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In vitro studies on optimal requirements for the growth of *Spironucleus vortens*, an intestinal parasite of the freshwater angelfish

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ABSTRACT: *Spironucleus vortens* were cultivated in either an artificial medium at different temperatures, or in medium at various pH conditions or supplemented with different bile concentrations at 25°C. Temperature, pH and bile requirements for the optimal growth of the parasite were determined. Parasites multiplied quickly at 28 and 31°C and reached maximum numbers on Day 4 of cultivation, whereafter they did not survive. At 25°C, parasites survived longer than those at 28 and 31°C with no difference in multiplication rate during the exponential phase. The longest survival period was seen at 22°C, although the growth rate of the parasite was not as high as those at 25°C. At a higher temperature of 37°C, no parasites were observed alive after the second day of cultivation. Optimal pH range for the parasite's growth was 6.5 to 7.5, with the highest cell number at pH 7.5. Parasites survived longest (15 d) at pH 6.0, although the maximum number of cells was lower than those at the optimal pH. Parasites were dead within 24 h at pH levels above 8.5 or below 5.5. All cultures supplemented with either bovine or fish bile yielded numbers of parasites lower than cultures with no bile. In addition, parasite growth was significantly suppressed in medium supplemented with higher concentrations of bile. These results indicate that the optimal condition for the *in vitro* cultivation of *S. vortens* is 25°C and pH 6.5 to 7.5 without supplementation with bile.

KEY WORDS: Hexamitid · Diplomonad · *Spironucleus vortens* · Angelfish · *Pterophyllum scalare* · *In vitro* · Cultivation · Protozoa

INTRODUCTION

Spironucleus spp. are parasitic flagellates found in both freshwater and saltwater fish. They have been recorded in marine gadoids, Atlantic cod and haddock (Poynton & Morrison 1990), and Atlantic salmon (Sterud et al. 1997). In freshwater fish, *Spironucleus* spp. have been recorded in grayling (Sterud et al. 1997), burbot (Sterud 1998a), and cichlids, including angelfish (Kulda & Lom 1964b, Specht et al. 1989, O'Brien et al. 1993, Poynton et al. 1995), and cyprinids (Molnár 1974). The parasite has been associated either locally in the digestive tract or systemically in various organs of the fish. They can cause an enteritis, especially of the posterior intestine, varying in severity from a diffuse lymphoplasmacytic infiltration to a severe intestinal necrosis (O'Brien et al. 1993). *Spironucleus* spp. may reach the blood stream and liver by invasion across the injured intestinal wall, and are capable of causing severe parasitemia in a host under stress conditions (Molnár 1974). It has been suggested that *Spironucleus* spp. cause systemic infection because they can invade intestinal mucosa and disseminate to other tissues (Siddall et al. 1992). In sea-caged Atlantic salmon *Salmo salar* L., *Spironucleus barkhanus* has been reported causing the death of fish, and the parasites were found in large numbers in most internal organs (Mo et al. 1990, Poppe et al. 1992).

Spironucleus vortens Poynton et al., 1995 was first successfully isolated from the freshwater angelfish *Pterophyllum scalare*. The organism can be maintained and propagated in artificial medium (Poynton et al. 1995). A few studies have suggested the *in vitro* growth requirements of fish hexamitids (Poynton et al.

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1995, Sterud 1998b), but none have examined the specific optimal requirements for growth of *S. vortens*. Although *S. barkhanus* cultivated in bile-supplemented medium have shown a slight difference in optimal temperatures between strains isolated from salmon and grayling (Sterud 1998b), the optimal pH and bile requirement for *Spironucleus* spp. are still unknown. In addition, most of the *in vitro* cultivation of diplomonads, including fish hexamitids, have been done in culture medium supplemented with bovine bile (Keister 1983, Buchmann & Uldal 1996, Sterud 1998b). To date, none of the fish hexamitids have been cultivated in medium supplemented with fish bile. In the present study, bile originating from cattle or fish was added to the culture medium and evaluated with temperature and pH for the optimal conditions maximizing the *in vitro* growth of *S. vortens*.

