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Non-invasive investigation of *Spironucleus vortens* transmission in freshwater angelfish *Pterophyllum scalare*

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ABSTRACT: *Spironucleus vortens* is a protozoan fish parasite of veterinary and economic importance in the ornamental aquaculture industry. Despite this, key aspects of the life cycle of this organism, including its mode of transmission, have not been fully elucidated. We developed a non-invasive method for quantifying *S. vortens* in freshwater angelfish, which was then used to investigate parasite transmission and aggregation within host populations. As previously observed for *S. meleagridis* and *S. salmonis*, motile *S. vortens* trophozoites were detected in host faeces using light microscopy. Species-level identification of these flagellates was confirmed using 16S rDNA PCR. Faecal trophozoite counts were significantly correlated with trophozoite counts from the posterior intestine, the primary habitat of the parasite. This novel finding allowed effective prediction of intestinal parasite load from faecal counts. Overall, faecal count data revealed that 20% of hosts harbour 83% of parasites, conforming to the Pareto Principle (80/20 rule) of parasite aggregation with implications for parasite transmission. Trophozoites survived for ≥36 d outside the host within faeces and remained motile at low pH (comparable with that of angelfish stomach). No putative *S. vortens* cysts were observed in cultures or faecal samples. This calls into question the commonly accepted hypothesis that a protective cyst is required in the life cycle of *S. vortens* to facilitate transmission to a new host.

KEY WORDS: Diplomonad · Spironucleosis · Protozoan · Parasite · Trophozoite · Cyst · Aquaculture

INTRODUCTION

Ornamental aquaculture production is a global multi-billion dollar industry. International export trades in this sector have increased at an average growth rate of 14% per annum since 1985 (FAO 2005), with world exports of fish and fishery products (food fish included) reaching US$102 billion in 2008 (FAO 2010). Disease is a major limiting factor in aquaculture production, causing losses of ca. 45% to the industry (FDA 2012). However, accurate diagnosis of many fish diseases requires sophisticated equipment, such as electron microscopy, and consultation with fish health experts for pathogen identification (Sterud & Poynton 2002, Noga 2010). A further complication is the lack of available data on basic aspects of pathogen biology, including the life cycle and transmission of some disease-causing agents. Hence, infections are often undiagnosed, and may lead to disease outbreaks and significant mortality of fish if control measures fail.

Diplomonads are a group of aerotolerant anaerobic or microaerophilic organisms which inhabit low-O_2 environments such as lake sediments and the intestinal tracts of animals (Biagini et al. 1998, Lloyd et al. 2002, Kolisko et al. 2010, Williams et al. 2011). Five species of piscine *Spironucleus* spp. are currently recognised: *S. salmonicida*, *S. barkhanus* and *S. torosa* infect marine food fish, whereas *S. salmonis* and *S. vortens*, the latter being the focus of the cur-

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rent study, infect freshwater ornamental fish (reviewed by Williams et al. 2011). First isolated from an angelfish in Florida (USA) in 1991 (Poynton et al. 1995), *S. vortens* is particularly prevalent in cichlids and cyprinids, including angelfish *Pterophyllum scalare* and discus *Symphysodon discus* (see Williams et al. 2011). The primary habitat of this parasite is the middle to posterior intestinal tract; however, motile trophozoites have also been isolated from other organs, including the liver, spleen and kidneys, indicating systemic spironucleosis. Recovery of this organism from head lesions has led to this parasite being described as the putative causative agent of hole-in-the-head disease (Paull & Matthews 2001).

Accurate identification of *Spironucleus* spp. has been greatly hampered by lack of appropriate experimental methods. Earlier studies, which relied on the use of light microscopy alone to identify diplomonads, resulted in the assignation of piscine diplomonads to the genera *Hexamita*, *Octomitus* and *Spironucleus*. Recent detailed ultrastructural work and molecular tools, however, have confirmed that all piscine diplomonads belong to the genus *Spironucleus* (see Poynton & Sterud 2002, Jørgensen & Sterud 2007). PCR and/or sequencing primers have now been designed for most *Spironucleus* spp. (reviewed by Williams et al. 2011). In the case of *S. vortens*, however, the only available primers indiscriminately amplify the 16S rRNA gene of most piscine *Spironucleus* species. Species-level identification of *S. vortens* therefore requires sequencing of 16S rDNA and transmission electron microscopy.

The life cycle of piscine *Spironucleus* spp. is poorly characterised, with most information being inferred from the distantly related mammalian diplomonad parasite *Giardia* (reviewed by Ankarklev et al. 2010). Like *Giardia*, the life cycle of *Spironucleus* spp. is thought to be direct, via the faecal–oral route, with transmission between hosts being facilitated by an infective cyst (Woo & Poynton 1995). The cyst form has been well-documented for *S. muris* and *S. meleagridis*, which infect rodents and birds, respectively (Januschka et al. 1988, Wood & Smith 2005). Cysts of the piscine diplomonad *S. salmonis* (host: salmon, trout) have also been documented *in vitro* and *in vivo* (reviewed by Williams et al. 2011), but in the case of *S. vortens*, have never been documented (Sangmaneedet & Smith 2000). Likewise, the cyst form has not yet been reported for *S. salmonicida* (host: salmon, trout), but a recent genomic survey identified in this parasite a *Giardia lamblia* cyst wall protein homologue as well as 2 enzymes involved in cyst wall assembly, glucosamine-6-phosphate isomerase and UDP-N-acetylglucosamine pyrophosphorylase (Andersson et al. 2007). For *S. meleagridis* and *S. salmonis*, live trophozoites are also expelled into fresh faeces (Tojo & Santamarina 1998, Philbey et al. 2002), and transmission of *S. salmonicida* trophozoites via skin lesions has been suggested (Poppe et al. 1992). The fact that *S. vortens*, *S. salmonicida* and *S. meleagridis* trophozoites are present in extra-intestinal systemic infections (Poppe et al. 1992, Paull & Matthews 2001, Bailey et al. 2010) indicates that trophozoites are able to tolerate fluctuations in O_2 tensions, pH and nutrition. Hence, it may be possible that *S. vortens*, *S. salmonicida* and *S. meleagridis* may not always require a cyst, with the trophozoite form instead responsible for transmission.

