1994

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Journal of Shellfish Research, Vol. 13, No. 2, 461–465, 1994.

THE *IN VITRO* LIFE CYCLE OF A *PERKINSUS* SPECIES (APICOMPLEXA, PERKINSIDAE) ISOLATED FROM *MACOMA BALTHICA* (LINNEAUS, 1758)

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ABSTRACT Using standard sterile techniques and a single medium previously described (Kleinschuster and Swink 1993), the *in vitro* culture of a *Perkinsus* species isolated from *Macoma balthica* was possible. Zoosporulation, the release of zoospores, and the reestablishment of secondary cultures from the zoospores completed an *in vitro* life cycle.

KEY WORDS: *Perkinsus* species, *Macoma balthica*, cell culture

INTRODUCTION

Species of the apicomplexan protozoan *Perkinsus* have been reported to cause major mortalities in bivalve populations and cross-transmission of *Perkinsus* species infections between certain bivalve species is possible (Goggin et al. 1989). Although a simple *in vitro* culture technique for the propagation of *Perkinsus marinus* Levine 1978 has been established (Kleinschuster and Swink 1993), techniques for the routine induction of zoosporulation and release of zoospores in quantity have not been available in recent years either *in vivo* or *in vitro*. Consequently, the role of the zoospore in the infection/invasion process has not been fully investigated.

We report herein simple methodology for the *in vitro* culture of a *Perkinsus* species isolated from *Macoma balthica* including vegetative propagation of the isolate, zoosporulation, and release of zoospores followed by the reestablishment of secondary cultures from the zoospores, thereby completing an *in vitro* life cycle.

MATERIALS AND METHODS

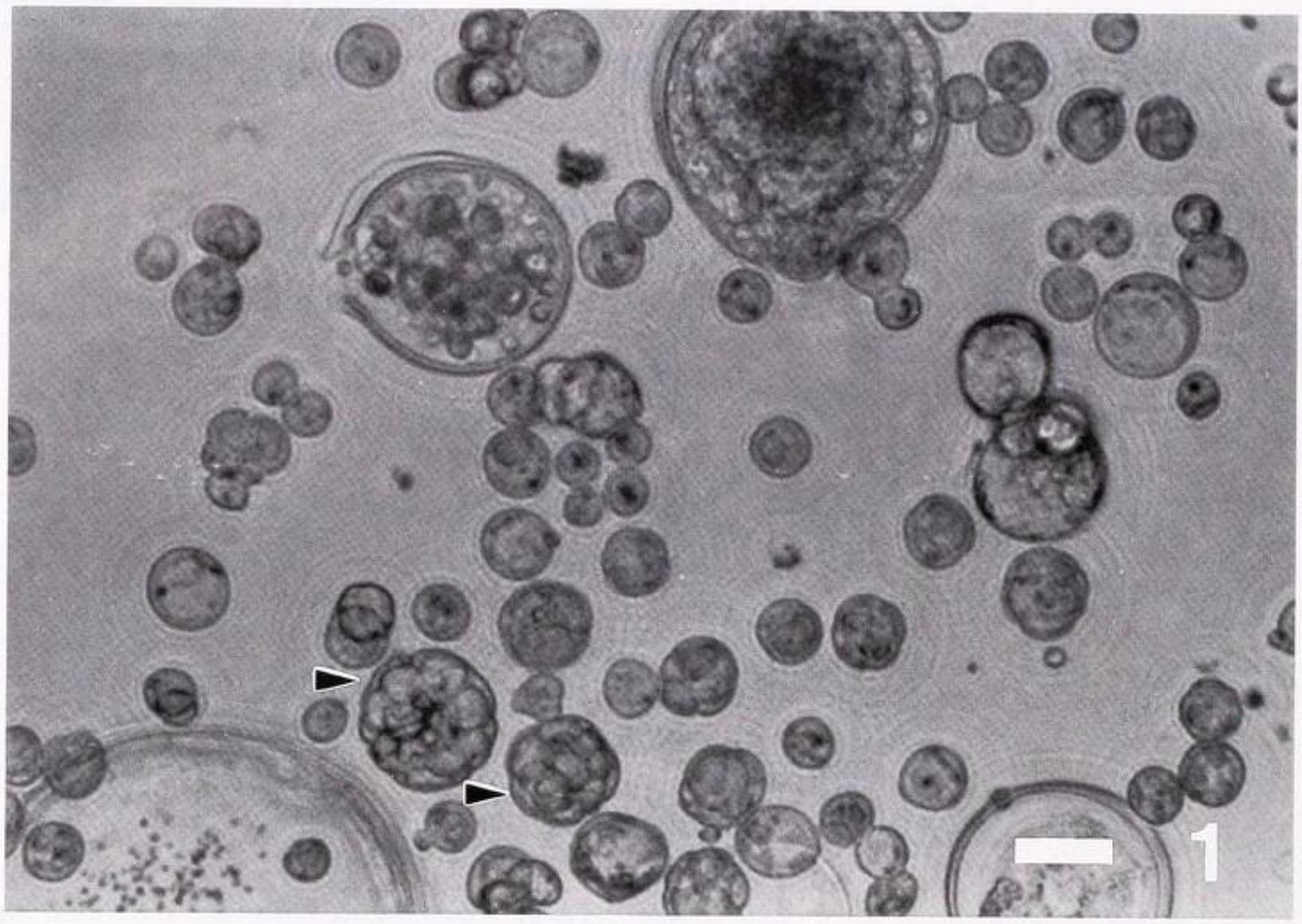
Infected clams were obtained from King's Creek, (14 ppt salinity), a tributary of the York River in Virginia, and maintained in aquaria at the Virginia Institute of Marine Science. Clams used as a source of *Perkinsus* species were transported to the Haskin Shellfish Research Lab and maintained in recirculating sea water at 12 to 15°C. Cells of the parasite were obtained from hemolymph aspirated from the blood sinuses of the adductor muscles. Immediately after aspiration, hemolymph and cellular components were transferred to 25 cm^2 T-flasks containing 1 to 2 ml of sterile sea water and appropriate antimicrobics (streptomycin, 0.2 mg ml^{-1}, penicillin, 200 U ml^{-1}, and amphotericin B 0.25 μg ml^{-1}) and held at room temperature for 3 hours. After this treatment, the isolation medium was replaced by a medium used for the *in vitro* culture of *P. marinus* (Kleinschuster and Swink 1993), and the cultures were kept at 28°C under ambient air. The medium was exchanged (50%) every 3 days. Phase contrast microscopy was used to obtain photomicrographs.

