segment using a 1 ml disposable syringe and 25-gauge needle, following sterilization of the cuticle with 70 % ethanol. More rarely fragments of excised infected tissue (gonad, midgut) were used to initiate cultures.
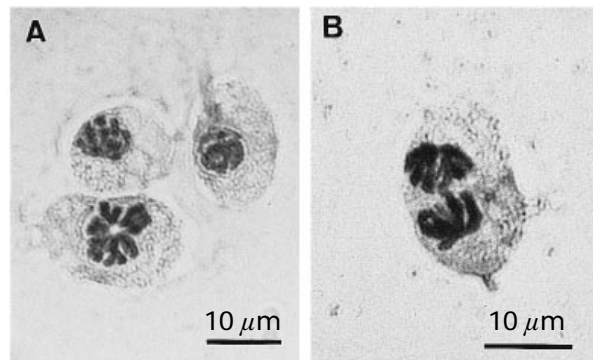


Fig. 2. Squashes of non-dividing (A) and dividing (B) sporoblasts showing prominent V-shaped chromosomes characteristic of syndinean dinoflagellates. Toluidine blue staining.

The parasite was isolated in culture in 3·5 cm well plates with 0·2 ml of infected haemolymph added to 5 ml of culture medium in each well. The culture medium consisted of 10 % foetal calf serum (Gibco) in autoclaved balanced *Nephrops* saline (NaCl 27·99 g/l; KCl 0·95 g/l; $CaCl_2$ 2·014 g/l; $MgSO_4$ 2·465 g/l; $Na_2SO_4$ 0·554 g/l; HEPES 1·92 g/l) adjusted to pH 7·8, with added gentamycin (25 $\mu$g/ml); initially the isolation medium contained penicillin (200 $\mu$g/ml) as additional protection against bacterial contamination. Cultures were incubated at 6–10 °C. Primary cultures were examined twice a week for contaminant bacteria, protists or fungi and heavily contaminated cultures were usually rejected; in the case of protist contaminants *Hematodinium* could be retrieved by dilution or serial washing of colonial stages.

### Subculture of sporogenic stages

*In vitro*, host-derived parasites multiplied and gave rise in due course to motile dinospores. Subcultures were made when parasites and host cells carpeted 2/3 of the floor of the well. When dinospores were produced they were diluted to achieve approximately the same distribution in fresh medium, and kept in the same well until germination took place.

### Subculture and growth of filamentous trophonts

On germination the dinospores gave rise to multiplicative filamentous trophonts. The parasite was maintained in this stage by serial subculture at 2–3
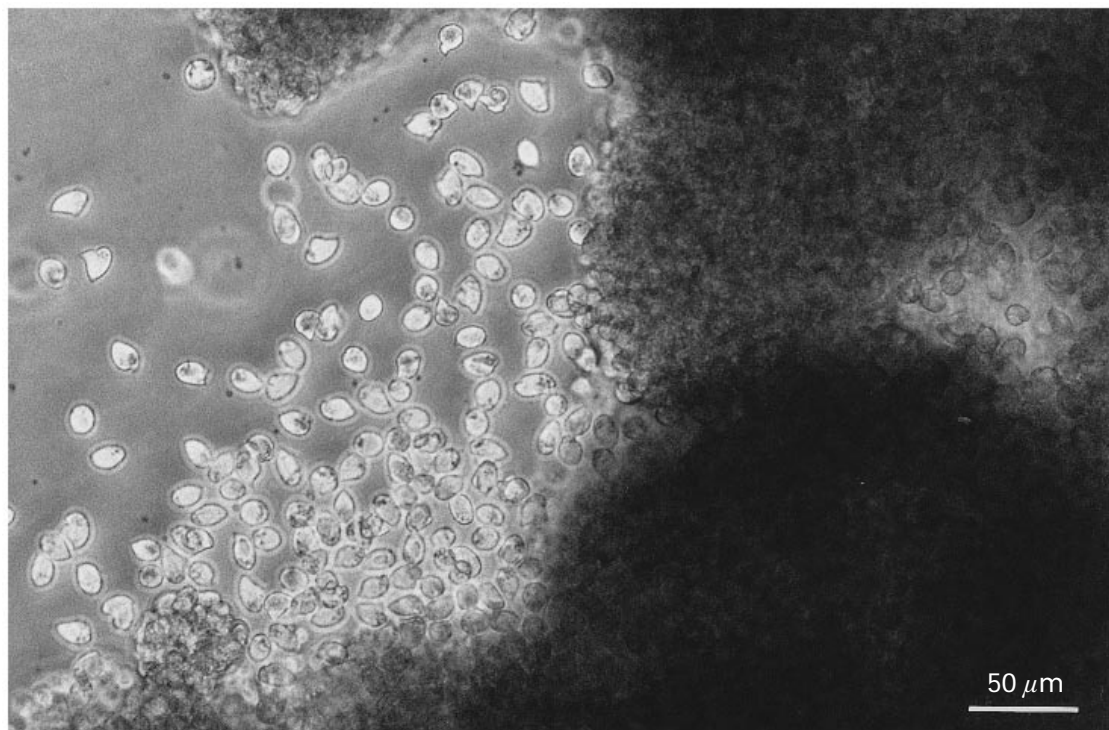


Fig. 3. Sporogenic mass from *in vitro* culture showing production of dinospores (macrospores). Phase contrast.
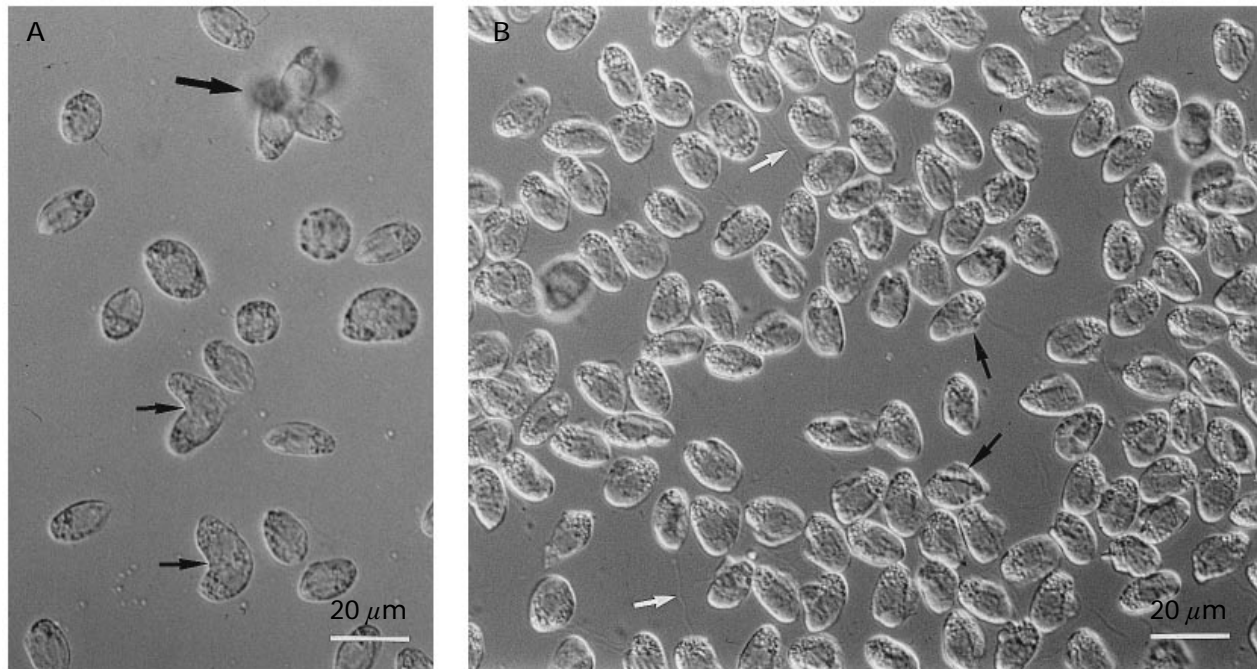
Fig. 4. (A) Final stages in the formation of macrospores by division of sporoblasts *in vitro*. The fission furrow passes from anterior to posterior end of the sporoblast (small arrows) and several dinospores may be formed simultaneously (large arrow). Glutaraldehyde fixation. Differential interference contrast. (B) Living macrospores, some showing long anterior flagellum (white arrows) and the pronounced keel (black arrows) that is characteristic of the mature macrospore. Differential interference contrast.
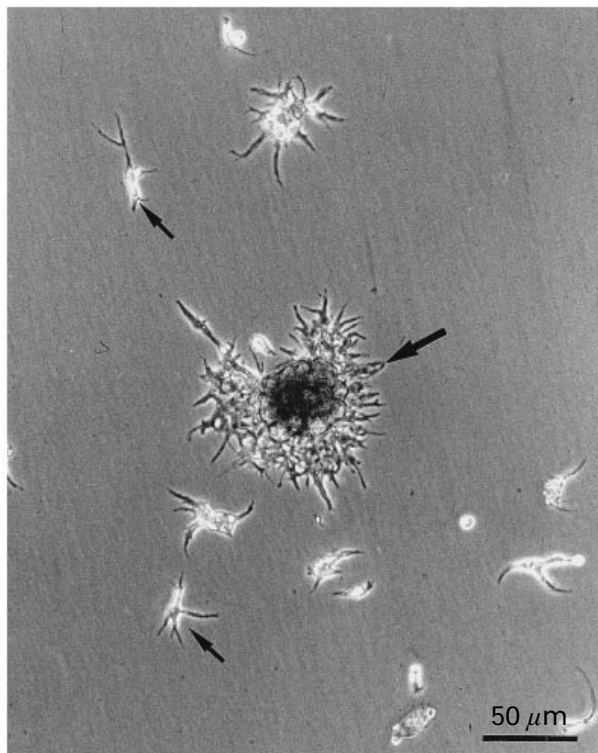


Fig. 5. Secondary sporonts in primary culture. Sporoblasts settle on the bottom of the well and put out anchoring filopodium-like extensions. Most of the sporonts shown here (small arrows) have one or two nuclei, but in the largest one (large arrow) several nuclei are present in a central cytoplasmic mass while the peripheral cytoplasm forms a flattened network of strands characteristic of the arachnoid sporont. Phase contrast.

week intervals. An approximately equal volume of fresh medium was added to the culture well and half the mixture transferred to a new well. The attached arachnoid phase could often be induced by diluting trophont cultures substantially, e.g. by transferring the contents of a culture well to 20 ml of fresh medium in a 50 ml Falcon flask. The arachnoid phase was subcultured by fragmentation achieved by vigorous pipetting of the medium.