The transmission potential of a pathogen is commonly measured using the basic reproduction rate, R_0, which represents the average number of secondary infections caused by an infected host during its lifetime. An R_0 value >1 typically marks an epidemic outbreak, whereas if the calculated R_0 value is <1, the outbreak will become extinct (Woolhouse et al. 1997). The reliability of this epidemic threshold value, however, has recently been questioned as it can lead to erroneous conclusions regarding the infectivity of the pathogen and lead to inappropriate medical interventions for pathogen control (Breban et al. 2007). For example, R_0 is affected by heterogeneities in pathogen transmission rates between individuals in a given population (Woolhouse et al. 1997). Such variation in pathogen transmission can be partially explained by the Pareto Principle, a statistical pattern known as the 80/20 rule, as proposed by Woolhouse et al. (1997). Host–parasite systems commonly abide to the 80/20 rule, whereby approximately 80% of the parasites are harboured by 20% of the hosts. Thus these top 20% of hosts account for the bulk of the population's transmission potential. Such over-dispersal amongst hosts has been documented for the major soil-transmitted helminths (*Ascaris lumbricoides*, *Trichuris trichiura*, *Necator americanus* and *Ancylostoma duodenale*), schistosomes (Hotez et al. 2006) and malaria (Bousema et al. 2012).

Overall, the current study aimed to provide novel information on the life cycle of *Spironucleus vortens*, specifically its mode of transmission. Three distinct outcomes arose from this study: (1) We developed a statistical modelling technique to non-invasively estimate the degree of intestinal colonization by *S. vortens* using trophozoite faecal counts (based on a previous study by Tojo & Santamarina 1998). This modelling method may be employed to replace cur-

rent invasive methods of determining parasite burden, which involve euthanasia of fish in order to examine intestinal contents (see Whaley & Francis-Floyd 1991, Sangmaneedet 1999, Jørgensen & Sterud 2004). (2) The distribution of *S. vortens* within a host population was assessed using the non-invasive parasite quantification method to test the 80/20 rule. (3) The role of the trophozoite in parasite transmission was examined using further faecal analysis (trophozoite survival), *in vitro* experiments (trophozoite tolerance to low pH) and an antibody-based technique (in the search for putative *S. vortens* cysts).

MATERIALS AND METHODS

Fish origin and general maintenance

Juvenile angelfish *Pterophyllum scalare* (N = 168, with an average length of ca. 5 cm) were obtained from J&K Aquatics (Taunton, UK). Fish were held individually for the duration of the experiment in 12 l plastic tanks, each filled with dechlorinated water, an air supply and a plastic tank decoration for cover. Fish were maintained at 24 ± 1°C with a 12:12 h light:dark cycle and fed on a flake food (TetraMin) diet. Complete water changes were performed at least once per week in order to maintain good water quality.

Parasite origins

***Spironucleus vortens* (ATCC 50386).** Trophozoite cultures were maintained in Keister's modified TYI-S-33 medium according to a method adapted by Williams et al. (2012). Briefly, cultures were maintained at 24 ± 1°C and sub-cultured at 48 h intervals by transferring 500 µl of a mixed log-phase culture into 10 ml of culture medium in 15 ml screw capped Falcon tubes (Greiner Bio-one), leaving a 5 ml head space. Cultures were monitored for microbial contamination by plating-out 100 µl of an exponentially growing culture on TYI-S-33 agar (0.5% w/v) and incubating under the above conditions for 5 d.

Spironucleus vortens Sv1. An infected angelfish (identified by the presence of *Spironucleus* trophozoites in faeces, August 2011) that was bred in England was euthanized by overdose of MS-222 (Sigma-Aldrich). The entire intestinal tract was removed by severing at the anterior (just after the stomach) and posterior (at the anus) ends, and transferred to 10 ml Keister's modified culture medium containing 0.5 mg ml^{-1} penicillin and streptomycin, 5 mg ml^{-1} gentamycin and 1 mg ml^{-1} collistin sulphate (all from Sigma-Aldrich), and incubated for 72 h at 24°C. This allowed trophozoites to migrate from the intestinal tract and into the surrounding culture medium through the open anterior and posterior ends of the gut. The organism was then sub-cultured 5 times as described above to allow an increase in trophozoite number and remove bacterial/fungal contamination. Finally, an unidentified ciliate contaminant derived from the fish gut was removed at room temperature (20°C) by low speed centrifugation at 120 × *g* (3 min). The resulting pellet, containing the axenic *Spironucleus* isolate, was aseptically transferred to fresh culture medium and maintained as described above, without antibiotics. Freezer stocks of the new isolate were prepared after a total of 6 subcultures by suspending ca. 10^6 cells ml^{-1} in 10% DMSO (v/v, Sigma-Aldrich) and cooling to −80°C in an isopropanol bath at a controlled rate of 1°C min^{-1}. Vials of frozen cultures were then transferred to liquid N_2 for long-term storage. Molecular identification of this new *Spironucleus* isolate (herein *Sv1*) was confirmed by PCR (see below). This new isolate was used alongside the ATCC strain in order to examine the occurrence of *S. vortens* cysts, as described below.

***Spironucleus barkhanus* (ATCC 50467).** Trophozoites from a liquid N_2 culture were thawed and incubated overnight in Keister's modified TYI-S-33 culture medium supplemented with 1 mg l^{-1} bile (bovine, sourced from a local slaughter house) at 20°C, according to Sterud (1998). This organism was used a positive control for the PCR assay described below.

Species-level identification of *Spironucleus vortens* using 16S rDNA PCR

A new primer pair, SV-1f (5′-TGT GGG AGA CTG TGC TCT TG-3′) and SV-1r: (5′-AGC ATA CTC CCC CAG GAA CT-3′), was designed to specifically amplify 158 bp of the 16S rRNA gene of *Spironucleus vortens* beginning at positions 704 (SV-1f) and 842 (SV-1r) of the GenBank Accession Number U93085 *S. vortens* gene sequence (Keeling & Doolittle 1997). These primers were designed using Primer3 (Rozen & Skaletsky 2000), and their suitability for PCR was confirmed using OligoAnalyzer version 3.1 (Primer Quest®: www.idtdna.com/Scitools). The Spiro-1f/1r primer pair from Jørgensen & Sterud (2004), which amplifies the 16S rRNA gene of most *Spironucleus* and *Hexamita* species, was employed as a positive

control, producing an amplicon of 1431 bp. Total DNA was extracted from the *Sv1* intestinal isolate, *S. vortens* ATCC strain (positive control), *S. barkhanus* ATCC strain (negative control) and dH_2O (negative control) using the DNeasy Blood and Tissue Kit (Qiagen). Each 10 µl PCR reaction consisted of 2 µl of extracted DNA, 1 µl of each primer (10 pmol μl^{-1}, Eurofins), 0.1 µl dNTPs (0.25 mM, Thermo Scientific), 0.6 µl $MgCl_2$ (1.5 mM, Thermo Scientific), 1 µl of PCR buffer (ABgene), 0.1 µl of *Taq* polymerase (0.05 U μl^{-1}, Thermo Scientific) and 4.2 µl of dH_2O. The PCR cycle was as follows: 95°C for 5 min, 95°C for 45 s, 56°C for 1 min, 72°C for 2 min, 72°C for 4 min and finally held at 4°C in a 96-well thermal cycler (Applied Biosystems). The denaturing, annealing and elongation steps were repeated for 35 cycles. Gel electrophoresis of the PCR products (2 µl each plus 1 µl loading dye, Applied Biosystems) and 100 kb DNA ladder was performed on 1.5% (w/v) agarose gels (Bioline) containing ethidium bromide (Invitrogen) with an applied voltage of 120 V. Gels were then visualized and imaged using an UV transilluminator (GelDoc-It).