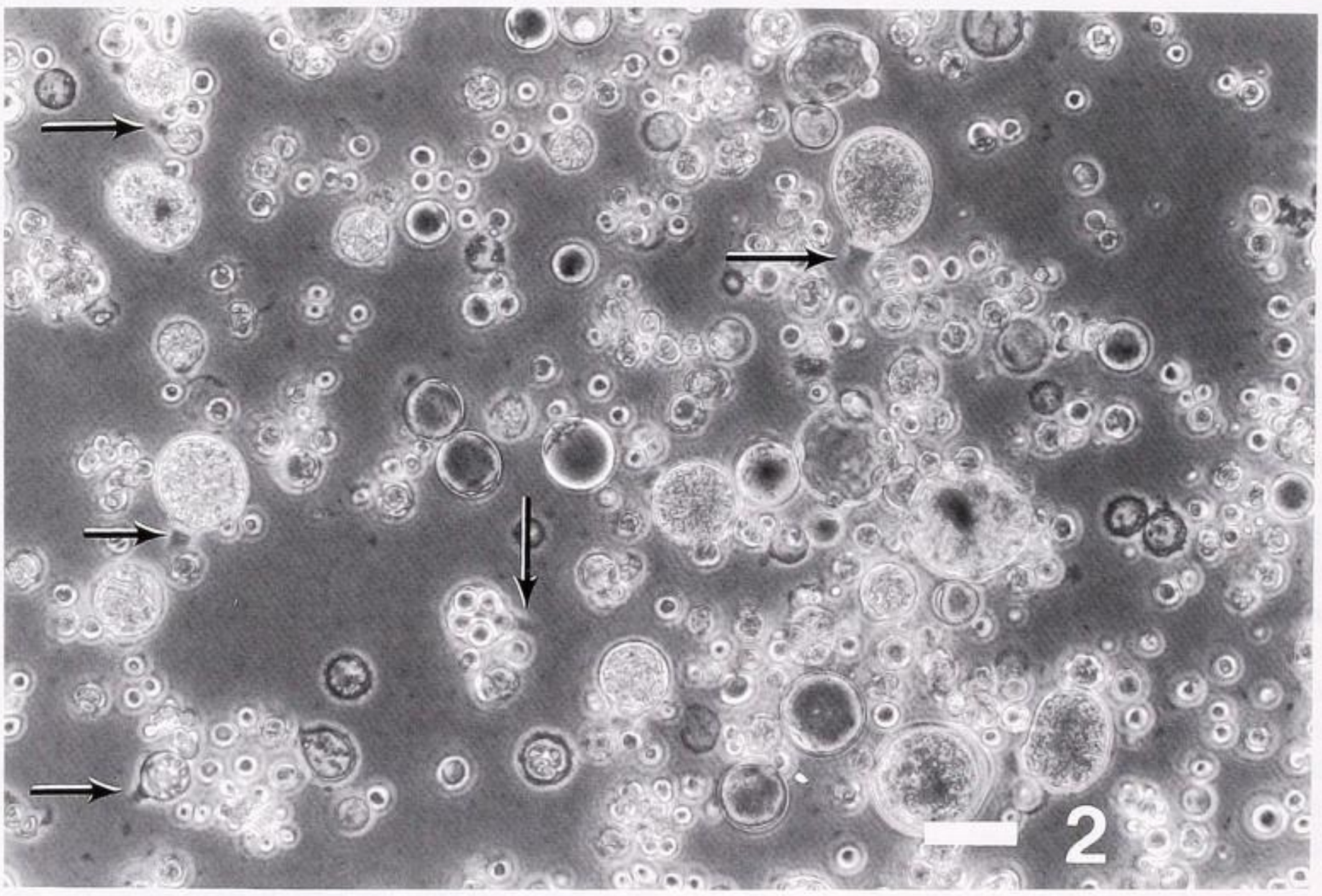
Routine roller bottle technique and subculturing were used to upscale the production of cultured cells. Aliquots of these cultures were centrifuged at low speed to pellet the organisms, which were then washed two times with sterile sea water and resuspended in T-flasks with sterile sea water to induce zoospore formation.

