Isolation and culture of *Sphaerothecum destruens* from Sunbleak (*Leucaspius delineatus*) in the UK and pathogenicity experiments in Atlantic salmon (*Salmo salar*)

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SUMMARY

The sunbleak (*Leucaspius delineatus*), a cyprinid fish native to continental Europe and now established in the UK, is experiencing population decline which appears to be linked to the spread of the invasive Asian cyprinid (*Pseudorasbora parva*). A population of sunbleak in the UK has previously been identified as infected with *S. destruens* at low prevalence. Because *Sphaerothaecum destruens* has, on occasion, caused severe disease in cultured and wild salmonids the aim of this work was to establish laboratory cultures of *S. destruens* from sunbleak in the UK and use these cultures in challenge experiments to determine if the UK isolate of *S. destruens* from cyprinid species is a potential threat to Atlantic salmon (*Salmo salar*). The first isolation and culture of *S. destruens* in the UK and from a cyprinid species is described. Cultured *S. destruens* spores from sunbleak are infective to EPC, CHSE and FHM cells, replicating most rapidly in FHM and EPC cells. Spores can be induced to zoosporulate in water forming motile, uni-flagellated zoospores. Challenge experiments indicated the spores are able to replicate and disperse in Atlantic salmon and are associated with increased mortality (up to 90%) when injected intraperitonealy.

Key words: rosette agent, cyprinids, 18S, IP injection, bath immersion, spores, zoospores.

INTRODUCTION

Sphaerothecum destruens, formerly known as the rosette agent, is an obligate intracellular parasite that causes disease and mortality in salmonid fish. This pathogen, which sits at the animal-fungal boundary, was previously grouped within the DRIP clade (Ragan *et al*. 1996), then the class Ichthyosporea (Cavalier-Smith, 1998) then the class Mesomycetozoea (Mendoza *et al*. 2002; Arkush *et al*. 2003). In a more recent revision of the classification of unicellular eukaryotes Adl *et al*. (2005) place this pathogen in the super-group Opisthokonta and refer to it as *Sphaerothecum destruens* (Mesomycetozoa; Ichthyosporea; Rhinosporideacae). *S. destruens* replicates by asexual division producing daughter cells of the spore stage which measure 2–6 μm in diameter. When transferred to deionized water these spores undergo internal division and then release up to 7 uniflagellated, motile zoospores of approximately 2 μm in diameter (Arkush *et al*. 2003).

Initially recorded in salmon in North America, *S. destruens* has been associated with sporadic severe infectious disease (occasionally mortalities up to 90%) of cage-reared Chinook salmon (*Oncorhynchus tshawytscha*) in North America (Harrell *et al*. 1986; Arkush *et al*. 1998) and in farmed Atlantic salmon (*Salmo salar*) in freshwater in California (Hedrick *et al*. 1989). The parasite from Chinook salmon has been established in cell culture in various cell lines and these cultures have been used to reproduce the disease in experimental challenges (Elston *et al*. 1986; Arkush *et al*. 1998; Mendonca and Arkush, 2004) in which a trend in host specificity was seen: chinook > coho >> rainbow > brown trout (*Salmo trutta*) with brook trout (*Salvelinus fontinalis*) almost refractory (Arkush *et al*. 1998). Two forms of the disease have been recorded in both the laboratory and natural infections; these are a limited nodular form with significant granulomatous response or a disseminated form with limited host response (Arkush *et al*. 1998; Andreou *et al*. 2011*a*). Apart from a few reported cultured salmonid outbreaks with high mortalities, the disease status, particularly in the wild, in North America is largely unknown.

Until recently the known host range of this parasite was limited to salmonids, but in 2005 in the UK the pathogen was discovered in a non-native cyprinid host, the sunbleak (*Leucaspius delineatus*) after experimental cohabitation with the topmouth gudgeon (*Pseudorasbora parva*), (Gozlan *et al*. 2005). The

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sunbleak, native to continental Europe, is experiencing population decline (Lelek, 1987; Mikschi *et al*. 1996) which has been proposed to be linked to the spread of the invasive Asian cyprinid the topmouth gudgeon (*Pseudorasbora parva*) (Gozlan *et al*. 2005). In species interaction studies, Gozlan *et al*. (2005) reported inhibition of spawning, wasting and then death in sunbleak co-habited with topmouth gudgeon or when exposed to their holding water. Detection in histological sections followed by subsequent PCR and sequence analysis confirmed the presence of S. *destruens* in the sunbleak leading these authors to postulate that the parasite was causing the declines and that, in the absence of visual evidence for the parasite in the topmouth gudgeon, this species acted as unaffected carriers. Given the potential of this parasite for causing severe disease in cultured and wild salmonids (and now possibly cyprinids) in the UK, this paper describes the development of cell cultures of the *S. destruens* from sunbleak in the UK and their use in challenge experiments to determine the pathogenicity of the isolate from sunbleak to Atlantic salmon by both intra-peritoneal injection of spores and bath immersion in zoospores.

MATERIALS AND METHODS

Animals

For the original isolation and for subsequent challenge experiments adult sunbleak (1 +, approx weight 2 g) were obtained from a freshwater pond in southern England (Park Pond, North Stoneham, Hampshire, UK, Grid Ref SU43301730). Atlantic salmon were obtained as eyed ova (Landcatch Natural Selection, Alloa, Scotland) and reared in-house. For challenge experiments salmon with an average weight of 9·91 g were used.

Isolation and culture of Sphaerothecum destruens *from sunbleak*

Chinook salmon embryo (CHSE-214, passage 275) cells (ECACC 91041114; Lannan *et al*. 1984), *Epithelioma papulosum cyprinid* (EPC, passage 34) cells (Fijan *et al*. 1983) and fat head minnow (FHM, passage 327) cells (ATCC CCL 42; Gravell and Malsberger, 1965) were maintained under standard subculturing conditions.