Detection and quantification of *Spironucleus vortens* trophozoites in angelfish faecal pellets by light microscopy

Fresh faecal pellets from angelfish were collected immediately after defecation from the bottom of clean plastic tanks using a plastic pipette. Preparation of faecal samples and estimation of parasite counts were conducted as previously described by Tojo & Santamarina (1998). Whole faecal samples (ca. 0.5 to 1 cm in length) were placed on a glass slide with a drop of fresh dechlorinated water and compressed using a 22 × 22 mm coverslip. These squash preparations were examined by light microscopy for the presence of *Spironucleus vortens* trophozoites, which were identified according to their length (5 to 10 µm), pyriform shape and characteristic rapid cell motility. The number of trophozoites per sample was qualitatively estimated by counting the number of parasites present for a 15 min period (400× magnification) until the whole sample had been examined (ca. 50 fields of view). Precise parasite counts were not possible due to intense trophozoite motility, which is a major characteristic employed in the identification of these organisms.

The PCR method described above, using the SV-1f/1r primer pair, was used to confirm *Spironucleus vortens* in the faeces of angelfish. To test for the sensitivity of the method for non-invasive *in vivo* diagnosis, we analysed 17 angelfish faecal samples, from fish with varying degrees of *Spironucleus* infection (as determined by faecal counts, see below). Unconcentrated tank water (1 ml) from a highly infected and uninfected fish was also tested for the presence of *S. vortens*, with fresh dechlorinated water used as an additional negative control.

Comparison of infection prevalence and parasite density via faecal and intestinal trophozoite counts

For non-invasive quantification of *Spironucleus vortens* trophozoites, 2 faecal samples (in order to account for variation in parasite shedding by the host over time, this was the maximum number of faecal samples produced by the angelfish in a single day) were collected from 20 fish. Later the same day, the fish were euthanized with an overdose of buffered MS-222 anaesthetic and dissected. The entire intestinal tract was removed and cut into 4 sections: anterior, anterior-middle, middle-posterior and posterior (according to Uldal & Buchmann 1996), and the total number of *S. vortens* trophozoites in each section was estimated through the intestinal wall, as for the faecal samples (squash preparation of intestinal sections under 400× magnification).

In order to model the relationship between faecal and intestinal trophozoite densities, trophozoite counts derived from angelfish faeces and the 4 angelfish intestinal sections were first incorporated into a generalized linear mixed model to account for the repeated faecal sampling of 1 individual. Since animal ID and faecal sample number did not have a significant effect on the faecal trophozoite count, the averages of the 2 faecal samples were then used as dependent variables in a negative binomial general linear model (GLM) with intestinal trophozoite counts in the 4 different gut locations as independent variables. Models were reduced using a stepwise deletion process with an α level of 0.05 and evaluation of Akaike's information criterion (AIC) values to assess the significance of model terms and overall model fit. The only independent variable which significantly correlated with faecal counts was the posterior intestine. Hence, the final model utilized $\log_{10}$-transformed faecal (dependent variable) and posterior intestinal (independent variable) count data and the square root link function. The model assumptions were checked for normal distribution (visual examination of histograms and Q-Q plots), heteroscedasticity (Fligner-Killeen test, $p > 0.05$),

overdispersion (theta = ~1) and proportion of variability in the dataset that is accounted for by the model (pseudo-r^2 ≥0.7). The final model was then used to predict the number of trophozoites in the posterior gut based on parasite counts in faeces.

Pareto Principle (80/20 rule)

The *Spironucleus vortens* trophozoite faecal quantification method described above was used to test the Pareto Principle that ca. 80% of the effects come from 20% of the causes. Hosts (N = 168, including uninfected individuals) were ranked from most to least infected according to semi-quantitative trophozoite faecal counts and the proportions of host (0 to 1, with 0.05 increments) plotted against the corresponding proportions of parasites. From the resulting line chart, the expected proportion of parasites, or transmission potential, was extrapolated from the top 20% of most infected individuals.

Survival of *Spironucleus vortens* trophozoites in faecal pellets

Fresh faecal samples were collected from angelfish immediately after defecation, transferred into 1 l plastic containers and incubated at 24°C for 0 (immediately counted after defecation), 1, 2, 3, 14 or 36 d in dechlorinated water (N = 28 faecal samples per time point). After incubation, samples were examined and the number of *Spironucleus vortens* trophozoites estimated as described above.

Survival of *Spironucleus vortens* trophozoites at varying pH *in vitro*

Survival of *Spironucleus vortens* in acidic pH was examined to determine whether trophozoites are able to survive transmission through the stomach. Log-phase *S. vortens* trophozoites from *in vitro* cultures (ATCC and *Sv1* strains) were inoculated into the wells of a 12-well plate to a final cell density of 2 × 10^6 to 3 × 10^6 cells $well^{-1}$ (counted by fixation of a 10 µl sample from a homogenous cell suspension in 1.5% v/v formaldehyde using an Improved Neubauer haemocytometer prior to well inoculation). Each well contained 2 ml of culture medium at different pH levels: pH 3, 4, 5, 6, 7 or 2 ml of distilled water (2 replicates of each pH per parasite strain). Trophozoite motility was used to monitor cell vigour using light microscopy (200× magnification). Cells were incubated on a shaker between observations (50 rpm) to mimic the motions of stomach peristalsis and observed every hour for 3 h. The transit time of food through the stomach in humans is ca. 2 h, whilst that of rats is far quicker (75% of stomach contents emptied in 15 min; Kararli 1995, Bodé et al. 2004). Hence, the transit time of food through an angelfish stomach is likely to be within the maximum incubation period selected of 3 h. Cell motility was ranked as follows: (–) immotile cells, (+) twitching cells (++), semi-motile/slow-moving cells and (+++) cells with normal/healthy motility.