Growth of the filamentous trophonts *in vitro* was quantified as follows: 0·5 ml of a well-established culture of filamentous trophonts was added to 30 ml of fresh medium in a 200 ml culture flask (Greiner), and the flask was placed on a rocking table (Luckham 4RT) at 10 °C. After 1 h and at intervals thereafter, 1 ml of the culture was centrifuged at 5000 rpm in a microfuge for 2 min; 900 $\mu$l of supernatant were removed and the pellet resuspended in the remainder. The 10× concentrated filamentous trophonts were then counted using a haemocytometer and 4 separate counts made on each occasion.

*Microscopy*

Light microscopy observations were made with a Leitz Diavert inverted microscope fitted with phase-contrast optics or with a Leitz Orthoplan microscope fitted with Differential Interference Contrast Device T. DAPI (4′,-6-diamidino-2-phenylindole, 10 $\mu$g/ml in phosphate-buffered saline) staining of nuclei was carried out on paraformaldehyde/glutaraldehyde-fixed (see below) material and examined with a
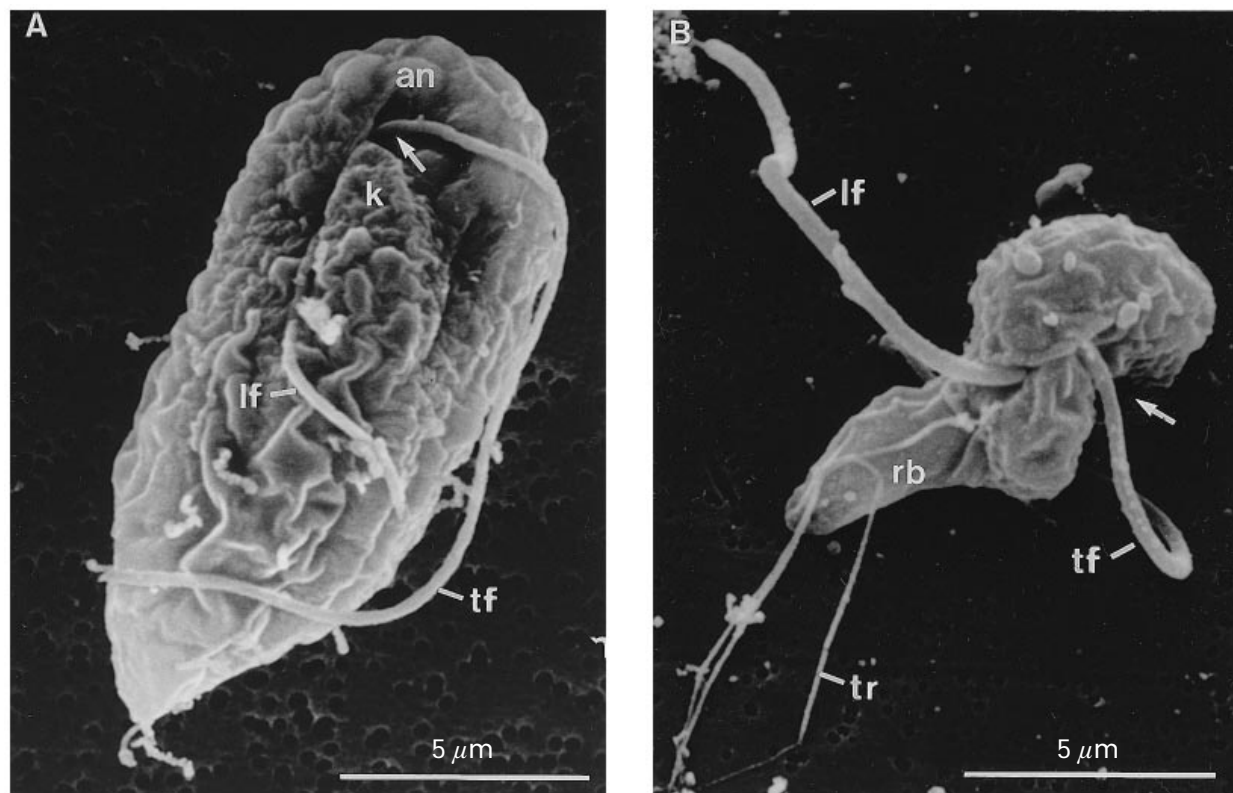
Fig. 6. Mature (4-day-old) dinospores from primary *in vitro* cultures. Scanning electron microscopy. (A) Macrospore. The longer transverse flagellum (tf) arises in a short groove (girdle, arrowed) close to the anterior end (an). The shorter longitudinal flagellum (lf) arises behind the transverse flagellum and runs initially along a straight sulcus which extends halfway along the macrospore. Collapsed amphiesmal alveoli result in wrinkling of the surface except at the anterior end where lipid globules cause external bulging. The keel (k) is pointing towards the viewer. (B) Microspore. The transverse flagellum (tf) runs initially in the girdle (arrow) which forms a deeply grooved channel around the microspore giving it a corkscrew shape; the longitudinal flagellum (lf), longer than the transverse, extends beyond the posterior end of the body. Note discharged trichocysts (tr). The smooth posterior projection houses the refractile body (rb).

Zeiss Axioskop microscope employing ultraviolet epifluorescence through a DAPI filter set. Chromosomes were visualized in similarly fixed squashes of cells stained with 1 % aqueous toluidine blue.

Transmission electron microscopy was carried out on parasites washed free of serum with *Nephrops* saline then fixed for 1 h at 4 °C in 2 % paraformaldehyde and 1 % glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) with 2 % sucrose and 1·5 % sodium chloride. After rinsing twice in similarly buffered 6·5 % sucrose solution, material was treated for 1 h in 1 % $OsO_4$ in rinsing solution, rinsed in distilled water and embedded in 1·5 % agar before block-staining in 0·5 % uranyl acetate in distilled water. After ethanol dehydration specimens were embedded in Spurr's resin. Thick sections (0·35–1·0 $\mu$m) were stained in 1 % aqueous toluidine blue for light microscopy; thin sections (silver interference colour) were stained with 2 % methanolic uranyl acetate (5 min) and Reynolds' lead citrate (5 min) before examination in a Zeiss 902 TEM operating at 80 kV. For scanning electron microscopy, material retained on 0·2 $\mu$m pore size polycarbonate membrane filters was acetone dehydrated, critical-point dried and mounted on stubs and sputter-coated with gold/palladium for viewing in a Philips 500 SEM operating at 12 kV.

RESULTS

This study is based on over 30 *in vitro* isolates. Details of the isolation of those maintained for more than 6 months are given in Table 1.