Sunbleak from the suspected positive site were netted in September 2006 brought to the laboratory and temperature manipulated to simulate an over-wintering period. For this the fish were initially maintained at 15 °C in freshwater for 10 days after arrival, then slowly acclimated to 9 °C and held at 9 °C for 1 month before returning to 15 °C for 2 months prior to sampling. Adult fish of approximately 2 g in weight were euthanized by an overdose of anaesthetic (120 mg/L benzocaine) followed by brain ablation. Fish were briefly dipped in 100% ethanol to sterilize, dabbed dry and then opened. Kidney and spleen were removed, dipped in 5% NaCl solution to remove surface contamination, rinsed briefly in culture medium (Eagles minimal essential medium (EMEM) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 5 μg/ml nystatin, 50 μg/ml gentamycin, 100 U/ml penicillin and 100 μg/ml streptomycin and buffered with 7·5% sodium carbonate) then placed in the appropriate volume of culture media to achieve a 1:10 dilution (generally tissues from 10 fish were pooled giving 0·5 g tissue in 4·5 ml of medium). Remaining carcasses were fixed in 10% neutral buffered formalin for subsequent histological analysis. Harvested tissue was incubated overnight in medium then homogenized with sand and a pestle in a mortar and briefly sonicated for 10 sec at 14 μm amplitude with a 23 kHz probe in a Soniprep ultrasonic disintegrator (MSE). The sand was allowed to settle out for 5 min at 4 °C then 80 μl of the homogenate was inoculated onto 24-h-old EPC, CHSE and FHM cell monolayers in 12·5 cm^2 culture flasks containing 4 ml of culture medium. Flasks were incubated at 15 °C for a maximum of 60 days. After initial isolation the *S. destruens* was subcultured by passage every 30 days. Culture medium containing released spores was removed from the flask, infected cell monolayers were briefly rinsed with sterile phosphate-buffered saline (PBS) then released from the flask by incubation with 0·25% trypsin-EDTA. Trypsinized cells were seeded at 1:2 dilution into new flasks with appropriate seeding density of fresh uninfected cells.

Molecular confirmation – PCR and sequencing

S. destruens spores from an infected EPC cell monolayer in a 75 cm^2 culture flask were harvested by scraping in 1 ml of medium. Cells and spores were pelleted by centrifugation for 5 min at 300 ***g***. The supernatant was removed and the pellet resuspended in 200 μl of lysis buffer G2 for automated extraction of DNA using an EZ1 DNA tissue kit and a Bio Robot EZ1 (Qiagen). Full-length small subunit (18S) ribosomal RNA gene DNA was amplified using primers NS1 and NS8 (White *et al*. 1990) synthesized by Sigma Genosys Ltd. Then 50 μl amplification reactions were prepared containing 2 μl of template DNA, 0·2 μM each primer, 0·25 mM each dNTP, 1x GoTaq DNA polymerase buffer (Promega), 2·5 mM $MgCl_2$ and 2·5 U Go Taq DNA polymerase (Promega). PCR amplification was performed in a DNA Engine 2 thermal cycler (M.J. Research). Cycling conditions were: 94 °C for 1 min. followed by 35 cycles of 94 °C for 1 min., 45 °C for 45 s then 72 °C for 1 min. with a final extension step of 72 °C for 10 min. PCR products were size separated through 1·2% agarose gels, visualized by ethidium bromide staining and UV transillumination

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(UVP). Products were excised from the gel, purified using a Wizard SV gel and PCR clean up kit (Promega), ligated into pGEM-T Easy cloning vector (Promega), transformed into JM109 *E.coli* cells (Promega) and grown on LB agar plates in the presence of 100 μg/ml ampicillin, 0·5 mM IPTG and 80 μg/ml X-gal. Plasmid DNA was prepared from selected positive colonies using the Wizard Plus Miniprep DNA purification system (Promega). Insert DNA was sequenced on both strands using the M13 universal sequencing primers and Big Dye Terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequencing reactions were analysed on an ABI Prism 3130×1 Genetic analyzer and edited, aligned and assembled into contigs and using Sequencer software (Gene Codes Corporation). Two independent 18S PCR amplification reactions were performed on the extracted *S. destruens* DNA and the products of these were independently cloned and 1 or 2 colonies from each separate PCR and cloning were fully sequenced. The consensus sequence was compared against the GenBank and EMBL sequence databases by BlastN search analysis (Altschul *et al*. 1997).

Induction of zoosporulation

After removal of released spores and media, infected cell cultures were harvested by scraping or trypsinization. Harvested cells were briefly sonicated for 10 sec, spores were then pelleted by centrifugation at 500 ***g*** for 10 min at 10 °C, rinsed twice in medium without FBS then re-suspended in sterile deionized water and incubated at 15 °C for 4 days until peak sporulation had occurred. Spore and zoospore counts were made with a haemocytometer.

Challenge experiments

Sunbleak for the challenge experiments were obtained in March 2007 from the same site that provided the fish used in the isolation and culture. Salmon (av. weight 9·91 g) and sunbleak (av. weight 1·91 g) were maintained in 30 litre tanks with 45 fish per tank. Fish were supplied with fresh well water at 12 °C with flow rates of 0·4 L min^{-1}, greater than 80% aeration and 12 h day-length (150–250 lux at water surface). Sunbleak and salmon were fed ground coarse and flake mix or Fry 03 diet respectively. Fish in a total of 5 tanks containing sunbleak and 5 tanks containing salmon were challenged by either intraperitoneal injection of 50 μl of culture medium containing 1×10^7 *S. destruens* spores (2 tanks for each species) or by bath exposure for 4 h to $1{\cdot}3\times10^5$ zoospores/ml (1 tank for each species). Spores were grown in and harvested from either CHSE or EPC cell cultures and used to challenge fish separately. The spore inoculum was similar to that used in previous experimental challenges with the North American isolates of *S. destruens* (Arkush *et al*. 1998). Sham IP challenges were performed using culture medium only (2 tanks for each species). Length and weight measurements were taken at the start of the challenge and on mortalities or samples taken at 1, 2 and 3 months post-exposure.

