Influence of host reproductive state on *Sphaerothecum destruens* prevalence and infection level

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SUMMARY

Sphaerothecum destruens is an obligate intracellular parasite with the potential to cause high mortalities and spawning inhibition in the endangered cyprinid *Leucaspius delineatus*. We investigated the influence of *L. delineatus*'s reproductive state on the prevalence and infection level of *S. destruens*. A novel real time quantitative polymerarse chain reaction (qPCR) was developed to determine *S. destruens*' prevalence and infection level. These parameters were quantified and compared in reproductive and non-reproductive *L. delineatus*. The detection limit of the *S. destruens* specific qPCR was determined to be 1 pg of purified *S. destruens* genomic DNA. Following cohabitation in the lab, reproductive *L. delineatus* had a significantly higher *S. destruens* prevalence ($P<0{\cdot}05$) and infection levels ($P<0{\cdot}01$) compared to non-reproductive *L. delineatus*. *S. destruens* prevalence was 19% ($n=40$) in non-reproductive *L. delineatus* and 41% ($n=32$) in reproductive *L. delineatus*. However, there was no difference in *S. destruens* prevalence in reproductive and non-reproductive fish under field conditions. Mean infection levels were 18 and 99 pg *S. destruens* DNA per 250 ng *L. delineatus* DNA for non-reproductive and reproductive *L. delineatus* respectively. The present work indicates that *S. destruens* infection in *L. delineatus* can be influenced by the latter's reproductive state and provides further support for the potential adverse impact of *S. destruens* on the conservation of *L. delineatus* populations.

Key words: *Leucaspius delineatus*, cyprinids, PCR, fish, invasive, rosette agent, salmonid.

INTRODUCTION

All organisms have limited energy resources that must be divided between key biological functions such as reproduction and growth (Simkova *et al*. 2005). Commonly, parasites negatively impact the fitness of their host (e.g. body condition, growth rate, reproductive status, reproductive output), increasing the evolutionary pressure on hosts to select for an effective immune system, which acts in defence of the invading organism (Simkova *et al*. 2008). Energetically, this anti-parasite immune defence is costly (Connors and Nickol, 1991; Lochmiller *et al*. 1993; Sheldon and Verhulst, 1996) with expected knock-on effects on life-history traits such as growth and reproduction (Simkova *et al*. 2008). Simultaneously, an increased investment in reproduction, for example, may decrease the immune defence efficiency and thus facilitate parasite infection (Sheldon and Verhulst, 1996; Skarstein and Folstad, 1996; Skarstein *et al*. 2001; Simkova *et al*. 2005, 2008).

Seasonal changes in immunocompetence, i.e., the ability to produce an effective immune response, and parasitism have been observed across numerous taxa, including birds and fish (Hamilton and Zuk, 1982; Nelson and Demas, 1996; Sheldon and Verhulst, 1996). Specifically, down regulation (decrease) of immunocompetence during the reproductive period has been well documented in fish such as roach *Rutilus rutilus* L., chub *Leuciscus cephalus* L. and Arctic charr, *Salvelinus alpinus* L. (Skarstein and Folstad, 1996; Skarstein *et al*. 2001; Kortet *et al*. 2003; Vainikka *et al*. 2004; Lamkova *et al*. 2007; Simkova *et al*. 2005, 2008). Male and female fish usually invest differently in reproduction, with males placing a higher investment in mate attraction through the exaggeration of sexual ornamentation during spawning, while females invest more in gamete production (Simkova *et al*. 2008).

Sexual ornamentation in males has been associated with increased steroid sexual hormones (such as testosterone) that can negatively influence the immune system (Slater and Schreck, 1993; Kurtz *et al*. 2007). The impact of steroid sexual hormones on male immunity is known as the immunohandicap hypothesis (Hamilton and Zuk, 1982). Evidence for this includes the negative effect of high testosterone levels on spleen weight (Fange and Nilsson, 1985), negative impact on innate immunity (Kurtz *et al*. 2007) and significantly higher parasite infection in testosterone-treated fish (Buchmann, 1997). Examples from the field include reproductive male

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S. alpinus being more heavily infected with macroparasites compared to 'resting' (non-reproductive mature *S. alpinus*), suggesting a reproductive cost to immunity (Skarstein *et al*. 2001).