For ultrastructural analysis, prezoosporangia, zoosporangia, and zoospores in sterile sea water were sent to N.C. State University in T-flasks. Contents of the flasks were decanted into a centrifuge tube, and after the bottom of the flask was scraped with a rubber policeman, the cells were pelleted at 1000 $\times$ g for 5 minutes. The medium was gently pipetted from the tube and replaced with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.8 (Azevedo 1989). After 1 hour of primary fixation at room temperature, the cells were rinsed in fresh buffer several times. The cells were pelleted after each rinse. After the final rinse, the cells were pelleted, the medium was removed, and the cells were resuspended in molten (approximately 50°C) 4% water agar and quickly centrifuged at 1000 $\times$ g for 30 seconds. Once the agar had solidified, the agar containing the cells was sliced into 1 mm thin slices with a razor blade and placed into 1% osmium tetroxide in the same buffer at room temperature. After 1 hour, the samples were rinsed three times in distilled water and subsequently dehydrated in a graded ethanol series, passed through 100% acetone, and infiltrated with Spurr resin (Dykstra 1993). Ultrathin sections were obtained, stained with methanolic uranyl acetate and lead citrate, and evaluated with a transmission electron microscope (TEM).

RESULTS

Low-density *in vitro* cultures of the *Perkinsus* isolate are represented in Figures 1 and 2. In nutrient-rich medium, vegetative