Histology

Sunbleak were euthanized by an anaesthetic overdose (120 mg/L benzocaine) and severance of the spinal column just posterior to the cranium. The visceral cavity was opened to allow access of fixative to the internal organs and whole fish placed in neutral buffered formalin (NBF). From salmon, following euthanasia, tissues including kidney, liver, spleen, heart, intestine and pyloric caecae as well as the head dissected in half longitudinally were immediately placed in NBF. Following fixation in NBF (for a minimum of 24 h) tissues were transferred to 70% industrial methylated spirit and processed to paraffin wax using a standard protocol and a vacuum infiltration processor. Embedded tissues were sectioned at 5 μm sections using a motorized rotary microtome, mounted on glass slides and stained with haematoxylin and eosin (H&E). Selected sections were subsequently stained with Gram stain to demonstrate the presence of *S. destruens*. Stained sections were examined using a Nikon Eclipse E800 photomicroscope and images captured using Lucia™ Screen Measurement System (Nikon, UK). Impression smears of affected liver tissue were air dried, fixed in 1% acetic methanol and stained with Gram stain.

Electron microscopy

Sphaerothecum destruens zoospores were filtered through a 0·1 μm ceramic syringe filter. The spores were fixed with 2·5% glutaraldehyde in 0·1 M sodium cacodylate buffer (pH 7·4) for 1 h at room temperature. Fixed spores were rinsed in 0·1 M sodium cacodylate buffer (pH 7·4) and post-fixed for 1 h in 1% osmium tetroxide in 0·1 M sodium cacodylate buffer. Spores were rinsed in 0·1 M sodium cacodylate buffer before dehydration through a graded acetone series. Filters were dried using a critical-point dryer, mounted onto aluminium stubs and sputter coated in 5 nm coating of gold. Filters were examined using a JEOL JSM-5200 Scanning Electron Microscope (SEM) and images taken using JEOL SemAphore software.

Statistics

Data were entered onto Excel spreadsheets and analysis of variance, regression and survival analysis was performed using 'anova', 'regress' and 'sts' commands in Stata 9.2. (Statacorp, 2007). Survival analysis was performed using log rank tests on Kaplan-Meier survival plots.

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RESULTS

Isolation and culture of Spaerothecum destruens *from sunbleak*

Isolation and culture of S*phaerothecum destruens* was achieved from 1 of 10 pools of 10 sunbleak kidney and spleen tissues after the homogenate was overlaid onto 24-h-old CHSE-214, EPC or FHM cell monolayers and incubated at 15 °C. Infected tissue culture cells were first observed by 12 days post-inoculation, with slightly greater abundance in FHM and EPC cultures than in CHSE cultures. Infection was apparent as small foci of phase bright cells containing blue/grey *S. destruens* spores within cells with gold-coloured released spores in the culture medium and resting on the monolayer (Fig. 1B, D and E). With continued incubation the foci of infection enlarged and eventually merged and the number of released spores in the medium increased. The infected cultures in EPC and CHSE cells were subcultured every 21 to 28 days depending on rate of infection within, and integrity of, the cell monolayer. The culture was not maintained in FHM cells because the rate of infection and monolayer degradation was rapid (7 days) compared to that in EPC and CHSE cells (approximately 21 and 35 days respectively).

PCR and sequence confirmation

PCR amplification of 18S small subunit ribosomal DNA produced a 1780 bp fragment which showed 99·94% identity (1 base mismatch over 1728 bases) to the BML *S. destruens* isolate (Accession no. AY267345). The cultured sunbleak *S. destruens* isolate has been called 'UK-Cefas1' and its 18S sequence lodged with EMBL/GenBank under Accession number FN996945.

Induction of zoosporulation

Harvested *S. destruens* spores from CHSE-214 or EPC cell cultures transferred to and incubated in deionized water were induced to zoosporulate producing small, motile, uni-flagellated zoospores (Fig. 1G and H). Preliminary work on zoosporulation efficiency and active motility at different incubation temperatures (Andreou *et al*. 2009) directed that, 4 days at 15 °C were chosen as the optimum incubation conditions to induce zoosporulation of as many spores as possible whilst maintaining viability (motility) of the resultant zoospores for as long as possible for use in zoospore challenge experiments.

Challenge experiments

Figure 2 (upper and lower) shows survival plots for salmon and sunbleak when challenged with *S. destruens* isolated from sunbleak. During the challenge there was a persistent fungal infection problem in the salmon, which was controlled by periodical 10 ppt seawater flushing, but which probably accounted for the mortalities in the sham IP-infected control tanks (21·4 and 12·8%) and contributed to some of the mortalities in the challenged salmon. However, survival plots for sham and *S. destruens* IP-injected salmon (Fig. 2 upper) diverged at approximately 40 days post-exposure and show a significant reduction in survival for the *S. destruens* injected salmon ($P<0·001$).

The pattern for sunbleak was similar, although the number of mortalities was less and the divergence between infected and control plots occurred later (Fig. 2 lower), after day 63. No difference in survival was found between the 2 control groups ($P>0·15$). The reduction in survival in the bath-exposed group was marginally significant when adjusted for multiple tests by Bonferroni adjustment ($P<0·03$) and compared to the very significant reduction in survival in the IP-exposed fish ($P<0·005$).

No difference was found in mortality or infectivity between spores generated from either cyprinid or salmonid cell cultures (EPC and CHSE-214 respectively) when injected into either sunbleak or salmon ($P>0·7$).