The energetic cost of high investment in gametes (also known as the energetic hypothesis) is expected to create a trade off between reproduction and quality of the immune system in females (Simkova *et al*. 2008). This has been confirmed for 17 species of cyprinid females including bream *Abramis brama* L., *R. rutilus* and topmouth gudgeon *Pseudorasbora parva* Temminck and Schlegel (see Simkova *et al*. 2008). Therefore, as females invest more in reproduction by producing larger gametes, they are expected to be more susceptible to infection compared to males (i.e. sperm is energetically less costly) (Nordling *et al*. 1998; Sanz *et al*. 2001). For example, female stone loach, *Barbatula barbatula* L., were more heavily parasitized with *Gyrodactylus* spp. and *Raphidascaris acus* during their reproductive season compared to male *B. barbatula* (Simkova *et al*. 2005).

An additional high energetic cost to reproduction is also expected for the sex which provides parental care and nest defence (Simkova *et al*. 2005). In the three-spined stickleback *Gasterosteus aculeatus* L., where males provide parental care and nest defence, males were more heavily parasitized with *Glugea anomala* cysts compared to females (Reimchen and Nosil, 2001; Arnold *et al*. 2003). Arnold *et al*. (2003) suggested that the observed sex biased parasitism could be the result of intense nest defence and parental care. Sex-biased parasitism would thus be expected in the sex providing parental care and nest defence.

Sunbleak *Leucaspius delineatus* is a small nest guarding cyprinid native to continental Europe and the only representative of its genus. *L. delineatus* has experienced significant declines in its native range and is currently listed on the IUCN red list of threatened species (WCMC, 1996). In *L. delineatus*, the cost of parental care, high courtship and aggression during reproduction in males and the high reproductive investment in females (i.e. batch spawning) could lead to reduced immunocompetence in reproductive individuals. *L. delineatus* has been found to be highly susceptible to the rosette agent *Sphaerothecum destruens* during its reproductive period with complete inhibition of *L. delineatus* spawning (Gozlan *et al*. 2005). However, infection of non-reproductive *L. delineatus* with *S. destruens* has not been investigated. *S. destruens* has been identified as a high-risk parasite for *L. delineatus* and a plausible cause for species decline throughout its native range (Gozlan *et al*. 2005, 2006, 2009). It is thus important to better understand this host-parasite association and, in particular, the effect of reproduction, as it can have direct implications for the species' conservation.

As an intracellular and unicellular parasite, the enumeration of individual *S. destruens* spores per host within fish tissues is particularly challenging. Currently, there is no established method to determine the parasite infection load. The only available method includes an estimation of infection severity in organ impression smears (Arkush *et al*. 2003) and histological sections. Molecular tools such as real time quantitative PCR have been used to estimate infection load of microparasites in aquatic organisms (Jones *et al*. 2003; Hallett and Bartholomew, 2006; Funk *et al*. 2007; Phelps and Goodwin, 2007). For example, Phelps and Goodwin (2007) used quantitative PCR to detect and quantify the microsporidian *Ovipeistophora ovariae* in the golden shiner *Notemigonus crysoleucas* Mitchill and showed an increase in spore release by *O. ovariae* during *N. crysoleucas*' spawning.

