Immunostaining of a ciliate protozoan causing significant mortality of farmed tuna: the development of a rapid identification technique which will enable improved farm management practices to be implemented to minimise fish mortality

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Immunostaining of a ciliate protozoan causing significant mortality of farmed tuna: the development of a rapid identification technique which will enable improved farm management practices to be implemented to minimise fish mortality

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South Australia Research and Development Institute

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NON TECHNICAL SUMMARY

1995/083 **Immunostaining of a ciliate protozoan causing significant mortality of farmed tuna: the development of a rapid identification technique which will enable improved farm management practices to be implemented to minimise fish mortality**

PRINCIPAL INVESTIGATOR: Dr B L Munday
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OBJECTIVES:

1. To develop the use of immuno-staining as a rapid diagnostic test which can be used on site (Port Lincoln) to accurately identify the ciliate protozoan of concern.
2. Using the diagnostic test developed, screen potential sources of infection associated with farming tuna (seawater and sediment around cages, organic matter fouling nets, feeds and feed preparation methodologies…etc).
3. Disseminate research and resultant recommendations on improved farm management strategies to tuna farmers so as to minimise mortalities of farmed fish.
4. To maximise the benefits obtained by the tuna farming industry from the presently funded position, Research Officer – fish health (National Training Company Scheme and TBOAA), based in Port Lincoln and the partnerships that have been established to facilitate research on industry priority topics.

The substantial southern bluefin tuna mortality event in April-May 1996 impacted on the delivery of the project objectives, particularly those associated with the application of the immuno-staining technique developed to:

- screen potential sources of infection associated with farming tuna; and based on this

- provide advice to farmers on how to improve farm management techniques so as to minimise infections.

The objective therefore became:

- To identify the causative organism for the disease and develop the use of immuno-staining as a rapid diagnostic test which can be used on site (Port Lincoln) to accurately identify the ciliate protozoan of concern.

NON TECHNICAL SUMMARY:

An immunofluorescent staining technique for the rapid detection of the ciliate protozoan Uronema sp. was developed during 1995 and 1996. The initial test was developed using seven cultures of Uronema sp. from various sources which were identified as Uronema nigricans by microscopical and histochemical techniques. These seven strains were maintained in a medium containing bacteria as their food source. Antisera against two of the strains were raised in rabbits and against one in sheep. These were then adsorbed with the bacteria and unrelated ciliates to eliminate cross-reactivity.

More recently, an axenic (bacteria-free) culture of Uronema sp. has been accomplished and high-titre antisera raised in rabbits for use in the immunofluorescent staining technique.

Arrangements have been made to have a monoclonal antibody to the axenic Uronema prepared at the Atlantic Veterinary College, University of Prince Edward Island in Canada. This will then provide a reliable supply of highly specific antibody for future research in this area.

The substantial southern bluefin tuna mortality event in 1996 impacted on the delivery of the project objectives, particularly those associated with the application of the immuno-staining technique developed to:

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- provide advice to farmers on how to improve farm management techniques so as to minimise infections.

KEYWORDS: **Uronema, aquaculture, southern bluefin tuna.**

Background

The southern bluefin tuna industry has been of significant economic importance for many years, however, the wild stock has now fallen to below 10 % of the original parent stock and the fish is considered to be a threatened species. To maintain the industry, and address the problem of falling quotas, wild tuna are captured then on-grown in sea-cages at Port Lincoln. The industry has grown exponentially, from the initial pilot programme in 1990, to a projected value this year (1996) of $90 million. However, as a result of capture and confinement the fish become susceptible to opportunistic pathogens, and a significant proportion of the stock is lost. In 1993 approximately 2.5% of the total number of farmed tuna died as a result of an infection by a ciliate protozoan, identified as a *Uronema* sp. A similar problem occurred in 1994 when some farms lost up to 10% of their stock.

Many parasitic ciliates are recognised as serious pathogens which cause severe economic loss to various aquaculture industries. It was considered imperative that this research be carried out in order to identify the etiological agent and to develop a rapid test for its detection, so that effective management practises can be established.

However, identification of ciliates is problematic, being traditionally based on certain morphological features revealed by silver staining techniques; in particular the number of ciliary rows, the position of the buccal cavity and the structure of the buccal ciliature. These structures are not always readily discernible and ciliate pathogens have, in the past, been mistakenly identified.

It was decided to develop a fluorescent antibody test (FAT) because this technique is quicker than histochemical stains, can be extremely specific and does not rely upon morphological data for its interpretation. This stain is widely used in the identification of bacterial and parasitic antigens in many

species as it is relatively inexpensive and can be carried out by technical staff with a minimum of training.

NEED

To minimise the substantial economic loss to the tuna farmers by establishing a method of rapid early detection of *Uronema* sp. at Port Lincoln.

OBJECTIVES

1. To develop the use of immuno-staining as a rapid diagnostic test which can be used on site (Port Lincoln) to accurately identify the ciliate protozoan of concern.
2. Using the diagnostic test developed, screen potential sources of infection associated with farming tuna (seawater and sediment around cages, organic matter fouling nets, feeds and feed preparation methodologies…etc).
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The substantial southern bluefin tuna mortality event in April-May 1996 impacted on the delivery of the project objectives, particularly those associated with the application of the immuno-staining technique developed to:

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The objective therefore became:

- To identify the causative organism for the disease and develop the use of immuno-staining as a rapid diagnostic test which can be used on site (Port Lincoln) to accurately identify the ciliate protozoan of concern.

METHODS

Part A: The Development of a Fluorescent Antibody Stain Using *U.nigricans* Grown in a Medium Containing Bacteria

In order to develop a fluorescent antibody stain the following areas had to be addressed:

1. The etiological agent needed to be identified
2. The etiological agent needed to be cultured in order to produce sufficient antigen for inoculation into a suitable animal
3. Antiserum had to be produced
4. The antiserum had to be tested to see if it was of use in a practical situation

It was established from pilot studies that it would be time consuming and difficult to remove the bacteria which were naturally associated with the *Uronema,* as they formed their food source. It was decided to grow the *Uronema* with their associated bacteria and then to remove the bacterial antibodies (which would be formed in conjunction with ciliate antibodies) by adsorption if necessary, at a later date.

Prior to this research very little was known about the etiological agent of the disease colloquially termed “swimmer syndrome” which was causing significant annual mortality in sea-caged tuna at Port Lincoln. The organism was identified as a scuticociliate of the Genus *Uronema*, but the exact species had not been assigned. The marine pathogenic species of *Uronema* was thought to be *U. marinum*, but this work (in close collaboration with Dr. P. O’ Donoghue) identified the species responsible for the disease at Port Lincoln as *U. nigricans.*

During the course of this research seven *Uronema* isolates were collected from pathological and environmental sources.

Origin of isolates

Isolate B came from a blenny (*Parablennius tasmanianus*) larvae tank at the National Key Centre for Research and Training in Aquaculture, Department of

Aquaculture, University of Tasmania at Launceston. This isolate was associated with the demise of the larvae and therefore may be pathogenic.
Isolate Q was obtained from Dr. P. O'Donoghue at the Department of Parasitology, University of Queensland. This isolate came from a moribund southern bluefin tuna (*Thunnus maccoyii* {Castelnau}) which died as a result of infection with an unidentified scuticociliate.
Isolate K from K. Rough, of the Tuna Boat Owners Association of Australia, Port Lincoln, South Australia, and was associated with the same outbreak as isolate Q, although it did not come from the same fish.
Isolate SH1 was derived from a skin lesion on a mature sea-horse (*Hippocampus abdominalis*) at the National Key Centre.
Isolate SH2 came from the body cavity of a dead juvenile sea-horse at the National Key Centre.
Isolate F came from a healthy larval flounder (*Rhombosolea tapirina*) tank at National Key Centre.
Isolate A was obtained from the routine cleaning of healthy juvenile flounder tank at National Key Centre.
Note: 50 µm sand -filtered sea-water is brought in to the National Key Centre from a variety of areas, therefore these isolates are likely to be heterogeneous.

Antibodies were produced in laboratory rabbits against the cultured isolates B and Q. These two strains were identified by observations of live cells in wet preparations with bright-field and phase-contrast microscopy as well as silver impregnation staining for morphometric measurements.

1. Identification

The morphology of all the cultured isolates was observed and compared in wet preparations. The length and width of live specimens of isolates B and Q were measured and the following characteristics of isolates B and Q were measured from protargol-stained specimens: length and width of cell; length of the oral membranelles and the paroral membrane; length of the nuclei; length of the oral apparatus and distance from the apex of the cell to the top

of M1. The number of kineties were counted and the position of the cytostome was noted (Tables I and II in Appendix).

In addition, measurements of isolate Q, made from smears produced directly from clinical material, were obtained from Dr. P. O'Donoghue at the University of Queensland (Table III in Appendix). These data were statistically analysed and compared with the literature values (Coppellotti, 1990; Dragesco and Dragesco-Kernis, 1986; Perez-Uz and Song, 1995; Thompson, 1964). Descriptions of each of these isolates are to be found in tables IV V and VI in the Appendix section.

a) Wet preparations for live observations

A drop of culture was placed onto a microscope slide and a cover slip applied. In order to facilitate measurement the slide was left for ~ 30 min to become slightly anoxic, which slowed the movement of the cells with many become stationary. Benzocaine was tried as a slowing agent but proved unsatisfactory as, although the cell movement became slower, the cells did not actually become stationary and many ruptured.

b) Vital stain for trichocysts and nuclear detail

Methyl-green pyronin (Foissner, 1991) was used to reveal detail of the nuclei and trichocysts. A drop each of culture and stain were mixed on a microscope slide, a coverslip applied and the preparation observed immediately. Because the organisms are stained while living they undergo many rapid changes as soon as they encounter the stain. Among those observed were: swelling and rupture; extrusion of the nuclei and the cellular contents; rapid absorption of stain and overstaining of the cytoplasm, which obscured nuclear detail.

c) Protargol stain for infraciliature measurements.

The procedure described by Foissner (1991) was successfully used. The cells were fixed for 15 min in Bouin's fluid using a ratio of fixative:sample fluid of 2:1. After fixation the cells were thoroughly washed in distilled water because residual Bouin's fluid inhibits the stain (Skibbe, 1994).

Specifications of microscopes which were used

Measurements were made of live organisms with an eyepiece graticule calibrated with a stage micrometer on a Nikon Alphaphot YS microscope

fitted with a 100/1.25 DL oil immersion objective. Morphometric data were measured from drawings made of protargol-stained material observed on a Zeiss Axsioskop microscope fitted with a 100/1.32 Fluotar oil immersion objective. Photographs and slides were taken using Kodak ASA 160 slide or print film on a Leitz Diaplan microscope with Leitz Wetzlar PL Fluotar 100/1.32 oil immersion objective.

Statistical analysis

A 1-way ANOVA (α 0.05) and Student's t-test for multiple comparison of means (α 0.05) were conducted, where necessary, on each characteristic. The data were checked for normality using a Shapiro-Wilk W test on the residuals; Cochran's test was used to determine homogeneity of variance. The data were assumed to be independent.

2. Culture

Large volumes of cultured isolates were necessary to provide the antigen for inoculation into rabbits, as positive controls for the IFAT and for immunological and morphological characterisation.

As pathogens *Uronema* sp are histophagous, devouring tissue and blood cells (Bassleer, 1983; Cheung et al., 1980); in their natural environment they are bactivorous, forming part of the marine food web (Berk et al., 1977; Solic and Krstulovic, 1994; Strom et al., 1993). Consequently, when collected from the environment the cultures are contaminated with bacteria. This is not a problem generally, as *Uronema* eat bacteria. Using bacteria as a food source maintains the *Uronema* in good condition as long as the bacteria are not allowed to grow to such an extent that the culture becomes anoxic.

a) Initial isolation

Samples of water from an aquarium containing Tasmanian blennies and *Uronema* sp. were inoculated into a series of dilutions of Oxoid brain heart infusion broth (BHI) in sea-water. This medium was used because *U. nigricans* has been shown to have a predilection for brain tissue which it preferentially devours in both tuna and blennies. The cultures were left at ambient temperature for 1d to allow for bacterial growth and then placed at

4°C. This temperature slows the growth of bacteria but still allows growth of *Uronema*.

A dilution of 1/10 v/v BHI in sea-water was found to support enough bacterial growth to ensure vigorous growth of *Uronema.* This dilution was used thereafter for isolation and subculture of all isolates. It proved adequate for the culture of isolates received from Dr. P. O'Donoghue, but later cultures did not thrive until this basic medium was supplemented with lysed blood and vitamins.

b) Routine culture

Oxoid brain heart infusion broth (BHI) was dissolved in 1/4 volume of distilled water, autoclaved and then made up to the appropriate volume with sterile, 0.2 μm filtered sea-water. This was used as a stock solution and diluted to 1/10 with sterile sea-water as required. The broth and the sea-water were autoclaved separately to avoid precipitation in the medium. Before use, 25 μL of lysed blood and 25 μL of vitamin solution (Appendix section) was added to 1 L of culture. The medium was dispensed aseptically into sterile 20 mL McCartney bottles.

Four replicate bottles were inoculated with 2 mL of high density culture, every 2 to 3 weeks, for each isolate. The newly inoculated cultures were left at ambient temperature overnight to allow for bacterial growth, and then placed at 4° C. *Uronema* continue to grow and divide at this temperature, but the bacteria are considerably slowed.

3. Antibody production

a) Preparation of immunogen using isolates B and Q

Large numbers of *Uronema* were grown with bacteria as food, and then the bacteria were removed as much as possible. The routine culture method was scaled up by the addition of 20 mL of the appropriate culture to 200 mL of fresh medium, allowing it to multiply for 3 d at room temperature before placing at 4° C for a further 3 d. The cultures were monitored daily to confirm the presence of numerous *Uronema.* Bacteria were killed by the addition of 1000 μg/mL each of ampicillin, streptomycin and neomycin.

In order to separate *Uronema* from killed bacteria the cultures were centrifuged at 1000 rpm for 5 min to sediment the large masses of flocculated debris which appears in these cultures, without damaging the *Uronema.* The cultures were then left for 2 h to allow the *Uronema* to swim back to the surface. It was found that these cultures could be left for up to 6 days at this stage without any apparent detriment. The *Uronema* were visible as a thin, milky line just below the surface of the culture. A Gram-stain of the culture medium indicated that few bacteria were present in culture-Q, but culture-B still appeared to have bacteria present. It was decided to proceed with both cultures as immunogens and compare the results.

The cultures were fixed by the addition of formalin to a final concentration of 0.5%, and left overnight at 4° C. After this time the cultures were centrifuged at 2000 rpm for 10 min with 3 washes in sterile sea water and the deposit harvested. Approximately 15 μL of deposit was obtained from a culture of 200 mL and was suspended in 1.5 mL of sterile PBS. For the preparation of the inoculum 0.5 mL of this suspension was added to 0.5 mL of Freund's complete adjuvant (FCA) for the first immunisation. The remaining 1.0 mL of suspension was frozen at -20° C to be added to 1 mL of Freund's incomplete antigen (FIA) 4 weeks later. A booster inoculation was needed so the procedure was repeated; 10 μL of antigen was suspended in 1 mL PBS and added to 1 mL FIA.

b)Rabbit inoculation protocol

Ethics committee approval was obtained to carry out this procedure.

1. A 10 mL pre-immunisation bleed was taken from each rabbit.
2. The rabbits were inoculated one week later with the antigen in FCA intramuscularly, at multiple sites.
3. Four weeks later the rabbits were inoculated as before, with the second dose of antigen emulsified in FIA (2 mL total).
4. Two weeks after the second inoculation the antibody titre was checked. Doubling dilutions from neat to 1/1024 were made for each antibody and the conjugate. IFAT procedure 1 was used to stain duplicate slides.

5. On the basis of the results obtained in step 4 the rabbits were given a booster in FIA 2 weeks later, and the antiserum re-titrated, in duplicate.
6. The antiserum was harvested ~ 8 weeks after the first inoculation.

4. Development of IFAT

a) IFAT protocol

Two procedures were used: procedure 1 was used to titrate the antiserum and to determine the best fixative for the adherence of *Uronema* to microscope slides, and procedure 2 was used in later tests when the method was modified to include blocking buffers to reduce non-specific fluorescence.
All microscope slides were cleaned in 1% HCl in 70% ethanol.

IFAT procedure 1

1. µL of *Uronema* culture was applied to a microscope slide, air dried and gently heat- fixed.
2. µL *Uronema* antiserum, appropriately diluted in phosphate buffered saline (PBS) was layered over the culture. **-**
3. The slides were incubated in a moist chamber at 37 ^{o}C for 30 min.
4. The slides were washed for 10 min with 3 changes of PBS, and drained.
5. 25µL of 1/40 conjugate (fluorescine-isothiocyanate [FITC] labelled anti-rabbit IgG; Sigma) was layered onto the culture and antibody mix.
6. The slides were incubated at 37 ^{o}C for 30 min.
7. Slides were washed for 10 min in 3 changes of PBS then drained.
8. Slides were counterstained with 2% Evan's Blue to quench autofluorescence.
9. Slides were mounted in 10% alkaline-buffered glycerol prepared as 90 mL glycerol + 10 mL 0.5 M bicarbonate, pH 9.6, which enhances and preserves fluorescence.