Table 1 shows the prevalence of infection in mortalities retrieved throughout the challenge and in fish sampled at 1, 2 and 3 months post-exposure. There was no evidence, by histopathology, of *S. destruens* in the mortalities or samples taken from control sham-infected salmon up to 3 months post-exposure. Out of the 39, 36 (92·3%) salmon mortalities that had been IP injected with *S. destruens* showed continued presence of the pathogen and 43 out of 45 (95·5%) sampled IP-injected salmon tested positive. There was no evidence of the parasite in mortalities or sampled fish from the salmon bath exposed to zoospores.

In the sunbleak 100% of all sampled sunbleak injected with *S. destruens* tested positive for the pathogen at all sampling points. Thirteen out of 14 (92·8%) IP-injected sunbleak mortalities and 3 out of 5 (60%) bath exposed sunbleak mortalities were positive for *S. destruens*. In the control sham-infected sunbleak, 5 out of 60 sampled and 1 out of 2 mortalities were positive for *S. destruens*, indicating a low level of prevalence (9·6%) of *S. destruens* in the sunbleak population used for these experiments.

Weight and standard length data were taken for each fish at the start of the challenge and from mortalities and samples taken post-exposure. The juvenile salmon grew throughout the study increasing in both length and weight. There was no indication that growth or condition of the juvenile salmon used in the study was affected by IP injection or bath exposure to spores or zoospores of *S. destruens* within the 3-month period of these challenges; however, the remaining salmon sampled at 84 days

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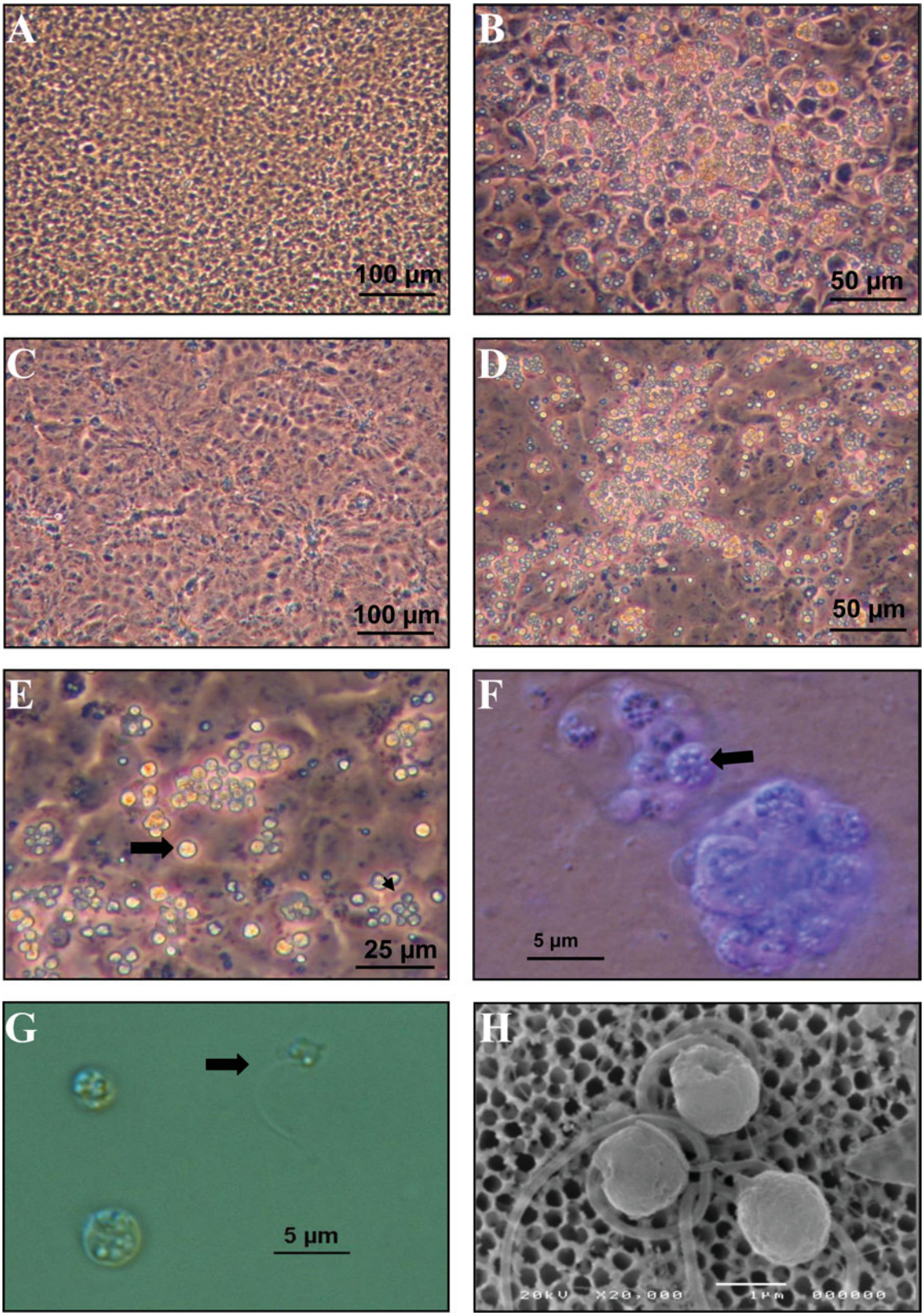

Fig. 1. *Sphaerothecum destruens* culture and life stage morphologies. (A and C) EPC and CHSE uninfected control cell cultures (X300 magnification). (B and D) *S. destruens* from sunbleak in EPC and CHSE cell culture (X600 magnification) under phase-contrast light microscopy, showing numerous foci of infection. (E) Infected CHSE cells at X1200 magnification, note gold-coloured phase bright released spores (arrow) and blue/grey coloured spores still within the cells (arrowhead). (F) Normarski differential interference microscopy image of spores within liver cells, with granules clearly visible within the spore (Scale bar = 5 μm). (G) Flagellated sporulated zoospore (arrow) in deionized water (Scale bar = 5 μm). (H) Scanning electron micrograph of flagellated zoospores (Scale bar = 1 μm).