In this study, the effect of *L. delineatus*' reproductive state on *S. destruens* prevalence and infection level was investigated. A real time quantitative PCR was designed and optimized in order to detect and quantify infection levels of *S. destruens*. From the work by Gozlan *et al*. (2005) and the energetic trade off between immunity and reproduction, it was predicted that the host's reproductive state should play an important role on the prevalence and infection level of *S. destruens* in *L. delineatus*. Higher prevalence and infection levels in reproductive *L. delineatus* were predicted. In addition, the difference in infection with *S. destruens* between the sexes was also investigated. No significant difference in *S. destruens* prevalence and infection levels between male and female *L. delineatus* was predicted, as both sexes experience high energy costs during their reproductive period (nest guarding and batch spawning respectively).

MATERIALS AND METHODS

Molecular analysis and light microscopy

DNA extraction. DNA was extracted using the Rodent tail protocol of the Qiagen DNeasy 96 Blood & Tissue kit. Extracted DNA from fish tissues was quantified in a spectrophotometer at 260 nm (NanoDrop ND-1000; Labtech). The extracted DNA was then diluted to 125 ng μl^{-1} and was stored at −70 °C until further analysis by real time PCR.

Real time PCR primer selection. In order to detect and quantify the parasite's infection levels, a real time quantitative PCR was designed and optimized for *S. destruens*. Oligonucleotides for the qPCR were designed using small subunit ribosomal DNA (18S rRNA gene) sequences of the 3 *S. destruens* isolates (SK-AY267346; BML-AY267345; WA-AY267344), *Dermocystidium salmonis* (U21337), *Ichthyophonus hoferi* (U25637) and *Diaphanoeca*

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grandis (DQ059033). These sequences were obtained from GenBank and aligned with Clustal X (Thompson *et al*. 1997). The 18S rRNA gene sequence of *Cyprinus carpio* (AF133089) was used as an out-group and a cyprinid reference. Primers were visually selected to regions of 18–19 nucleotides showing 100% complementarily to the 3 *S. destruens* isolate sequences. Primer sequences had a minimum of 3 mismatches with sequences of related organisms. The melting temperatures, percentage guanine and cytosine content and potential to form secondary structures were evaluated for each primer. A single primer pair was then subjected to a basic local-alignment-search-tool (BLAST) search against all the nucleotide sequences stored in GenBank to confirm its specificity to *S. destruens*. The selected primer pair included the forward primer 5′-GGA TTT TGG GAT AAG CTT-3′ and reverse primer 5′-GT AAA AGT CCC AAA CTC-3′.

qPCR reaction conditions. The Platinum® SYBR® Green qPCR SuperMix-UDG kit (Invitrogen) was used for this assay. The reaction conditions included 20 μl reaction volumes containing 10 μl of Platinum® SYBR® Green qPCR SuperMix-UDG kit mix, 1 × bovine serum albumin, 0·4 μM of each primer and 250 ng of template DNA.

Cycling conditions. All reactions were performed in a Rotor Gene™ 6000 (Corbett Research) and were analysed with Rotor Gene™ 6000 version 1.7.75 software. A standard curve was generated from a 1 in 10 serial dilution of purified *S. destruens* DNA (10 ng to 1 fg) obtained from cell culture. Cycling conditions consisted of an initial denaturation cycle at 95 °C for 2 min followed by 45 cycles of 95 °C for 30 sec and 35 sec at 53 °C. A melting temperature curve was calculated at the end of the run at a temperature range of 55 to 99 °C.

Limits of detection. In order to quantify the amount of genomic *S. destruens* in samples and determine the detection limit of the qPCR, a standard curve was generated using a 10-fold serial dilution of *S. destruens* genomic DNA extracted from cell culture. Serial dilutions were prepared in sterile water to produce template concentrations of 10 ng to 1 fg. To simulate the screening of *S. destruens*-infected samples, an additional standard curve was produced by spiking serial dilutions of genomic *S. destruens* DNA (10 ng to 1 fg) with 270 ng ml^{-1} carp *Cyprinus carpio* kidney genomic DNA. qPCR was also performed on 270 ng and 540 ng of *C. carpio* genomic DNA to detect possible non-specific amplification by the qPCR primers. The correlation coefficient was calculated for both standard curves (Rotor-Gene 6000 v 1.7). Negative controls used in this assay included genomic *C. carpio* DNA and sterile water. For the purpose of this study, the detection limit was defined as the lowest genomic *S. destruens* DNA concentration in the PCR reaction. The optimal fluorescent threshold was determined automatically by the Rotor Gene™ 6000 version 1.7.75 (Corbett research) with a set upper bound threshold of 0·38. This threshold varied for each reaction (minimum value: 0·0056 and maximum value: 0·379) but did not alter the PCR efficiency and detection limits.