*Sporogenesis* in vitro

In the late stage of infections from which cultures were initiated, the parasites circulating in the milky-white haemolymph were found by phase-contrast microscopy to be subspherical syncytia (5–14 $\mu$m in diameter), with refractile appearance and irregular contour, each containing 1–5 nuclei. After several minutes in the culture well, the host haemocytes were easily distinguished because they had spread on the floor of the well, while the parasites had not (Fig. 1). The number of haemocytes present in infected haemolymph reflected the infection status of the
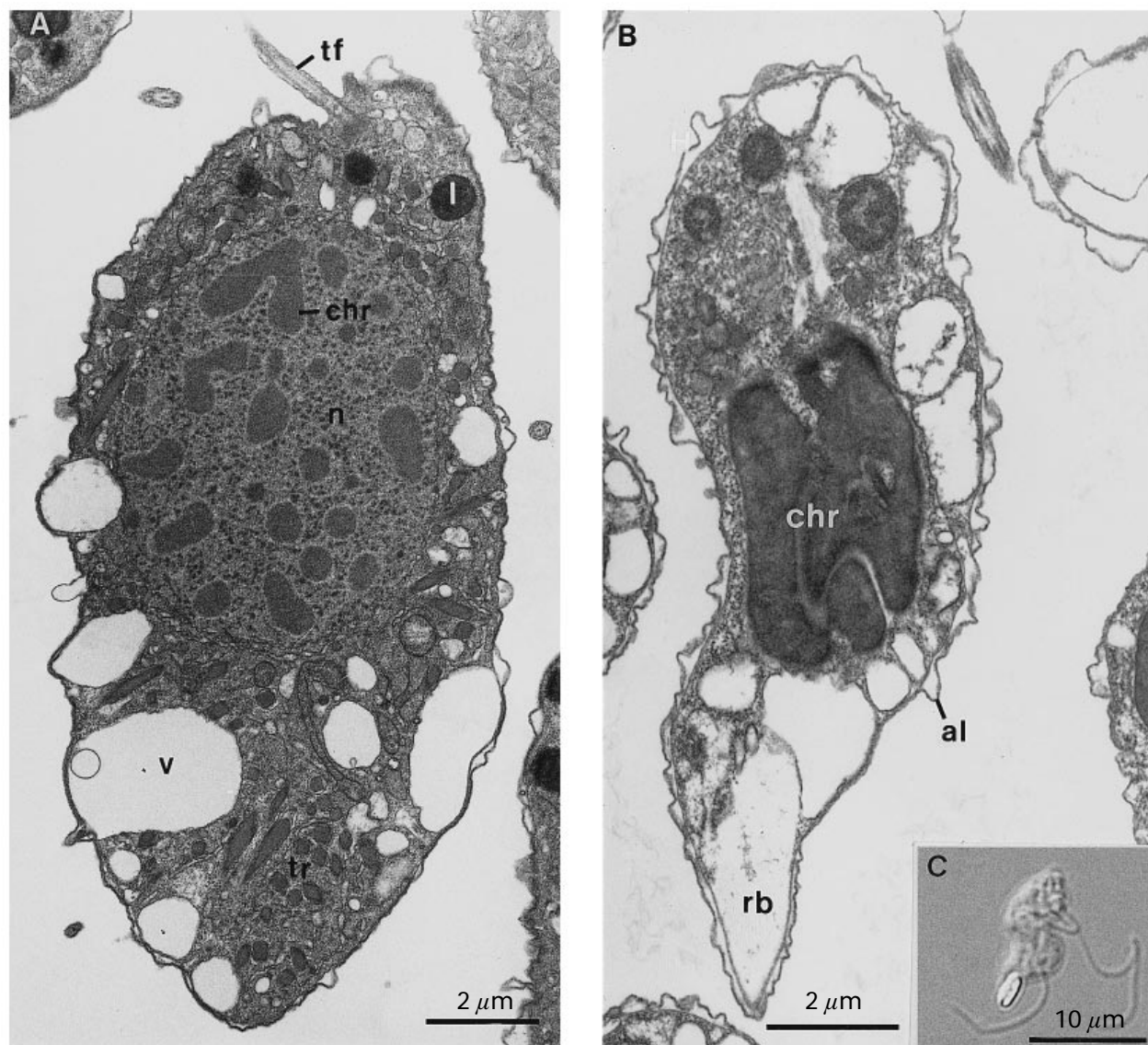
Fig. 7. Mature (4-day-old) dinospores from primary *in vitro* cultures. (A and B) Transmission electron microscopy of longitudinal sections. (A) Macrospore. The base of the transverse flagellum (tf) is situated at a distance from the nucleus (n) which shows condensed chromosome profiles (chr). Lipid droplets (l), peripheral vacuoles (v) and trichocysts (tr) are abundant in the cytoplasm. (B) Microspore. The condensed chromosomes (chr) entirely fill the nucleus; swollen alveoli (al) give a corrugated profile to the dinospore surface. The refractile body (rb) contained in the posteriormost vacuole is not readily visualized here. (C) The refractile body is clearly visible in this light micrograph of a glutaraldehyde-fixed microspore, 3 weeks after sporogenesis. Differential interference contrast.

lobster. In infection stages I and II 30–80 % of the total cells were haemocytes, whereas in infection stages III and IV only 3–25 % of the cells were haemocytes (Field & Appleton, 1995).

Circulating parasites from late-stage infections were found to have the character of sporoblasts, i.e. they would eventually give rise by division to flagellated dinospores in the crustacean host. Toluidine blue-stained squashes of sporoblasts in division showed the 5 V-shaped chromosomes (Fig. 2). The fine structure of these sporoblasts has been described previously (Field *et al.* 1992; Appleton & Vickerman, 1996). They contained abundant trichocysts and swollen cisternae of the nuclear envelope

and endoplasmic reticulum containing filamentous material tentatively identified as flagellar hairs destined for the transverse flagellum of the dinospore. Their nuclei showed highly condensed chromosomes and their surface had the characteristic dinoflagellate amphiesma (alveolate) structure.

*In vivo*, sporogenesis was more or less synchronous in the sporoblast population, the spores escaping through the gills. *In vitro* the circulating sporoblasts gave rise to dinospores (Fig. 3) after a period of days to weeks. The time-course of dinospore production is indicated in Table 1. Two patterns of development were observed. In the first, the sporoblasts increased in number by fission, often forming spherical masses
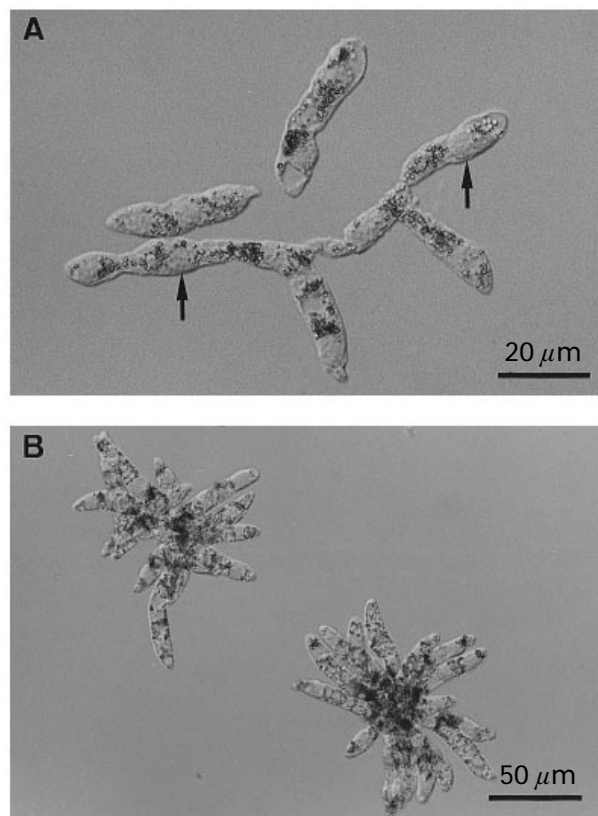
Fig. 8. Filamentous trophonts. Osmium vapour fixation. (A) Single filaments. Clustered lipid globules (black) in cytoplasm outline clearer spaces (arrowed) that correspond to nuclei. One filament shows branching. (B) Gorgonlocks configurations of filaments.
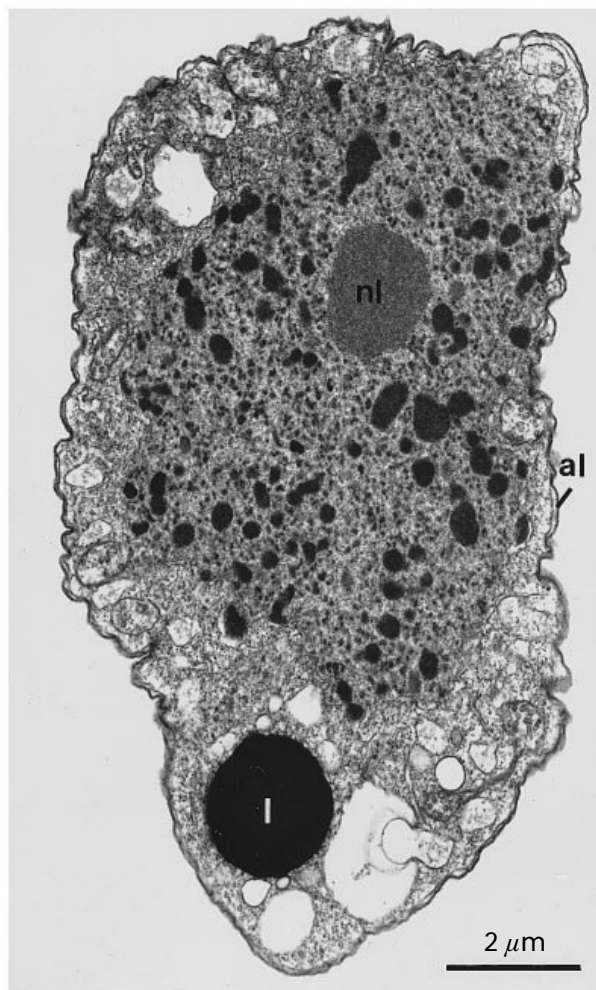


Fig. 9. Filamentous trophont. Transmission electron microscopy of transverse section. Note the surface corrugations are independent of cortical alveoli (al). In the nucleus, chromosomes are extended (cf. Figs 7A, B and 16). l, Lipid; nl, nucleolus.

or sheets of multinucleate cells which eventually broke up to release spore mother cells; each of which gave rise to 4 or more flagellated dinospores (Fig. 4A, B). In the second pattern of development the sporoblasts gave rise to a branched network of filaments with nuclei scattered along their length (Fig. 5), and noticeably attached to the substratum. Such attached syncytia we shall refer to as secondary sporonts: their development will be referred to later after describing primary sporont production.