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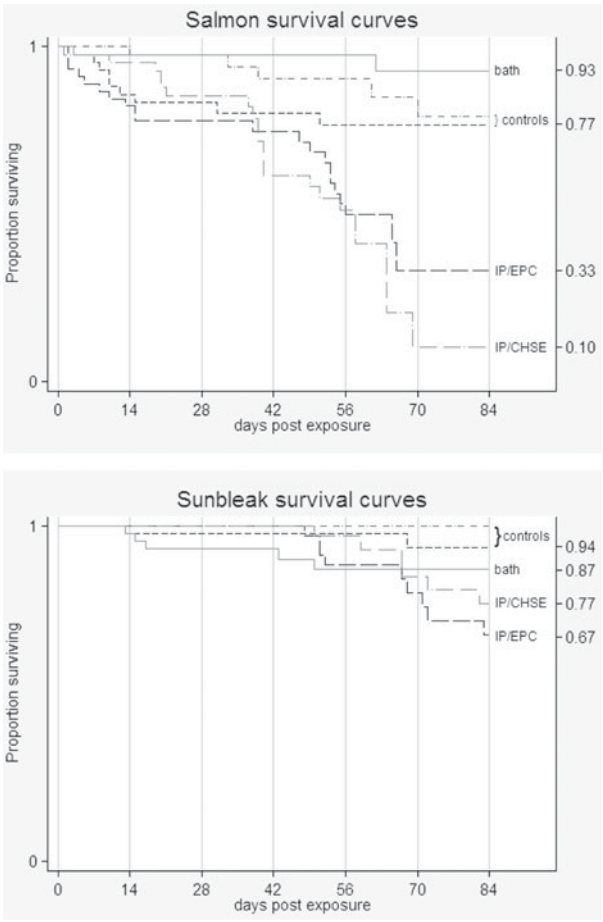

Fig. 2. Survival plots for *Sphaerothecum destruens* challenge experiments. Salmon (upper) and sunbleak (lower) were IP injected with 50 μl of culture medium containing 1×10^7 *S. destruens* spores grown in EPC or CHSE cells or sham infected with culture medium only (dashed lines). Solid lines indicate fish exposed by bath for 4 h to $1{\cdot}3 \times 10^5$ *S. destruens* zoospores/ml.

post-exposure, both pathogen exposed and sham controls, showed decreased condition. Analysis of covariance between the time-intervals showed no tank effect whilst time 4 (84 days post-exposure) was significantly different, $P < 0{\cdot}001$. The adult sunbleak did not grow during the study, but lost weight after day 29 and hence lost condition (Fig. 3). The same analysis of covariance gave a negative regression coefficient on time, $P < 0{\cdot}02$. This loss of condition was not limited to sunbleak exposed to *S. destruens*. Mortalities of salmon had the same mean weight overall as sampled fish (14·7 *vs* 14·8 g), whereas mortalities of sunbleak were substantially lighter than sampled fish (1·34 *vs* 1·85 g, *t*-test $P < 0{\cdot}001$).

Histopathology

IP-injected salmon mortalities and apparently healthy samples taken up to 3 months post-exposure showed no external signs of disease. At 1 month post-challenge 9/10 salmon injected with *S. destruens* grown in EPC cells (group 1) and 10/10 salmon

Table 1. Prevalence of infection in mortalities and sampled fish during experimental challenge with *Sphaerothecum destruens*

(Salmon and sunbleak were IP injected with 50 μl of culture medium containing 1×10^7 *S. destruens* spores grown in EPC or CHSE cells or sham infected with culture medium only (Con). The number positive/number sampled is given. Prevalence based on histopathology by examination of fixed, H&E stained slides.)

Treatment		Salmon	Sunbleak
29-day sample	*i.p. S.d.* EPC	9/10	10/10
	i.p. S. d. CHSE	10/10	10/10
	i.p. con. EPC	0/10	0/10
	i.p. con. CHSE	0/10	1/10
	Bath	0/10	1/10
56-day sample	*i.p. S.d.* EPC	10/10	10/10
	i.p. S. d. CHSE	10/10	10/10
	i.p. con. EPC	0/10	0/10
	i.p. con. CHSE	0/10	0/10
	Bath	0/10	0/10
84-day sample	*i.p. S.d.* EPC	3/4	10/10
	i.p. S. d. CHSE	0/1	10/10
	i.p. con. EPC	0/10	2/10
	i.p. con. CHSE	0/10	2/10
	Bath	0/10	1/10
Mortalities[a]	*i.p. S.d.* EPC	18/21	9/9
	i.p. S. d. CHSE	18/18	4/6
	i.p. con. EPC	0/9	1/2
	i.p. con. CHSE	0/5	0/0
	Bath	0/2	3/5

[a] Retrieved throughout the 3 month trial. Bath – fish exposed by bath to *S. destruens* zoospores.

challenged with CHSE derived *S. destruens* (group 2) displayed pancreatitis associated with the presence of the parasites. A few *S. destruens* cells were seen in kidneys, which displayed a proliferation of haemato-poietic tissue. This was not seen in group 2 fish. At 2 months post-challenge all fish from both groups were infected. Internally there were numerous plaques of pale tissue on the external surfaces of the liver and the spleen and extensive inflammation and haemorrhaging around the pyloric caecae (Fig. 4A). These plaques were superficial inflammatory lesions containing many intensely eosinophilic *S. destruens* spores (Fig. 4B). Smaller lesions were detected within the hepatic parenchyma (Fig. 4B) where they increased in size and formed granulomatous lesions surrounded by attenuated hepatocytes and inflammatory cells (Fig. 4C). Impression smears of affected liver tissue stained with Gram stain revealed the presence of numerous *S. destruens* spores of different sizes, characterized by the presence of intense Gram-positive staining cytoplasmic granules (Fig. 4D). In the inflamed areas of adipose and pancreatic tissues surrounding the pyloric caecae there was extensive replication of spores and replace-ment of pancreatic tissue with inflammatory cells and spores (Fig. 4E). Infection of the kidney was