Putative *Spironucleus vortens* cysts

Encystment of *Spironucleus vortens in vitro.* To investigate whether *S. vortens* encystment could be induced, 4 methods were tested that are based on successful encystment protocols for the related diplomonad *Giardia* and other *Spironucleus* spp. These were as follows: (1) starvation: post-stationary phase cultures (i.e. >72 h) incubated in phosphate-buffered saline (pH 7.2) or distilled water (Uldal 1996); (2) *Giardia* encystment medium: *S. vortens* trophozoites (ATCC and *Sv1* strains) were grown to late log phase in culture medium, before being transferred to encystment medium, consisting of culture medium supplemented with 1 M NaOH (Sigma-Aldrich), 0.25 mg ml^{-1} bovine bile (obtained from local slaughter house) and 5 mM lactic acid (Sigma-Aldrich, Gillin et al. 1989); (3) bile salts: taurocholic acid and glycocholic acid (both from Sigma-Aldrich) in culture medium (1, 2, 4, 8 and 16 mM); (4) fatty acids: oleic acid and myristic acid (both from Sigma-Aldrich), in culture medium (2, 1, 0.5, 0.25 and 0.125 mM). Approximately 10^6 *S. vortens* trophozoites were incubated with all treatments for 72 h in 12-well plates (2 ml volume for each treatment) and examined by light microscopy every 24 h for the presence of putative cysts using the trypan blue (0.4% w/v, Sigma-Aldrich) exclusion assay to identify viable cells.

Morphological identification of putative *Spironucleus vortens* cysts *in vivo.* There are no commercially available cyst-specific stains for the identification of *Spironucleus*. However, Januschka et al. (1988) showed that the cysts of *S. muris* and *Giardia microti* have similar cyst wall protein compositions. Therefore, the GiardiaCel kit (Cellabs) was used to examine angelfish faecal samples for the presence of *Spironucleus* cysts. Fresh (n = 10) and 2 wk old (n = 10) faecal samples (the latter incubated in aquarium

water in closed Eppendorf tubes) were collected from angelfish and fixed, permeabilized and stained according to the manufacturer's instructions. The kit contained a *Giardia*-specific cyst wall primary antibody, which binds to CWP1, a protein component of the *Giardia* cyst wall (Chatterjee et al. 2010). To visualize the cyst, a fluorescent fluorescein isothiocyanate (FITC)-tagged anti-mouse secondary antibody was used (λ_{ex} 490 nm, λ_{em} 530 nm). Cells were imaged at 200× and 600× magnification using confocal microscopy (Leica TCS SP2 AOBS). A *Giardia*-positive slide, supplied with the kit, was used as a positive control. *S. vortens* trophozoites (ATCC strain) from 5 d and 2 wk old *in vitro* cultures were also examined in the same way.

Statistical analysis

All statistical analyses described above were conducted using R (version 2.15.1; R Development Core Team 2012). To compare faecal and intestinal trophozoite counts, mean density was used, and is defined as the mean number of parasites counted per sample, with zero counts included.

RESULTS

Identification of *Spironucleus vortens* trophozoites in fresh angelfish faecal pellets

The SV-1f/1r primer pair specifically distinguished *Spironucleus vortens* from *S. barkhanus* (Fig. 1a) and confirmed the presence of *S. vortens* in angelfish faecal samples (Fig. 1b). The smaller DNA fragment