MATERIALS AND METHODS

Parasite cultivation. Cryopreserved *Spironucleus vortens* were obtained from the American Type Culture Collection (ATCC No. 50386, Manassas, VA, USA). The parasites were thawed by immersion of the vial into water at 35°C for 2 min and the suspension (0.5 ml) immediately added to a 16 × 125 mm sterile screw-capped glass tube containing 13 ml culture medium. The culture tube was tightly closed and placed on a 15° horizontal slant in an incubator without light at 25°C. The parasites were propagated and subcultivated every 6 d by transferring 0.1 ml of parasite suspension to a sterile tube with 13 ml fresh culture medium.

Culture medium. The flagellates were cultivated in sterile TYI-S-33 medium (ATCC No. 350-X) at pH 6.8 supplemented with the antibiotics, penicillin (2000 U ml^{-1}) and gentamicin (50 µg ml^{-1}).

Parasite cell counting. The concentration (average cell number ml^{-1}) of *Spironucleus vortens* in culture medium was determined daily by automatic cell counter (CASY®1; model TTC, Schärfe System GmbH, Germany). The parasite suspension was gently mixed and aliquots of 5 or 10 µl were added into counter containers with 10 ml PBS, pH 7.4.

Determination of optimal temperature. Culture medium (2 ml, pH 6.8) with *Spironucleus vortens* concentration of 5000 cells ml^{-1} was established in 4 ml screw-capped glass tubes. The parasites were incubated and allowed to grow in thermostat-regulated incubators without light at 22, 25, 28, 31, 34 and 37°C. The cell cultures at each temperature were performed in triplicate. Concentrations of *S. vortens* were determined every 24 h until no parasites were observed alive.

Determination of optimal pH. Parasite cultures (30 ml, 5000 cells ml^{-1}) were established in 50 ml sterile tissue culture flasks. The pH of cultures was adjusted gradually from the original pH (6.8) to the appropriate final incubation pH: 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5 or 10.0 within 4 d of culture initiation. The pH of cultures were measured daily by a pH meter (Accumet® pH meter 10, Fisher Scientific, Pittsburgh, PA) and were adjusted to the final incubation pH by sterile 0.1 N NaOH or 0.1 N HCl. The larger volume of culture media used in this experiment allowed for the aseptic removal of a small sample of media for pH determination, thus preventing the contamination of the primary experimental culture with a non-sterile pH probe. The culture flasks were tightly capped and incubated without light at 25°C. The average cell numbers of 3 aseptically removed subsamples were determined daily by the automatic cell counter as previously described.

Determination of bile requirement. Parasite cultures (2 ml, 5000 cells ml^{-1}) with different concentrations (0.00, 0.05, 0.20, 0.40, 0.80, 1.60, 3.20 and 6.40 mg ml^{-1}) of bovine bile (Sigma Chemical Co., No. B-8381) were established in 4 ml screw-capped glass tubes. Parasite cultures at each bile concentration were performed in triplicate and allowed to grow at 25°C. Concentrations of *Spironucleus vortens* were determined every 24 h until no parasites were observed alive. In addition to bovine bile, fish bile collected directly from the gall bladder of hybrid *Oreochromis aureus* was pooled and used as a supplement in the culture medium. Parasite cultures were established containing fish bile concentrations of 0 (control), 1.0, 2.0, 4.0, 6.0, 8.0, 10.0 and 12.5%. Procedures for evaluating the parasite's fish bile requirement were done in the same manner as described for the bovine bile investigation.

Criteria for determining optimal condition. Optimal conditions for the growth of the parasites were based on a combination of factors which included average cell number ml^{-1}, growth rate, survival time, and cell conditions (motility and morphology). The motility and shape of the parasites were observed under a compound microscope. Initially, the optimal temperature for growth of the organism was evaluated using previously reported culture conditions (Poynton et al. 1995), then the optimal pH and concentration of bile were determined based on this optimal temperature. Any condition of temperature, pH and bile that yielded high numbers of active motile flagellates with a long survival period was considered an optimal condition.