qPCR specificity. The specificity of the two qPCR primer pairs was tested using a PCR amplified *D. salmonis* DNA section inserted in the pGEM® – T (Promega). The *D. salmonis* insert had a length of 830 bp and included the binding areas of the two primer sets designed for *S. destruens*. Cross-amplification of the *S. destruens*-specific real time primers was tested on 4 ng of *D. salmonis* DNA.

qPCR detection capacity. The detection capacity of the *S. destruens*-specific qPCR was tested further through the analysis of genomic DNA samples from 10 *S. destruens*-positive organs confirmed through histological examination (Gram's and Haematoxylin & Eosin stain).

Light microscopy. All fish classified as positive by real time PCR were also assessed by light microscopy in order to (a) visually confirm the presence of the parasite and (b) determine the level of agreement between real time PCR and histology. Histological analysis consisted of staining with Haematolylin & Eosin and Gram's stain.

Determinination of the effect of the reproductive state of Leucaspius delineatus *on* Sphaerothecum destruens

Co-habitation experiments were carried out in 2006 for a total of 60 days each during *L. delineatus*'s reproductive (May–July) and non-reproductive season (October–December). In total, 250 *L. delineatus* were collected by seine netting from Stoneham lakes (Eastleigh, England: 50°57′14″N; 1°22′56″W) on each of 2 occasions in May 2006 and October 2006. *L. delineatus* were transferred to the laboratory in 90 L bins of water with a supply of oxygen. The maximum transport time was 2 h. On arrival, *L. delineatus* were sorted by sex (through visual inspection: females are larger than males and have a prominent ovipositor) and were then placed in 70 L aquaria (length × width × height = 90 × 30 × 30 cm). The sex ratio per aquarium was approximately 1:1 (this was confirmed through histological examination of the gonad at the end of the experiment). Maximum stocking density per aquarium was 35 fish. The weight (measured to the nearest 0·1 g) and fork length (measured to the nearest 0·1 cm) of 70 *L. delineatus* (35 females and 35 males) was recorded prior to the experiments' onset.

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In order to determine the prevalence of *S. destruens* in Stoneham Lakes, an additional 100 *L. delineatus* were randomly collected and euthanized straight after capture following Home Office (HO) guidelines using a lethal overdose of 2-phenoxyethanol anaesthetic and severance of the spinal cord using a sterile blade. These samples were stored at −70 °C for further molecular analysis.

Leucaspius delineatus *co-habitation set-up*. A sample of 100 *L. delineatus* from Stoneham Lakes was co-habited in 2 × 3-tank re-circulating 70 L aquaria on 2 occasions; during the reproductive and non-reproductive season of *L. delineatus* ($n = 2$ groups of fish per season). The water used in the re-circulating systems was dechlorinated tap water and fish were introduced into the system after being rinsed with clean water. The flow rate was approximately 2 L/min. Water was filtered through a biological filter with gravel substrate, and was then redistributed to the aquaria. In this experiment, fish in different tanks shared the same water supply but remained physically isolated from one another. Fish were fed twice a day with commercial flake food (Nutrafin MAX, Hagen) and kept under natural light conditions at room temperature. Temperature was recorded hourly using a temperature recorder (Tinytag Splash and Aquatic, OmniInstruments, Dundee, UK). Each re-circulating system had its own nets and cleaning devices. Water temperature in the aquaria was monitored throughout the duration of the experiments. Mean water temperature was 25 °C ± 1·5 during the reproductive season and 19 °C ± 1·3 during the non-reproductive period.