IFAT procedure 2

1. Air-dried, heat-fixed smears were flooded with 2% bovine serum albumin (BSA).
2. The slides were drained and *U. nigricans* antiserum, diluted with 1% BSA in PBS, was added (dilution was varied, depending on the test).
3. The slides were incubated at 37^{o} C for 30-40 min in a humidity chamber.

4. The slides were washed in 3 changes of PBS for 10 min.
5. The slides were drained and FITC conjugate diluted 1/40 with 1% BSA in PBS was added.
6. The slides were incubated at 37° C for 30 min in a humidity chamber.
7. Then they were counterstained with 2% Evan's Blue for 5 min.
8. Finally the slides were rinsed in PBS, drained and mounted in alkaline buffered glycerol.

Negative controls. The following negative controls were included for all IFAT procedures: pre-immune serum in place of immune serum to screen for non-specific activity; PBS instead of immune serum to assess autofluorescence. A completely unrelated organism (*Paramoeba* sp.) was also stained. Fluorescence in any of these negative controls renders the test uninterpretable.

Positive controls. The individual isolates were used as positive controls for their specific antiserum in all IFAT procedures. A positive result was taken as peripheral bright green fluorescence and semi-quantitatively scored as; + = faint fluorescence to ++++ = intense fluorescence (0 = no fluorescence). At least 10 microscope fields were examined for each set of conditions.

b) Checking for cross-reactivity of antiserum and assessment of autofluoresce

A variety of ciliates was obtained from the routine washing out of a healthy juvenile flounder tank. This material was fixed in 1% formalin and used as required. A proportion was stained with protargol to broadly classify the taxa present. Heterotrichs, Stichotrichs, Peritrichs and Hymenostomatia could be distinguished. This ciliate assemblage was stained with 1/50 to 1/400 diluted antiserum, unabsorbed for ciliates, to determine the degree of cross-reactivity with other ciliates, using IFAT protocol 2.

Autofluorescence

Both *Uronema* and other marine ciliates fluoresce when excited with blue light of the wavelength (450 - 470 nm) used in the IFAT. This was successfully quenched by counterstaining with Evan's Blue. With heat-fixing,

many of the ciliates detached from the slide during staining. Mixing them with egg albumin, air-drying and then heat-fixing reduced this problem.

c) Adsorption of cross-reacting ciliate antibodies

Antiserum diluted to 1/50 was adsorbed with a 50μL of a ciliate mix containing 4 x 10^{10} ciliates/mL. The mixture was incubated overnight at 37°C (optimum temperature for mammalian IgG reactions) and then placed at 4°C overnight. The suspension was inverted at times to ensure that the antiserum had ample exposure to the ciliates. The suspension was next centrifuged at 3000 rpm for 10 min to sediment the ciliate antigen/antibody complexes. The antiserum was finally sterilised by filtration through a 0.2μm millipore filter.

d) Adsorption of interfering bacterial antibodies

Bacterial antibodies were adsorbed by adding a suspension of the feed bacteria to the antiserum. These bacteria were cultured by taking a sample from the *Uronema* culture, plating onto sea-water nutrient agar plates and incubating at ambient temperature (~ 20°C) for 2 d. A suspension containing 2 x 10^{10} colony forming units (CFU)/mL was made in sterile PBS. 125μL of this suspension was added to 5mL of neat and 1/100 diluted anti-B and anti-Q antisera, and incubated at 37°C overnight. After incubation 1mL was removed, centrifuged, filter sterilised and titrated using IFAT procedure 2. Each antiserum and bacterial mixture was incubated at 4°C for a further 5 d. At daily intervals 1 mL of antiserum was removed, centrifuged and filter sterilised. The antiserum was then retitrated, in duplicate, using IFAT procedure 2.

e) Retesting with adsorbed antiserum

The IFAT was repeated with the ciliate mix and the *Uronema* isolates to test the success of adsorption with 1/50 to 1/400 adsorbed diluted antiserum. The test was carried out in duplicate and positive and negative controls were included. Also, the ciliate assemblage and isolate B were mixed together and stained to observe whether, after adsorption, sufficient anti-B remained to distinguish *Uronema* from other ciliates.

RESULTS

Part A: The Development of a Fluorescent Antibody Stain Using *U.nigricans* Grown in a Medium Containing Bacteria

1. Identification

a) Live observations

From the examination of numerous wet preparations, each comprising observations of over 100 cells, the only observable difference between isolates was size and number of food vacuoles. Figures 1 to 7 depict isolates B, Q, SH1, A, K, SH2 and F respectively. Isolate B measured 34.6 μm long by 17.4 μm wide, isolate Q was slightly smaller, being 26.8 by 14.7 μm. In all other aspects these organisms appear to be identical.

The body is roughly pear-shaped with a distinct indentation at the cytostome which is clearly observed in lateral views of the organisms (Fig. 4), but not in dorsal (Fig. 6) or ventral views (Fig. 1). The anterior pole (ap) is flat with a distinct slope, especially discernible in the lateral views (Figs. 3 and 4). The endoplasm is transparent with numerous spherical, greenish food vacuoles (fv) (Fig. 3, large cell, Fig. 2 dividing cell and Fig. 6). The number of food vacuoles is variable; some cells having considerably more with a corresponding increase in body size (Fig. 3, large cell). Bilobed, lamellar vesicles (lv), thought to function in membrane recycling, are also present (Fig. 2). A contractile vacuole (cv) is clearly observed in the posterior of the cell (Figs. 3, 4 and 5). The pellicle is colourless and ridged between the ciliary rows (c) (Fig. 3). Somatic cilia (sc) of approximately 5 μm in length and a long caudal cilium (cc) about 10 -15 μm in length were noted. These numerous characteristics were summarised in composite drawings made from > 100 observations (Figs. 8, 9 and 10).

Behaviour

These organisms are bactivorous and, when feeding within a bacterial mass, are extremely pleomorphic. They stretch out and undulate their way through small gaps; round up, or assume irregular shapes while moving, and slowly rotate within the food mass. When not in dense bacteria they swim rapidly,

rotating about their longitudinal axis with a corkscrew movement, stopping frequently to feed. The caudal cilium seems to function as a steering device as the cell appears to pivot around this when changing direction. They use their somatic cilia, especially the anterior ones, to hang onto the edge of food clumps and to each other. They create a vortex with the oral ciliature to facilitate feeding and, when stationary, the somatic cilia appear to beat towards the oral area enhancing prey capture.

b) Vital stain

In the methyl-green pyronin stained cells, blue macronuclei are visible in preparations of each isolate (Figs. 11 to 17). Dark-staining nucleoli are visible in the macronucleus of isolates A and B (Figs. 11 and 12 respectively) and the smaller micronucleus can be distinguished (Figs. 11, 12, 14 and 16). Trichocysts can be seen in the cell pellicle (Fig. 14). Destructive processes such as cell rupture and extrusion of contents, occurred more quickly in some isolates than others. Isolate Q (Fig. 13), K (Fig. 14) and SH1 (Fig. 16) swelled and ruptured within seconds. A large vacuole can be seen forming just prior to cell rupture in isolate Q; in isolate SH1 the cell contents can be seen emanating from the cell; in isolate SH2 the macronucleus is extruding. In isolate F (Fig. 15) the stain can be seen entering the cell, possibly at the cytostome and the cell membrane is beginning to balloon out.

c) Protargol stain observations and measurements

Protargol staining of isolates B and Q revealed the infrastructural details (Figs. 18 to 22). Composite diagrams (Figs. 23, 24 and 25) showing the salient features were drawn from observations on > 50 specimens.

For isolates B and Q, measurements of salient identification features are tabulated in Appendix 2. Between 10 and 15 evenly spaced, bipolar somatic kineties were counted in > 50 specimens. The first kinety (K1) and last (Kn) curve around the buccal apparatus, clearly visible in figures 19, 20 and 21. K1 lies to the right of the buccal apparatus and starts at the level of the second kinetosome position of the other kineties, which was visible in views of the anterior pole (Fig. 23). Each kinety contains between 20 - 30 kinetosomes which are paired in the anterior part of the cell. The pairing, in most

specimens, starts at the level of the 5th kinetosome from the posterior end and is easiest to see in the wider, posterior part (Fig. 20; top cell), The pairs are not easy to distinguish in the narrower anterior of the cell. The caudal cilium arises from a central kinetosome and has a dark staining granule either side, giving the impression of 3 kinetosomes (Fig. 21). Buccal infraciliature, typical for *Uronema* was observed (Figs. 19, 20, 21 and 22). Three oral membranelles were distinguished (Figs. 19 and 21): M1 appeared to be a single row of 4 kinetosomes (Fig. 19), M2 a double row of 4 kinetosomes (Fig. 21) and M3 was triangular in shape, but the individual kinetosomes were not discernible (Fig. 21). The undulating membrane begins at a level half-way along M2, has a central indentation and curves slightly at the posterior end around the cytostome (Figs. 19, 21 and 22). The cytostome is always slightly above the equator of the cell (Fig. 19). A non-ciliated scuticum consisting of between 3 to 5 kinetosomes in the form of a letter Y, is present at the end of the undulating membrane (Fig. 22). A cytoproct, visible as a faint, irregular line, is present between K1 and Kn, posterior to the scuticum (Fig. 19).

Statistical analysis

The statistical analysis using the measured characteristics from the protargol stains (Tables I, II, III, IV, V & VI in Appendix section) indicated that the data was normal and variance was homogeneous for each characteristic, except length of oral apparatus and width. Heterogeneity of variance increases the risk of false rejection of the hypothesis (type 1 error) (Sokal and Rohlf, 1987); therefore, it would only affect the data for width as this was the only characteristic which was significantly different ($p < 0.05$). However, in this case a type 1 error is unlikely because $p < 0.0004$ for this characteristic which strongly indicates that the difference between the means is real and cannot be attributed purely to random variation. By 1-way ANOVA, there is no significant difference between any of the morphological characteristics measured for isolate Q and B ($p > 0.05$). The characteristics with no significant difference ($p > 0.05$) between the clinical specimen and isolates B and Q were: length, length of oral apparatus (LOA) and distance from the

apex of the cell to the top of M1 (AOP). These are the most important characteristics for differentiating between these organisms and indicates that this isolate is not significantly different from isolate B and Q ($p > 0.05$). This is not surprising as this clinical organism is in fact a specimen of isolate Q taken directly from a brain smear. The 1-way ANOVA results are summarised in Table 1.

Table 1: Summary of statistical analysis using 1-way ANOVA and Student's t-test for multiple comparisons of means, on isolates B and Q, and clinical specimen.

	ISOLATE					
	Clinical		Q		B	
Characteristic	mean	SE	mean	SE	mean	SE
Length	27.3 [a]	1.2	24.8 [a]	0.9	25.3 [a]	0.56
Width	14.0 [a]	1.26	9.3 [b]	0.5	9.3 [b]	0.2
LOA	11.6 [a]	0.86	10.6 [a]	0.3	10.6 [a]	0.29
AOP	3.0 [a]	0.18	3.3 [a]	0.31	3.3[a]	0.063
M1	1.4 [a]	0.085	2.0 [b]	0.11	1.8 [b]	0.09
M2	2.5 [a]	0.09	1.8 [b]	0.11	1.8 [b]	0.12
M3	0.96 [a]	0.05	0.8 [b]	0.08	0.6 [b]	0.05
PO	0.88 [a]	0.35	6.2 [b]	0.25	7.0 [b]	0.19

Note: Means within a row, sharing a common superscript, are not significantly different; ($P>0.05$).

Abbreviations: LOA; length of oral apparatus
AOP; apex to oral apparatus
M; membranelle
PO; paroral membrane

Kinety number varied between 12 - 14 for the clinical isolate, and 11 - 15 for isolates B and Q.

In these studies the organisms were found to achieve variable size depending on the age of the culture and the trophic state. Well nourished organisms increased their size by increasing the number of food vacuoles (Figs. 2 and 3). These differences may affect the position of the cytostome, that is it could seem to move upwards relative to the rest of the cell as it fills up with food

vacuoles (P. O'Donoghue, pers. comm). In small cells the paroral membrane is located more posteriorly, than in large cells (Thompson, 1964). There would also be a concomitant relative relocation of the oral ciliature. It would appear therefore, that detailed morphological studies are not an entirely reliable means of differentiating between the species, which highlights the usefulness of immunological methods as identification tools.

2. Culture

The method used has been successful at maintaining all cultures to the present time.

3. Antibody production and 4. Development of IFAT

Titre of anti-B and anti-Q antisera

Table 2: Titration of anti-B antiserum with isolate B; first bleed

	Antiserum Dilution										
Conjugate Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1/20	+++	++++	+++	+++	++	+	-	-	-	-	-
1/40	+++	+++	++	++	+	+	-	-	-	-	-
1/80	++	++	++	+	+	-	-	-	-	-	-
1/160	++	+	+	+	-	-	-	-	-	-	-
1/320	+	+	+	+	-	-	-	-	-	-	-

Table 3: Titration of anti-B antiserum with isolate B; second bleed

	Antiserum Dilution										
Conjugate Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1/40	+++	+++	++++	++++	++++	++++	+++	+++	++	+	-

Table 4: Titration of anti-Q antiserum with isolate Q; first bleed

	Antiserum Dilution										
Conjugate Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1/20	+++	++++	+++	++	+	+	-	-	-	-	-
1/40	++	++	++	+	-	-	-	-	-	-	-
1/80	++	++	++	+	-	-	-	-	-	-	-
1/160	++	++	+	-	-	-	-	-	-	-	-
1/320	-	-	-	-	-	-	-	-	-	-	-

Table 5: Titration of anti-Q antiserum with isolate Q; second bleed

	Antiserum Dilution										
Conjugate Dilution	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1/40	+++	+++	++++	++++	++++	+++	++	+	-	-	-

++++	Intense Fluorescence
+++	Strong Fluorescence
++	Moderate Fluorescence
+	Faint Fluorescence
-	No Fluorescence

The end-point of the titration was taken to be the last dilution giving +++ fluorescence.

Because the initial titres were low (1/8 for anti-B antiserum; Table 2, and 1/4 for anti-Q antiserum; Table 3) a booster inoculation of 10 μL of pelleted *Uronema* in 1 mL PBS plus 1 mL FIA was given. Two weeks later the antibody titre was checked. An improved response was obtained from both rabbits. Anti-B antiserum titre was 1/128 (Table 4) and anti-Q antiserum was 1/32 (Table 5). Background fluorescence was evident with both antibodies, especially in high concentrations of antiserum.

Best adherence and fixation method for *Uronema*

Unfixed, air-dried slides produced the best results for all isolates. *Uronema* cells were least distorted and best preserved. Gentle heat fixation produced the next best results with only occasional rupture of the cells. Acetone fixation gave a fairly good result, but there was a lot of background fluorescence due to the rupture of some cells. Methanol fixation was highly destructive, few whole *Uronema* remained, and *Uronema* contents caused abundant background fluorescence. Considerable fluorescence due to bacteria was noted in all samples. As bacterial cells are much smaller (approximately 2 - 3 μm long) than the target organism (approximately 30 μm long) they are not likely to be confused with it.

Adsorption of antisera with bacterial antigens

Table 6: Effect of adsorption of bacterial antibodies; anti-B antiserum and isolate B

	Antiserum Dilution										
Time - Days	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1	+++	+++	++++	++++	++++	++++	++++	++++	+++	++	+
2	+++	+++	++++	++++	++++	++++	++++	++++	+++	++	+
3	+++	+++	++++	++++	++++	++++	++++	++++	+++	++	+
4	+++	+++	++++	++++	++++	++++	++++	++++	+++	++	+
5	+++	+++	++++	++++	++++	++++	++++	++++	+++	++	+

Table 7: Effect of adsorption of bacterial antibodies; anti-Q antiserum and isolate Q

	Antiserum Dilution										
Time - Days	1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024
1	+++	+++	++++	++++	++++	+++	+++	++	+	-	-
2	+++	+++	++++	++++	++++	+++	+++	++	+	-	-
3	+++	+++	++++	++++	++++	+++	+++	++	+	-	-
4	+++	+++	++++	++++	++++	+++	+++	++	+	-	-
5	+++	+++	++++	++++	++++	+++	+++	++	+	-	-

Table 8: Effect of adsorption of bacterial antibodies on bacteria background fluorescence with anti-B and anti-Q antisera

	Antiserum Dilution	
Time - Days	1/1	1/100
1	+++	+
2	++	-
3	++	-
4	++	-
5	++	-

++++	Intense Fluorescence
+++	Strong Fluorescence
++	Moderate Fluorescence
+	Faint Fluorescence
-	No Fluorescence

The adsorption of bacterial antibodies surprisingly resulted in an increase in antibody titre for both antibodies, indicating that these antibodies may have had a blocking effect. Anti-B antiserum increased from 1/128 to 1/156, anti-Q

antiserum increased from 1/32 to 1/64. The background and bacterial fluorescence was removed after 2 d absorption in high dilution, but there was little change using neat antiserum.