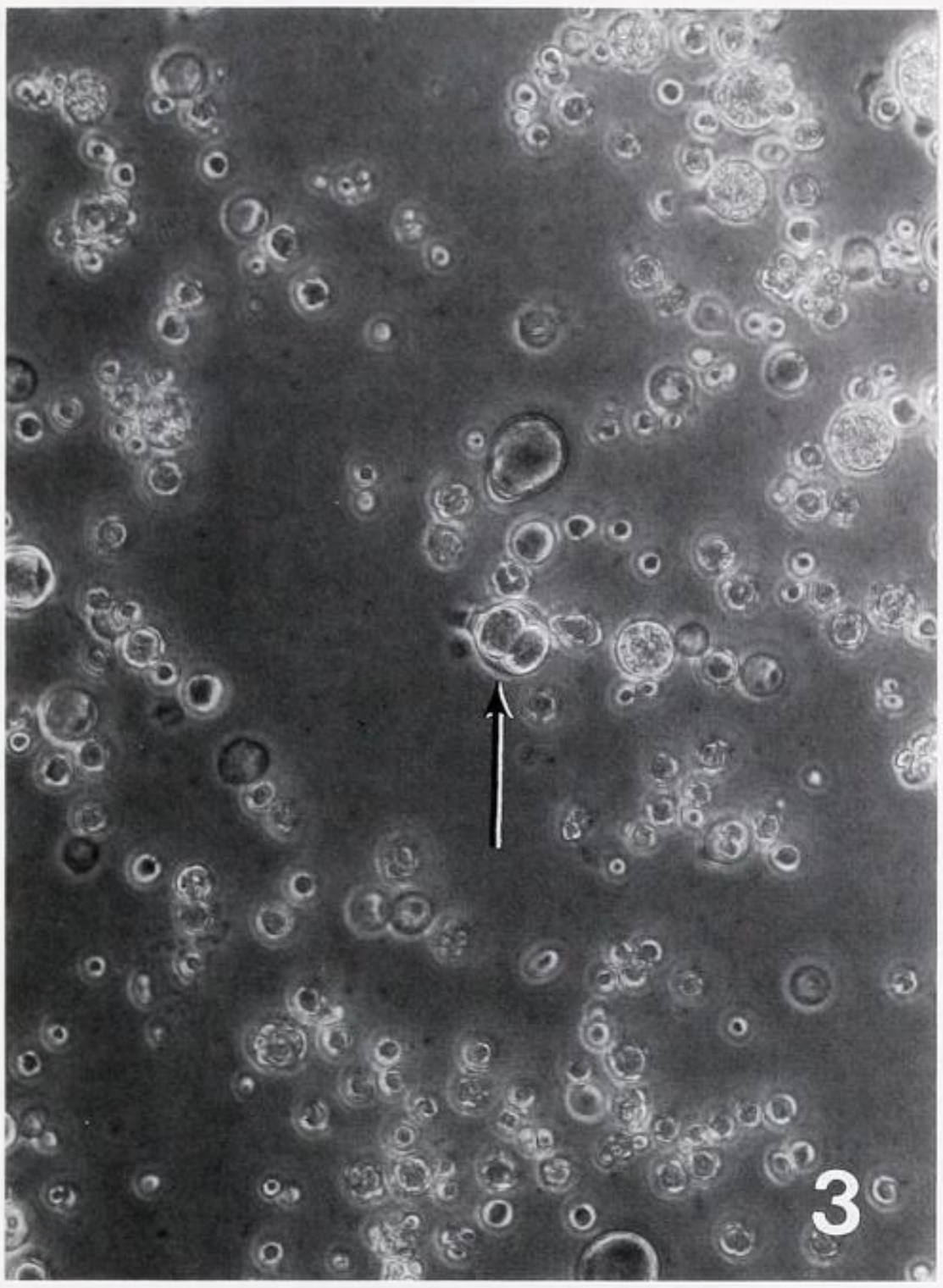


Figures 1 and 2. Photomicrographs of low-density *in vitro* cultures of a *Perkinsus* species isolated from *M. balthica*. Note schizonts typical of *Perkinsus* species (arrowheads) in nutrient-enriched medium and the discharge tubes of zoosporangia present in sea water cultures (arrows). Scale bar, 0.05 mm (Fig. 1) and 0.1 mm (Fig. 2).

reproduction was vigorous and typically consisted of schizonts in various stages of merogeny; meronts and merozoites were evident (Fig. 1). Generally, vegetative stages of this isolate were larger and more ovoid than *P. marinus* under similar conditions; however, merogeny appeared to be similar. A typical low-density sea water culture is represented by Figure 2. Various presporulation stages were evident, including prezoosporangia and zoosporangia with discharge tubes.

Several developmental stages of induced zoosporulation are seen in Figures 3 to 7. Typical two-, four-, and eight-cell stages of developments are seen in Figures 3 to 5, respectively. Discharge tubes were routinely evident at the two-cell stage (Fig. 3),