*Dinospore dimorphism*

Dinospores produced *in vitro* (as *in vivo*) were uninucleate and of 2 types, differing in size and form, termed macrospores (16–20 $\mu$m long, 8·5–11·5 $\mu$m in width, $n = 25$) and microspores (11–14 $\mu$m long, 4·5–6·5 $\mu$m in width, $n = 25$). Each spore type had 2 heterodynamic flagella, the anterior flagellum encircling the anterior end of the body in beating and providing the principal propulsive force, the rudder-like posterior flagellum trailing behind. Microspores were intermittently much faster in their movement than macrospores. By scanning electron microscopy, the longitudinal flagellum was seen to lie initially in a groove, the sulcus, as in other dinoflagellates; the anterior flagellum could also be seen arising in a

shallow groove, close to that of the longitudinal flagellum (Fig. 6A), but it was not confined in an equatorial cingulum (girdle) as in the dinokaryote dinoflagellates. The free region of the longitudinal flagellum was longer in the microspore (Fig. 6B). Discharged trichocysts were commonly observed associated with the dinospore surface (Fig. 6B).

In transmission electron micrographs of sections, lipid droplets were prominent at the anterior end of both spore types (Fig. 7A, B), often causing bulging of the macrospore surface (Fig. 6A). Clear vacuoles with sparse (or no) electron-dense granular contents were abundant (Fig. 7A, B) and trichocyst profiles, often in bundles, were common throughout the cytoplasm. In the dinospores, the flagellar bases appeared to be dissociated from the nuclear envelope (Fig. 7A), whereas in the non-flagellated stages the barren basal bodies were present in an intucking of the nuclear envelope (cf. Ris & Kubai, 1974).

Some days after their release from the sporoblast, the dinospores were observed to develop more
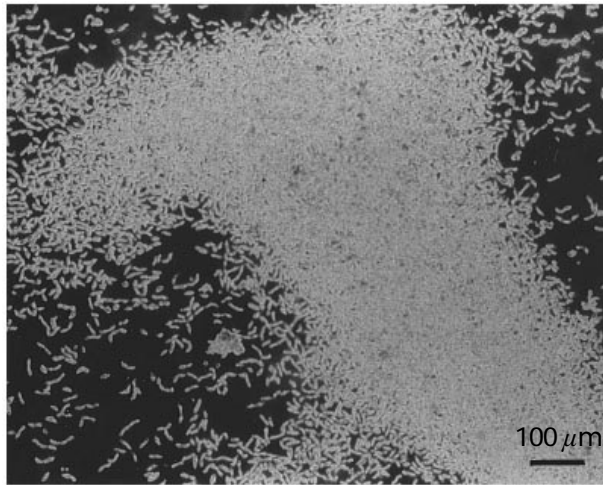
Fig. 10. Trophont cloud. Filamentous trophonts multiply in an aggregated mass in the centre of a 3·5 cm well. Phase contrast.

pronounced morphological differences. Whereas in the bullet-shaped macrospore the sulcus was straight, in the microspore it formed a deep channel spiralling around the body (Fig. 6B); in the macrospore the ridge between the flagellar grooves developed into a prominent keel (Figs 4B and 6A). A striking refractile inclusion in a vacuole at the posterior extremity of the microspore was another distinguishing feature of this spore type (Fig. 7C). Condensation of the chromosomes in the nucleus was clearly more pronounced in microspores than in macrospores (Fig. 7B).

## Germination of dinospores

After a period of about 5 weeks in fresh medium, longer in the case of some macrospore isolates, shorter for some microspores (see Table 1), both
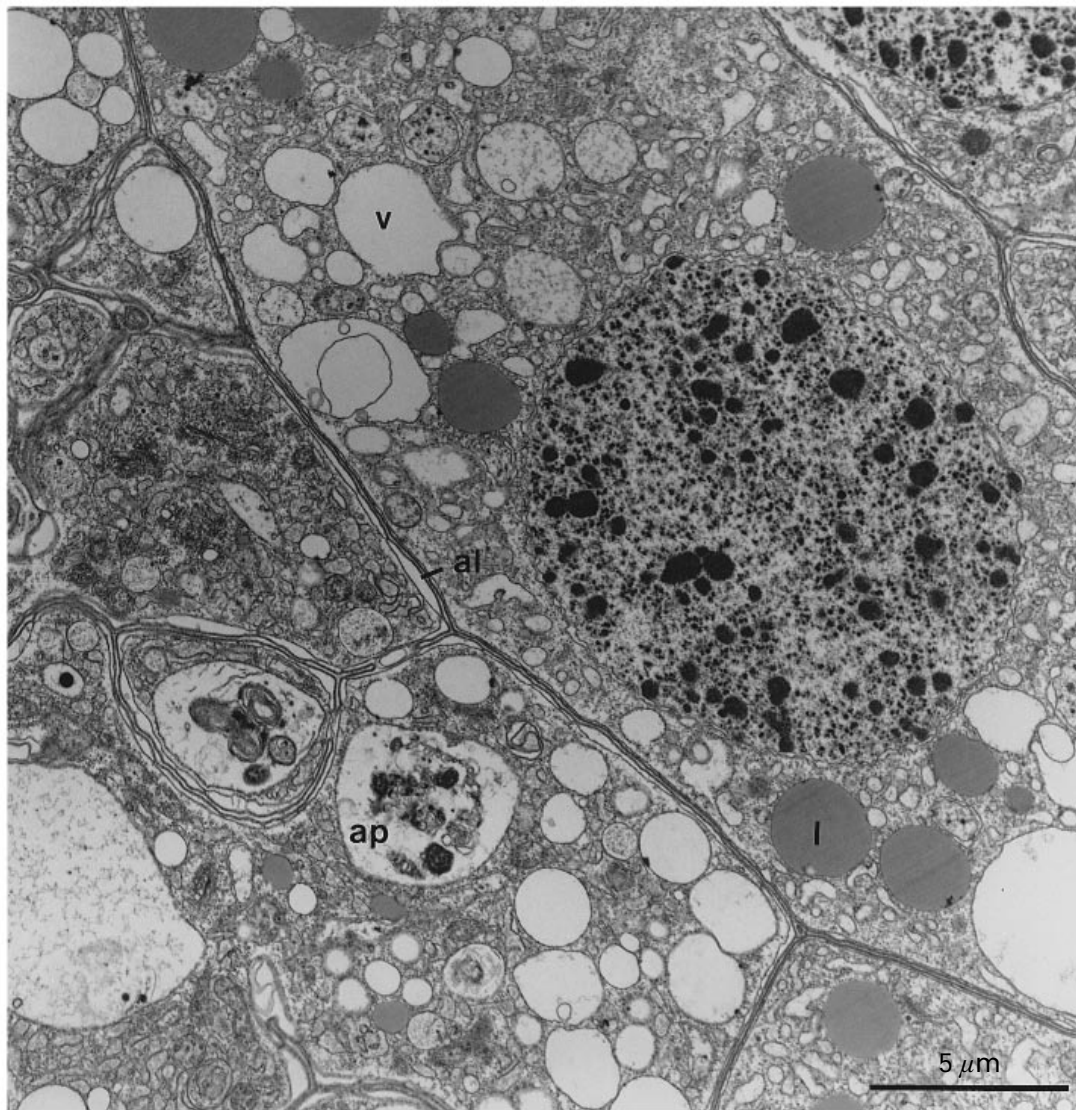


Fig. 11. Clump colony. Transmission electron micrograph of section of part of colony showing interdigitation of packed trophonts, each delimited by its alveolate surface (al). The nucleus shows typical trophont structure. Autophagosome-like structures (ap) may represent early degenerative changes. l, Lipid; v, vacuoles.
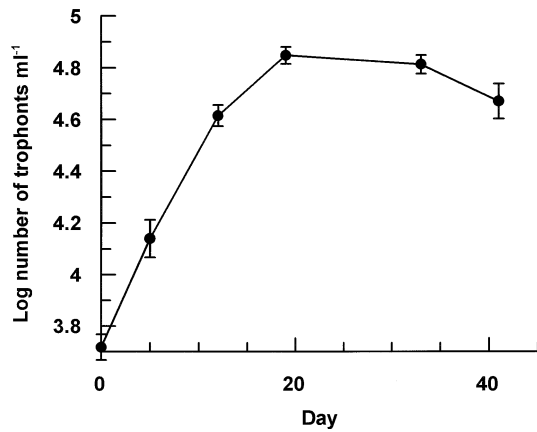
Development of Hematodinium

Fig. 12. Representative growth curve of filamentous trophonts in foetal calf serum–*Nephrops* saline medium. Points show geometric mean $\pm 2$ s.e. ($n = 4$).

types of spore became immotile and settled on the bottom of the culture well. If spore density was adjusted to produce a monolayer, both types of spore were shown to transform into square cells and then undergo growth and nuclear division to produce multinucleate filaments; no fusion of motile spores was discerned, and each type of spore was found to be capable of germination independent of the other.

*Growth of the filamentous trophont phase*

The unattached 12–50 $\mu$m-long filaments produced

on germination of the dinospores represent the principal multiplicative form obtained in culture and are designated trophonts by analogy with the growth phase in other parasitic dinoflagellate life-cycles. DAPI staining of filaments showed that the majority were uninucleate (50%) or binucleate (43%) and that relatively few (7%) had 3 or more nuclei. Healthy filamentous trophonts (Fig. 8A) displayed a profoundly wrinkled surface (Fig. 9) and showed lateral flexing and squirming movements: in the latter, constrictions passed along the filaments as cytoplasm and nuclei were shunted backwards and forwards along them. These movements were sufficient to effect slow translation and could be reversibly inhibited in the presence of 2 $\mu$g/ml Cytochalasin D, suggesting an actin-based motility. Transmission electron microscopy of filaments (Fig. 9) showed that they contain nuclei with extended chromosomes, clustered lipid globules (also prominent by light microscopy, see Fig. 8A) and clear vacuoles, but not trichocysts or flagellar hair vesicles. Micropores, a paranuclear Golgi apparatus and a mitochondrial network are present in this as in other stages (cf. Appleton & Vickerman, 1996).