Adsorption of antisera with cross-reacting ciliate antigens

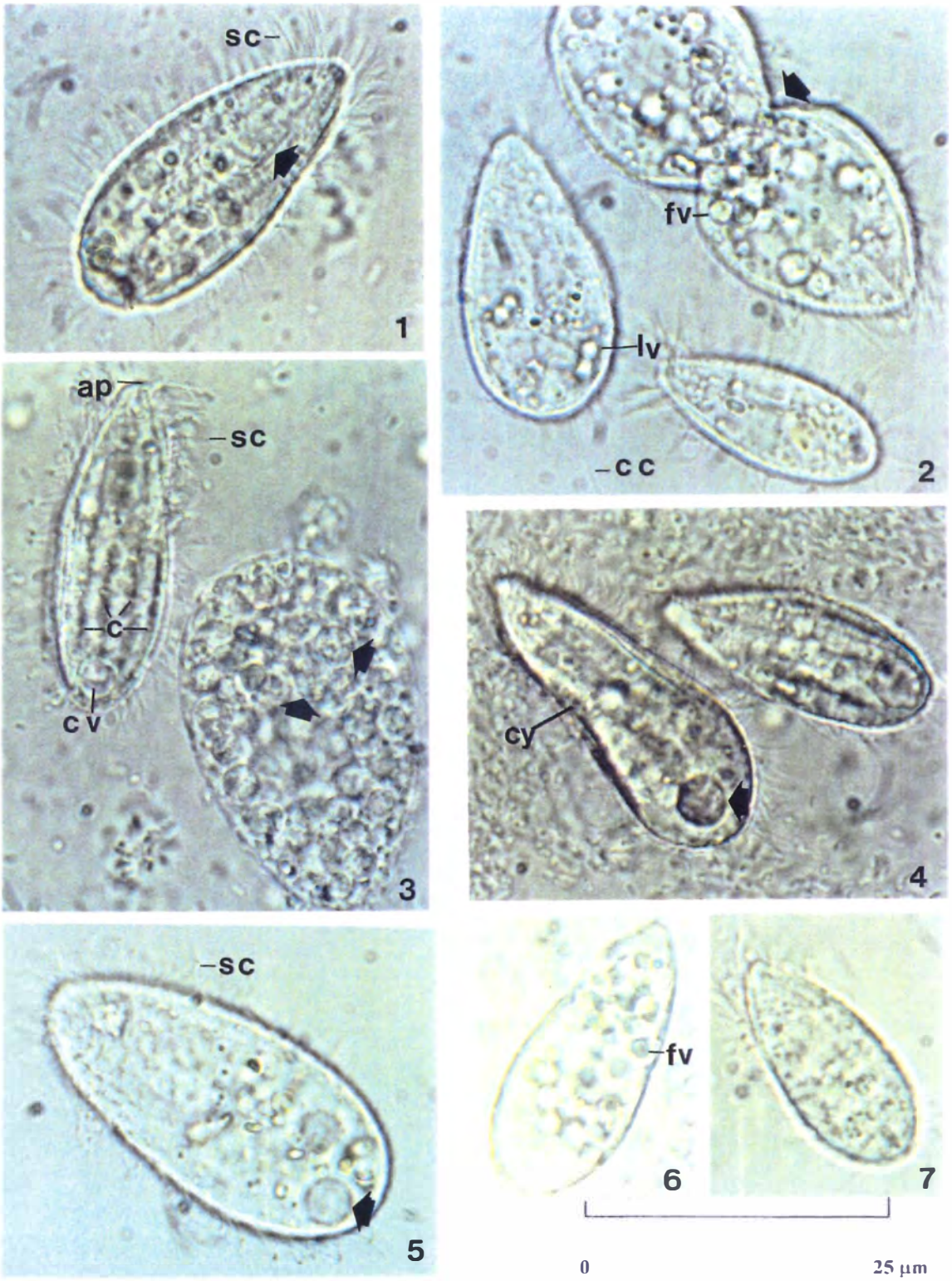
Table 9: Effect of adsorption of cross-reacting ciliate antibodies on both antisera

	anti - B				anti - Q			
	unadsorbed		adsorbed		unadsorbed		adsorbed	
Dilution	B	ciliates	B	ciliates	Q	ciliates	Q	ciliates
1/50	++++	+++	+++	+	++++	++	++	-
1/100	++++	+++	++	-	+++	++	+	-
1/200	+++	+++	++	-	++	++	-	-
1/400	++	+++	-	-	+	++	-	-

Adsorption of the antisera (previously adsorbed with the bacteria) removes the ciliate cross-reacting antibodies from both anti-B and anti-Q antisera (Table 9).

Unadsorbed antiserum stains both the ciliate assemblage and *Uronema*. However, after adsorption of antiserum with mixed ciliates, only *Uronema* are stained with the antiserum.

In Figures 26 and 27 it can be seen that adsorbed anti-B antiserum diluted to 1/50 stained *Uronema* effectively, but the ciliate assemblage remained unstained. *Uronema* are easily differentiated from the other ciliates.



Figures 1 -7: Live organisms from wet preparations; magnification 1000 x. Fig. 1: isolate B; paroral membrane visible (arrow). Fig. 2: isolate Q; note variation in size. A dividing cell is present (arrow) with food vacuoles visible (fv). Lamellar vesicles (lv) can be discerned. A caudal cilium (cc) is visible. Fig. 3: isolate SH1; size variation between cells is due to numerous food vacuoles (arrows) in right- hand cell. Ciliary rows (c) visible in left-hand cell. Somatic cilia (sc) are indicated. The contractile vacuole (cv) is visible. Fig. 4: isolate A; left-hand cell is distorted due to bacterial mass. Contractile vacuole clearly visible (arrow). Indentation at cytostome (cy). Fig. 5: isolate K; contractile vacuole (arrow). Fig. 6: isolate SH2. Fig. 7: isolate F.

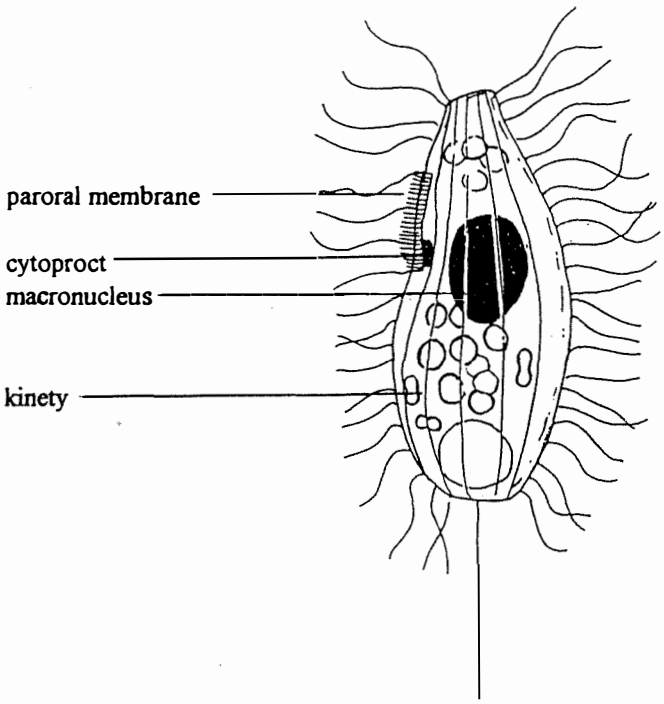


Fig. 8

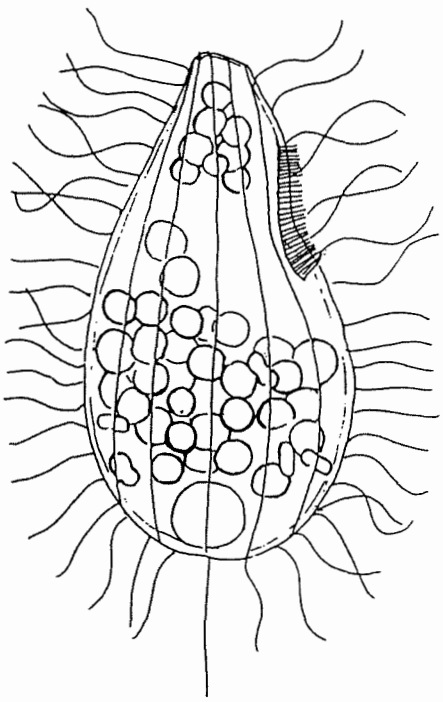


Fig. 9

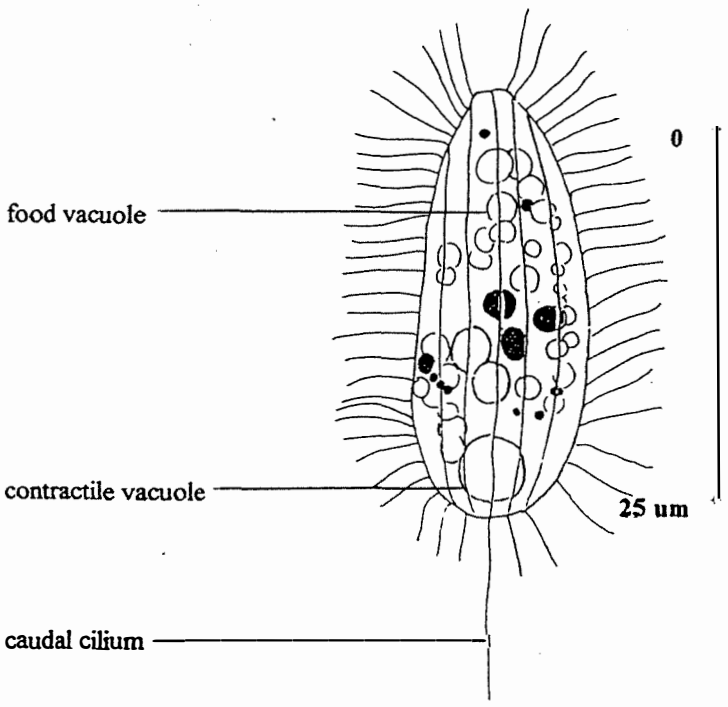
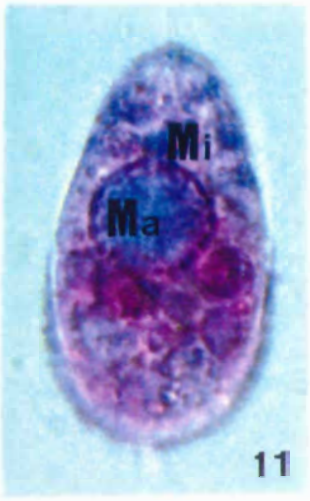
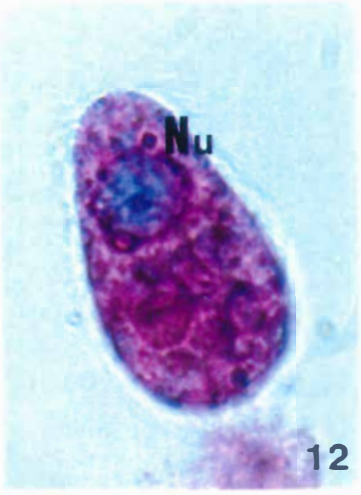
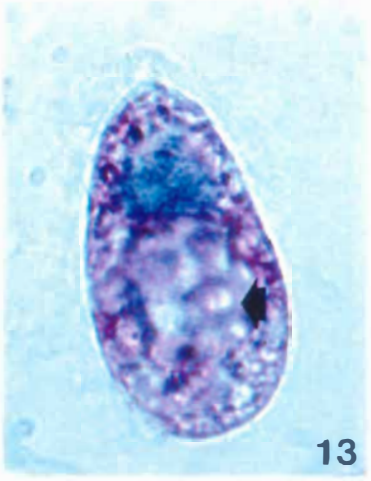


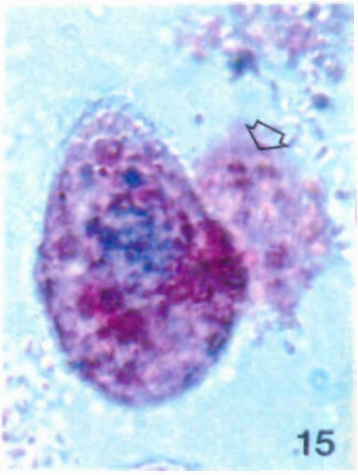
Fig. 10

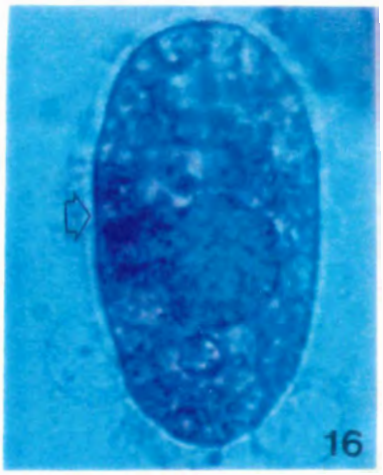
Figures 8, 9 and 10. Representative drawings made from wet-preparation observations of > 100 organisms from one culture. Note the difference in size. Left lateral view (Fig. 8); right lateral view (Fig. 9) and dorsal view (Fig. 10).

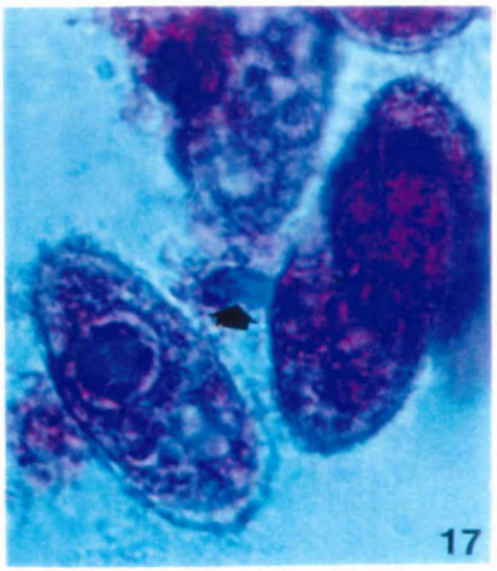




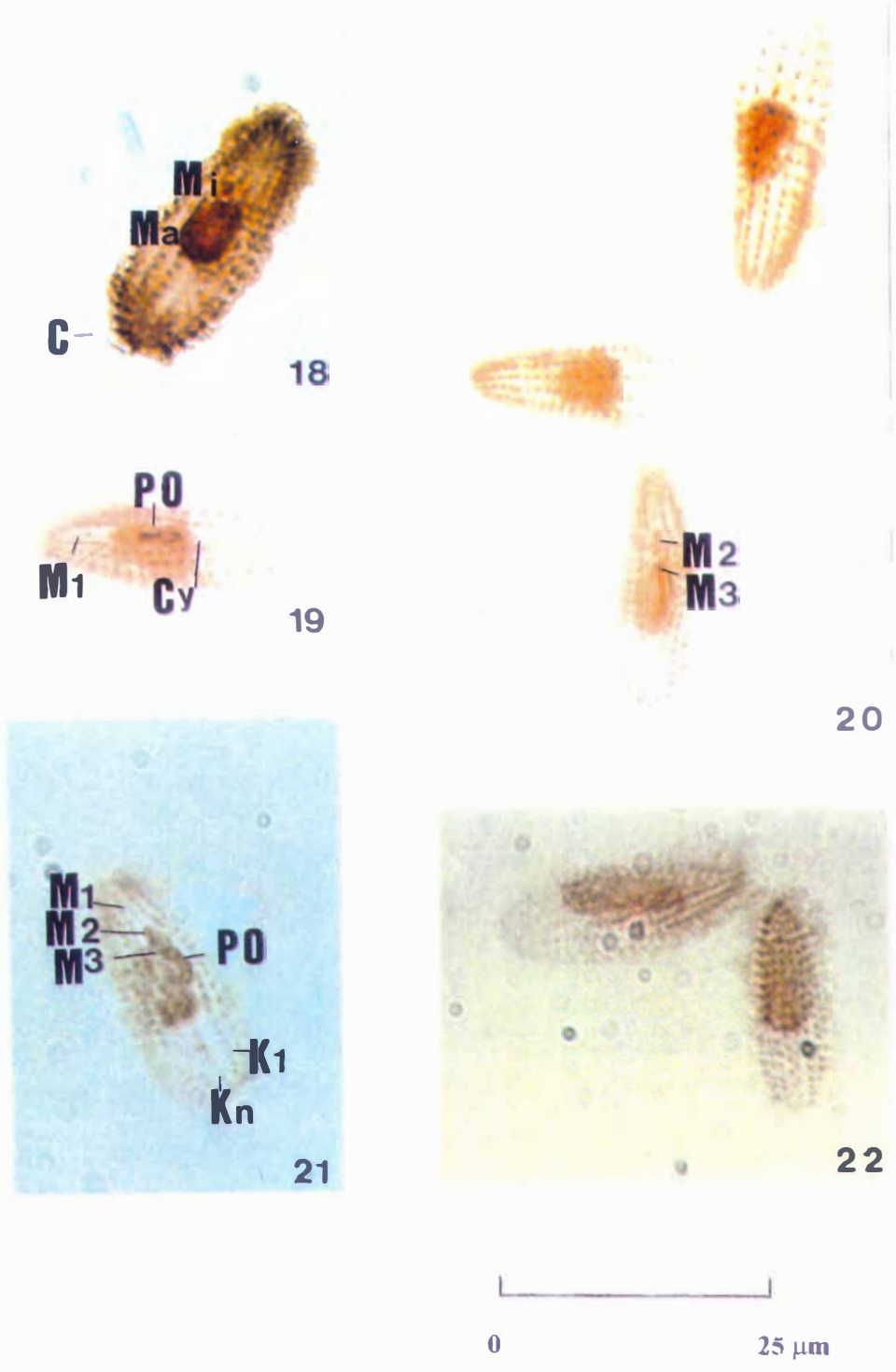








Magnification 1000 x.
Figures 11 to 17: Methyl-green pyronin stains. Figs. 11 and 12: isolate A and B respectively: macronucleus (Ma) with prominent nucleoli (Nu) and anterior micronucleus (Mi). Fig. 13: isolate Q; rapid swelling is occurring in the centre of the cell (arrow). Fig. 14: isolate K; trichocysts can be seen in the cell pellicle (arrow). Fig. 15: isolate F; cell contents extruding (arrow). Fig. 16 : isolate SH1; cell is pale; area to right (arrow) indicates where the stain is being taken up. Fig. 17: isolate SH2; extruded nucleus visible (arrow).