Figures 3 to 6. Photomicrographs of a low-density sea water culture of a *Perkinsus* species isolated from *M. balthica*. Represented are typical two-, four-, and eight-cell stages of zoosporulation (arrows). Figure 6 exhibits a sporangium with two discharges tubes (arrows). Blurred background images are motile zoospores. Scale bar, 0.1 mm.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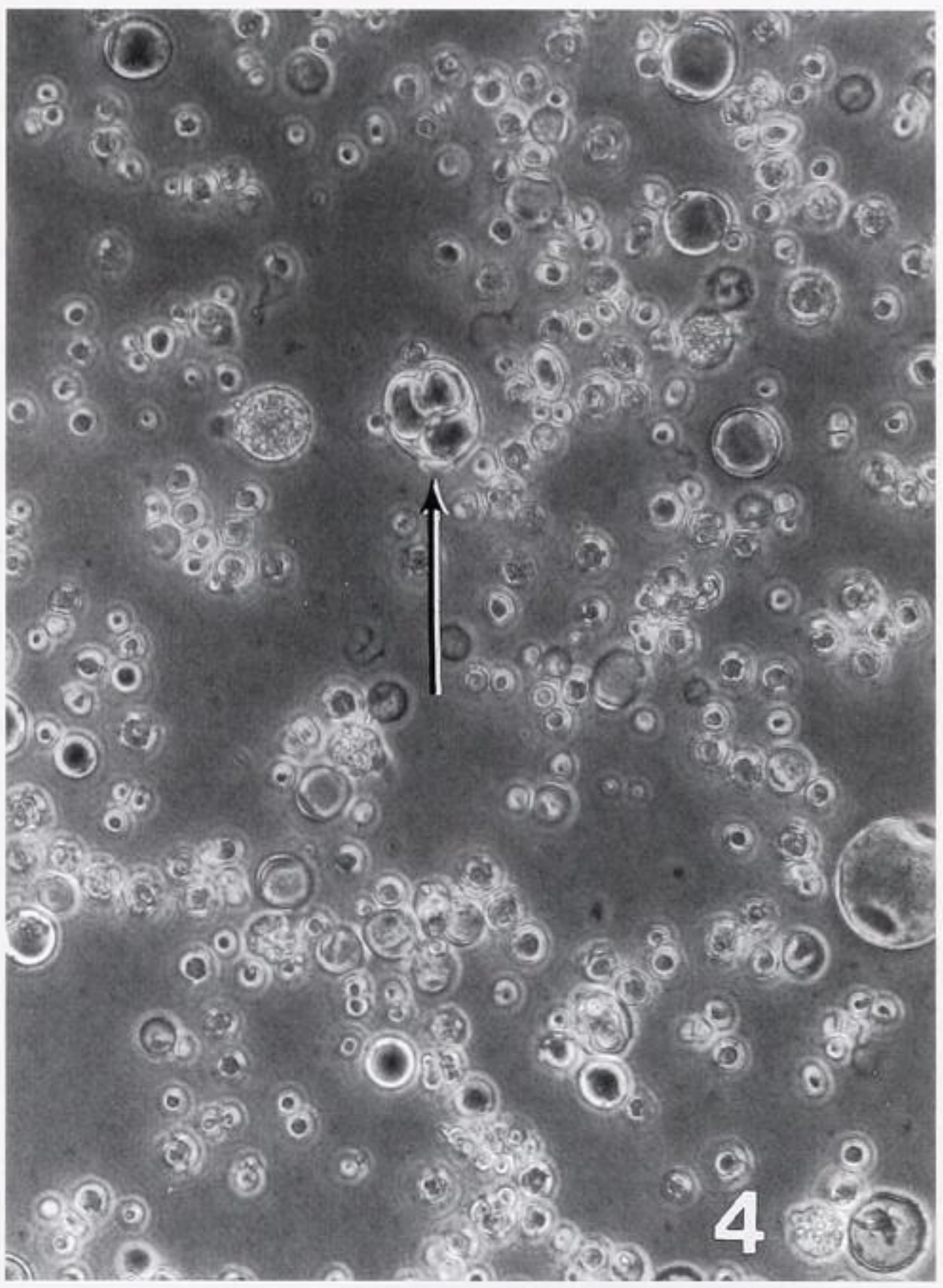
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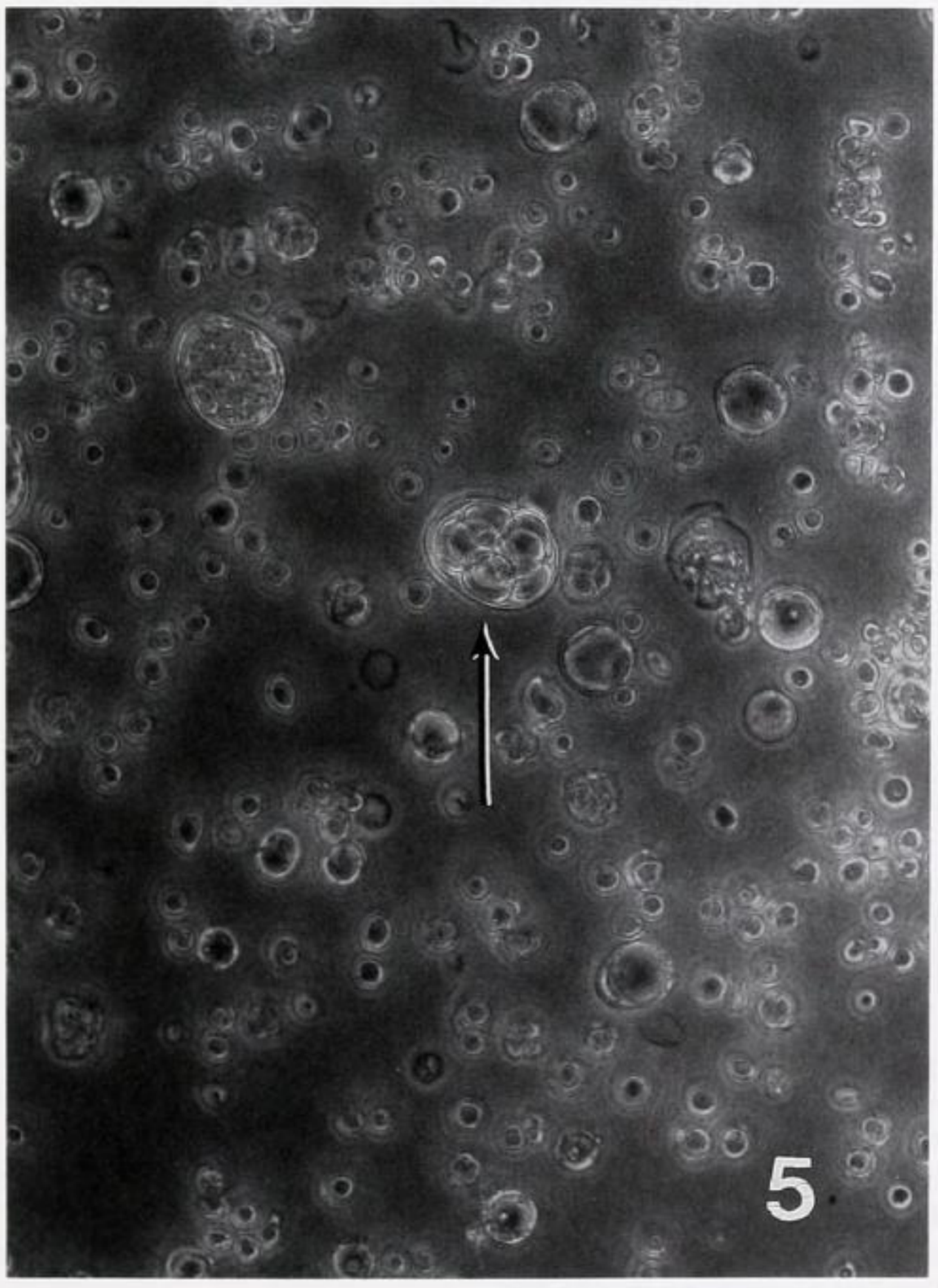
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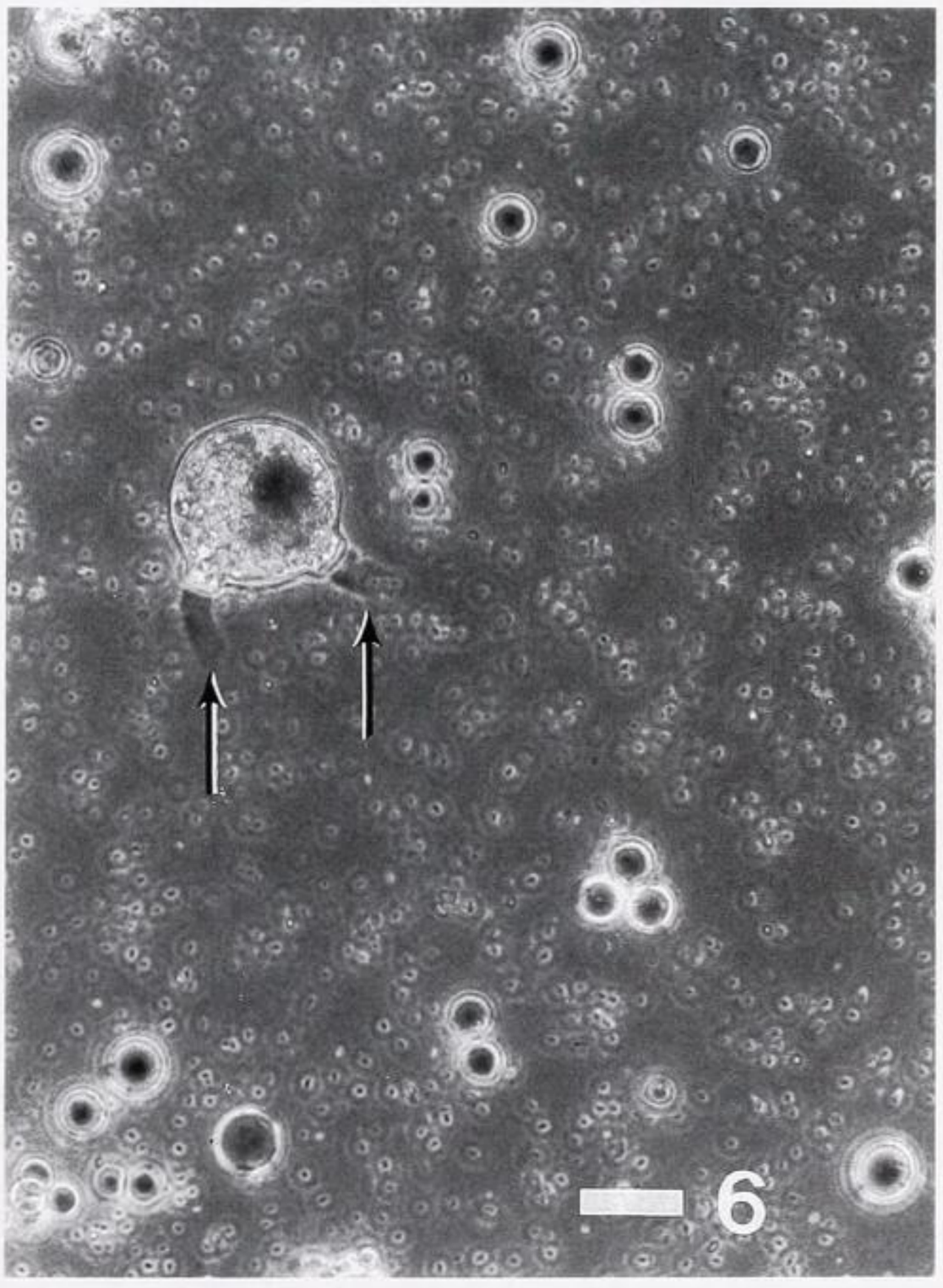
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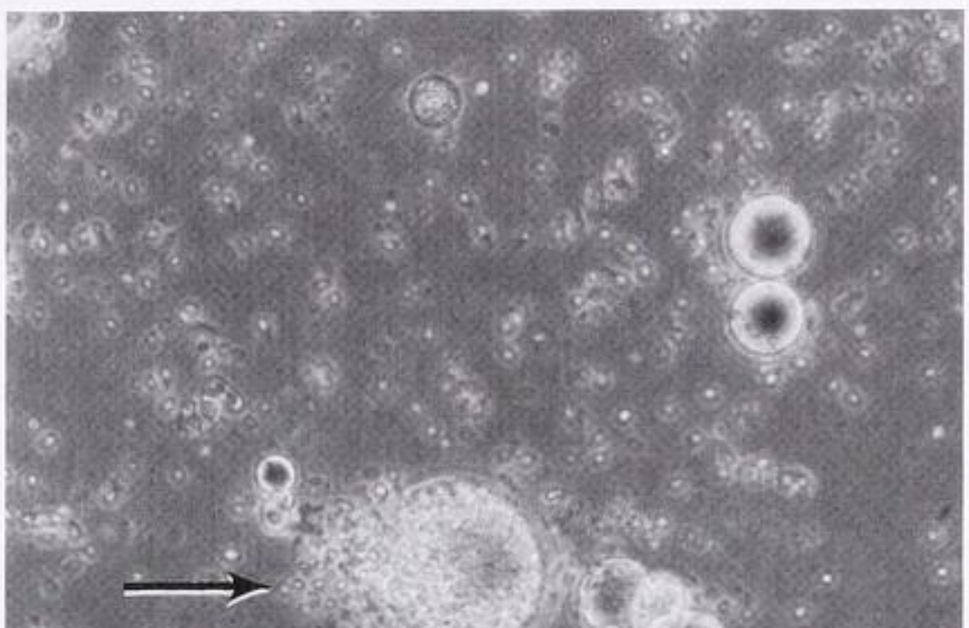