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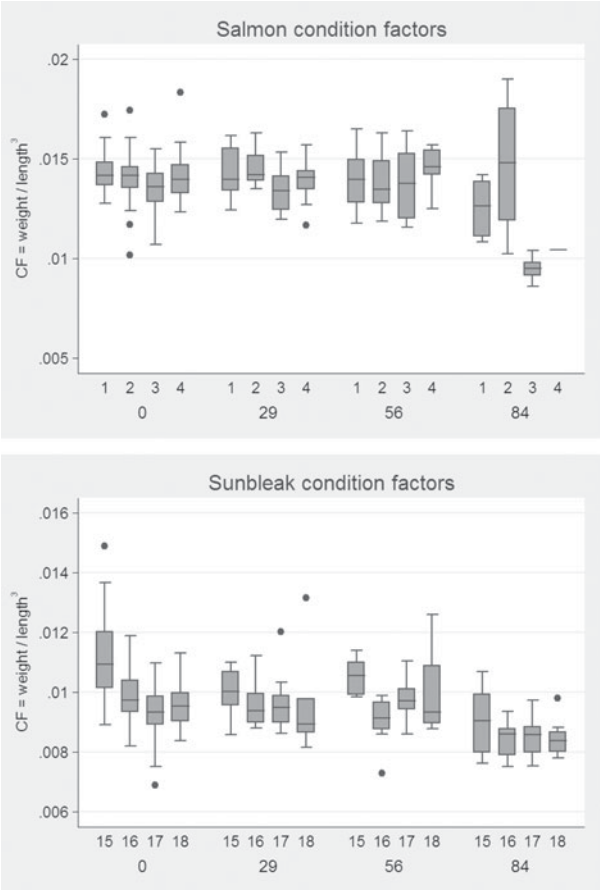

Fig. 3. Condition factor of challenged fish. Box plots indicating the condition factor of salmon (upper, groups 1–4) or sunbleak (lower, groups 15–18) at the start (0) and 29, 56 and 84 days post-challenge with *Sphaerothecum destruens* are shown. Fish were IP injected with 50 μl of culture medium containing 1×10^7 *S. destruens* spores grown in EPC (groups 2 and 16) or CHSE (groups 4 and 18) cells or sham infected with 50 μl of culture medium only (groups 1, 3, 15 and 17). Condition factor is based on weight and standard length ($n = 45$ for all groups at time 0, $n = 10$ for all groups at 29 and 56 days post-exposure and $n = 10$ for groups 15–18 at 84 days post-exposure; $n = 10$, 4, 10 and 1 for groups 1–4 respectively at 84 days post-exposure).

observed in several fish with numerous *S. destruens* spores visible within melanomacrophages (Fig. 4F). Other organs and tissues including the spleen, heart, choroidal rete and cranial connective tissue also harboured inflammatory lesions associated with the parasite. Similar histopathology was seen in sunbleak injected with *S. destruens*. In particular, pancreatitis, severe inflammation of the spleen and renal interstitial haematopoietic tissue with a marked proliferation of the *S. destruens* spores was seen in several fish.

DISCUSSION

S. destruens was successfully isolated from a population of non-native sunbleak in Southern England and established in cell culture. The parasite was cultured from a single infected fish out of a total of 126 pooled or individual fish sampled over a 1-year period, indicating a low prevalence in the population studied.

Previously, the *S. destruens* from Chinook salmon in the United States was readily established in cell culture in the Chinook salmon embryo cell line (CHSE-214) and in Chum salmon (*O. keta*) heart cells (CHH-1; Elston *et al.* 1986; Arkush *et al.* 1998). In contrast Coho (*O. kisutch*), Sockeye (*O. nerka*) and Atlantic salmon cell lines, (CSE-119, SSE-5 and AS cells respectively) were much less readily infected and Rainbow trout (*O. mykiss*) cells (RTG-2) were refractory to infection (Elston *et al.* 1986). Sequence analysis of a near complete 18S small subunit ribosomal RNA gene of the UK isolate from sunbleak indicated almost complete homology (99·94% identity over 1780 bp fragment including primer sequence) with previously described *S. destruens* isolates from the USA. Analysis of the less conserved ribosomal internal transcribed spacer (ITS) sequence between isolates from the USA and UK suggests a degree of geographical isolation between isolates from the USA and UK (Gozlan *et al.* 2009). It might be expected then, that the *S. destruens* from sunbleak in the UK, having been isolated from a cyprinid and showing some sequence divergence, would not grow as well in CHSE cells and may not infect salmonids. As reported here, *S. destruens* from sunbleak in the UK was isolated and grew from the first passage in FHM and EPC cells and also in CHSE cell cultures but with faster replication evident in the cyprinid cell lines (data not shown). Hence in cell culture at least the sunbleak isolate is not cyprinid specific.