Statistical analysis. Growth rates of *Spironucleus vortens* during the exponential phase were analyzed and compared by a SAS statistical program (SAS Institute Inc., Cary, NC). Numbers of parasites during the exponential phase were transformed to a ln-growth

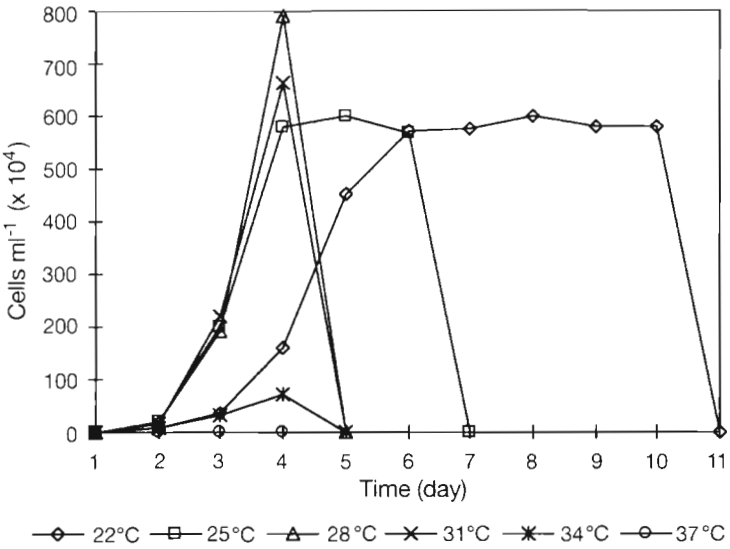

Fig. 1. *Spironucleus vortens*. Growth of the parasite cultivated at different temperatures (°C)

curve in order to stabilize the variances for analysis purposes. The exponential increases (slope) of growth and the number of parasites during the exponential phase were analyzed and compared by Tukey's HSD at $\alpha = 0.05$.

RESULTS

Optimal temperature

Growth of *Spironucleus vortens* cultivated under different temperatures is shown in Fig. 1. No parasites were observed alive in the culture at 37°C after 24 h of culture initiation. At 28, 31 and 34°C, parasites were observed alive for only the first 4 d of cultivation. The cultured cells, except those at 34 and 37°C, started multiplying with a lag phase followed by an exponential phase. The lag phases and the exponential phases of cultures at 25, 28 and 31°C were 1 d shorter than those at 22°C. The cultures at 22, 25, 28 and 31°C yielded maximum cell numbers of 6.0, 6.0, 7.91 and 6.7 million cells ml^{-1}, respectively. The cultured cells at 25°C reached maximum cell numbers within 5 d, while those at 22°C reached the same cell number within 8 d. However, cells in the cultures at 22°C were observed alive up to 10 d of the cultivation.

The ln-transformed growth curves of *Spironucleus vortens* during Days 2 to 4 of cultures are shown in Fig. 2. An equation representing the growth of each curve is ln(cell no.) = $\beta_0 + \beta_1$ day. The growth rates (β_1) at 22, 25, 28, 31, and 34°C during Days 2 to 4 were 1.52, 1.71, 1.84, 1.86, and 1.02, respectively. The parasites multiplied faster at 22, 25, 28 and 31°C than those at 34°C. There was no significant difference between the proportional increase in cell numbers of cultures at 22, 25, 28 and 31°C. Although the parasites multiplied at 22°C with no difference in growth rate from those at 25, 28, and 31°C, the number of parasites at 22°C during Days 2 to 4 was significantly lower (Table 1). The parasites cultured at 22 and 34°C had similar cell numbers on the second and third day of cultivation; however, by Day 4 the number of parasites at 22°C was significantly higher than those at 34°C.