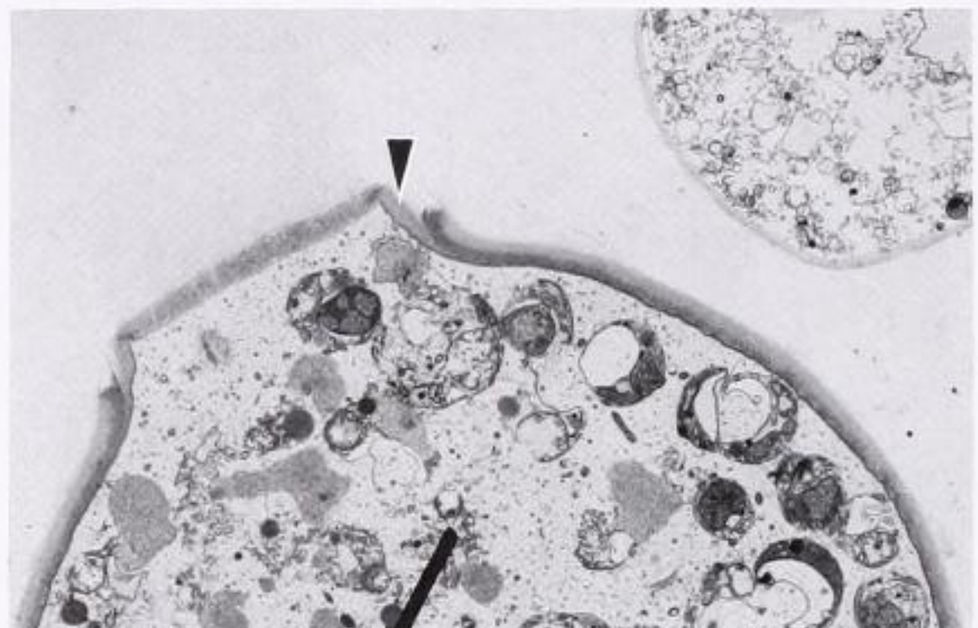
and multiple discharge tubes were not uncommon (Fig. 6). Successive karyokineses and cytokineses resulted in the formation and release of motile zoospores (Fig. 7). The blurred and dotted backgrounds of Figures 6 and 7 represent discharged and motile zoospores.