In the challenge experiments undertaken and described here intra-peritoneal injection of high doses of cultured sunbleak *S. destruens* spores into salmon and sunbleak allowed for the establishment of an infection with replication and spread of *S. destruens* spores which significantly contributed to increased mortality. No difference was observed in subsequent mortality (or infectivity) when CHSE or EPC grown *S. destruens* was used to challenge both species. Since similar results were observed for the two different spore sources for both salmon and sunbleak, the mortality seen is not likely to be the result of a tank effect but rather the result of infection, i.e. experimental replication within the tank (45 fish IP injected) is supported by replication between tanks. Over the 3-month period of challenge in these experiments the salmon continued to grow and salmon maintained their condition until at the last sample (3 months post-exposure) when a reduction was seen in both pathogen-exposed fish and sham-exposed controls. The challenged sunbleak, whilst not appearing overtly emaciated, did show loss of condition throughout the challenge period. This

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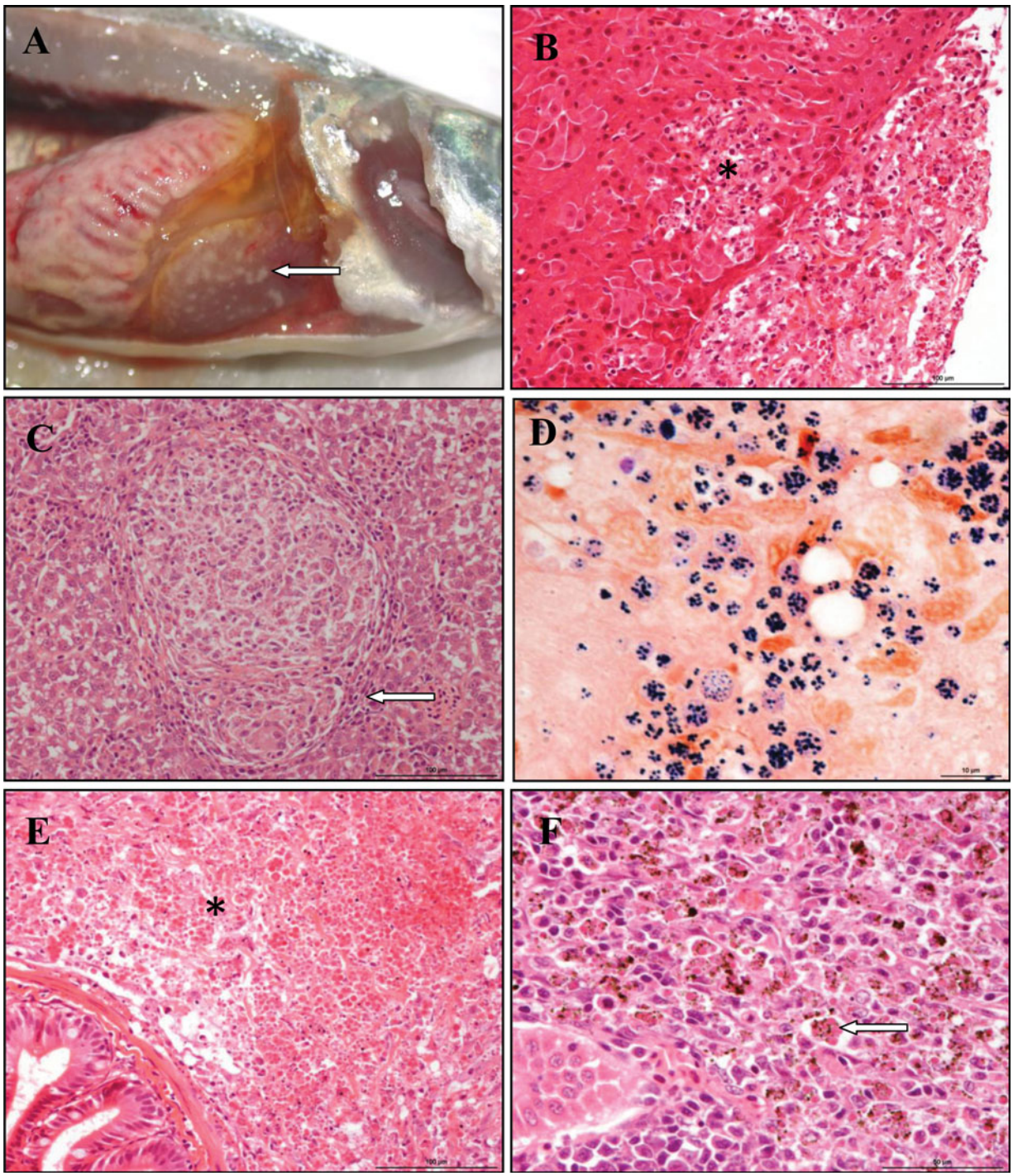

Fig. 4. Gross and microscopic pathology of salmon following intraperitoneal injection of *Sphaerothecum destruens*. (A) Dissection showing conspicuous plaques on the surface of the liver (arrow) and haemorrhaging of the pyloric caecae. (B) Perpendicular section through a plaque on the surface of a liver showing focal inflammation of the serosa associated with the presence of numerous *S. destruens* cells. Additional small foci of inflammation can be seen within the hepatic parenchyma (*). H&E, Scale bar = 100 μm. (C) Lesion within the liver with numerous *S. destruens* cells eliciting a granulomatous response. Note the lymphocytic infiltration of the surrounding liver tissue (arrow). H&E, Scale bar = 100 μm. (D) High-power view of an hepatic lesion. Note the granular Gram-positive staining of cellular constituents of *S. destruens* cells. Gram stain, Scale bar = 50 μm. (E) Extensive replacement of adipose and pancreatic tissue surrounding the pyloric caecae with numerous *S. destruens* cells of variable size (*). H&E, Scale bar = 100 μm. (F) High-power view of haematopoietic renal tissue showing infiltration of inflammatory cells associated with the presence of numerous *S. destruens* cells, many of which are within host melanomacrophages (arrow). H&E, Scale bar = 50 μm.

amounted to approximately a 1% decline in condition and was observed in both *S. destruens* exposed and sham-exposed controls. In relation to condition factor, tank to tank variation was negligible until the 84 day sample for salmon at which point numbers sampled were low in some tanks due to ongoing