Figures 18 to 22. Isolates B and Q, protargol stained; magnification 1000 x. Fig. 18: isolate B showing macronucleus (Ma) micronucleus (Mi) and caudal cilium (C). Fig. 19: ventral view (through cell) of isolate B demonstrating membranelle 1, (M1), paroral membrane (PO) and cytopyge (Cy). Fig. 20: top 2 cells are dorsal views of isolate B with 6 ciliary rows visible; the lower cell is a ventral view (taken through cell) of isolate B showing membranelles 2 (M2) and 3 (M3); 5 ciliary rows are evident. Fig. 21: ventral view of isolate Q showing M1, M2, M3, PO, K 1 and Kn. Fig. 22: dorsal (right hand cell) and ventral views (left-hand cell) of isolate Q with 7 kineties visible.

Fig. 23

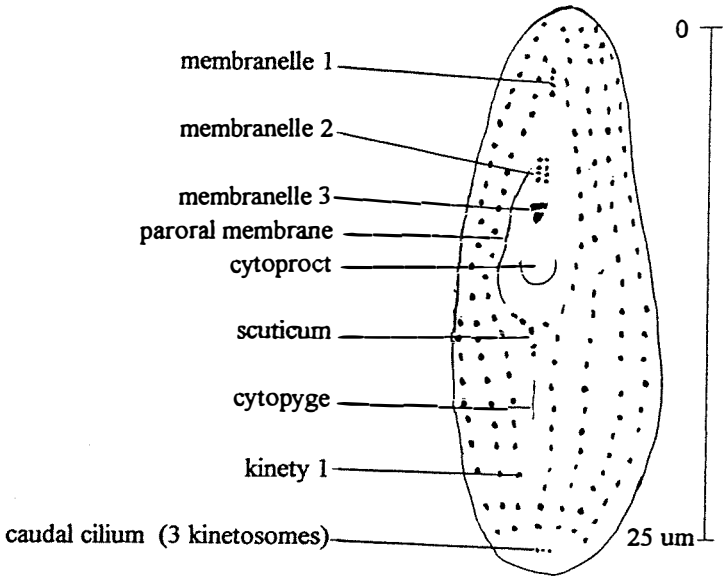


Fig. 24

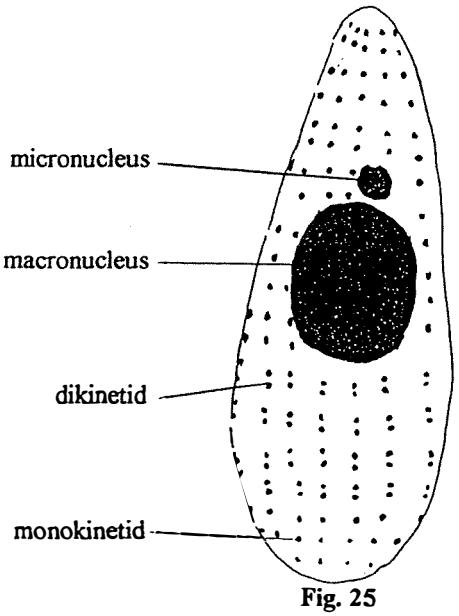
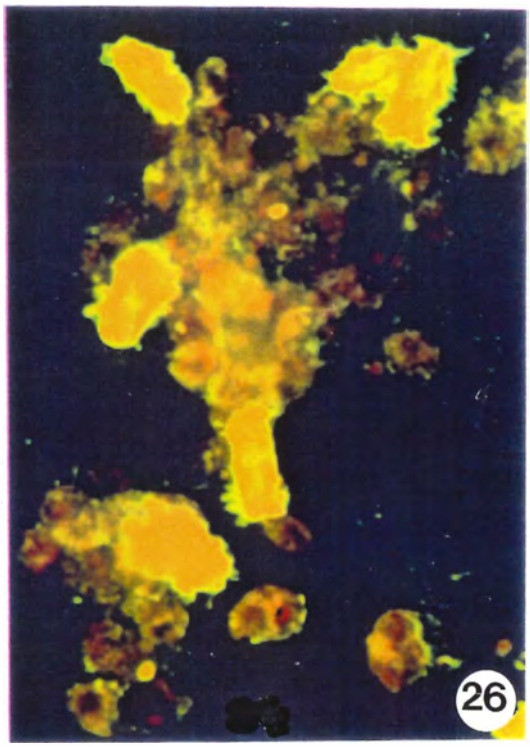


Fig. 25

Figs. 23, 24 and 25. Composite drawings made from numerous protargol-stained, cultured specimens. Fig. 23: naked anterior pole, demonstrating number of kineties. Fig 24: ventral view. Fig 25: dorsal view.

Figures 26 and 27 the effect of staining *Uronema* and ciliate assemblage, together, with anti-B antiserum diluted to 1/50 adsorbed and unadsorbed for ciliates. Both slides counterstained. Fig. 14: unadsorbed anti-B antiserum showing *Uronema* and ciliate assemblage, both fluorescing. Fig. 15: adsorbed anti-B showing only *Uronema* fluorescing.



METHODS

Part B: The Development of a Fluorescent Antibody Stain Using Axenic *Uronema* sp.

1. Culture

Axenic culture media.

Axenic culture of *Uronema* sp. had never been previously documented and earlier efforts to cultivate *Uronema* sp. in the absence of bacteria were unsuccessful. The method for the axenic culture of *Mesanophrys chesapeakensis* (Messick and Small, 1996) was used. In this method the ATCC Culture Medium 1651 MA Medium was modified by substituting the listed vitamins with RPMI 1640 vitamins, by filter sterilisation and by medium supplementation with 10% (v/v) foetal calf serum (see Appendix section).

Origin of isolates

Isolate T was submitted by K. Rough and was sampled from the brain of a diseased sea-caged southern bluefin tuna (SBT) at Port Lincoln. Isolate Q was one of the original isolates from Part A and was being maintained in routine culture medium (RCM) (see Appendix section), with bacteria as a food source.

Culture technique

The isolate T was inoculated directly into the axenic culture media upon receipt and kept at room temperature and examined by microscopy and culture onto horse blood agar (HBA) daily. Isolate Q was being maintained in RCM with bacteria as a food source and it was therefore necessary to remove the bacteria before inoculating the axenic culture medium.

Removal of bacteria

To establish which antibiotic or antibiotics to use to remove the bacteria, the medium supporting the growth of isolate Q was cultured onto HBA and also onto HBA containing 30 mg/L of ampicillin. Colonies from the HBA only agar were selected and plated onto purity plates. These cultures were then tested for sensitivity to ampicillin on Oxoid sensitest agar by disc diffusion.

Routine culture medium (RCM) and axenic culture medium (ACM) were then prepared with the addition of 5 mg/L of ampicillin and then 10 mL aliquot's placed into sterile 15 mL Kimble™ tubes.

Duplicate tubes of RCM with ampicillin (RCMA) were inoculated with 100µl of Q culture drawn from the surface of the medium because in this medium the ciliates are characteristically negatively geotropic. These cultures were mixed for 30 min on tube rotator, centrifuged at 3000 rpm, left to stand for 1 h at room temperature and the surface of the medium column examined for the presence of *Uronema* sp. in a wet preparation under x100 magnification. Once the presence of the ciliates had been established, the medium was subcultured for the presence of viable bacteria onto HBA and above the procedure was repeated twice using RCMA tubes and twice using ACM with ampicillin (ACMA) in the tube.

Cloning of the ciliates

Three methods of collecting a single ciliate in order to initiate cloned cultures were attempted. These were :

1. The use of capillary tubes to distribute drops of media onto petri dishes. Drops containing single ciliates were subsequently transferred to 200µL of medium (BHI) contained in individual wells of 96-well microtitre plates.
2. Serial dilution of a stock culture in microtitre plates until only 1 ciliate was visible/evident in a well.
3. The silicon oil plate procedure (SOPP) (Soldo and Brickson, 1993). 200µL of dilute suspension (approx. 1000 cells/mL was added to 3 mL of silicon oil (heat sterilised at 160°C for 1 h). The oil was mixed on a vortex for 20-25 seconds then contents immediately poured onto a petri dish. On examination with a dissecting microscope (Olympus® camera lucida) many resultant microdroplets contained individual ciliates which were subsequently collected with a pasteur pipette (drawn through a flame to reduce the diameter to 100-200µm) and placed in 150 µL of medium (BHI). The microtitre lids were taped shut and wells were stored in the dark at ambient temperatures (18-22^{o}C). As densities grew additional medium was added to the wells in 50µL aliquot's. After 3 days ciliates were transferred to McCartney bottles with 3 mL of medium and incubated at ambient

temperature for 24h. Finally, culture bottles were filled to 25 mL and refrigerated.

Maintaining cultures

The ACM being highly nutritious for the ciliates was also at risk of bacterial contamination. It was therefore necessary to locate the experiment in a laminar flow work station dedicated for the project to lessen the risk. Fresh ACM was prepared fortnightly, 10 mL aliquoted into 15 mL sterile Kimble™ tubes and the cultures subcultured each week with regular checks for the absence of bacteria by subculture onto HBA and microscopy to determine the health of the ciliate.

2. Antibody production

Preparation of immunogen using isolate T

After 8 wks of culture there was sufficient cloned axenic *Uronema* sp. isolate T to prepare an antigen for raising the axenic antisera in rabbits. Four tubes showing dense (>100 ciliates/mL) growth were centrifuged at 3000 rpm for 10 min, the supernatant drawn off, and the pellet washed with 10 mL of sterile PBS (pH 7.2). The pellet was reconstituted with 5 mL of sterile PBS, 4 drops of 10% formal saline added to kill the ciliate and 5 mL of Montanide ® ISA 50 added as an incomplete adjuvant.

Rabbit inoculation protocol

Ethics committee approval was obtained to carry out this procedure.

1. A 10 mL pre-immunisation bleed was taken from each rabbit.
2. Two rabbits were inoculated one week later with the antigen intramuscularly, at multiple sites.
3. Four weeks later the rabbits were inoculated as before, with the second dose of antigen (2 mL total).
4. Two weeks after the second inoculation, bleed and reinoculate as before.
5. Check for antibody in six weeks post inoculation sample using the IFAT method.
6. Bleed again 8 weeks after the first inoculation and check for antibody.
7. Bleed out rabbits at 10 weeks.

IFAT protocol

1. 20 μl of *Uronema* culture was applied to microscope slide and air dried.
2. 20 μl *Uronema* antiserum, appropriately diluted in phosphate buffered saline (PBS) was layered over the culture..
3. The slides were incubated in a moist chamber at 37°C for 30 min.
4. The slides were washed for 10 min with 3 changes of PBS, and drained.
5. 20 μl of 1/20 conjugate (fluorescine-isothiocyanate [FITC] labelled anti-rabbit Ig (Silenus laboratories) was pipetted onto the *Uronema* sp. and antibody mix.
6. The slides were again incubated at 37°C for 30 min.
7. Slides were washed for 10 min in 3 changes of PBS then drained.
8. Slides were counterstained with 2% Evan's Blue for 2 min.
9. Slides were mounted with Difco Bacto FA mounting fluid and examined under epifluorescence.

3. Field trip to Port Lincoln

On August 11, 1996 a trip was made to Port Lincoln the purpose of which was to deliver the reagents and materials necessary for the *Uronema nigricans* IFAT, including antisera to non-axenic sheep, FITC-conjugated anti-sheep Ig, positive control slides, pipette tips in boxes, syringe filters and syringes. It was also intended to demonstrate the IFAT technique to Ms Rough.

RESULTS

Part B: The Development of a Fluorescent Antibody Stain Using Axenic *Uronema* sp.

1. Culture

Axenic culture media

Once the isolates of *Uronema* sp. were established, it was found that the axenic culture medium easily sustained growth of the ciliate.

Origin of isolates and culture technique

Uronema sp. isolate T, when cultured into ACM, initially showed very small sluggish ciliates and no growth on HBA. After 7 d the ciliates became large and highly motile and there was still no growth on HBA so this culture was deemed to be axenic. An axenic version of isolate Q resulted from treatment of the non-axenic culture with antibiotic (see result section for the removal of bacteria).

Removal of bacteria

The bacterial cultures taken from the RCM of isolate Q gave a mixed growth of three types of gram negative bacteria on HBA and no growth on the HBAA, suggesting that all the bacteria were sensitive to ampicillin. This was confirmed by the disc diffusion test and ampicillin was chosen as the antibiotic to be incorporated in the treatment tube.

After 3 treatments with RCMA and 2 treatments with ACMA (see Table 1), ciliates were identified in RCMA broth two, transferred to ACMA broth one and later found to give no bacterial growth on culture. The ciliates isolated from ACMA broth one were then transferred to ACM for routine maintenance of culture.

Table 1. Results of treatment with ampicillin for the removal of bacteria

Treatment	Culture	*Uronema* sp.	Comment
RCMA one	Positive	Positive	Discarded
RCMA two	Negative	Positive	To ACMA one
RCMA three	Negative	Negative	To ACMA two
ACMA one	Negative	Positive	To ACM
ACMA two	Negative	Negative	Discarded

Cloning of the ciliates

Neither manipulation of individual ciliates with capillary tubes, nor serial dilution of stock cultures were successful in initiating cloned cultures. The SOPP method was successful and, after transfer of a single ciliate to a microtitre well, a number of ciliates were visible under the dissecting microscope after 3-5 days. Therefore this culture was deemed the clone of the original ciliate. Both T and Q isolates were cloned.

Maintaining cultures

Bacterial contamination was the major problem with maintaining the axenic *Uronema* sp. isolates T and Q. Any contamination was clearly evidenced by a rapid change in the opacity of the media and these cultures discarded.

2. Antibody production

Rabbit inoculation and IFAT

Results of the titration of the anti-sera using the IFAT from both rabbits is shown in Table 2

Table 2. Titration of the axenic antisera

	Rabbit one	Rabbit two
Pre dose	Negative	Negative
6 weeks post dose	1:256	1:128
8 weeks dose	1:256	1:64
10 weeks post dose	1:128	< 1:64
12 weeks post dose	< 1:64	< 1:64

3. Field to Port Lincoln

The reagents and materials required to perform the *Uronema nigricans* IFAT were delivered and the technique was demonstrated to Ms Rough. The axenic *Uronema nigricans* rabbit antisera has also been supplied by mail.

TECHNICAL SUMMARY

This sub-project has seen the successful development of a rapid test for the potential detection of *Uronema nigricans* in SBT and the environment. This was first accomplished by identifying the etiological agent by microscopical and histochemical techniques using live observations, vital staining for trichocysts and nuclear detail and protargol staining for infraciliature measurements. Next, antisera to two of the non-axenic strains (Q and B) were raised in rabbits and to Q in sheep and an immunostaining technique using the indirect immunofluorescent test was established. The antisera was able to detect and presumptively identify *Uronema* sp. in both pathological and environmental samples. The IFAT is a quick test and smears could be made *in situ* and stained later. The test is robust; fluorescence persisted for 9 months, even when the slides were left at ambient temperature. However, as a practical tool there was still much to be done regarding the development of the test and the sample collection procedures.

Currently, work is under way on two isolates (Q and T) that have been cloned and are axenic. These are being maintained in axenic culture media under special conditions to avoid potential contamination. Good results were obtained with antisera against axenic *Uronema nigricans* prepared in rabbits when diluted up to 1: 256. This reagent was sent to Ms Rough for further evaluation of samples from infected SBT and environmental samples. Tests to exclude cross-reactivity with other environmental ciliates, especially from Port Lincoln, still need to be performed.

So that there will be a reliable source of antibody for the future, it is also anticipated that a monoclonal antibody to one of the axenic isolates will be prepared.

BENEFITS

The SBT farming industry in South Australia will directly benefit from this part of the research into the rapid detection of *Uronema* sp. in SBT and in the

environment, which involved the identification of the causative organism, production of the specific antisera and development of the immunofluorescent technique. This could then be used to identify carrier fish or to determine the environmental reservoir of the pathogen.
Indirectly, there will be a broad benefit bought about by this research, due to the increased scientific knowledge about this disease in SBT known as "swimmer syndrome" which was causing considerable annual mortality in sea-caged SBT at Port Lincoln. Prior to 1996 the etiological agent had not been documented as a cause of disease in tuna.