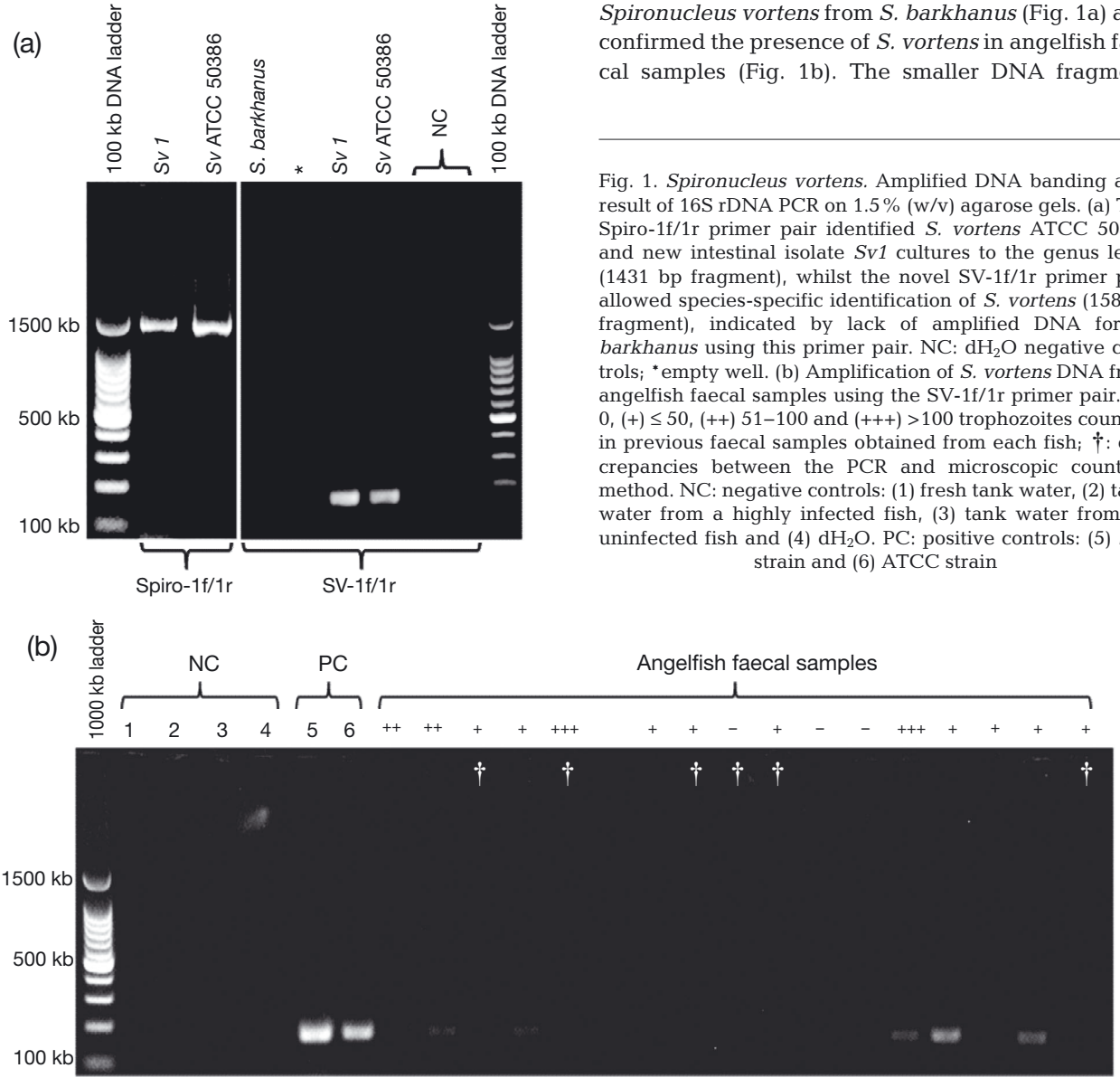

Fig. 1. *Spironucleus vortens.* Amplified DNA banding as a result of 16S rDNA PCR on 1.5% (w/v) agarose gels. (a) The Spiro-1f/1r primer pair identified *S. vortens* ATCC 50386 and new intestinal isolate *Sv1* cultures to the genus level (1431 bp fragment), whilst the novel SV-1f/1r primer pair allowed species-specific identification of *S. vortens* (158 bp fragment), indicated by lack of amplified DNA for *S. barkhanus* using this primer pair. NC: dH_2O negative controls; *empty well. (b) Amplification of *S. vortens* DNA from angelfish faecal samples using the SV-1f/1r primer pair. (−) 0, (+) ≤ 50, (++) 51–100 and (+++) >100 trophozoites counted in previous faecal samples obtained from each fish; †: discrepancies between the PCR and microscopic counting method. NC: negative controls: (1) fresh tank water, (2) tank water from a highly infected fish, (3) tank water from an uninfected fish and (4) dH_2O. PC: positive controls: (5) *Sv1* strain and (6) ATCC strain

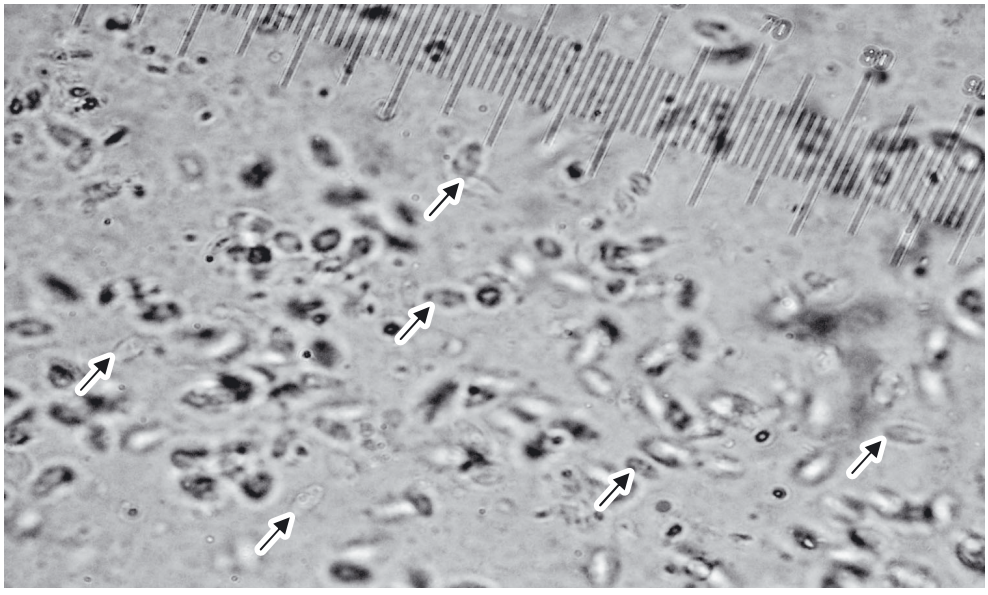

Fig. 2. *Spironucleus* sp. Numerous (ca. 70) motile trophozoites in the faeces of an angelfish. Note the characteristic length (ca. 5–10 µm) and pyriform shape of parasites, indicated by arrows (400× magnification, 1 eye piece unit = 2.5 µm)

generated from the new primer pair was easy to amplify, and therefore the test was more sensitive than the previously documented Spiro-1f/1r primer pair (see Jørgensen & Sterud 2004; data not shown), which only identifies *Spironucleus* to the genus level.

Single-celled, pyriform-shaped, highly motile flagellates with rapid motion were observed at 400× magnification mostly towards the centre of the faecal squash preparations (Fig. 2). These organisms were ca. 5 to 10 µm in length, abundant and were found freely swimming amongst the faecal matter. Numerous intracellular inclusions, putative vacuoles and/or bacteria, were observed within these organisms, which have been characteristically observed in *Spironucleus* trophozoites (Poynton & Sterud 2002). These cytoplasmic inclusions are less numerous in laboratory cultured isolates.

The mean density of *Spironucleus vortens* trophozoites in faecal samples of all fish (N = 168) was 68, with a range of 1 to 1322. Differences were observed in the sensitivity of the molecular and microscopic approaches for identifying *S. vortens* trophozoites. This is illustrated in Fig. 1b, whereby no *S. vortens* DNA amplification was apparent in faecal samples of fish previously deemed to be highly infected with *S. vortens* (>100 trophozoites counted) by the microscopic method. However, not enough replicates were performed in order to directly compare the accuracy of the microscopic and molecular methods.

Correlation between faecal and intestinal trophozoite counts

The variation between faecal counts taken from the same fish over time is illustrated in Fig. 3. The largest difference between 2 faecal samples from the same host was 685 trophozoites, with a range of 135 to 820 parasites counted. However, this is an extreme example indicating an occasional outlier that was infrequently encountered. The prevalence of infection in the intestinal tract of angelfish (N = 20) was 100%, with a mean density of 3096, ca. 46× greater than that

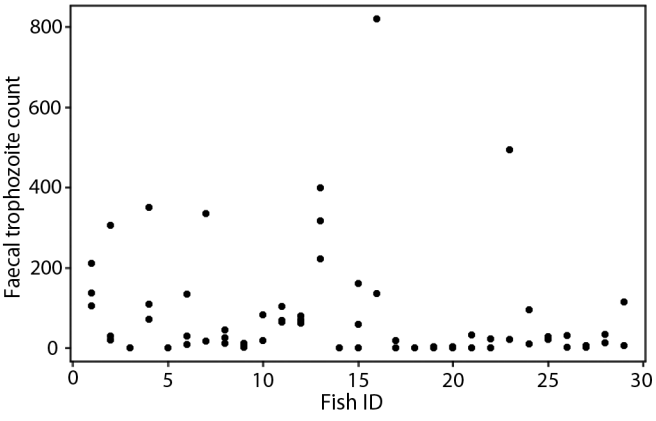

Fig. 3. *Spironucleus* sp. Variability in the total number of trophozoites observed per faecal sample (n ≥ 2) at ×400 magnification for each fish (29 in total)