A representative TEM photomicrograph of a mature prezoosporangium with typical thickened cell wall, discharge tube, and multiple prezoospores is seen in Figure 8.

A TEM photomicrograph of a zoospore displaying apicomplexan structures, including rhoptries, a conoid, and subpellicular microtubular and microneme-like organelles is seen in Figure 9. The kinetosome substructure was the same as in *P. marinus* (Per-





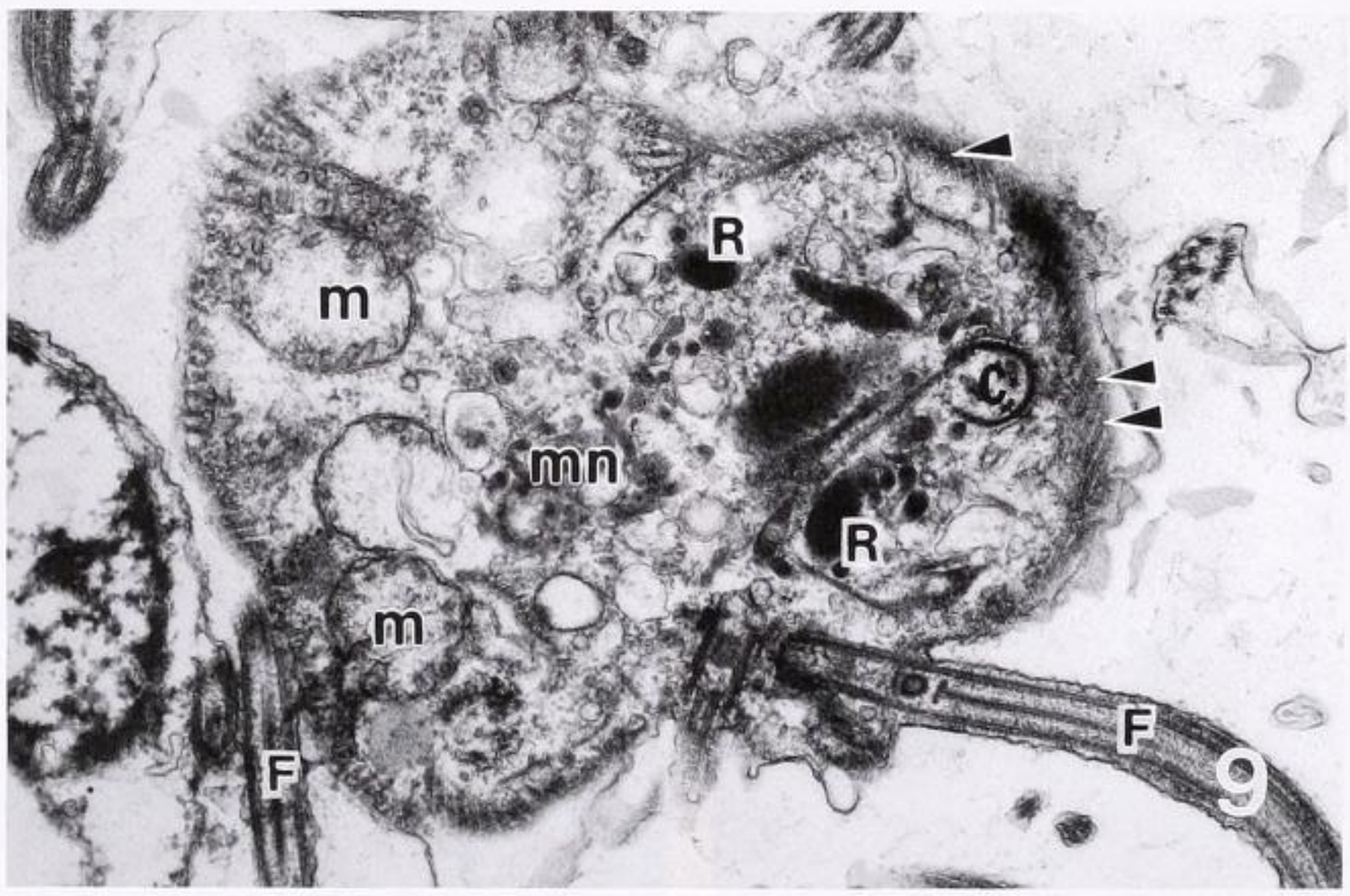


Figure 7. Photomicrographs of a low-density sea water culture of *Perkinsus* species isolated from *M. balthica*. Notice discharge of zoospores from zoosporangium (arrow). Blurred background images are motile zoospores. Scale bar, 0.1 mm.

Figure 8. Zoosporangium of a *Perkinsus* species containing zoospores (arrows). Note the thick zoosporangial wall and the discharge tube and plug of wall material derived from the inner layer of the zoosporangial wall (arrowhead). Original magnification, ×2,240.

Figure 9. Zoospore of a *Perkinsus* species showing apicomplexan structure: Conoid (C), rhoptries (R), subpellicular microtubules (arrowheads), microneme-like organelles (mn), flagella (F), and mitochondrion (m). Original magnification, ×22,800.

kins 1988). Whole mounts of zoospores negatively stained in 0.5% aqueous uranyl acetate exhibited filamentous mastigonemes and spur-like structures identical to those of *P. marinus* (Perkins 1991).

In general, this *Perkinsus* species exhibited more rigorous growth and reproduction than *P. marinus* under similar culture conditions and had a very short doubling time (approximately 20 hours in log phase). Additionally, sea water–induced zoospores were readily returned to the vegetative-propagation state by substitution of the sea water with nutrient-enriched medium and cultured as described above.

DISCUSSION

Isolation and *in vitro* propagation of a vigorous *Perkinsus* species will facilitate studies of the basic biology of this parasite. The ability to induce zoospores and their subsequent release directly from vegetative cultures without the use of the Ray thioglycolate technique (1952) together with the reestablishment of vegetative cultures from zoospores may provide an impetus toward our understanding of this organism's parasitic profile through experimental manipulation. Although species identification was not an objective of this study, in consideration of the morphologial characteristics of this isolate as seen in the host, as well as the possibility of cross-species infection, it is suggested that this organism may be *Perkinsus atlanticus*, which has been described from *Ruditapes decussatres*, a Portuguese clam (Azevedo 1989). Because of the ease of culturing this parasite through an *in vitro* life cycle, it may be appropriate to develop this system as a model for the study of *Perkinsus* species/host interactions.

ACKNOWLEDGMENTS

This work is identified as Hatch Project No. 32100 and is identified as paper No. D-32100-6-94 by the New Jersey Agricultural Experiment Station and paper No. 94-25 by the Institute of Marine and Coastal Sciences, Rutgers University; contribution No. 1901 of the School of Marine Science and Virginia Institute of Marine Science, College of William and Mary.

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