INTELLECTUAL PROPERTY

Intellectual property will be the public domain.

FURTHER DEVELOPMENT

1. The polyclonal antibody against axenic cultures of *Uronema nigricans* requires assessment for possible cross-reactivity with other ciliates.

2. A monoclonal antibody to *U. nigricans* is to be developed at the University of Prince Edward Island, Canada. This will greatly enhance the sensitivity of the test.

3. The IFAT can potentially be used to screen the environment for management and epidemiological purposes. However, controlled investigations are required to validate its use for this purpose.

4. SBT serum from known infections is needed in order to determine the nature of the SBT immune response to this infection. Serological tests may determine disease prevalence rates which will aid in disease management and epidemiological studies.

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APPENDIX

Routine culture medium

1. Brain heart infusion broth (BHI) (OXOID).
2. Lysed sheep red blood cells (RBC).
3. Accomin* vitamin complex containing vit. B1, B6, B12, lysine and ferric phosphate.

A stock solution of BHI was made to 3x concentration, following manufacture's instructions, in distilled water. This was diluted to a final concentration of 1/10 with sterile sea-water just before use. 25 μL each of lysed RBC and Accomin* were added per 500 mL of dilute BHI. The medium was dispensed aseptically into sterile McCartney bottles.

Accomin* is a registered trademark of the American Cyanamid company, Lederle Laboratory division, Cyanamid Australia Pty. Ltd., 5, Gibson Road, Baulkham Hills, NSW, Australia.

Axenic culture medium

10x stock solution

Base solution
100g proteose peptone (Difco #0120-01)
100g of Bacto-tryptone (Difco #0123-01)
10g of yeast RNA (Sigma #R-6625)
Dissolve in 200 mL of distilled water at 80^{o}C.
Asolectin emulsion
2 g of L-a-phosphatidylcholine Type II-s (Sigma #P5638)
Dissolve in 200 mL of distilled water at 80^{o}C
Add to the base solution with constant stirring for up to an hour
Cool and add 20 mL of vitamin solution RPMI 1640 (Sigma #R-7256)
Make the volume made up to 1L.
Prefilter through Whatman's No. 1 paper then filter through 0.45μ and finally filter through a 0.2μ filter to sterilise. Freeze in 10 mL aliquot's

Working solution

10 mL of thawed stock solution

10 mL of foetal calf serum (heat inactivated at 56 ^{o}C for 30 minutes)

80 mL of artificial seawater.

Filter (0.22 µ) sterilise into a sterile glass bottle

Table I

BIOMETRIC CHARACTERISATION

Sample ..... **Blenny Isolate**

Species ..... ***Uronema*** Stains ..... **Protargol**

Character	Measurements (um)										x	M	SD	CV	Min	Max	n
Body																	
- length	26.0	27.5	22.6	28.3	23.5	25.0	26.4	25.5	24.0	24.0	25.3	25.3	1.81	7.2	22.6	28.3	10.0
- width	9.4	10.8	8.9	9.9	8.9	9.4	8.4	8.9	9.4	8.9	9.3	9.2	0.67	7.3	8.4	10.8	10.0
- length (live)	38.0	32.0	32.0	30.0	35.0	40.0	38.0	35.0	35.0	31.0	34.6	35.0	3.34	9.7	30.0	40.0	10.0
- width (live)	19.0	15.0	16.0	15.0	15.0	20.0	20.0	21.0	18.0	15.0	17.4	17.0	2.46	14.1	15.0	21.0	10.0
Macronuclear segments																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10.0
- length	6.1	8.0	6.6	8.0	6.6	6.6	8.4	9.4	8.5	7.5	7.6	7.8	1.07	14.1	6.1	9.4	10.0
Micronuclei																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10.0
- length	1.4	1.4	1.4	1.4	1.7	1.4	1.6	1.4	1.4	1.3	1.4	1.4	0.12	8.2	1.3	1.7	10.0
Oral Kineties																	
- Apex-oral apparatus	3.0	3.8	2.8	3.0	4.2	3.3	2.4	4.2	3.3	2.6	3.3	3.2	0.63	19.4	2.4	4.2	10.0
- Length-oral apparatus	10.6	11.4	11.0	11.6	8.8	10.3	11.2	10.4	9.8	11.3	10.6	10.8	0.86	8.1	8.8	11.6	10.0
- Length- PO/UM	8.0	7.0	6.1	7.1	6.5	7.5	7.5	7.0	6.1	6.8	7.0	7.0	0.61	8.8	6.1	8.0	10.0
- M1	2.4	1.9	1.7	1.5	1.5	1.5	1.7	1.8	2.1	1.8	1.8	1.8	0.29	16.1	1.5	2.4	10.0
- M2	1.4	2.0	2.1	1.3	2.0	1.5	2.0				1.8	2.0	0.34	19.4	1.3	2.1	7.0
- M3	0.8	0.7	0.6	0.8	0.6	0.5	0.5				0.6	0.6	0.13	19.8	0.5	0.8	7.0
Somatic Kineties																	
- No. kineties	>12																
- No. basal bodies K1	>20																
- Length cilia*	5.0										5.0	5.0			5.0	5.0	1.0
Length -caudal cilium*	15.0										15.0	15.0			15.0	15.0	1.0

Table II

BIOMETRIC CHARACTERISATION

Sample ..... **Tuna Isolate**

Species ..... ***Uronema*** Stains ..... **Protargol**

Character	Measurements (um)										x	M	SD	CV	Min	Max	n
Body																	
- length	25.0	27.8	29.2	26.4	25.5	19.8	25.0	22.6	21.6	25.0	24.8	25.0	2.82	11.4	19.8	29.2	10.0
- width	9.4	10.8	12.7	11.3	8.9	8.0	8.0	9.0	11.3	9.4	9.9	9.4	1.57	15.9	8.0	12.7	10.0
- length (live)	32.0	24.0	25.0	22.0	31.0	32.0	25.0	25.0	28.0	24.0	26.8	25.0	3.68	13.7	22.0	32.0	10.0
- width (live)	13.0	15.0	15.0	12.0	14.0	15.0	12.0	18.0	18.0	15.0	14.7	15.0	2.11	14.4	12.0	18.0	10.0
Macronuclear segments																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10.0
- length	8.0	9.0	8.5	9.0	9.9	10.4	7.5	7.5	9.0	8.5	8.7	8.8	0.95	10.8	7.5	10.4	10.0
Micronuclei																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10.0
- length	1.4	1.4	1.0	1.1	1.4	1.2	1.7	1.2	1.5	1.1	1.3	1.3	0.22	16.6	1.0	1.7	10.0
Oral Kineties																	
- Apex-oral apparatus	3.4	3.8	3.3	2.3	2.7	5.2	3.0	2.8			3.3	3.2	0.89	26.9	2.3	5.2	8.0
- Length-oral apparatus	9.7	10.8	10.4	11.5	10.1	11.8		9.9			10.6	10.4	0.80	7.6	9.7	11.8	7.0
- Length- PO/UM	6.1	6.6	6.1	7.1	4.9	6.1		6.4			6.2	6.1	0.67	10.9	4.9	7.1	7.0
- M1	2.4	1.9	2.2	2.1	2.2	1.5	1.8	1.7			2.0	2.0	0.30	15.3	1.5	2.4	8.0
- M2	1.8	1.8	1.7	2.4	1.9	1.9	1.5	1.4			1.8	1.8	0.30	16.8	1.4	2.4	8.0
- M3	0.9	0.8	0.7	0.7	0.7	1.2		0.5			0.8	0.7	0.22	27.9	0.5	1.2	7.0
Somatic Kineties																	
- No. kineties	>12																
- No. basal bodies K1	>20																
- Length cilia	approx 5																
-																	
- somatic cilia	3.0	5.0	4.0	3.0							3.8	3.5	0.96	25.5	3.0	5.0	4.0
Length -caudal cilium	approx 10																
(from live observation)																	

Table III

BIOMETRIC CHARACTERISATION

Sample ..... **URO - B 1/2 URO - B2 1/2** (Data obtained from Dr. P. O'Donoghue)

Species ..... ***Uronema*** Stains ..... **Protargol**

Character	Measurements (um)										x	M	SD	CV	Min	Max	n
Body																	
- length	30.0	28.0	26.0	25.0	20.0	34.0	27.0	32.0	25.0	26.0	27.3	26.5	3.97	14.6	20.0	34.0	10
- width	18.5	13.0	15.2	9.5	7.1	20.0	12.5	17.0	12.0	15.0	14.0	14.0	3.98	28.5	7.1	20.0	10
Macronuclear segments																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10
- length	5.8	5.4	6.0	4.6	4.2	6.4	5.3	6.0	5.5	6.0	5.5	5.7	0.68	12.4	4.2	6.4	10
Micronuclei																	
- no.	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0			1.0	1.0	10
- length	0.8	1.0	0.8	0.7	0.6	1.1	0.9	0.8	0.9	1.0	0.9	0.9	0.15	17.5	0.6	1.1	10
Oral Kineties																	
- Apex-oral apparatus	3.0	2.5	3.2	4.20	2.1	3.4	2.7	3	3.4	2.8	3.0	2.9	0.58	19.3	2.1	4.2	10
- Length-oral apparatus	14.0	10.8	9.7	8.7	7.2	16.5	12.2	12.4	13.0	12.0	11.7	12.1	2.69	23.0	7.2	16.5	10
- Length- PO/UM	9.8	8.8	8.7	7.6	6.4	10.4	9.4	8.9	9.0	9.1	8.8	9.0	1.12	12.7	6.4	10.4	10
- M1	1.6	1.4	1.4	1.2	1.0	2.0	1.4	1.4	1.2	1.6	1.4	1.4	0.27	19.3	1.0	2.0	10
- M2	2.8	2.6	2.4	2.2	2.0	3.1	2.6	2.6	2.4	2.6	2.5	2.6	0.31	12.1	2.0	3.1	10
- M3	1.1	1.0	1.0	0.8	0.7	1.2	1.0	1.0	1.0	0.8	1.0	1.0	0.15	15.7	0.7	1.2	10
Sumatic Kineties																	
- No. kineties	12.0	12.0	12.0	12.0	12.0	14.0	12.0	12.0	14.0	12.0	12.4	12.0	0.84	6.8	12.0	14.0	10
- No. basal bodies K1	18.0	16.0	18.0	17.0	16.0	19.0	18.0	16.0	18.0	18.0	17.4	18.0	1.07	6.2	16.0	19.0	10
- Length cilia	3.1	3.0	3.1	2.8	2.8	3.4	3.0	3.1	3.0	2.8	3.0	3.0	0.19	6.2	2.8	3.4	10
Length -caudal cilium	7.6	7.8	7.8	8.0	7.1	8.2	7.5	7.4	7.6	7.8	7.7	7.7	0.31	4.1	7.1	8.2	10

Table IV

DESCRIPTION OF SPECIES	
Sample Code:	Blenny isolate
Order:	Scuticociliatida (Small 1967)
Family:	Uronematidae (Thompson 1964)
Genus:	*Uronema* (Dujardin 1841)
Species:	
DIAGNOSIS (summary of distinguishing characteristics)	
Size (in vitro)	35 um long, 17 um wide
Shape	pear-shaped, indentation at cytostome, blunt anterior end, rounded posterior
Organelles	numerous greenish food vacuoles, clear terminal contractile vacuole
Oral ciliature	paroral membrane visible; three membranelles
Somatic ciliature	distinct ciliary rows visible, caudal cilium conspicuous.
TYPE LOCALITY	
Location	Key Centre, Launceston, Tasmania
Habitat	Blenny larvae tank
DESCRIPTION (general features)	
Size variation	variable in length and width, shape remains constant when free-swimming
Shape variation	pleomorphic when moving through bacterial food mass
Flexibility	flexible; undulating movement through bacterial food mass
Movement	corkscrew, rotating around longitudinal axis; pivots around caudal cilium
Macronuclei	1 only, central to anterior, visible in live specimen as a granular body
Micronuclei	1 only, usually associated with macronucleus
Contractile vac.	terminal; pore not seen
Trichocysts	present
Pellicle	colourless, ridged between ciliary rows
Colour	colourless
Food Vacuoles	numerous, greenish, spherical, sometimes bilobed
Cytosome	located above equatorial line; fibrils evident; paroral membrane plus 3 membranelles
Oral kineties	difficult to distinguish if ciliated or not
Somatic kineties	anterior pole non -ciliated
Other features	scuticum evident at posterior end of paroral membrane, looks ciliated
DIFFERENTIAL DIAGNOSIS: (similarities/differences to related species)	
	Conforms to *U.nigricans*

Table V

DESCRIPTION OF SPECIES	
Sample Code:	Uronema; Queensland isolate
Order:	Scuticociliatida (Small 1967)
Family:	Uronematidae (Thompson 1964)
Genus:	*Uronema* (Dujardin 1841)
Species:	
DIAGNOSIS (summary of distinguishing characteristics)	
Size (in vitro)	26 um long, 15 um wide
Shape	pear-shaped, indentation at cytostome, blunt anterior end, rounded posterior
Organelles	numerous greenish food vacuoles, clear terminal contractile vacuole
Oral ciliature	paroral membrane visible; three membranelles
Somatic ciliature	distinct ciliary rows visible, caudal cilium conspicuous.
TYPE LOCALITY	
Location	Pt. Lincoln, South Australia
Habitat	Forebrain of southern bluefin tuna
DESCRIPTION (general features)	
Size variation	variable in length and width, shape remains constant when free-swimming
Shape variation	pleomorphic when moving through bacterial food mass
Flexibility	flexible; undulating movement through bacterial food mass
Movement	corkscrew, rotating around longitudinal axis; pivots around caudal cilium
Macronuclei	1 only, central to anterior, visible in live specimen as a granular body
Micronuclei	1 only, usually associated with macronucleus
Contractile vac.	terminal; pore not seen
Trichocysts	present
Pellicle	colourless, ridged between ciliary rows
Colour	colourless
Food Vacuoles	numerous, greenish, spherical, sometimes bilobed
Cytosome	located above equatorial line; fibrils evident; paroral membrane plus 3 membranelles
Oral kineties	difficult to distinguish if ciliated or not
Somatic kineties	anterior pole non -ciliated
Other features	scuticum evident at posterior end of paroral membrane, looks ciliated
DIFFERENTIAL DIAGNOSIS: (similarities/differences to related species)	
	Conforms to *U.nigricans*

Table VI

DESCRIPTION OF SPECIES	
Sample Code:	URO-B
Order:	Scuticociliatida Small 1967
Family:	Uronematidae Thompson 1964
Genus:	*Uronema* Dujardin 1841
Species:	
DIAGNOSIS (summary of distinguishing characteristics)	
Size (in vitro)	
Shape	ovid, slightly flattened dorso-ventrally
Organelles	numerous, green food vacuoles
Oral ciliature	indistinct membranelles although paroral membrane can be seen
Somatic ciliature	uniform somatic cilia - conspicuous caudal cilium
TYPE LOCALITY	
Location	Port Lincoln, South Australia
Habit	forebrain of southern bluefin tuna
DESCRIPTION (general features)	
Size variation	quite variable in length and width, robust when fed
Shape variation	not so variable in shape,ovoid whether slim or robust
Flexibility	inflexible
Movement	forward curving ventrally, short reverse avoidance
Macronuclei	1 only; located central or slightly anterior
Micronuclei	not visible without staining
Contractile vac.	terminal, pore located near end of K2
Tricocysts	present
Pellicle	colourless, slightly ridged along kineties
Colour	colourless
Food Vacuoles	green, spherical vacuoles
Cytosome	several fibrils evident
Oral kineties	paroral membrane, plus 3 membranelles. M1 not ciliated
Somatic kineties	some 12-14 meridians
	anterior end non-ciliated disc
Other features	small scuticum at posterior end of PO
	longitudinal cytopract between first and last kineties
DIFFERENTIAL DIAGNOSIS: (similarities/differences to related species)	
conforms to small *U.marinum* (but fewer kineties and oral apparatus closer to apex of ciliate)	
conforms to *U.nigricans* (in size, shape and infraciliature)	

Bull. Eur. Ass. Fish Pathol. 16(3),104, 1996.

THE DEVELOPMENT OF A FLUORESCENT ANTIBODY STAIN TO IDENTIFY A *URONEMA* SP. (CILIOPHORA: SCUTICOCILIATIDA) IMPLICATED IN FATAL ENCEPHALITIS IN SOUTHERN BLUEFIN TUNA (*THUNNUS MACCOYII*)

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Abstract

Polyclonal antisera, designated anti-Q antiserum and anti-B antiserum, were produced in rabbits against *Uronema* sp. isolated from diseased tuna (Q) and dead blenny larvae (B). Extraneous bacterial antibodies and cross-reacting ciliate antibodies were present in the antiserum and were removed by adsorption, enabling *Uronema* sp. to be easily differentiated within a marine ciliate assemblage. Adsorbed anti-B antiserum, diluted to 1/50, was used in indirect fluorescent antibody tests (IFATs) and detected ≥ 10 *Uronema* sp. in a 1 L sample of seawater and approximately 10 presumptive *Uronema*/25 µL in sediment samples from a tuna sea-cage. In addition, a variable staining pattern was demonstrated with different isolates of *Uronema* sp. The results indicate that this test is able to detect both cultured and environmental *Uronema* sp. and may have an application both as an environmental monitoring tool and a means of speciating *Uronema*.