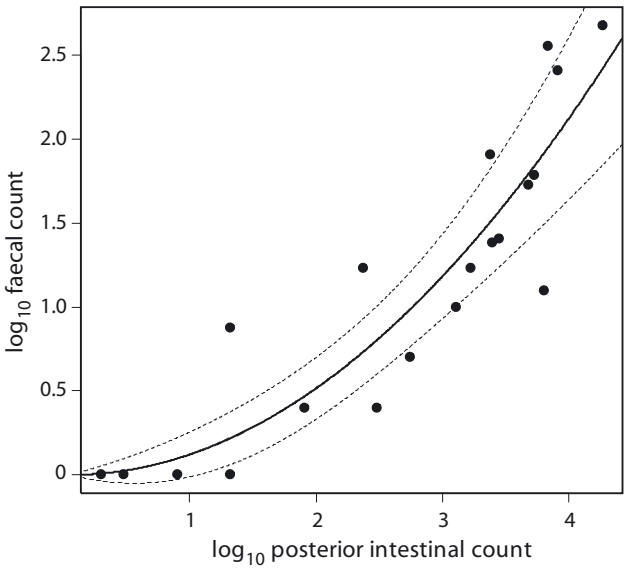

Fig. 4. *Spironucleus vortens.* Log_{10}-transformed faecal trophozoite count vs. posterior intestinal trophozoite count. Continuous line: predicted values of posterior counts for a given faecal count, derived from a negative binomial general linear model (Z = 3.979, p < 0.001, theta = 0.308 and pseudo-R^2 = 0.7); this prediction curve was overlaid on top of a scatterplot of the actual count data. Dashed lines: upper and lower SEs of the model

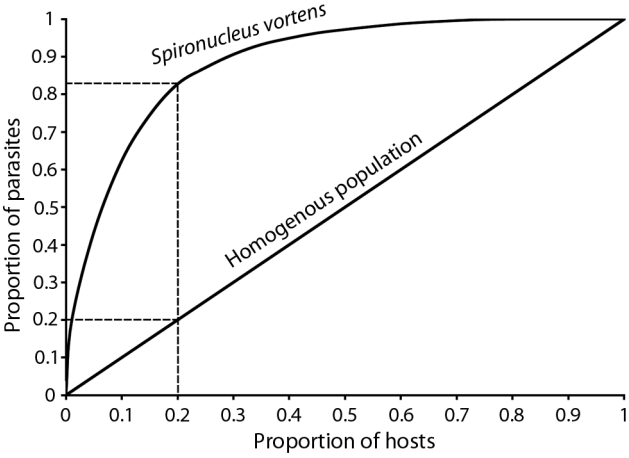

Fig. 5. *Pterophyllum scalare* infected by *Spironucleus vortens.* Fitted line plot (continuous line) of the proportion of hosts (N = 168), ranked from most to least infected with *S. vortens*, against the proportion of parasites in order to test the Pareto Principle (or 80/20 rule) of parasite aggregation in a host population. The distribution of a homogeneous population is also indicated. Dashed lines: expected proportion of parasites present in 20% of the hosts

of faecal samples (mean density 68, see previous section), with a range of 1 to 18430 in the posterior section (the intestinal section which had the highest parasite load). There was a significant positive correlation between average faecal trophozoite counts and posterior intestinal trophozoite counts (negative binomial GLM, Z = 3.979, p < 0.001, AIC = 44.60, pseudo-R^2 = 0.7; Fig. 4). Although individual faecal counts, i.e. count 1 or count 2, when incorporated individually into the model, also significantly correlated with posterior intestinal counts, the overall fit of both of these models was reduced with regards to the proportion of variability in the dataset that was accounted for by the models (pseudo-R^2 = 0.58 and 0.50 for count 1 and count 2, respectively). Hence, obtaining the average of at least 2 faecal counts greatly increases reliability when predicting posterior intestinal trophozoite numbers.

Aggregation of *Spironucleus vortens* infection in angelfish

Fig. 5 shows that 20% of angelfish hosts harbour approximately 83% of parasites, and thus transmission potential of the population. Of the total host population, 85% harboured 100% of the parasites.

Survival of *Spironucleus vortens* trophozoites in faeces and varying pH

Spironucleus vortens trophozoites survived in the angelfish faecal pellets for a minimum of 36 d (the final time point examined during this experiment). Between 1 and 16 *S. vortens* trophozoites were observed in the 36 d old faecal samples, with a mean density of 2. This was a decline of 90% compared to the Day 0 samples (mean density 19.2, range 1 to 130).

Spironucleus vortens trophozoites (both the *Sv1* and ATCC strains) survived for a minimum of 3 h in culture medium at pH 5 and 7 indicated by motility of the organisms. Parasite motility in dH_2O and at pH 6 was reduced to twitching (ATCC strain) or no motility (*Sv1* strain) after 3 h. Motility of the *Sv1* new intestinal isolate also diminished greatly following 3 h incubation in pH 5 and 7 culture media. However, for cells incubated in culture medium at pH ≤4, cell motility immediately ceased for both strains and trophozoites became irregular in morphology, eventually resulting in lysis of cells (Table 1).