Table 1. *Spironucleus vortens*. Average number of parasites ($\times 10^4$ cells ml^{-1}) from Days 2 to 4 of culture at different temperatures. Back-transformed means within a row (day) followed by different letters are significantly different at $\alpha = 0.05$ according to Tukey's HSD

Day	Temperature (°C)				
	22	25	28	31	34
2	7.18^a	18.84^b	20.04^b	16.05^b	9.38^a
3	34.52^a	198.68^b	192.92^b	212.72^b	31.77^a
4	149.73^a	580.03^b	790.95^b	663.91^b	71.83^c

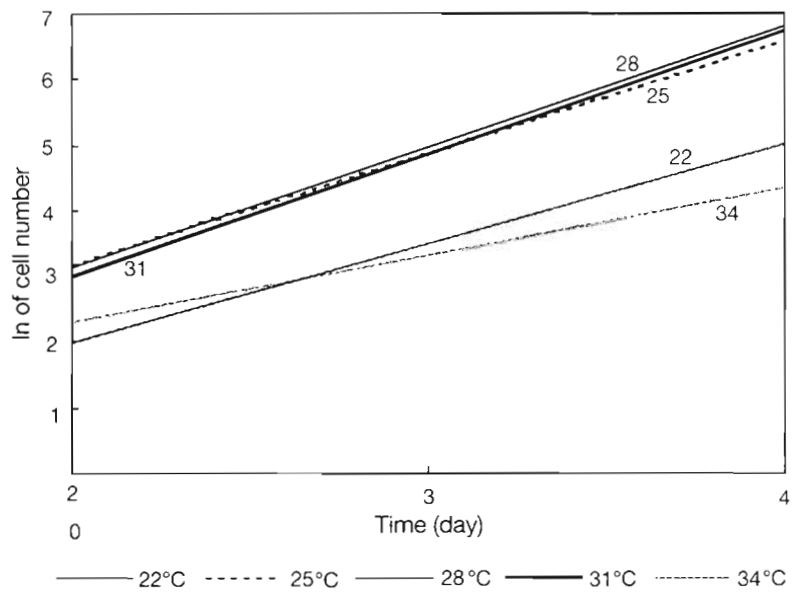

Fig. 2. *Spironucleus vortens*. Transformed growth curves of the parasite cultivated at different temperatures (°C)

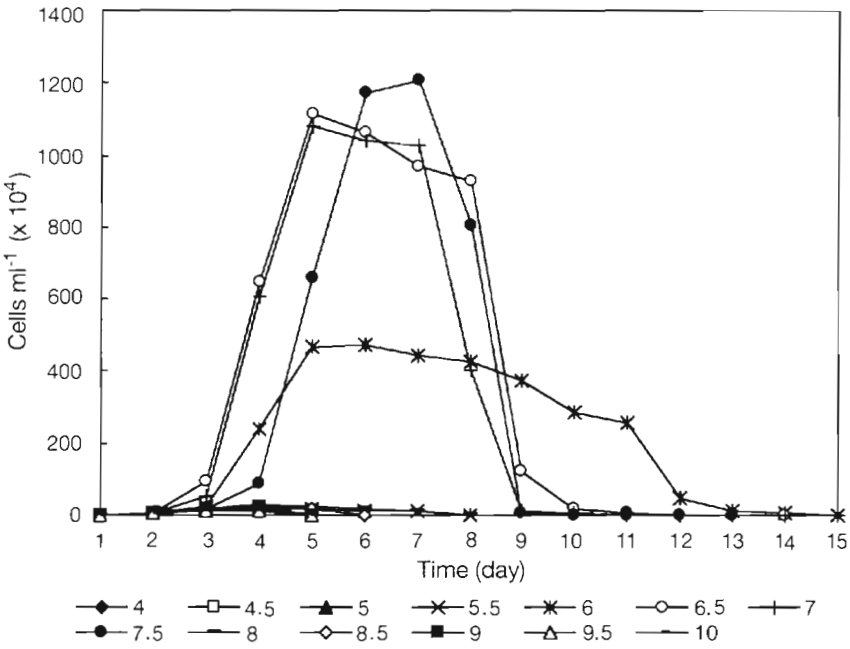

Fig. 3. *Spironucleus vortens*. Growth of the parasite cultivated under different pH conditions

Under microscopic observation, *Spironucleus vortens* cultivated at temperature ranges from 22 to 31°C were not different in shape. The majority of cell populations were pyriform in shape with a lower percentage of multinucleated trophozoites. A high percentage (12 to 17%) of abnormal trophozoites was seen in the culture at 34°C. The parasites at each temperature actively moved forward in the culture medium, and were less active only the last few days of cultivation.