Introduction

Scuticociliates, consistent with *Uronema* spp. have been reported in infections of various marine fish species in aquaria and aquaculture. Whereas larval fish suffer generalised infection, juveniles and adults usually have extensive myoliquefaction, although other lesions, mainly involving the gills or skin, have been reported (Cheung et al., 1980; Yoshinaga and Nakazoe, 1993; Dykova and Figueras, 1994). Also, infection with *Uronema* sp. is regarded as a significant cause of mortality in captive southern bluefin tuna at Port Lincoln, South Australia (Munday, 1993). This paper reports the development of an indirect fluorescent antibody test (IFAT) to identify this organism for diagnostic and epidemiological purposes.

Materials and Methods

a) Isolation of Uronema sp.

Isolates of *Uronema* sp. were obtained from diseased tuna (K and Q), blenny larvae (*Parablennius tasmanianus*)(B) and sea horses (*Hippocampus abdominalis*)(SH1, SH2), as well as from tanks containing healthy juvenile and larval flounder (*Rhombosolea tapirina*)(A, F). All isolates were maintained on their natural microbiota by culture in Oxoid brain-heart infusion broth, diluted to 1:10 with sterile sea-water, and supplemented with 25 µL/L lysed sheep blood and 25µL/ L Accomin* ®(mixed vitamin solution). Cultures were left at ambient room temperature (17 - 21 °C) overnight to permit rapid bacterial growth, and then placed at 4 °C for 2 - 3 weeks.

b) Antiserum preparation

Uronema antigen was prepared from isolates Q and B, by adding 1mg/mL each of ampicillin, neomycin and streptomycin to 200 mL of culture to kill the bacteria, which were then removed centrifugally. Three formalin-fixed inocula were prepared from these cultures and administered intramuscularly into rabbits according to standard procedures (Stolen et al., 1990) and the antisera harvested. Pre-immunisation sera were taken for use as negative controls. This procedure was authorised by the Ethics Committee (Animal Experimentation) of the University of Tasmania.

c)Purification of antisera

Bacterial antibodies were adsorbed by adding 125 µL of a 2 x 10^{10} cells/mL suspension, prepared from the natural microbiota, to 5 mL of 1/2 diluted antiserum. The mixture was incubated, with frequent mixing, at 37 °C for 24 h, and then at 4 °C for a further 24 h. After incubation the suspension was centrifuged at 3000 rpm for 10 min and then sterilised by filtration through a 0.2 µm filter. Cross-reacting ciliate antibodies were similarly adsorbed using 50 µL of a marine ciliate concentrate containing 4 x 10^{10} ciliates/mL, obtained from a larval flounder tank.

d)Test methodology

All dilutions were made in sterile phosphate buffered saline pH 7.4(PBS) containing 1% bovine serum albumin. 25 µL drops of *Uronema* culture were air-dried and fixed onto cleaned slides by gentle heating. The slides were stained following a standard procedure (Lam and Mutharia, 1994) with 30 - 60 min incubation times. Slides were counterstained with 2% Evan's blue, mounted in 10% alkaline-buffered glycerol (pH 9.6) and examined at x100 to x200 magnification using a Zeiss epifluorescence microscope. The antibody titre was taken as the last dilution giving strong, apple-green, peripheral fluorescence.

e) Use of antiserum.

Anti-B antiserum diluted to 1/50 was used to:-

1) determine the limits of sensitivity of the antibody as an environmental screening agent. Four replicate 1 L samples of 0.2 µm-filtered sea water were inoculated with 1000, 100, 10, or 1 *Uronema*/mL and fixed overnight with a final concentration of 1% formalin. Each sample was concentrated a thousand-fold by supernatant removal after two centrifugation steps. The final 1 mL amounts were stained in tubes, following the same protocol as the slide IFAT. Six *Uronema* counts were made, in an improved Neubauer counting chamber for the 1000 and 100 *Uronema*/mL inocula, and in 10 µL drops for the 10 and 1 *Uronema*/mL inocula.

2) stain each isolate to determine the usefulness of the antibody as a means of differentiating between *Uronema* sp. and/or strains.

3) stain environmental samples. Slides prepared from approximately 25 µL of sediment, obtained from under a tuna sea-cage, were provided heat fixed from Pt. Lincoln, South Australia. The whole slide was scanned and any fluorescent ciliates, of the same size and shape as *Uronema* sp., were counted.

Table 1: Titre of anti-B and anti-Q antisera pre- and post- adsorption of bacterial and cross-reacting ciliate antibodies

	Anti-B	Anti-Q
Initial titre	1/128	1/32
After adsorption of bacterial antibodies	1/256	1/64
After adsorption of cross-reacting ciliate antibodies	1/50	0

Statistical analysis.

Methods used were Cochran's, the Shapiro-Wilk W test and regression analysis (statistical software package JMP 2.0).

Results

When raw antisera were used in the IFAT there was cross-reactivity with other ciliates and the natural microbiota. Adsorption with bacterial antigens produced an enhanced titre, suggesting that the bacterial antibodies had a blocking effect. In contrast, adsorption with ciliate antigens produced a marked drop in titre indicating the removal of antibody to shared ciliate epitopes (Table 1).

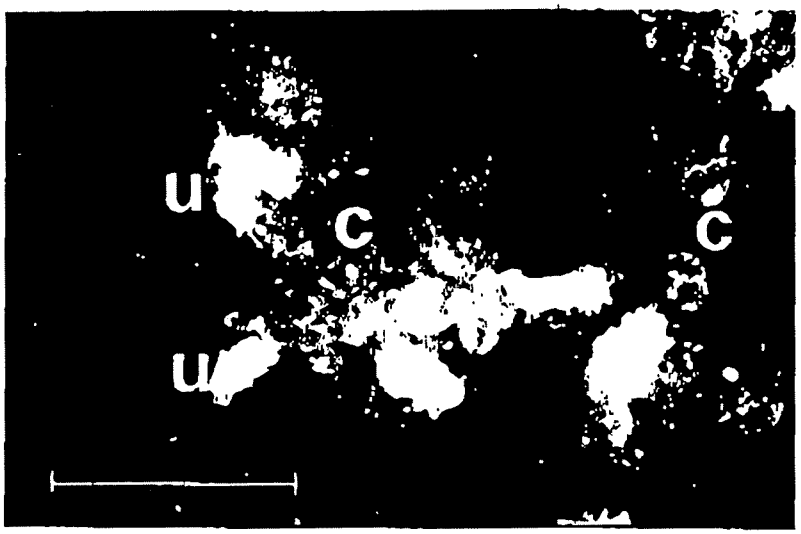

Figure 1 *Uronema* sp. (u) and marine ciliate assemblage (c), showing cross-reactivity with ciliate mixture and specific fluorescence with *Uronema* sp. Scale bar = 100 µm.

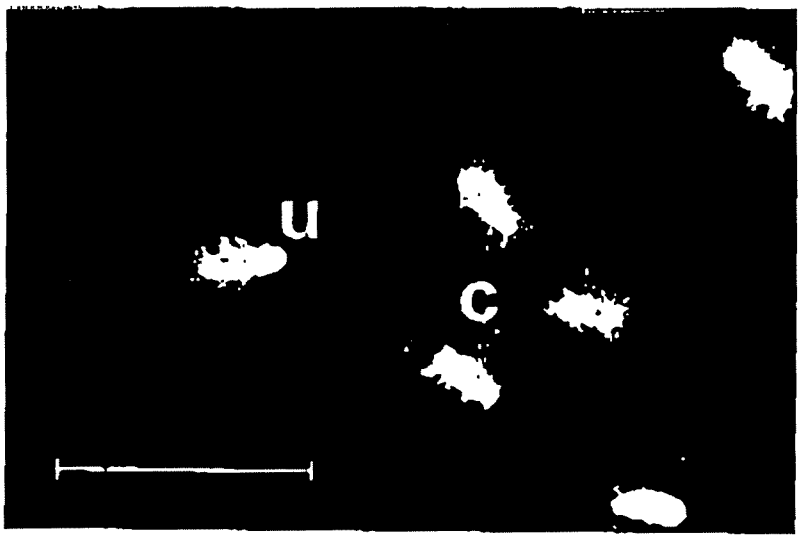

Figure 2 *Uronema* sp. (u) are easily distinguished from other ciliates (c) as cross-reacting antibodies have been removed. Scale bar = 100µm.

The effect of adsorption of cross-reacting ciliate antibodies is demonstrated with unadsorbed anti-B antiserum (Fig. 1) and adsorbed anti-B antiserum (Fig. 2).This test is rapid and robust, taking only 2 h to obtain a result with fluorescence persisting for more than 9 months.

In the recovery experiment regression analysis (r^2 = 0.94 p < 0.001) indicated a strong linear relationship between the numbers of *Uronema* inoculated and numbers recovered. The antiserum was able to detect down to 10 *Uronema*/mL in a 1 L sea water sample (Fig 3).

Direct counts of fluorescent, presumptive, *Uronema* sp. on slides of sediment samples from Port Lincoln revealed > 10 organisms per slide. This equates to >400 *Uronema* sp./mL of sediment.

When stained with adsorbed anti-B antiserum, a different response was obtained with each isolate, ranging from a trace of fluorescence with isolates Q and K, slight fluorescence with isolates SH2 and F, strong fluorescence with isolates B and A and, interestingly, the strongest fluorescence with isolate S111. In addition, a variable staining response was observed between individual organisms within a culture.

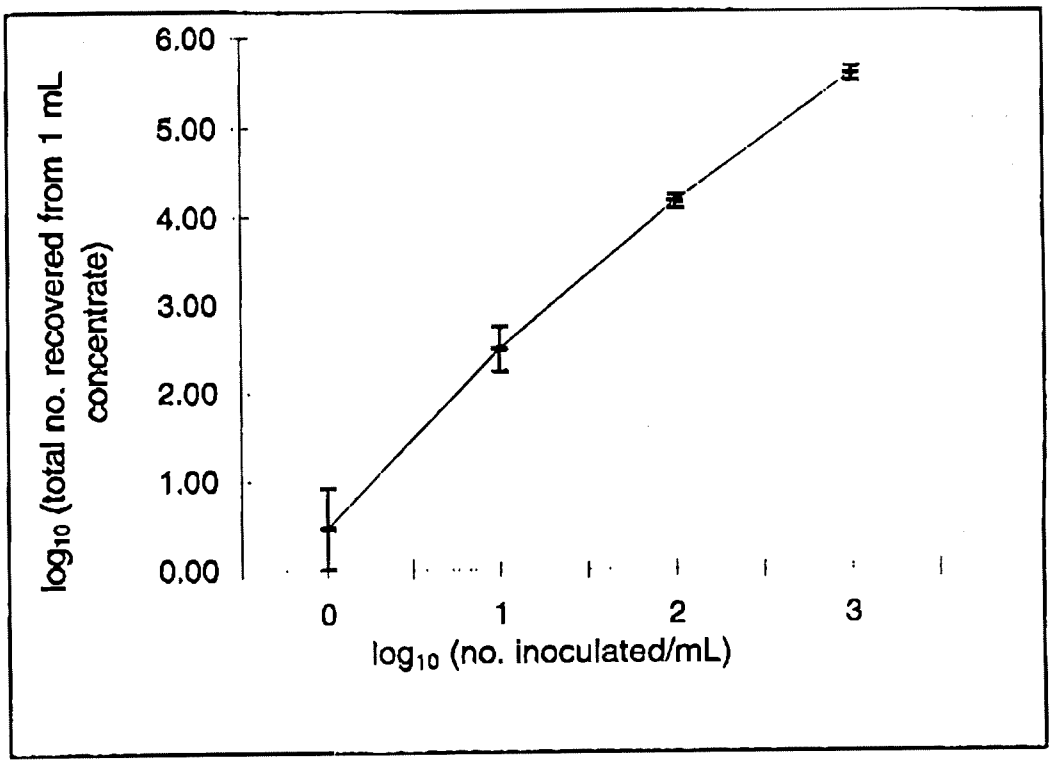

Figure 3 Log_{10} (*Uronema*/mL) inoculated into 1 L of sea water, plotted against log_{10} (*Uronema*/mL) concentrate recovered from 1 L of sea water. Bars show standard error of the mean of six counts.

Discussion

The demonstrated cross-reactivity of *Uronema* sp. with other marine ciliates is not surprising in view of the fact that ciliates share many structural proteins, particularly epiplasmins (Grain, 1986). Because of this, systematic adsorption and screening with type specimens would be necessary to fully verify the specificity of the adsorbed antiserum. The observed blocking effect by bacterial antibodies suggests that they were cross-reactive with ciliate epitopes, which has not been previously reported, even though cross reactivity between ciliate monoclonal antibodies and bacterial epitopes has been documented (Dohra *et al.* (1994).

By fixing and concentrating 1 L of sea water anti-B antiserum was able to detect ≥10 *Uronema*/mL. As sediment samples were found to contain approximately 400 presumptive *Uronema*/mL, it is likely that the IFAT would be useful as a monitoring tool.

A variable staining pattern, both within and between isolates, was obtained with adsorbed anti-B antiserum. Within culture variation indicates that immunologically different strains were present and/or organisms were expressing a different antigenic profile due to different physiological states. The variation between cultures suggests the occurrence of different strains of *Uronema* which is conceivable because the cultures were derived from different sources. Future work should address this issue by reducing

the variability in the cultures and testing them in defined physiological states. Attempts should be made to achieve axenic culture (Hanna and Lilley, 1974) to remove bacterial interference. Cloning cultures (Soldo and Brickson, 1993) would make it possible to ascertain if the difference in immunological reactions observed within and between cultures is due to different biotypes, different species or merely variations in physiological states. As morphological data is difficult to interpret (Perez-Uz, 1995), the response to the antiserum may form the basis for a rapid speciation test if these data can be supported by genetic analysis or other taxonomic procedures.

Acknowledgments

The authors are grateful to Kirsten Rough, of the Tuna Boat Owners Association, Port Lincoln, for providing the sediment samples and *Uronema* isolate K, Dr. P. O'Donoghue, Department of Parasitology, University of Queensland, for providing *Uronema* isolate Q, and to Mr. B. Day and Mr. D. Kunde, Department of Applied Biomedical Sciences, University of Tasmania for their advice.

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Fatal encephalitis due to the scuticociliate *Uronema nigricans* in sea-caged, southern bluefin tuna *Thunnus maccoyii*

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ABSTRACT: A syndrome characterized by atypical swimming behaviour followed by rapid death was first reported in captive southern bluefin tuna *Thunnus maccoyii* (Castelnau) in the winter of 1993. The cause of this behaviour was found to be a parasitic encephalitis due to the scuticociliate *Uronema nigricans* (Mueller). Based on parasitological and histological findings, it is proposed that the parasites initially colonise the olfactory rosettes and then ascend the olfactory nerves to eventually invade the brain. Possible epidemiological factors involved in the pathogenesis of the disease include water temperature (<18°C) and the immune status of the fish.

KEY WORDS: Southern bluefin tuna · *Thunnus maccoyii* · Scuticociliate · *Uronema nigricans* · Encephalitis · Neuritis

INTRODUCTION

Capture and fattening of southern bluefin tuna (SBT) for the Japanese sashimi market is a recent innovation in Australia and is entirely localised to Port Lincoln in South Australia. Wild SBT in the range of 15 to 35 kg bodyweight are captured, usually by purse-seine netting in the southern summer/fall (December to May) and placed in sea-cages where they are fed frozen 'bait' fish, predominantly pilchards *Sardinops* spp.

Once accustomed to captivity, the fish fatten quickly and harvesting commences within 3 mo of capture with all fish being removed from the sea-cages prior to the intake of the next batch of tuna.

Individual, market-ready fish have a value of $A1000–2000 and the total value of the industry has increased from $A1.8m in 1991-92 to $A64.0m in 1994–95.

Apart from immediate and delayed mortalities associated with capture and towing in special cages, SBT have proven to be very robust and disease problems were minimal in 1991 and 1992. However, in the winter/spring (June–October) of 1993 significant mortalities were reported in association with unusual clinical signs. This previously undescribed syndrome is the subject of this report.

MATERIAL AND METHODS

Fish. Clinically affected *Thunnus maccoyii* (Castelnau), colloquially termed 'swimmers', were all captive and had been held for periods of 3 to 8 mo. Appropriate samples for comparative purposes were collected from unaffected captive tuna.

Clinical parameters. Information on the proportion of fish affected and clinical signs were provided by farm operators. Clinically affected fish were also observed by some of the authors.

Clinical pathology. Blood samples from affected and normal tuna were collected by cardiac puncture into plain and EDTA and fluoride-oxalate treated tubes.

Haematocrits and differential leucocyte counts were undertaken at the Tuna Boat Owners Association laboratory at Port Lincoln.

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Blood samples were submitted to Veterinary Pathology Services, Adelaide, for estimation of haemoglobin and glucose in whole blood, and creatine phosphokinase, alanine aminotransferase, protein, calcium, magnesium, sodium, chloride, potassium and cortisol in serum.