After growth by increase in length the filaments branched and developed constrictions at branching points and along their lengths, that later become points of severance (Fig. 8A). Filaments multiplying in this way were gregarious and formed centripetal aggregates (trophont clouds; Fig. 10) in the culture wells, or sheets extending into the medium above; in
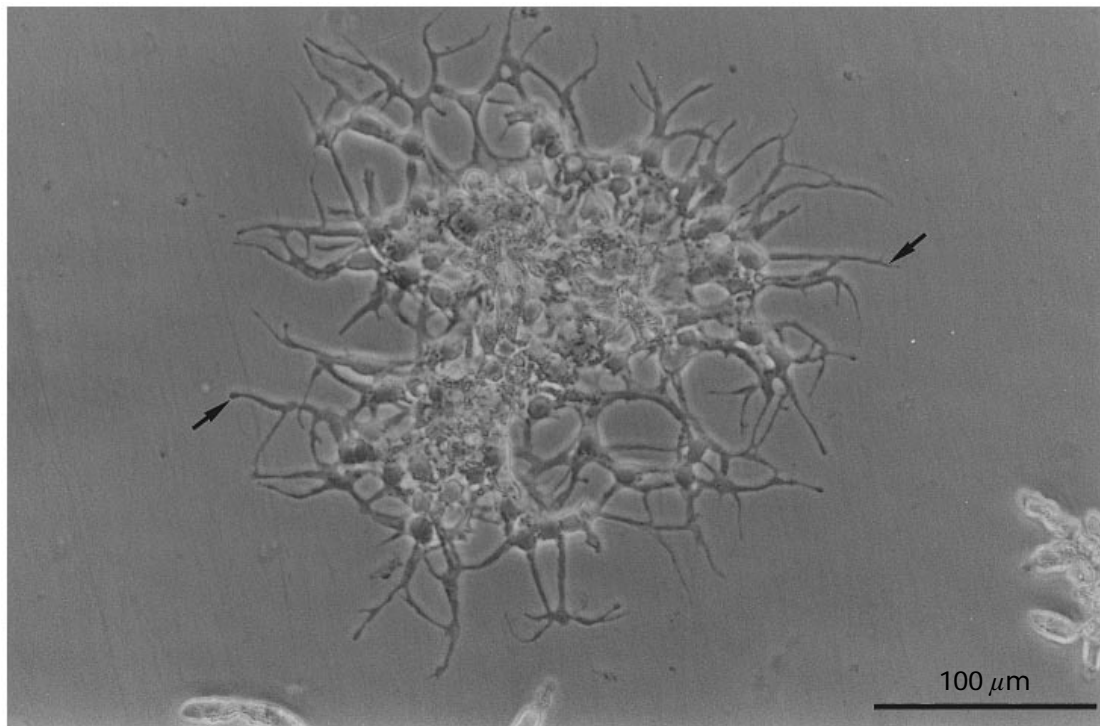


Fig. 13. Arachnoid trophonts derived from filamentous trophonts in a well culture. Nucleated islands of cytoplasm are linked by cytoplasmic strands; peripheral spike-like processes (arrowed) appear to have a role in anchorage to the substratum. Phase contrast.
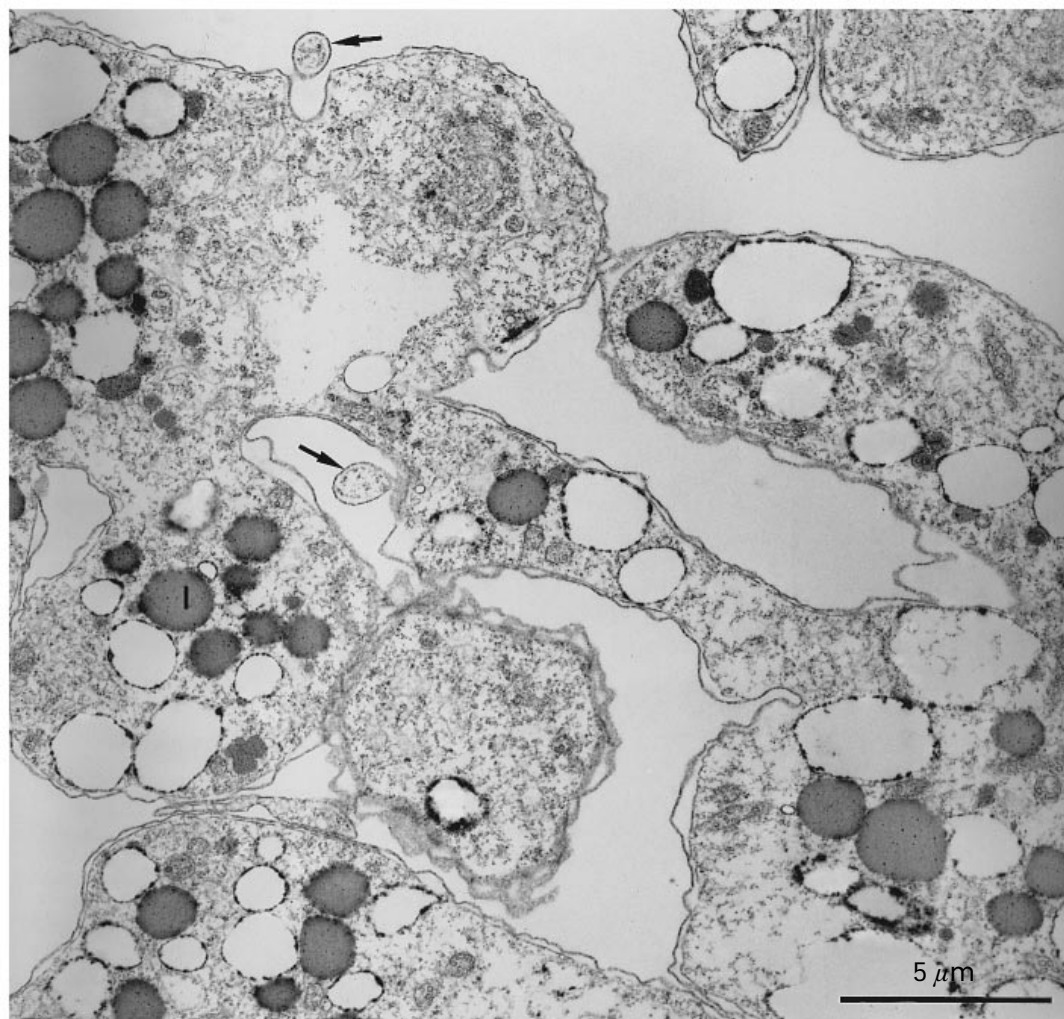
Fig. 14. Arachnoid trophont. Transmission electron micrograph of part of plasmodium showing cytoplasmic islands and strands covered in alveolate cortex. Some profiles (arrowed), however, are delimited by a single membrane and these are believed to represent the anchoring spikes. The vacuoles have peripheral inclusions. Trichocysts are absent. l, Lipid.

older cultures the aggregated isotropic trophonts extended in spoke fashion from a central hub-like mass to the edge of the well and along its perimeter.

One specific growth pattern of the multiplying trophonts around the edge of the centripetal cloud where the filaments were less densely packed, was in colonies with the filaments radiating from a central point (Fig. 8B); the writhing movement of the filaments recalled the snake locks of the mythical gorgon and so we refer to such colonies as 'gorgonlocks'. Trophonts growing in 50 ml flasks tended not to grow in clouds, but readily adopted the gorgonlock colony form. Left for 3 weeks or more, the gorgonlock colonies condensed and became spherical or flattened masses referred to as clump colonies and formed from interdigitated syncytia which retained the trophont structure. Transmission electron micrographs of sections of clump colonies showed the dense tissue-like form of such structures (Fig. 11). If not transferred to fresh medium the clump colonies increased in size but became excessively vacuolated

and senescent; subculture within a week or so of their formation, however, enabled them to regenerate filamentous trophonts.

Subcultured at 2-week intervals, the parasites maintained growth in the filamentous trophont form. The social habits of the trophonts plus their variable size and number of nuclei per filament made meaningful estimates of growth rate in the trophont phase very difficult to obtain; dispersal to facilitate counting appeared to inhibit growth in wells. Fig. 12 shows a growth curve obtained from trophonts grown as dispersed filaments in a flask on a rocking table.