Optimal pH

Growth of *Spironucleus vortens* cultivated under different pH conditions is shown in Fig. 3. Parasites had no difference in growth rates during the first 2 d of cultivation. The fastest growth rates were observed between pH 6.5 and 7.5. The parasites cultivated at pH 6.5 and 7.0 were similar in growth patterns during the first 6 d of cultivation, and they had a 1 d shorter period of multiplication compared to those incubated at pH 7.5. However, the highest average cell number (1.21×10^7 cells ml^{-1}) was observed in culture at pH 7.5. The lowest pH at which the organisms had a moderate growth rate and a long survival period was 6.0. Parasites survived only a few days at pH 5.5, 8.0 and 8.5 and were killed within 24 h at a pH above 8.5 or below 5.5.

Bile requirements

Spironucleus vortens in medium (pH 6.8) supplemented with bovine bile concentrations lower than 3.2 mg ml^{-1} multiplied slowly during the first 3 d of cultivation. Parasites multiplied gradually with the highest growth rate during Days 4 to 5. The cultured cells in the medium supplemented with bile concentrations lower than 1.6 mg ml^{-1} had similar replication rates after Day 5 of cultivation. All cultures supplemented with bovine bile had maximum numbers of cells lower than the control culture with no bile (Fig. 4). Cell growth was suppressed at high concentrations (3.2 and 6.4 mg ml^{-1}) of bovine bile. The numbers of cells at these higher concentrations of bile gradually decreased from the first day of cultivation until no cells were observed alive after Day 5.

The parasites were also suppressed in medium supplemented with fish bile (Fig. 5). The average numbers of parasites ml^{-1} at all concentrations of fish bile were lower than those in the control culture with no bile. Parasites cultivated in fish bile-supplemented (1 to 8%) medium multiplied slowly during the lag phase,

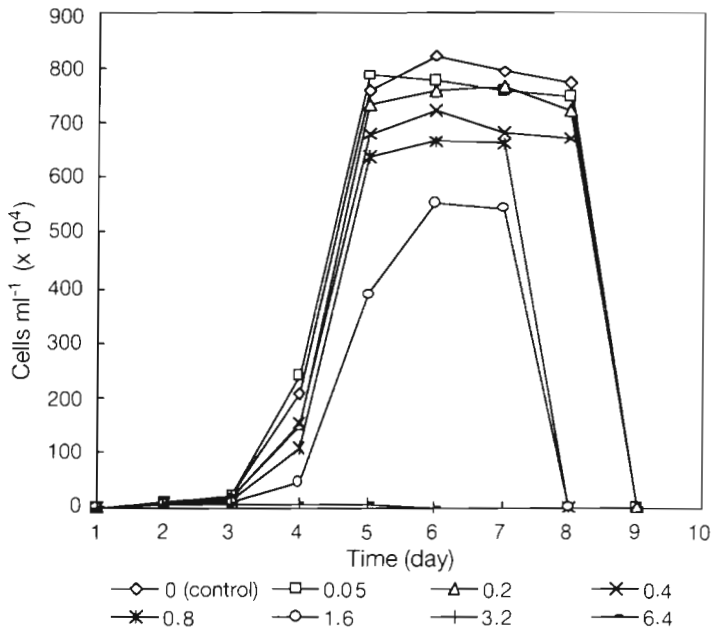

Fig. 4. *Spironucleus vortens*. Growth of the parasite cultivated in different concentrations (mg ml^{-1}) of bovine bile