Results for affected and unaffected fish were compared for significant differences using the Student's *t*-test.

Macroscopic and microscopic pathology. Most affected fish were collected alive although a small number of presumptive 'swimmers' were retrieved within 6 h of death from the bottom of the net pens. Because affected fish still had a monetary value, full necropsies were only undertaken on a small number. The other diseased tuna and all normal fish were examined by a modified necropsy technique which permitted collection of selected samples. Some difficulty was experienced in obtaining the tissues of choice (central nervous system) because in fish for the sashimi market the brain and spinal cord are immediately destroyed by trephining a hole in the skull and passing a wire down the spinal canal.

Minimal samples for histological examination were olfactory rosettes, olfactory nerves and brain. Additional highly desirable samples were spinal cord and optic nerves. Other samples taken depending on circumstances were gills, heart, liver, pancreas, spleen, head and tail kidney and skeletal muscle.

Tissues were fixed in 10% formal saline then trimmed and embedded in paraffin wax, before sectioning at 5 µm and staining with haematoxylin and eosin.

Parasitology. Live ciliates were observed in wet preparations of cerebrospinal fluid and brain using normal saline or seawater as diluent where necessary. Examination was at 100 and 400× using bright-field, phase-contrast and Nomarski interference-contrast illumination.

Culture of these protozoans from central nervous tissues was achieved using brain heart infusion broth diluted 1/10 v/v with sterile 0.2 µm filtered seawater. For each 1 l of this medium 25 µl of lysed blood and 25 µl of vitamin solution (Accomin, Cyanamid) was added. Marine environmental bacteria were added to the broth to provide a food source and these were encouraged to grow by leaving the culture at room temperature overnight before reducing the temperature to 4°C to prevent bacterial overgrowth (Watts 1995).

Axenic culture of central nervous tissue taken aseptically was performed using a modified marine axenic medium (Messick & Small 1996).

Live ciliates were examined unstained and stained with methyl-green pyronin to record vital characteristics and patterns of motility. Clinical isolates and cultured ciliates were fixed in Bouin's fluid, washed in distilled water and subsequently stained by silver proteinate (protargol) impregnation using standard techniques (Foissner 1991). Stained ciliates were examined by light microscopy, measured using a calibrated eyepiece graticule, drawn with the aid of a camera lucida and photographed.

RESULTS

Epidemiological information

Clinical cases of 'swimmer' syndrome have only been reported between May and November when water temperatures fall below 18°C with most cases occurring at temperatures below 15°C. In 1993 the number of affected tuna was reported as 5 to 10% of the fish remaining after June of that year. Mortalities in 1994 and 1995 were reportedly lower, but, because of the high value of individual fish, were still of concern.

Clinical signs

Affected tuna were in good condition and usually died within 2 to 8 h of being first detected, although the occasional fish survived for 24 to 72 h. Typically, the fish came to the surface, turned light blue and swam vigorously around the cage. Eventually, the fish ceased compulsive swimming and exhibited short bursts of forward motion with their heads out of water, followed by periods of sinking, before once again coming to the surface and then repeating the process. Finally, the fish sank and died at the bottom of the netpen.

Clinical pathology

Significant variations from presumptive normal values were found for serum protein, magnesium, sodium, chloride, cortisol (elevated) and potassium (lowered) and blood glucose (elevated). These alterations were considered to be related to general stress resulting from the disease process and were of no direct diagnostic value. Mean values for normal and affected fish are shown in Table 1.

Macroscopic lesions

Specific changes have been restricted to the olfactory rosettes and brain. In affected fish the olfactory rosettes were frequently darkened and the brain exhibited variable degrees of softening/liquefaction.

Table 1. *Thunnus macroyii*. Clinical pathology results for normal and diseased (swimmer) tuna. Significant differences shown by $^{*}p < 0.01$, $^{**}p < 0.001$

Parameter (unit)	Normal Mean (SE)	n	Swimmer Mean (SE)	n
Haemaglobin (g l^{-1})	15.94 (0.26)	10	16.23 (0.25)	4
Haematocrit (%)	47.40 (0.78)	10	50.00 (1.11)	4
Creatine phosphokinase (U l^{-1})	629.90 (136.56)	10	568.20 (99.42)	10
Alanine aminotransferase (U l^{-1})	13.00 (5.82)	10	3.60 (0.35)	10
Protein (g l^{-1})	53.30 (0.78)	10	60.10 (0.89)*	10
Magnesium (mmol l^{-1})	0.84 (0.01)	10	1.92 (0.21)*	10
Sodium (mmol l^{-1})	192.10 (0.90)	10	206.60 (2.00)*	10
Potassium (mmol l^{-1})	4.33 (0.21)	10	2.28 (0.16)**	10
Chloride (mmol l^{-1})	148.90 (0.75)	10	177.60 (1.27)**	10
Calcium (mmol l^{-1})	3.29 (0.03)	10	3.51 (0.05)	10
Cortisol (nmol l^{-1})	15.70 (5.48)	10	1195.50 (17.57)**	10
Glucose (mmol l^{-1})	6.94 (0.27)	10	14.31 (0.61)**	10
Heterophils (% range)	4.61–11.26	10	1.29–9.15	10
Monocytes (% range)	1.19–5.92	10	2.58–3.27	10
Lymphocytes (% range)	48.68–51.66	10	56.86–61.94	10
Eosinophils (% range)	2.63–7.29	10	0–3.92	10
Thrombocytes (% range)	17.26–38.16	10	26.80–34.19	10

Microscopic lesions

Lesions of the brain parenchyma were restricted to the forebrain where pyriform organisms were found in varying numbers in brain tissue. Peripheral cilia were detected in a few organisms and a macronucleus was visible (Fig. 1). In some lesions there were numerous ciliates containing erythrocytes and food-granules present in areas of liquefaction (Fig. 2). At the other extreme, organisms were rare and they contained fewer food granules and there was no liquefaction of the brain parenchyma. In both instances, there was no host response apart from the presence of compound granular corpuscles in areas of liquefaction.

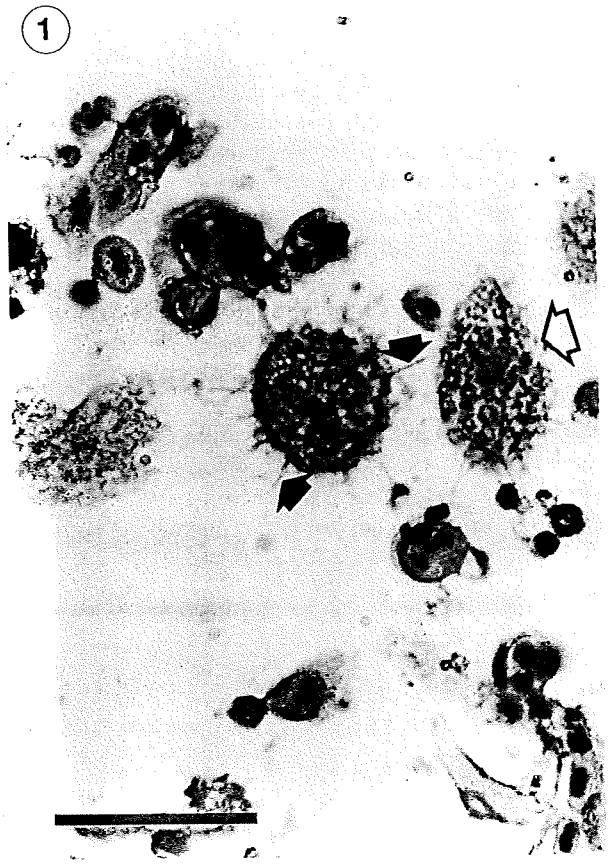


Fig. 1. *Uronema nigricans* in brain of southern bluefin tuna *Thunnus maccoyii*. Note tear drop shape in longitudinal section (open arrow) and presence of cilia in parasite cut in cross section (solid arrows). H&E. Scale bar = 25 µm

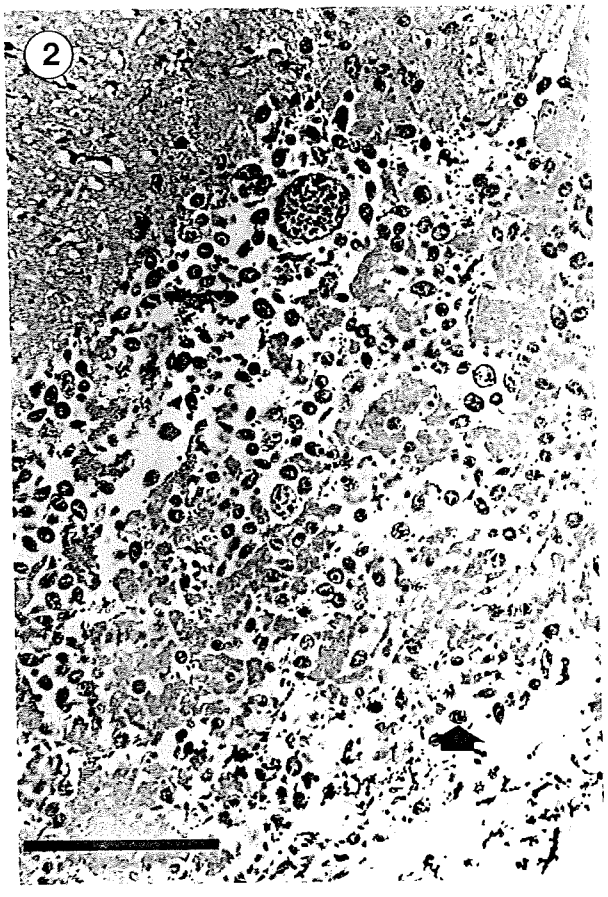


Fig. 2. Area of liquefactive necrosis in forebrain of bluefin tuna *Thunnus maccoyii*. Note numerous scuticociliates (solid arrow). H&E. Scale bar = 250 µm

Contrary to the situation in other fish (Ferguson 1989) lateral ventricles were present in the brain and there frequently was a mild to moderate, lymphocytic ependymitis. In some instances haemorrhage was associated with the parasitic invasion. Similar lesions were also found in the third ventricle.

Focal meningitis was a common finding. This varied from a mild lymphocytic invasion accompanied by oedema to thickening of the meninges with associated lymphocytic reaction. Variable numbers of ciliates were consistently found in these lesions and usually they contained relatively few food granules (Fig. 3).

Lesions were consistently found in the olfactory nerves. The most common finding was the presence of linear necrosis often with accompanying lymphocytic infiltration. Ciliates were usually associated with the lesions (Fig. 4). In some instances focal zones of necrosis led to partial or even complete destruction of the nerve—in the latter instance there was thrombosis of blood vessels and invasion by macrophages. Inflammatory lesions were consistently found in the perineural tissues. This inflammation was usually lymphocytic in nature but in one, apparently longer-standing lesion, there were macrophages and eosinophilic granule cells in the thickened, fibrotic tissues (Fig. 5).

In the olfactory rosette specific lesions were only found in the axis. In those instances where there was significant necrosis, ciliates were present in moderate numbers. Otherwise there was an inflammatory response consisting of oedema, lymphocytes, plasma cells, macrophages and eosinophilic granule cells and only a few, scattered ciliates (Fig. 6).

Parasitology

Culture

The ciliate grew readily in brain heart infusion with marine bacteria, but care was needed to ensure that bacterial overgrowth did not occur and lead to elimination of the protozoans. Axenic organisms grew more slowly (in the order of approximately 25% the rate of non-axenic culures).

Live observation

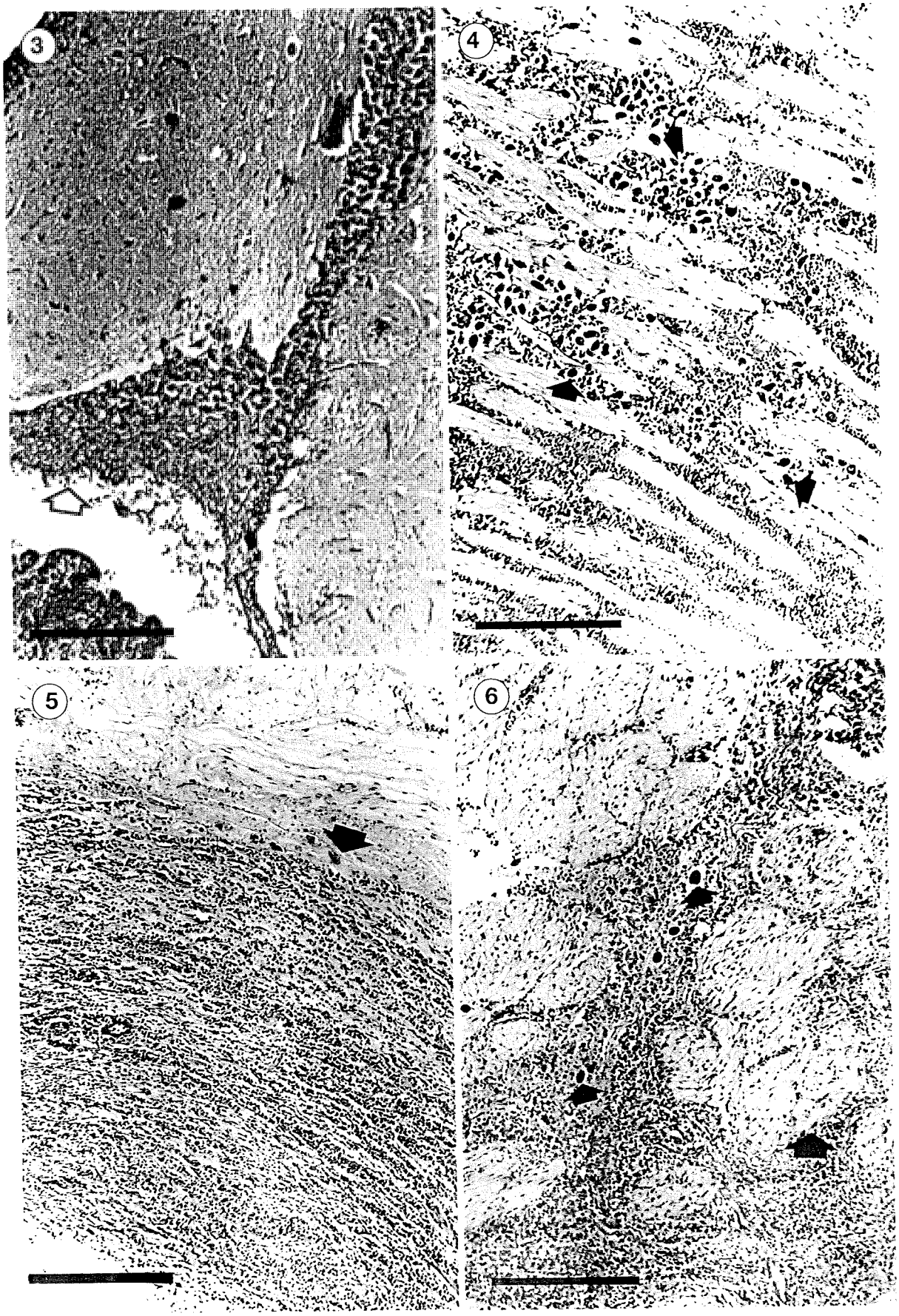
Numerous ciliates were detected in cerebrospinal fluid recovered from the brain cavities and olfactory nerves of infected tuna. The ciliates ranged from 22 to 32 µm in length by 12 to 18 µm in width and were pyriform in shape with a slightly flattened and oblique anterior margin (Fig. 7).

The ciliates were uniformly covered with short somatic cilia except for the anterior pole which remained naked. All ciliates possessed a prominent caudal cilium approximately twice the length of the somatic cilia. The oral ciliature was inconspicuous in unstained preparations and was located in a slight subapical depression extending backwards to the equator. Supravital staining with methyl-green pyronin revealed the presence of a large spherical macronucleus and a small micronucleus located in the anterior to middle portion of the cell. The ciliate pellicle contained numerous trichocysts distributed over the entire surface. Ciliates recovered from tuna were robust and granular in appearance due to the presence of numerous pale-green and red food vacuoles located mainly in the posterior half of the body (Fig. 8a). Bilobed lamellar vesicles were also occasionally observed in the cytoplasm. The ciliates contained a single translucent contractile vacuole located near the posterior pole. Cultured ciliates were more slender in appearance and usually contained fewer food vacuoles (Fig. 8b). The ciliate pellicle was colourless and ridged between somatic kineties (Fig. 8a, b). The ciliates were highly motile in wet mounts and they exhibited a smooth swimming motion while slowly rotating about their long axes in a corkscrew pattern. The ciliates were somewhat elastic in shape and were observed to distend and stretch their way through small gaps. They were actively bactivorous and stopped frequently to feed on bacterial masses. They were also observed to congregate and browse over particulate material but it is not known whether this represented histophagous behaviour. Cyst formation was not observed even when wet mounts became depleted of tissue or dried out.

Silver impregnation

The ciliates readily stained with protargol revealing details of their oral and somatic ciliature (Figs. 8 to 10).