Attempted induction and identification of *Spironucleus vortens* cysts

No viable cyst-like structures were observed after incubation in any of the 4 cyst-induction media tested. After incubation, there was a marked increase

Table 1. *Spironucleus vortens.* Survival of *S. vortens* (ATCC and *Sv1*) trophozoites over time in culture medium of varying pH and distilled water, indicated by (+++) healthy motility, (++) semi-motile, (+) twitching motility and (–) immotile cells. Cells were shaken between each observation

Time (h)	pH 4	pH 5	pH 6	pH 7	dH_2O
ATCC strain					
0	–	+++	+++	+++	+++
1	–	+++	+++	+++	+++
2	–	+++	++	+++	++
3	–	+++	+	+++	+
Sv1 strain					
0	–	+++	+++	+++	+++
1	–	++	++	++	+++
2	–	+	+	++	++
3	–	+	–	+	+

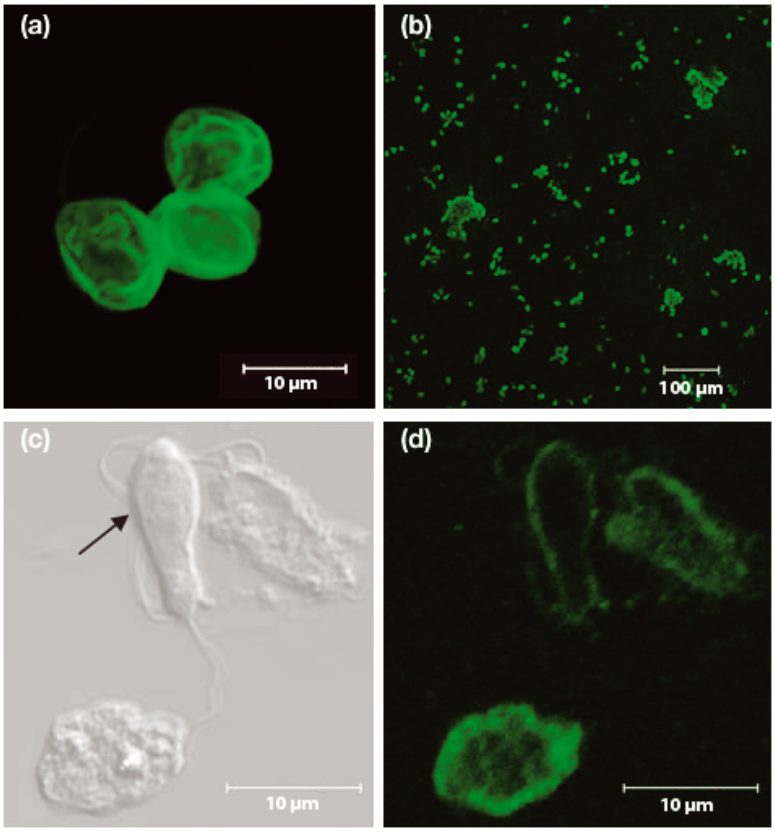

Fig. 6. *Giardia* sp. and *Spironucleus vortens.* Staining of diplomonad trophozoites (ATCC strain) with GiardiaCel antibody. (a) *Giardia* cysts (positive control), (b) 2 wk old *S. vortens* culture, (c) differential interference contrast image of 5 d old *S. vortens* culture (arrow indicates an intact trophozoite with visible flagella) and (d) fluorescence FITC image of (c). The debris at the bottom left hand side of images (c) and (d) is a non-viable (dead) trophozoite from the culture

in lysed cell debris (i.e. aggregations of organelles) and trypan blue was taken up by all remaining intact cellular structures, indicating dead or dying cells. Fig. 6a shows the characteristic staining of the *Giardia* cyst wall, which was used as a positive control in the search for putative *S. vortens* cysts. No *Spironucleus* cysts were identified in any of the angelfish faecal samples. There was a high degree of cross-reactivity of the *Giardia* cyst wall antibody with other matter found within the faecal samples, but no stained structures were observed that were similar in morphology to *Giardia* and *S. muris* cysts (see Januschka et al. 1988). Interestingly, however, *S. vortens* trophozoites from 5 d old and 2 wk old cultures were positively labelled with this antibody (Fig. 6b–d). Unlike the *Giardia* cysts, intact trophozoites were labelled only around the periphery of the cell.

DISCUSSION

Using non-invasive methods, this study presents compelling evidence to support trophozoite- (rather than cyst-) mediated transmission of *Spironucleus vortens* in angelfish.

Non-invasive semi-quantitative estimation of *Spironucleus vortens* intestinal load

The characteristic rapid motility of *Spironucleus vortens*, as well as its pyriform shape and typical length of ca. 5 to 10 µm, allowed easy identification of *S. vortens* from squash preparations of angelfish intestinal sections and faecal pellets during this study. The presence of parasites in samples was confirmed using novel SV-1f/1r primers, which provided species level identification of *S. vortens*. Intestinal dissections revealed that all fish examined (N = 20) were infected with *S. vortens*. Density of infection was greatest in the posterior intestine, which is comparable with previous observations by Poynton et al. (1995) and Sangmaneedet (1999).

As a non-invasive method of quantifying infection, faecal trophozoite counts were used as a tool to predict the degree of intestinal colonization by

Spironucleus vortens. Faecal trophozoite counts have been used previously by Tojo & Santamarina (1998) as a means of estimating *S. salmonis* infections in salmonids. The current study confirms the feasibility of employing faecal trophozoite counts to estimate the degree of intestinal colonization, with faecal counts correlating significantly with trophozoite counts observed in the posterior intestine of angelfish. It is important to note, however, that the mean density of posterior intestinal trophozoite counts was 46× greater than the faecal trophozoite counts. Hence, care must be taken whilst interpreting faecal samples where no trophozoites are detected for both the microscopic and molecular identification methods, as this may be due to human counting error, reduced parasite motility in the sample or differential parasite shedding by the host over time. The latter is true of *Giardia*, for which periodicity in cyst excretion by the host has been reported (McGlade et al. 2003). We therefore recommend that a minimum of 2 faecal samples are analysed from the same fish in order to estimate intestinal colonization.

Aggregation of *Spironucleus vortens* in angelfish

Parasite aggregation of *Spironucleus vortens* in hosts was investigated using data from faecal trophozoite counts. In the current study, angelfish *S. vortens* aggregation matched the 80/20 rule, with 20% of the hosts harbouring 83% of parasites. These highly infected hosts, termed 'supershedders', account for the bulk of the transmission potential (see Lloyd-Smith et al. 2005). Woolhouse et al. (1997) observed a similar aggregation pattern for the trypanosomatid and apicomplexan protozoan parasites *Leishmania* and *Plasmodium*, respectively. The same authors suggested that control measures should focus on this 20% core of highly infected individuals. Hence, the non-invasive trophozoite quantification method developed during the current study could be applied in aquaculture to identify and treat the top 20% of infected individuals in order to efficiently control disease outbreaks. This would be especially useful for broodstock or other highly prized angelfish and discus.