One macrospore-derived isolate has now been serially cultured over a 5-year period in the foetal calf serum medium and shows no sign of expiring. A microspore-derived isolate has been maintained similarly for almost the same length of time (Table 1). The parasite has remarkable survival potential *in vitro*. Old cultures have on occasion been resurrected as much as 12 months after they were initiated.
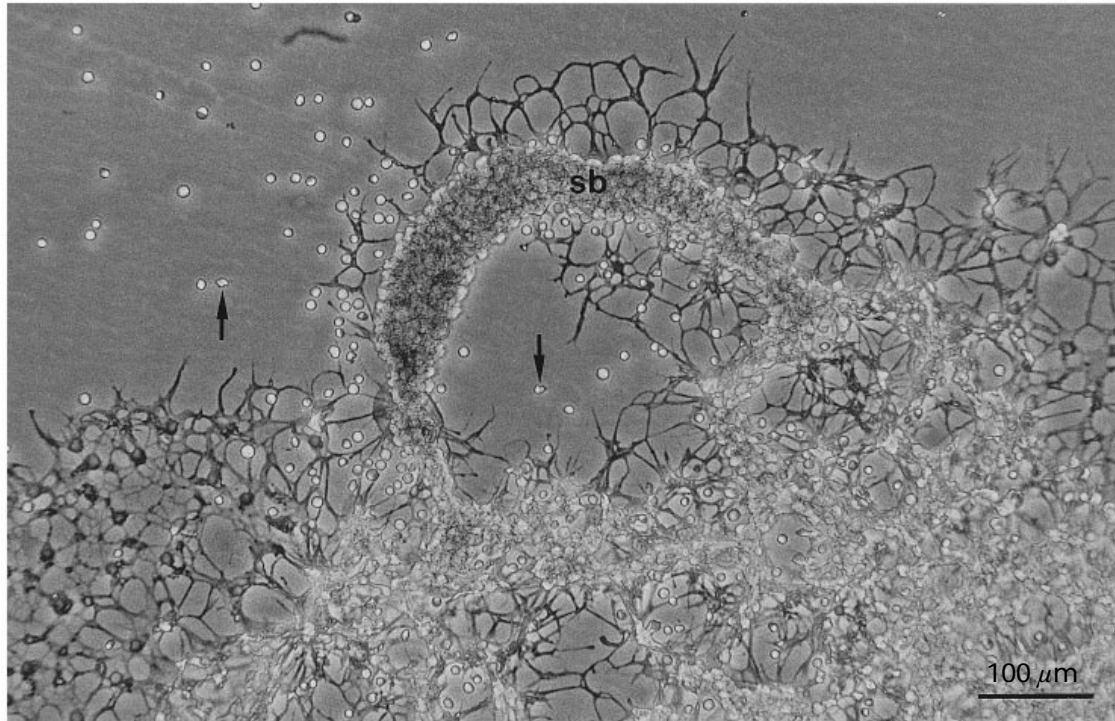
Fig. 15. Arachnoid sporont. Four weeks after isolation *in vitro*. Macrospores (arrowed) are being released from sporoblasts (sb) piled high around a central 'crater' of 1 of the 3 arachnoids undergoing fusion in this micrograph. Phase contrast.

### Development of the arachnoid trophont phase

Under certain conditions, as yet undefined (though sudden dilution of the trophont cultures with excess fresh medium appears to be effective), the filamentous trophonts, often as gorgonlock colonies, adhere to the substratum. Then, through progressive nuclear division and growth, and branching and anastomosis of filaments, they formed elaborate syncytial networks (plasmodia) fringed by anchoring spikes (Fig. 13). Such plasmodia constitute the arachnoid (spider-web-like) phase of the developmental cycle. These arachnoid forms expanded progressively outwards on the base of the culture vessel and coalasced with one another when their spike-like marginal extensions met. In this way they occasionally formed a single plasmodium that covered the floor of the well. Pseudopodial activity was not discernible in arachnoid trophonts. Should the arachnoids be dislodged and fragmented by repeated pipetting of the culture well contents, they could be subcultured by transfer to fresh medium and resettled and started to grow as arachnoids again.

Transmission electron micrographs of early arachnoid plasmodia showed that their cytoplasmic and nuclear structure was similar to that of the filamentous trophonts. Their cytoplasm was packed with clear vacuoles with peripheral electron-dense inclusions and lipid globules but the alveolar surface was punctuated not only by micropores, but also by cytoplasmic extensions which had but a single surface membrane (Fig. 14). The latter profiles were identified with the attachment spikes.

### Sporoblast production by the arachnoid sporont

The arachnoid trophonts transformed into sporonts which gave rise to dinospores. Sporoblasts (sporogenic syncytia) were usually generated from the centre of the arachnoid which became progressively raised from the substratum to give the plasmodium a volcanic crater-like appearance, with aggregations of sporoblasts piling up around the rim (Fig. 15). Sometimes several centres of sporoblast production were encountered on a sporont. Further development of the sporont involved the release of the sporoblasts which corresponded structurally to the circulating haemolymph stages used to initiate the cultures.

Transformation of the arachnoid trophont into a sporont was marked by the appearance of numerous trichocysts in the cytoplasm and marked condensation of chromosomes in the nuclei (Fig. 16). Scanning electron micrographs of the surface of sporont arachnoids (Fig. 17) showed the presence of numerous surface particles which were interpreted as discharged trichocysts. In sporoblasts also, trichocysts and flagellar hair vesicles were readily visible in transmission electron micrographs of sections. Sporoblasts could settle on the substratum, giving rise to secondary sporont arachnoids. Fig. 5 shows stages in the development of secondary sporonts from circulating sporoblasts on primary isolation.
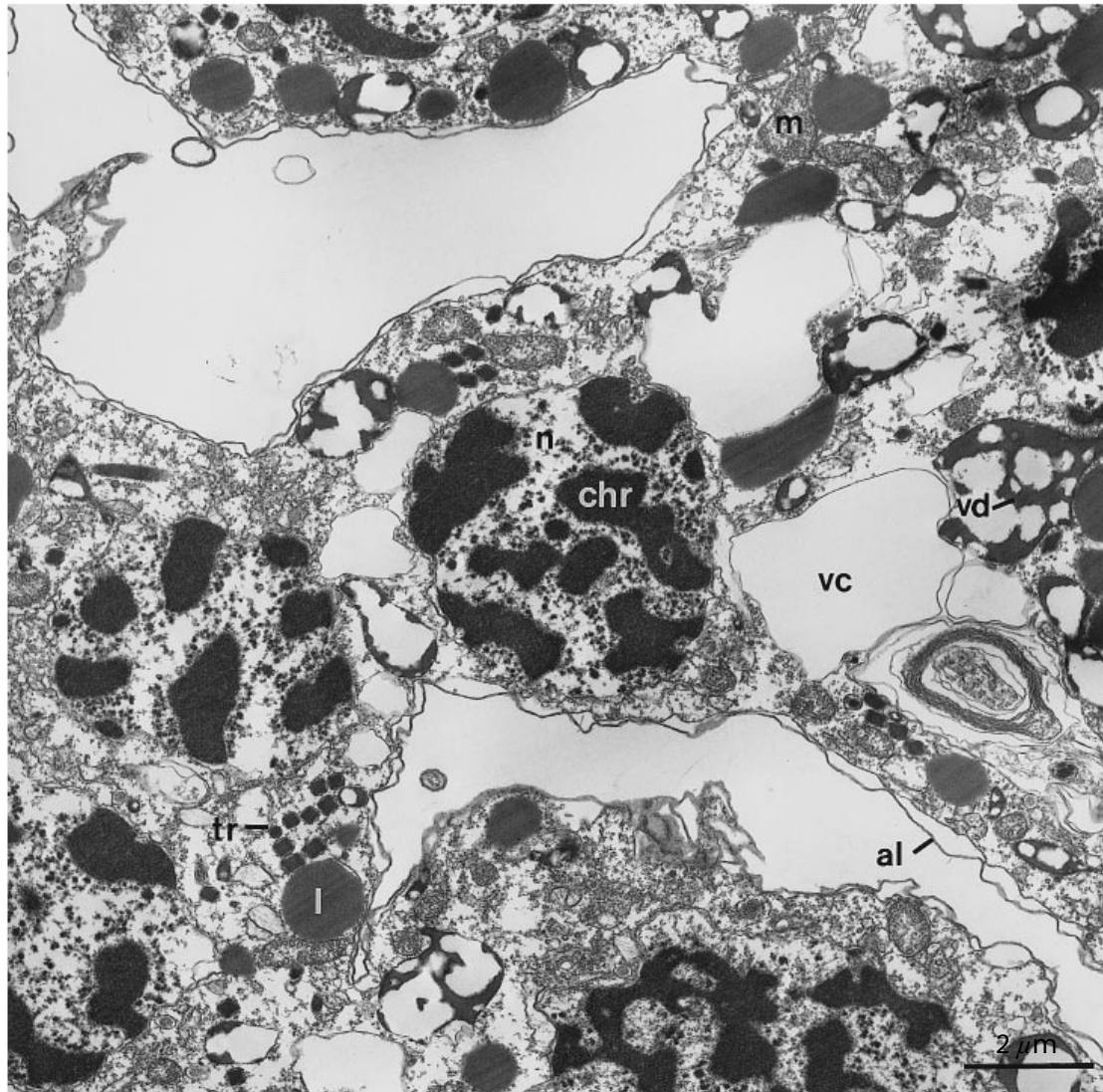
Fig. 16. Arachnoid sporont. Transmission electron microscopy. Horizontal section through part of secondary sporont. Note very condensed chromosomes (chr) in nuclei (n, cf. trophont nucleus in Fig. 9), abundant trichocysts (tr) in cytoplasm, also heavy electron-dense deposit in many of vacuoles (vd) while others (vc) remain clear. The latter can be distinguished from spaces between cytoplasmic islands and strands by their lack of alveolar lining (al). m, Mitochondrion; l, lipid.

To date, a second bout of sporogenesis *in vitro* has occurred only in the long-lived macrospore-derived Isolate 1. Dinospores were released directly from arachnoid sporonts and resembled microspores in size. They rarely assumed the corkscrew shape of first-generation microspores, however, and were much more sluggish in their movement. Nevertheless, they germinated in due course to give rise to a generation of filamentous trophonts which appeared to be just as viable as their predecessors, giving rise in their turn to arachnoids which underwent sporogenesis. One sporogenic line has now been maintained separately for 2 years, with subculture at 3-weekly intervals. In all other isolates maintained in culture, trophont arachnoids did not differentiate into sporonts.