Figs. 3 to 6. *Thunnus maccoyii* infected with *Uronema nigricans*. Fig. 3. Meningitis of tuna forebrain (open arrow) associated with numerous *U. nigricans*. H&E. Scale bar = 250 µm. Fig. 4. Olfactory neuritis associated with *U. nigricans* (small solid arrows). H&E. Scale bar = 250 µm. Fig. 5. Marked olfactory perineuritis in bluefin tuna. A few *U. nigricans* are present within the nerve tissue adjacent to the inflammatory reaction (solid arrow). H&E. Scale bar = 250 µm. Fig. 6. Inflammatory response to scuticociliates (small solid arrows) in the olfactory rosette of a bluefin tuna. Note branch of olfactory nerve (large solid arrow). H&E. Scale bar = 250 µm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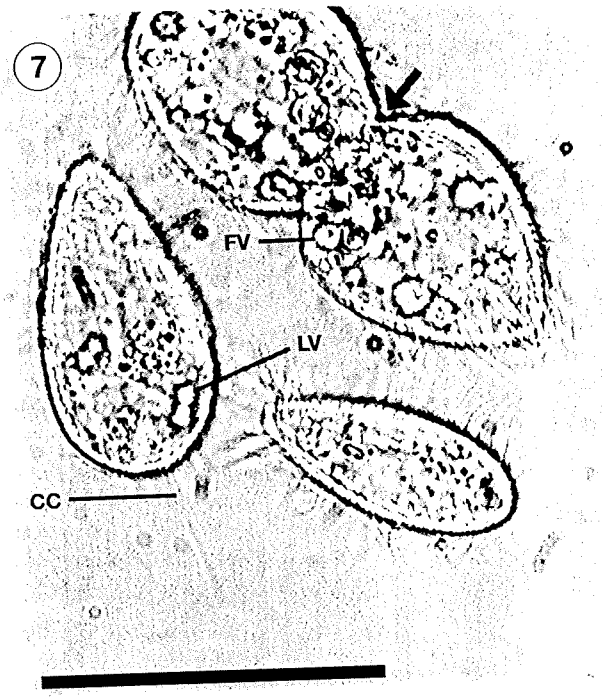


Fig. 7. *Uronema nigricans* in *Thunnus maccoyii* cerebrospinal fluid. Live organisms of variable size in a wet preparation. Note a dividing cell (arrow) containing numerous food vacuoles, one of which is labelled (FV). A lamellar vesicle (LV) is indicated in the cell on the left and the caudal cilium (CC) is clearly visible. Scale bar = 25 µm

Their key morphometric characteristics are given in Table 2. Fixed and stained ciliates were variable in size ranging from 19.8 to 34.0 µm in length by 7.1 to 20.0 µm in width. They contained distinctive somatic ciliature consisting of 12 to 14 longitudinal kineties arranged in meridional rows. The first and last kineties curved around the buccal apparatus and all meridians terminated before reaching the anterior or posterior poles (Fig. 8c). The first kinety was slightly shorter than the remainder and terminated near the contractile vacuole pore. Each kinety contained 24 to 30 kinetosomes which were paired in the anterior two-thirds of the cell. Only the posterior 2 to 4 kinetids were monokinetids whereas the remainder were dikinetids. The somatic cilia measured on average 3.2 µm in length and the caudal cilium 7.7 µm in length (Table 1). The caudal cilium arose from a kinetosome located between 2 parasomal sacs located at the posterior pole (Fig. 8c). The oral ciliary field was always located in the anterior half of the body, beginning on average 3.1 µm from the anterior pole and extending 11.2 µm backwards to the equator. The oral apparatus consisted of an elongate paroral (undulating) membrane on the right and 3 shorter membranelles on the left (Fig. 8c). The first membranelle appeared as a single row of 4 kinetosomes and the second membranelle as a double row of 4 kinetosomes.

The first membranelle did not appear to be ciliated. Individual kinetosomes were not discernible in the third membranelle or the undulating membrane. The former was triangular in shape and the latter was elongate, beginning adjacent to the second membranelle and curving posteriorly around the cytostome. A non-ciliated scuticum consisting of 3 to 5 kinetosomes was located below the cytostome in line with the undulating membrane. A cytoproct was visible as a faint irregular line located posterior to the scuticum. The ciliates contained a single ovoid macronucleus measuring on average 7.1 µm in diameter located around the middle of the cell next to a small ovoid micronucleus measur-

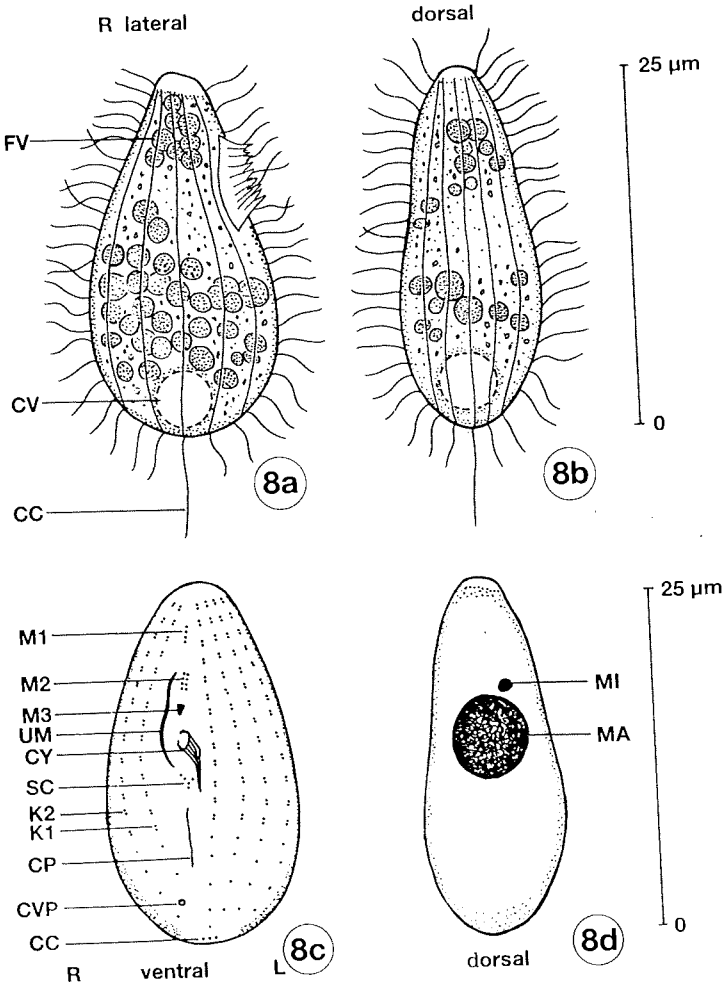


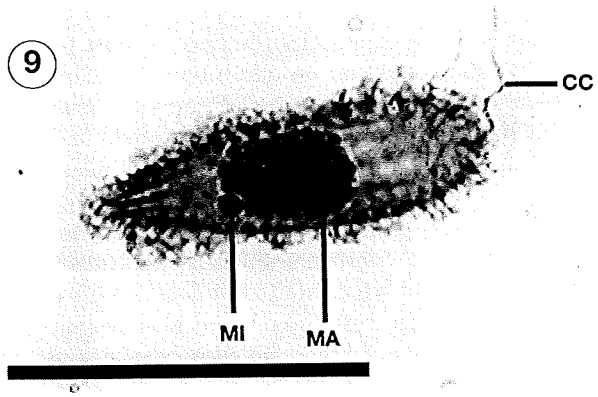
Table 2. *Uronema nigricans*. Morphometric characterization of scuticociliate recovered from the forebrain of southern bluefin tuna *Thunnus maccoyii*. SD: standard deviation; CV: coefficient of variation; Min: minimum; Max: maximum; n: number of observations

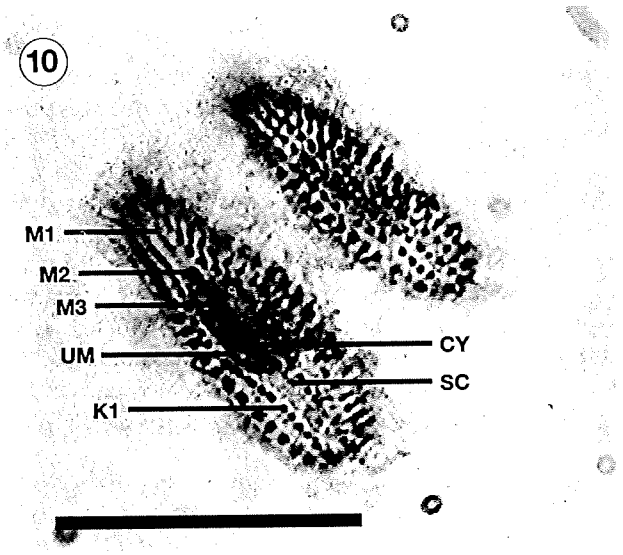
Character	Mean	SD	CV	Min	Max	n
Body dimensions						
Length (µm)	26.1	3.59	13.8	19.8	34.0	20
Width (µm)	11.9	3.62	30.3	7.1	20.0	20
Nuclei						
Macronucleus diameter (µm)	7.1	1.83	25.7	4.2	10.4	20
Micronucleus diameter (µm)	1.1	0.29	26.8	0.6	1.7	20
Somatic ciliature						
Total number of kineties	12.4	0.84	6.8	12	14	10
Length of somatic cilia (µm)	3.2	0.60	18.5	2.8	5.0	14
Length of caudal cilium (µm)	7.7	0.31	4.1	7.1	8.2	10
Oral ciliature						
Distance from apex to oral ciliary field (µm)	3.1	0.73	23.2	2.1	5.2	18
Length of oral ciliary field (µm)	11.2	2.14	19.1	7.2	16.5	17
Length of undulating membrane (µm)	7.7	1.63	21.1	4.9	10.4	17
Length of first membranelle, M1 (µm)	1.7	0.40	23.8	1.0	2.4	18
Length of second membranelle, M2 (µm)	2.2	0.48	21.6	1.4	3.1	18
Length of third membranelle, M3 (µm)	0.9	0.20	22.1	0.5	1.2	17

ing 1.1 µm in diameter (Fig. 8d). On the basis of their morphological characteristics (summarized by Thompson & Evans 1968, Dragesco & Dragesco-Kernéis 1986, Foissner et al. 1994), the ciliates were identified as belonging to the species *Uronema nigricans* (Mueller 1786) Florentin 1901.

DISCUSSION

Uronema-like scuticociliates, previously regarded as being environmental scavengers, are increasingly being recognised as important opportunistic pathogens of marine fish.





Figs. 8 to 10 (facing page and above). *Uronema nigricans* from southern bluefin tuna *Thunnus maccoyii*. Fig. 8. (a) Right lateral view of robust ciliate recovered from forebrain. (b) Dorsal view of slender ciliate following *in vitro* culture. (c) Ventral view of protargol impregnated ciliate showing oral and somatic infraciliature. (d) Dorsal view of silver stained ciliate showing nuclei. Scale bars = 25 µm. CC: caudal cilium; CP: cytoproct; CV: contractile vacuole; CVP: contractile vacuole pores; CY: cytostome; FV: food vacuoles; K1: first kinety; K2: second kinety; L: left side; MA: macronucleus; MI: micronucleus; M1, M2, M3: first, second and third membranelles; R: right side; UM: undulating membrane; SC: scuticum. Fig. 9. A protargol-stained cell showing the micronucleus (MI), macronucleus (MA) and caudal cilium (CC). Scale bar = 25 µm. Fig. 10. Protargol-stained cells. Ventral view demonstrates membranelles 1, 2 and 3 (M1, M2, M3). The undulating, or paroral membrane (UM), cytoproct (CY) and scuticum (SC) are also visible. The first kinety is indicated (K1). Scale bar = 25 µm

In finfish, they have been implicated in disease of all growth phases. Munday (1996) reported generalised infections leading to severe mortalities in larval marine fish. Heavy mortalities due to histophagous ciliates were reported in juvenile turbot *Scopthalmus maximus* by Dykova & Figueras (1994) and significant disease has been reported in flounder (Yoshinaga & Nakazoe 1993) and sea bass (Dragesco et al. 1995). Subadult and adult fish affected by scuticociliasis usually suffer from a parasitic myositis (Cheung et al. 1980, Bassleer 1983, Munday 1996), but seahorses may be more affected by inflammatory lesions of the skin and gill (Cheung et al. 1980, Munday 1996). However, the type of pathology found in SBT appears to be unique except for a single case in a yellowtail kingfish *Seriola lalandi* captured in a tuna cage (Rough unpubl.).

Scuticociliates have also been associated with clinical disease in crabs, lobsters and prawns (Morado & Small 1995, Cawthorn et al. 1996) and clam *Venecrupis* sp. larvae (G. Maguire pers. comm.) thus indicating the great potential importance of these pathogens in marine aquaculture.

In many instances the protozoans associated with particular outbreaks have not been fully identified. Cheung et al. (1980) classified the organism present in aquarium fish as *Uronema marinum*. The ciliates present in SBT were clearly hymenostomes with well-defined oral and somatic ciliature, the former comprising an undulating membrane and 3 membranelles. They possessed a distinctive scuticum located below the oral ciliary field and were readily identified as scuticociliates (Corliss 1979). They belonged to the suborder Philasterina because their undulating membranes were shorter than the other oral structures and the membranes were not reinforced by ribbed walls (Small & Lynn 1985). Of the 12 constituent families, the ciliates belonged to the family Uronematidae because the undulating membrane and 3 membranelles were aligned with the long axis of the body and the anterior pole was nonciliated and flattened. The ciliates were differentiated from 5 other genera and assigned to the genus *Uronema* on the basis of the location of the cytostome which was always anterior to the equator (Small & Lynn 1985).

Over 25 *Uronema* spp. have previously been described, all originally being reported as free-living organisms in freshwater or marine habitats. Of these, only 10 species have been described (or redescribed) using silver impregnation techniques (cf. Pérez-Uz & Song 1995). The morphological characteristics of the ciliates recovered from the tuna conformed to those previously reported for the species *U. nigricans* by Thompson & Evans (1968), Dragesco & Dragesco-Kernéis (1986), Song (1991), Foissner et al. (1994) and Pérez-Uz & Song (1995). They could be differentiated from all other *Uronema* spp. on the basis of their size, number of somatic kineties, alignment of caudal cilium complex, position of contractile vacuole, length and position of oral apparatus and location of cytostome. They were most similar to *U. marinum* except that the latter were larger in size, contained more somatic kineties and the oral apparatus was located further from the anterior pole (Thompson 1964, Dragesco & Dragesco-Kernéis 1986, Coppellotti 1990, Foissner et al. 1994). Several authors have previously suggested that *U. nigricans* and *U. marinum* be synonymized due to their superficial similarities (e.g. Hoare 1927, Kahl 1931) whereas others have suggested that they be retained as separate species on the basis of their occurrence in freshwater and marine habitats respectively (Thompson & Evans 1968). However, *U. nigricans* has been recorded from both freshwater and marine habitats and various isolates have exhibited good growth in brackish and seawater (Pérez-Uz 1995). Pending further characterization studies, we regard them to be separate species on the basis of their morphological differences and do not consider them to be restricted to specific habitats. The ciliates detected in the tuna were therefore identified as *U. nigricans* (Mueller 1786) Florentin 1901.

Presumptive diagnosis of the disease in SBT can be made by examining wet preparations of CSF and brain. However, although a fluorescent antibody test has been developed for cultured and environmental organisms (Watts 1995), this is not suitable for clinical material because of autofluorescence of host tissues. Definitive diagnosis can be made by microscopic examination of histological slides of nervous tissues. As clinical pathology only reflects general stress and perturbed osmoregulation, it is of no specific diagnostic value.

The proposed pathogenesis of the disease in SBT is that *Uronema nigricans* initially parasitizes the olfactory rosette at which stage the host mounts a vigorous inflammatory response. If the host response is inadequate, the ciliate then invades branches of the olfactory nerve present in the axis of the olfactory rosette. Even though there is still some host response to invasion of the olfactory nerve the migration of *U. nigricans* is probably then inexorable, ending in invasion of the brain, which causes locomotor dysfunction and, ultimately, death.

Epidemiological factors which may be implicated in the initiation of the disease in SBT are water temperature and host immune status.

The 'swimmer' syndrome has not occurred when water temperatures have exceeded 18°C. SBT maintain a body temperature of about 24°C, even in much colder water (A. Smart pers. comm.), so it is possible that the parasite, which would be expected to have an

optimum growth temperature of about 25°C (Parker 1976), would be preferentially attracted to the fish under conditions of relatively low water temperatures (<18°C).

Studies undertaken by Fitz-Gerald & Bremner (1997) have shown that the pilchards fed to captive SBT are highly oxidised. When this is taken in the context of the work of Obach & Baudin Laurencin (1992), who demonstrated immunosuppression in turbot fed a diet high in oxidised oils, it is likely that the tuna become immunocompromised as a result of the type of diet they receive. Whether or not other factors, such as the stress of captivity, could also affect immunocompetence is more difficult to decide. The immune status of captive SBT is now under investigation by the authors.

Attempts to control the disease in SBT will focus on improving the environmental conditions and optimising the immune system of the tuna by dietary manipulations and/or the addition of immunomodulators to the diet.

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