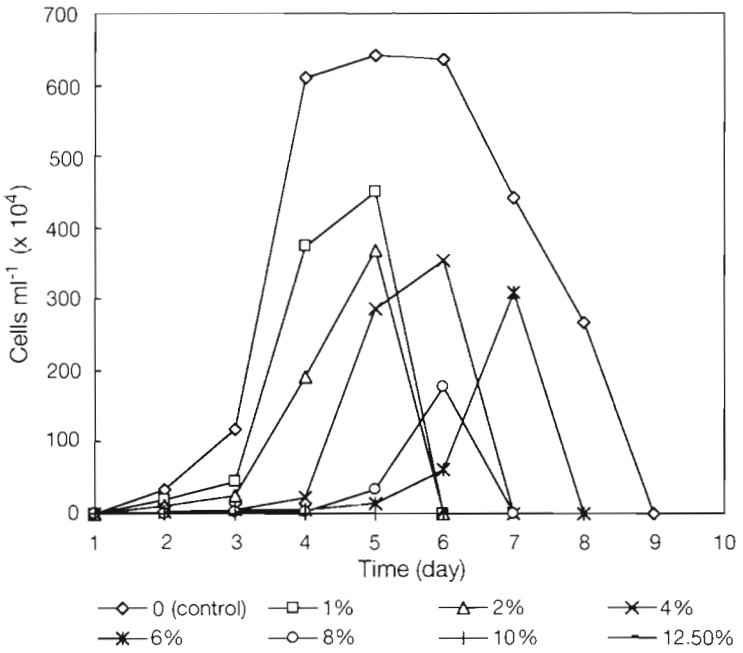

Fig. 5. *Spironucleus vortens*. Growth of the parasite cultivated in different concentrations (%) of fish bile

and no cells were observed alive after they reached peak numbers. In cultures supplemented with 10% fish bile, the highest number of parasites was 6.3×10^3 cells ml^{-1}, and only 200 cells ml^{-1} of parasites were observed alive on the last day of cultivation. At 12.5% fish bile supplementation, the number of parasites in cultures gradually decreased and no cells were observed alive on Day 4 of cultivation.

DISCUSSION

Spironucleus vortens are highly motile flagellates. They actively move forward in the culture medium at temperatures ranging from 22 to 34°C. Although *S. vortens* has been reported to grow and reproduce between 25 and 31°C in culture medium (Poynton et al. 1995), the present study suggests that the range of temperatures is broader, extending from a low of 22°C to a high of 34°C. At 22°C the parasites are less active via microscopic observation than those at the higher temperatures, but survive longer. This suggests that the long-term risk of maintaining a *S. vortens* infection in angelfish may be increased when the environmental temperature is decreased to 22°C.

In the artificial medium, normal pyriform-shaped trophozoites were the dominant form seen throughout the cultivation period at all temperatures, and they have been reported to be the typical form of the free flagellate found in the intestinal lumen of angelfish (Poynton et al. 1995). The dividing trophozoites which resembled bifurcated poles (V-shaped cells) were found in the highest number on the second day of cultivation. The dividing trophozoites did not always move forward in one direction, but sometimes in an opposite direction. Irregular (abnormal) multinucleated trophozoites were also found in low numbers throughout the cultivation period, with a higher percentage (12 to 17%) in the culture at 34°C, suggesting that high temperature (34°C) is a factor inducing failure of cytokinesis of the parasite resulting in a decrease in the number of new free-flagellated trophozoites. Cysts were not observed in any of the cultures. To date, cysts of fish hexamitids are very rare either in cell culture or in host feces. They were reported in one early study of *Octomitus salmonis* in trout (Moore 1922), but were not detected in recent studies (Kulda & Lom 1964a, Kent et al. 1992, Tojo & Santamarina 1998). Therefore, it is probable that trophozoites, not cysts, of fish hexamitids play an important role in transmission in a natural condition.