Sampling and sample processing

The fish were checked twice daily, at which time any mortalities present were collected and dissected. At 14-day intervals, 10 non-moribund *L. delineatus* were randomly sampled across the 3 tanks of each re-circulating system. In order to keep the number of fish sampled from each tank equal, tanks from which 4 fish were sampled were alternated with each sampling period. Fish were euthanized according to HO guidelines. The weight and fork length of each fish were recorded prior to dissection. Each fish was dissected using individual dissecting boards. Dissection tools were thoroughly wiped with 90% ethanol and flamed between dissections. Tissues collected included the gill, liver, kidney, gonad and posterior intestine of sampled and dead fish. Each organ sample was divided, preserving one half at −70 °C for molecular analysis (DNA extraction and qPCR) and the other half in 10% neutral-buffered formalin for histological analysis.

The Fulton's condition factor (K_F) was used to determine the somatic condition of *L. delineatus* and was calculated according to Ostlund-Nilsson *et al.* (2005): $K_F = (W/FL) * 10^5$. W was the weight in grams and FL the fork length of the fish in millimeters. Fish condition was considered separately for each sex as they were expected to naturally differ, especially during the reproductive season of *L. delineatus* when female fish were gravid with eggs.

Statistical analysis

All statistical analyses were performed using SPSS 14.0 (SPSS Inc., Chicago, Illinois, USA). Data are given as means ± standard deviation (S.D.) unless otherwise stated. The Mann-Whitney *U*-test was used to test for differences in *S. destruens*' prevalence, infection level and fish condition between *L. delineatus* in the reproductive season and *L. delineatus* in the non-reproductive season. Difference in prevalence and infection level between the sexes was also investigated.

Disease prevalence was calculated as: (number of *S. destruens*-positive fish / total number of fish tested) × 100. Parasite infection levels were quantified using real time PCR as picograms (pg) of *S. destruens* DNA per 250 ng of *L. delineatus* DNA. The total infection level per fish was calculated as the sum of all infection levels across the 5 organs considered here.

RESULTS

Real time PCR optimization

Using a 10-fold serial dilution of genomic *S. destruens* DNA, the detection limit of the *S. destruens*-specific qPCR was determined to be 1 pg of purified *S. destruens* genomic DNA both in the presence and absence of *C. carpio* genomic DNA. The PCR amplicon was 83 bp long with a melting temperature of 86 °C. Mean correlation coefficients were high both in the presence and absence of common carp DNA (0·99 and 0·97 respectively). The inclusion of *C. carpio* genomic DNA did not interfere with the amplification of *S. destruens* genomic DNA. There was no observable non-specific amplification when the qPCR was performed on 270 ng of *C. carpio* genomic DNA and *D. salmonis* DNA.

Six of the 10 *S. destruens* positive by histology samples were also confirmed as positive by qPCR and 4 of the 5, *S. destruens* negative by histology samples were detected positive by qPCR. Real time PCR was successfully performed on the DNA of 460 organs, with a mean R^2 value of 0·99 ± 0·01. All extraction-negative samples were detected as negative during PCR.

Light microscopy

Only 15% of reproductive *L. delineatus* ($n = 13$) and 33% of non-reproductive *L. delineatus* ($n = 6$) that had