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mortality. Although spawning was not monitored and assessed as an experimental parameter, nor was it encouraged by the correct temperature manipulation during holding, many sunbleak on dissection were gravid females. Andreou *et al*. (2011*b*) assessed, by quantitative PCR, the prevalence and level of infection of *S. destruens* in sunbleak when in both reproductive and non-reproductive states (by co-habitation and sampling at different times of year). Prevalence and level of infection was observed to be increased in fish during their reproductive season with no significant difference between male or female individuals. Whether the gravid female sunbleak infected with *S. destruens* in these and our experiments would have proceeded to spawn successfully was not tested. The spore dose used for the intraperitoneal injection of salmon was similar to that used in work previously undertaken in America with US isolates of *S. destruens* in various salmonid species (Arkush *et al*. 1998). In our experiments, designed to test the pathogenicity of the UK sunbleak isolate in salmon, the sunbleak were acting as positive controls to confirm the pathogenicity of the cell culture grown *S. destruens*, based on the fact that the pathogen was originally isolated from this species. In the challenge experiments the sunbleak received an identical dose to the salmon. Given the large difference in size between the two test species, one might have expected to have observed a higher rate of infection and mortality in the sunbleak relative to the salmon based on this higher dose (by body weight). In fact the prevalence of infection generated by this route of exposure was similar and nearly maximal for the two species (95·5 and 100% in sampled salmon and sunbleak respectively). That we did not observe higher mortality in the sunbleak might lead one to speculate that the salmon were significantly more susceptible to disease (as opposed to just infection) than sunbleak or that the pathogen may have a salmonid host preference *in vivo*. However, there are other confounding parameters such as age and sexual maturity that were different between the two test species populations that make direct comparison like this only speculative at this stage; further experimentation, including inoculation with lower doses, would be required to confirm such hypotheses.

Although the zoospore stage has been postulated to be infective (Arkush *et al*. 2003) this has not yet been proven. In our preliminary experiments bath emersion in zoospore suspension for 4 h failed to elicit disease or detectable spore replication in either sunbleak or salmon for a period up to 3 months post-exposure. It is possible that a longer exposure of pathogen to fish may have elicited a different response. The induction of stress or skin abrasion or the use of the so called 'ami-mori' process have been shown to assist with the establishment of infections via emersion challenges with zoospores for various species of the aquatic fungal pathogen *Saprolegnia* (Howe and Stehly, 1998; Grandes *et al*. 2001). Such processes may assist the development of infections in fish emersion exposed to *S. destruens* zoospores. Longer exposures to pathogen in static bath emersion may also add potential water quality stress, assisting the establishment of infection. This remains the subject of further work. It also remains to be seen whether more natural routes of infection such as bath immersion in spores or ingestion of infected material represent possible risks for transmission of *S. destruens* from sunbleak to wild or cultured salmon. Experiments with bath exposure of spores to other cyprinid species (Andreou, 2011*b*) support the preliminary evidence from cell culture and injection challenge and indicate that *S. destruens* is proving to have an increasingly broad species-specificity.

Atlantic salmon are clearly susceptible to infection, develop disease and suffer mortality after experimental IP injection (this work) and there has been 1 report of natural infection in farmed Atlantic salmon in the US (Hedrick *et al*. 1989). Despite this, to the best of our knowledge, naturally occurring infections of Atlantic salmon with this pathogen have not been reported from either wild populations or aquaculture facilities in the UK. Aquaculture facilities undergo both routine fish health examinations and investigations in response to reported disease outbreaks and examinations to date have not identified *S. destruens* infections from aquaculture facilities. The histopathology caused by *S. destruens* in diseased fish is easily identifiable by expert histopathologists. A low level of pathogen that does not cause disease, as proposed for topmouth gudgeon (Gozlan *et al*. 2005) is, of course, more difficult to detect. However, given the apparent susceptibility of Atlantic salmon to disease caused by this pathogen this would suggest that UK salmon aquaculture facilities have either not been infected with *S. destruens* or that the conditions for infection to cause disease have not been experienced. Since the majority of salmon production in the UK is located in Scotland where, to date, sunbleak and topmouth gudgeon populations have not become established, the opportunity to transmit disease from these freshwater cyprinid species to salmon in aquaculture will have been very limited. The impact to wild fish is more difficult to estimate given that mortalities are rarely available for analysis unless significant mortality events involving large numbers of fish occur. The disease status in the wild in the UK, as in America, is therefore largely unknown. Health screens and investigations into reported disease outbreaks of wild fish have also been a feature of the management practices of UK environmental departments for many years and as yet the pathogen has not been attributed as the cause of major disease outbreaks nor routinely identified. In the period since the initial discovery of *S. destruens* in the UK in 2005 until the end of November 2011 the number of

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combined investigations into relevant farmed and wild species in which a histopathological screen was undertaken includes a minimum of 585 Atlantic salmon, over 2700 other salmonids and over 3500 various cyprinids in England and Wales (source – Cefas, Fish Health Inspectorate database). Since first identification in the mid-1980s this pathogen has caused only sporadic and relatively few reported episodes of disease in the US. Taken together this might indicate that for Atlantic salmon, if the pathogen is present, the risk of infection may be relatively high but the likelihood of developing disease causing significant acute mortality events is relatively low. Other possible non-salmonid hosts or vectors of the pathogen in the US have, to date, not been identified. The prevalence of S. *destruens* in a single known established population of non-native sunbleak and topmouth gudgeon in southern England appears low (this work and Andreou *et al*. 2011*a*) but prevalence in other established populations of these species in the UK is as yet undetermined. It follows therefore that the infection pressure on populations of other species (including Atlantic salmon) co-habiting the water bodies or exposed to river water downstream is unknown. Further work to determine the presence or absence of *S. destruens* in these populations and develop robust emersion challenge methods with *S. destruens* spores and zoospores in Atlantic salmon for establishment of epidemiological parameters such as minimum infectious dose, effect of fish age, stocking density, infection coefficient, persistence and other transmission parameters would be instrumental in fully determining the risk of this pathogen to Atlantic salmon in the UK.

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