The optimal temperature for *Spironucleus vortens in vitro* growth in this study was determined to be 25°C because the parasites had a high replication rate, good motility, and extended survival period of up to 6 d. Although at 22°C the organisms survived longest and had no difference in growth rate compared to those at 25°C, they were less active throughout the culture period and required a longer period to reach a maximum number. At 28 and 31°C, the parasites had a high replication rate, reached maximum numbers by Day 4, but did not survive after that time. Either a shortage of nutrients and/or increased waste products released from live or dead cells are factors that may have contributed to the death of the parasites. In addition, temperature was considered to be the primary factor in the death of *S. vortens* in cultures maintained at 34°C or higher. The parasite's growth was significantly suppressed and was lethal within 24 h at 37°C.

Fish are poikilothermic animals which adapt to changes in water temperature. Freshwater angelfish *Pterophyllum scalare* are indigenous in a tropical zone, South America: Amazon River (Axelrod 1985), and have been successfully bred and maintained in captivity under warmwater conditions. The most suitable temperature for raising angelfish ranges from 22 to 30°C (Mills et al. 1988). Therefore, the present study indicates that an appropriate temperature for raising angelfish is also suitable for facilitating the growth of *Spironucleus vortens*. It has been suggested that high temperatures (above 28°C) can sometimes help in

controlling hexamitid infections (Bassleer 1983). The present study supports this suggestion in that the trophozoites of *S. vortens* are suppressed and rapidly killed at high temperature. Thus, maintaining higher water temperatures may help decrease the number of trophozoites in the host and might minimize the rate of infection.

There have been few studies reporting the optimal conditions for fish hexamitids. The optimal temperature and pH for *Hexamita salmonis* isolated from rainbow trout were 10°C and pH 7.5 to 8.0 (Buchmann & Uldal 1996). *Spironucleus barkhanus* isolated from grayling and salmon have a suitable temperature range for growth from 5 to 20°C (Sterud 1998b). Both of these organisms are parasites of coldwater fish. In the present study, the suitable temperature range for *S. vortens* is 22 to 25°C, which is higher than the previously mentioned species. However, optimal pH (6.5 to 7.5) for the growth of *S. vortens* is close to that reported for *H. salmonis*. Thus, it is obvious that different species of hexamitids have different optimal conditions for growth due to the adaptation of the hosts to environmental conditions.

Bile concentrations between 0.03 and 0.96 mg ml^{-1} have been reported to stimulate growth of *Hexamita salmonis* cultivated in artificial medium (Buchmann & Uldal 1996). However, the present study demonstrated that bile was not a requirement for the growth of *Spironucleus vortens*. In addition, the growth of the parasite was significantly suppressed at higher concentrations of either bovine (3.2 and 6.4 mg ml^{-1}) or fish (10 and 12.5%) bile. *Spironucleus* sp. have been reported frequently to cause diseases in many aquarium fishes (Molnár 1974) including the freshwater angelfish (O'Brien et al. 1993). The parasites are normally found in the intestine (O'Brien et al. 1993, Poynton et al. 1995), but may be found in other organs including the gall bladder (Molnár 1974, Sterud 1998b). However, the results of this study indicate that *S. vortens* is less tolerant to bile, therefore suggesting that it may only accidentally enter the gall bladder during heavy intestinal or systemic infections.

The *in vitro* environmental conditions such as temperature, pH and concentration of bile are all important factors in maximizing or suppressing the growth of the parasite. In this study, a temperature of 25°C and pH between 6.5 and 7.5 were considered as the optimal conditions for the parasite growth. However, *Spironucleus vortens* cultivated in culture medium at 25°C during routine cultivation (13 ml) and in the pH experiment (30 ml) survived longer than those cultivated in the temperature experiment (2 ml). Thus, the amount of nutrient (medium) provided to the organisms may also be considered an important factor in the growth and survival requirements.

Spironucleus vortens has been found mostly in the middle to posterior region of angelfish's intestine (Poynton et al. 1995). The present study is useful in explaining the microhabitat of *S. vortens*. The anterior intestine, which presumably contains a higher concentration of bile, is not suitable for long-term parasite survival and the number of parasites should be lower in this region. Conversely, the number of parasites should be higher in the middle and posterior intestine due to the lower concentration of bile in these areas.

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