Transmission potential of *Spironucleus vortens* trophozoites

A small proportion of expelled *Spironucleus vortens* trophozoites (ca. 10% of the number recovered from fresh faecal samples) survived for 36 d in angelfish faecal pellets. At this stage, it is not clear whether individual trophozoites survived for this period or whether cell division occurred; however, this is the first observation of prolonged survival of diplomonad trophozoites in host faeces. This suggests that *S. vortens* trophozoites are able to use substrates present in the faeces and withstand fluctuations in nutrients, temperature and O_2 tensions for long-term survival. Millet et al. (2010) found that *S. vortens* consumes O_2 at a rate of 62 nmol min^{-1} per 10^7 cells in a closed reaction vessel, suggesting a highly effective O_2 scavenging mechanism. *S. vortens* is only capable of surviving in water for a limited time (10 h, *in vitro* study; Millet et al. 2011), a finding which was confirmed during the current study whereby trophozoite motility was dramatically reduced after 3 h. Hence, faecal matter is likely to confer a substantial degree of protection for the trophozoite against the external environment. For example, the presence of O_2-consuming gut bacteria and saprophytic organisms will result in the removal of O_2 from faeces, generating a microaerobic habitat. Indeed, trophozoites were mainly found towards the centre of faecal samples where O_2 tensions would be at their lowest (pers. obs.). *S. salmonis* trophozoites have also been observed in the faeces of salmonids by Tojo & Santamarina (1998); however, prolonged survival of these trophozoites in trout faeces was not investigated in this previous study. The fact that *S. vortens* and *S. salmonis* cluster together in 16S rDNA phylogenies suggests that they are closely related, and may explain the similarities observed in the presence of trophozoites in the faeces of their hosts (Fard et al. 2007).

Kent et al. (1992) showed that trophozoites of a marine diplomonad, later described as *Spironucleus salmonicida* (Jørgensen & Sterud 2006), could be transmitted via water-borne exposure and gavage of Atlantic and Chinook salmon. *In vitro* studies, also conducted by Kent et al. (1992), revealed that these trophozoites survived poorly in freshwater (<5 min) and seawater (<4 h); thus it was suggested that host mucus or faeces confers protection to the trophozoite from osmotic stress. In the same study, no *S. salmonicida* cysts were detected (Kent et al. 1992). Hence, transmission of *S. salmonicida* and *S. vortens* is likely to be similar, directly facilitated by trophozoites.

As *Spironucleus vortens* trophozoites can survive for extended periods in angelfish faecal pellets, they potentially represent a transmission stage to new hosts, but if ingested they would have to withstand the acidic pH of the stomach. *S. vortens* trophozoites generally remained motile at pH 5–7, but motility

instantly ceased at pH ≤4, eventually resulting in cell lysis. Reduced motility of trophozoites over time at pH 6, as compared to that at pH 5 and 7, is intriguing. This may be explained by the possible presence of pH-sensitive metabolic or antioxidant enzymes in *S. vortens*, and requires further investigation. The stomach pH of freshwater angelfish has not been well characterised; however, that of *Holacanthus passer*, a marine angelfish species, has been reported to range from 2 to 5 (Martínez-Díaz & Pérez-España 1999). In another cichlid, *Oreochromis niloticus*, stomach pH levels range from 0.9 to 7 (Getachew 1989), being more acidic after feeding due to the secretion of gastric fluids for digestion. Hence, at the more alkaline end of the scale it may be plausible that *S. vortens* trophozoites can survive passage through the stomach. Indeed, *Spironucleus* trophozoites have previously been documented in the stomach of hosts, including mice (Fain et al. 2008), Siamese fighting fish (O'Brien et al. 1993) and angelfish (Sangmaneedet 1999), with that of mice being confirmed by molecular identification. As some fish, including angelfish (pers. obs.) are coprophagic (Bailey & Robertson 1982), it is highly likely that *S. vortens* trophozoites would be readily consumed and, given the appropriate conditions, may survive passage to the intestine. Indeed, Sangmaneedet (1999) showed that oral infection of angelfish with *S. vortens* trophozoites resulted in intestinal colonization by this parasite.

Spironucleus vortens encystment?

It has been proposed that cyst-like structures confer long-term survival of *Spironucleus* species outside the host (Woo & Poynton 1995). However, despite various methods being employed during the current study to induce encystment in *S. vortens*, no viable cyst-like structures were identified. Hence, under the conditions described, neither starvation (in spent culture medium and water) nor putative chemical triggers of encystment, e.g. bile and lactic acid, induced encystment of *S. vortens in vitro*. The latter chemical triggers have previously been documented as encystment-induction factors of the related diplomonads *S. salmonis* and *Giardia intestinalis* (see Gillin et al. 1989, Uldal 1996). For some protists, e.g. *Entamoeba histolytica*, it has been notoriously difficult to induce encystment *in vitro*, despite the confirmed presence of cysts *in vivo*. For *E. histolytica*, reactive O_2 species from the host immune system or intestinal microbiota have been suggested as potential triggers of encystment, a stress factor that may not be present to the same degree *in vitro* (Aguilar-Díaz et al. 2010). As *S. vortens* occupies a similar niche to *E. histolytica* (i.e. posterior intestine), this may explain the failure to induce encystment *in vitro* during this study. As a result, angelfish faecal samples were also analysed for the presence of putative *S. vortens* cysts. Due to morphological similarities between the cyst walls of *G. muris* and *S. muris* (see Poppe et al. 1992), a cyst wall antibody from *G. intestinalis* was employed to identify *S. vortens* cysts in angelfish faeces. Again, no such structures were identified, either indicating a lack of cysts or non-specificity of the *Giardia* antibody with *S. vortens* cysts. Interestingly, however, *S. vortens* trophozoites (ATCC strain) were found to be positively labelled with the antibody. The antibody has been previously shown not to react with *Giardia* trophozoites (WB strain, in culture since 1978; Chatterjee et al. 2010), which suggests that either the cell membrane of *S. vortens* trophozoites shares a similar epitope with the *Giardia* cyst wall, perhaps as a constituent of a protective pellicle, or that these trophozoites were in the process of encysting. Completion of the genome sequence of *S. vortens* will provide further insight into the presence or absence of encystment-specific genes in this organism.

CONCLUSION

In conclusion, our results call into question the existence of a cyst stage in the life cycle of *Spironucleus vortens*. This is especially relevant in aquaculture where host density, and therefore transmission potential via the faecal–oral route, is high. The non-invasive methods (faecal counts and 16S rDNA PCR) employed in this study require further investigation to determine their applicability in aquaculture for reliable diagnosis of *S. vortens* infection and subsequent control of spironucleosis. Prolonged survival of *S. vortens* trophozoites in faeces outside the host is a novel discovery that requires further physiological elucidation in terms of the capacity of the organism to tolerate high fluctuations in O_2 tensions and nutrition status.

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