All stages of development displayed the typical dinoflagellate amphiesma or alveolate cortex. Trichocysts were absent from the trophont stages but appeared on differentiation of the arachnoid trophont to the sporont; they were present throughout sporogenesis and occurred in both types of spore. The sporoblasts contained swollen cisternae of the nuclear envelope and endoplasmic reticulum containing filamentous material tentatively identified as flagellar hairs but these were less conspicuous in the cytoplasm of the mature spores (Fig. 7B).

DISCUSSION

The life-cycles and taxonomy of the syndinean dinoflagellates are at present poorly known yet they are potentially important parasites that can do much
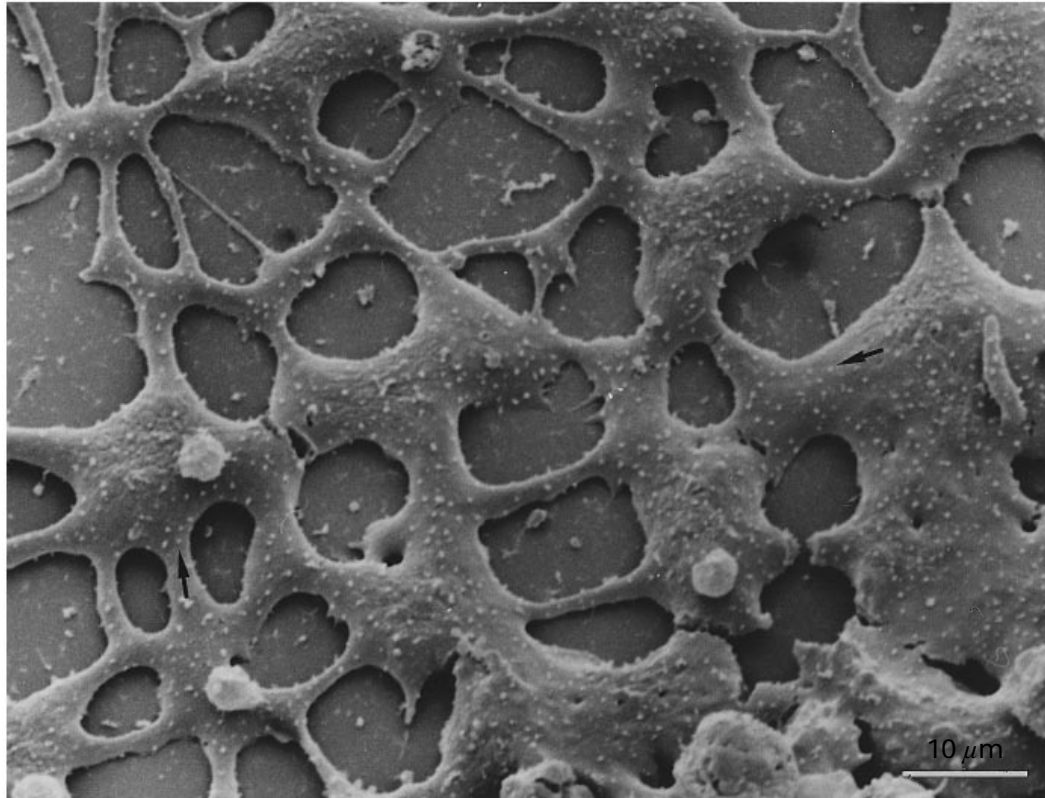
Fig. 17. Arachnoid sporont. Scanning electron microscopy. Part of secondary sporont showing cytoplasmic islands and interconnecting strands. The small particles (arrowed) are believed to represent trichocyst discharge sites and are absent from arachnoid trophonts.

harm to crustacean fisheries. Although syndinean basic biology has several interesting facets, only the nuclear division processes of those forms that are intracellular parasites of other protists (Hollande, 1974; Ris & Kubai, 1974) have been studied in any detail and mainly in contrast to those of the better-known dinokaryotes.

We describe here a simple procedure for the *in vitro* cultivation of *Hematodinium* sp., an extracellular syndinean parasite of decapod crustacea associated with mortality of *Nephrops norvegicus*. The parasite has been serially cultured in the filamentous trophont stage over a period of 5 years, but other developmental stages have been produced *in vitro* (Fig. 18), and these shed some light on the enigmatic developmental cycle in the natural host and on the species problem in *Hematodinium*.

Chatton & Poisson (1931) in their original account of *Hematodinium perezi* described only motile filamentous ('vermiform') stages with 2–8 nuclei, which they found circulating in the haemolymph of portunid crabs. Apart from Newman & Johnson (1975) and Messick (1994), who found parasites that were elongate and motile in crab hosts, subsequent reports of *Hematodinium* have described only round and immotile parasites in the haemolymph (Maclean & Ruddell, 1978; Myers *et al.* 1987; Hudson & Shields, 1994; Taylor & Khan, 1995). We also have observed mostly immotile rounded forms in *Nephrops norve-*

gicus haemolymph (Field *et al.* 1992), but have shown here that motile filamentous multinucleate forms, as described by Chatton and Poisson, do occur in the developmental cycle *in vitro*, indeed using the present culture system, these are the principal multiplicative forms obtained. We suggest that these represent the early-developing trophont phase of all species of *Hematodinium*, and have shown here that they arise from both macrospores and microspores on germination. We have shown elsewhere that this stage is able to take up colloidal gold by endocytosis via cortical pores, similar in structure to the micropores of the Apicomplexa. These pores are inserted in the typically dinoflagellate amphiesma which is composed of membrane-bound alveolar sacs underlying the surface membrane. The trophont phase does not contain trichocysts (Appleton & Vickerman, 1996).

As yet little is known of the early stages of development of the *Nephrops* parasite, but these stages most probably occur in the July to December season, when patently-diseased animals are absent from trawls, but when examination of the tissues of healthy animals using a *Hematodinium*-specific polyclonal antibody reveals latent infection with the parasite (Field & Appleton, 1996). The location of filamentous trophonts in these early infections is as yet undetermined, however. The significance of the colonial gorgonlocks colonies, and the spherical

Filamentous trophonts (1)

Gorgonlocks
trophont
colony (2)

Clump colony (3)

Microspore
(9)

Macrospore
(10)

Arachnoid
trophont (4)

Sporogenesis (8)

Secondary
arachnoid
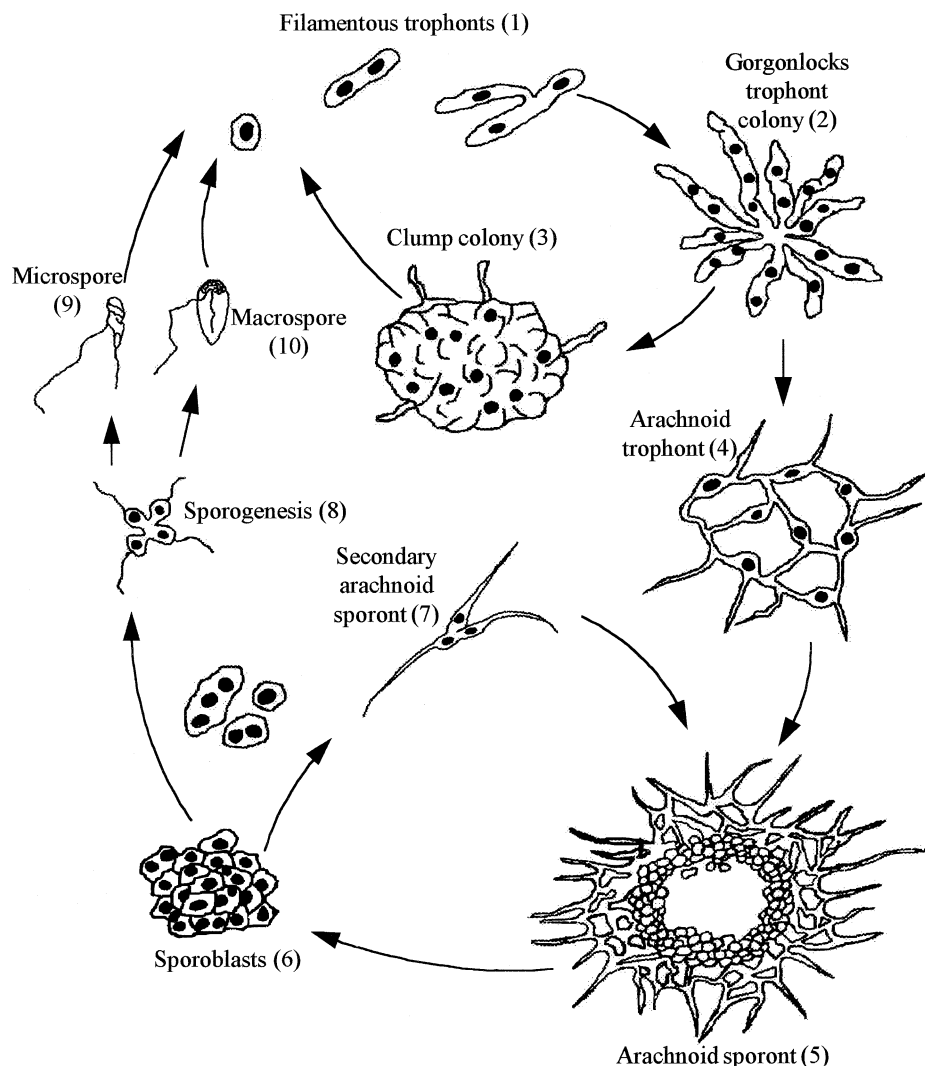sporont (7)

Sporoblasts (6)

Arachnoid sporont (5)

Fig. 18. Schematic diagram of developmental cycle of *Hematodinium* sp. from *Nephrops norvegicus* in *in vitro* culture. The principal multiplicative form *in vitro* is the multinucleate filamentous trophont (1), which undergoes growth, branching and fragmentation. In older cultures, multi-branched filaments form radiating gorgonlocks colonies (2) which may undergo compaction to form more spherical clump colonies (3) or attach to the substratum and become flattened arachnoid trophonts (4). The latter are capable of outward growth and fusion with one another. The syncytial arachnoid becomes a sporont when it synthesizes trichocysts and generates masses of sporoblasts from its raised centre (5). Detached multinucleate sporoblasts (6) may settle to become secondary arachnoid sporonts (7) if introduced into fresh medium, otherwise they generate flagellated dinospores (8), either microspores (9) or macrospores (10). Both types of spore germinate several weeks later, giving rise to a new generation of filamentous trophonts.

masses of interlocked trophonts that frequently arise from them, is also at present uncertain. Flexing and stretching movements are more evident in the filamentous trophonts of the gorgonlocks colonies than in the isolated filaments. It is possible that this motility confers on the trophont the ability to migrate between tissues.

Failure to find filamentous trophonts in the haemolymph may be a consequence of the practice of examining only patently diseased animals or of a relatively short period in development when such forms actually circulate in the haemolymph. The circulating subspherical parasites encountered by most workers we interpret, not as trophonts but as sporoblasts characteristic of a relatively late stage in

infection. We propose that these are the product of a sedentary sporont (arachnoid) phase derived from the filamentous trophonts, as observed in our *in vitro* cultures. Such sporonts probably line the haemal sinuses, shedding the sporoblasts into the haemolymph. They have been detected as multinucleate fluorescent networks in smears of the hepatopancreas (Field & Appleton, 1995). As noted here the sporoblasts can settle down and give rise to secondary sporonts *in vitro*, and it is possible that similar metastasis of the sporont occurs *in vivo*, helping to disseminate the parasite throughout the body. We cannot comment on the possibility that sporonts may be able to revert to trophont status. Filamentous trophonts have occasionally been seen in primary

cultures alongside sporonts or sporogenic syncytia, but whether these were present as rare circulating parasites in the original inoculum or arose secondarily from sporonts we are unable to say.

The rarity of a second bout of sporogenesis in our culture system suggests that haemolymph-derived sporogenic syncytia that give rise to dinospores *in vitro* have become committed to spore production, perhaps while still in the host, while the *in vitro*-generated sporont does not often encounter the environmental stimulus that elicits this commitment. The simultaneous sporulation of parasites in an infected host is striking, sometimes leaving no trace of parasites in the tissues while the haemolymph teems with dinospores (R. H. Field & P. L. Appleton, unpublished observations), and in marked contrast to the release of dinospores over several months observed in the *in vitro* system. Attempts are being made to determine the nature of the stimulus triggering dinospore formation.

We cannot, from the work described here, comment on what determines the type of spore produced (i.e. whether macrospore or microspore), but can comment on the fate of the spores produced. The possibility that the 2 types of spore represent anisogamous gametes can now be discounted as either appears to be capable of initiating a new developmental cycle *in vitro*. The likelihood of isogamous conjugation between identical macrospores or microspores being required for initiation of a new cycle also seems remote but cannot be completely discounted because it has not been observed. The possibility that a latent mud-dwelling hypnospore stage (present in the life-cycle of free-living dinokaryote dinoflagellates, where it develops from a zygote) occurs in the life-cycle of *Hematodinium* also seems remote, because dinospores appear to be able to develop directly into trophonts. The possibility of an intermediate host being involved in the life-cycle cannot at present be discounted, however, as it is not inconceivable that dinospores could initiate the trophont phase in, for example, a benthic amphipod and that, on predation by *Nephrops*, the sporogenic phase might be instituted in the decapod. The most likely pattern of infection of *Nephrops* would seem to be ingestion of the dinospores, possibly as a result of the suspension-feeding activities of the lobster (Loo, Baden & Ulmestrand, 1993), and initiation of the developmental cycle after penetration of the gut wall by the dinospore; the time-scale of germination is unlikely to allow this to occur in the gut itself. An alternative route might be via the thin or broken cuticle during moulting. Eaton and coworkers (1991) reported experimental infection of tanner crabs following injection of naturally generated dinospores of their *Hematodinium* sp. So far, however, we have not been successful in infecting *Nephrops* by this or any other route (Vickerman, 1994).

REFERENCES

APPLETON, P. L. & VICKERMAN, K. (1996). Presence of apicomplexan-type micropores in a parasitic dinoflagellate, *Hematodinium* sp. *Parasitology Research* **82**, 279–282.

CACHON, J. & CACHON, M. (1987). Parasitic dinoflagellates. In *The Biology of Dinoflagellates* (ed. Taylor, F. J. R.), pp. 571–610. Blackwell Scientific, Oxford, UK.

CHATTON, É. & POISSON, R. (1931). Sur l'existence, dans le sang des Crabes, de Péridiniens parasites: *Hematodinium perezi* n. g., n. sp. (Syndinidae). *Comptes Rendus des Séances de la Société de Biologie, Paris* **105**, 553–557.

EATON, W. D., LOVE, D. C., BOTHELO, C., MEYERS, T. T., IMAMURA, K. & KOENEMAN, T. (1991). Preliminary results on the seasonality and the life cycle of the parasitic dinoflagellate causing bitter crab disease in Alaska Tanner crabs (*Chionoecetes bairdi*). *Journal of Invertebrate Pathology* **57**, 426–434.

FENSOME, R. A., TAYLOR, F. J. R., NORRIS, G., SARGEANT, W. A. S., WHARTON, D. I. & WILLIAMS, S. G. L. (1993). A classification of fossil and living dinoflagellates. *Micropalaentology* Suppl. 7.

FIELD, R. H. & APPLETON, P. L. (1995). A *Hematodinium*-like dinoflagellate infection of the Norway lobster *Nephrops norvegicus*: observations on pathology and progression of infection. *Diseases of Aquatic Organisms* **22**, 115–128.

FIELD, R. H. & APPLETON, P. L. (1996). An indirect fluorescent antibody technique for the diagnosis of *Hematodinium* sp. infection of the Norway lobster, *Nephrops norvegicus*. *Diseases of Aquatic Organisms* **24**, 199–204.

FIELD, R. H., CHAPMAN, C. J., TAYLOR, A. C., NEIL, D. M. & VICKERMAN, K. (1992). Infection of the Norway lobster *Nephrops norvegicus* by a *Hematodinium*-like species of dinoflagellate on the west coast of Scotland. *Diseases of Aquatic Organisms* **13**, 1–15.

HOLLANDE, A. (1974). Étude comparée de la mitose syndinienne et de celle des péridiniens libres et des Hypermastigines: infrastructure et cycle évolutif des Syndinides parasites de Radiolaires. *Protistologica* **10**, 413–451.

HUDSON, D. A. & SHIELDS, J. D. (1994). *Hematodinium australis* n. sp., a parasitic dinoflagellate of the sand crab *Portunus pelagicus* from Moreton Bay, Australia. *Diseases of Aquatic Organisms* **19**, 109–119.

LOO, L.-O., BADEN, S. P. & ULMESTRAND, M. (1993). Suspension feeding in adult *Nephrops norvegicus* (L.) and *Homarus gammarus* (L.) (Decapoda). *Netherlands Journal of Sea Research* **31**, 291–297.

MACLEAN, S. A. & RUDDELL, C. L. (1978). Three new crustacean hosts for the parasitic dinoflagellate

*Hematodinium perezi* (Dinoflagellata: Syndinidae). *Journal of Parasitology* **64**, 158–160.

MESSICK, G. A. (1994). *Hematodinium perezi* infections in adult and juvenile blue crabs *Callinectes sapidus* from coastal bays of Maryland and Virginia, USA. *Diseases of Aquatic Organisms* **19**, 77–82.

MEYERS, T. R., KOENEMAN, T. M., BOTELHO, C. & SHORT, S. (1987). Bitter Crab Disease: a fatal dinoflagellate infection and marketing problem for Alaskan tanner crabs *Chionoecetes bairdi*. *Diseases of Aquatic Organisms* **3**, 195–216.

NEWMAN, M. W. & JOHNSON, C. A. (1975). A disease of blue crabs (*Callinectes sapidus*) caused by a parasitic dinoflagellate, *Hematodinium* sp. *Journal of Parasitology* **61**, 554–557.

RIS, H. & KUBAI, D. F. (1974). An unusual mitotic mechanism in the parasitic protozoan *Syndinium* sp. *Journal of Cell Biology* **60**, 701–720.

SHIELDS, J. D. (1994). The parasitic dinoflagellates of marine crustaceans. *Annual Review of Fish Diseases* **4**, 241–271.

TAYLOR, D. M. & KHAN, R. A. (1995). Observations on the occurrence of *Hematodinium* sp. (Dinoflagellata: Syndinidae), the causative agent of bitter crab disease in Newfoundland snow crab (*Chionoecetes opilio*). *Journal of Invertebrate Pathology* **65**, 283–288.

VICKERMAN, K. (1994). Playing at being Pasteur. *International Journal for Parasitology* **24**, 779–786.

WILHELM, G. & MIALHE, E. (1996). Dinoflagellate infection associated with the decline of *Necora puber* crab populations in France. *Diseases of Aquatic Organisms* **26**, 213–219.