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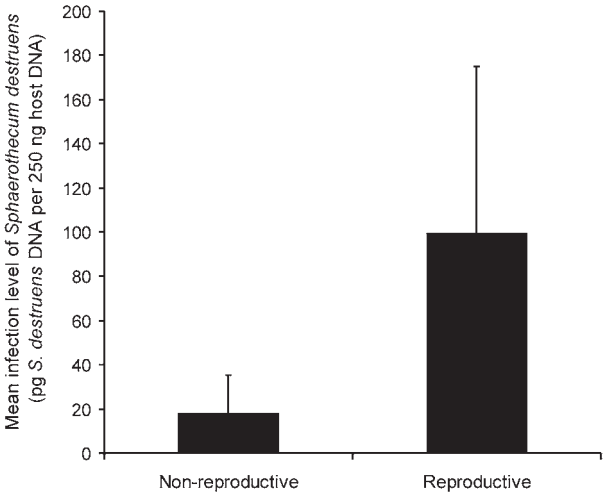

Fig. 1. Mean infection level (pg *Sphaerothecum destruens* per 250 ng sunbleak *Leucaspius delineatus* DNA) for reproductive and non-reproductive parasitized *L. delineatus*. Infection levels were determined by real time PCR of the 18S rRNA gene of *S. destruens* for the kidney, liver, posterior intestine, gonad and gill of *L. delineatus*. Means ± S.E. are given ($n=6$ and $n=13$ for non-reproductive and reproductive *L. delineatus* respectively).

tested positive using qPCR were also found to be positive by light microscopy. Overall, qPCR detected 3 times more positive samples in non-reproductive *L. delineatus* and 6 times more in reproductive *L. delineatus* compared to histological methods.

Sphaerothecum destruens *prevalence in the wild source population*

In order to determine an increase in parasite prevalence as a result of the experimental treatment, *S. destruens* prevalence in *L. delineatus* (using the kidney as the organ of choice) was determined at the start of each experiment. *S. destruens* prevalence in *L. delineatus* was found to be 2% (95% Confidence Intervals (CI): −0·8 to 5) during the reproductive season and 1% (95% CI: 1 to 2) during the non-reproductive season by real time PCR. Prevalence in the two sampling periods did not significantly differ (Mann-Whitney *U*-test; $n=200$, $P=0{\cdot}56$).

Under experimental conditions, reproductive *L. delineatus* had a significantly higher *S. destruens* prevalence (Mann-Whitney *U*-test; $n=72$, $P<0{\cdot}05$) and infection levels (Mann-Whitney *U*-test; $n=72$, $P<0{\cdot}01$) compared to non-reproductive *L. delineatus*. *S. destruens* prevalence was 19% ($n=40$) in non-reproductive *L. delineatus* and 41% ($n=32$) in reproductive *L. delineatus*. Mean *S. destruens* infection levels were 18 and 99 pg *S. destruens* DNA per 250 ng of *L. delineatus* DNA for non-reproductive and reproductive *L. delineatus* respectively (Fig. 1). There was no significant difference in *S. destruens* prevalence (Mann-Whitney *U*-test; $n=13$, $P=0{\cdot}13$) and infection level (Mann-Whitney *U*-test; $n=13$, $P=1$) between infected reproductive female and male *L. delineatus*.

Overall, female and male *L. delineatus* in the reproductive season had significantly lower condition factors when compared to their non-reproductive counterparts (Females: Mann-Whitney *U*-test; $n=38$, $P=0{\cdot}01$. Males: Mann-Whitney *U*-test; $n=34$, $P<0{\cdot}05$). However, there was no significant difference in the condition factor of parasitized reproductive male and reproductive female *L. delineatus* (Mann-Whitney *U*-test; $n=13$, $P=0{\cdot}23$). When the condition factor of parasitized reproductive female and male *L. delineatus* were compared to their respective non-infected reproductive counterparts there was no significant difference in condition (Reproductive females: Mann-Whitney *U*-test; $n=14$, $P=1$. Reproductive males: Mann-Whitney *U*-test; $n=18$, $P=0{\cdot}437$).

S. destruens prevalence and infection levels differed between reproductive and non-reproductive *L. delineatus* (Tables 1 and 2). The parasite was present in all 5 organs in reproductive fish with varying prevalence whereas it was only present in 3 organs with equal prevalence in the non-reproductive fish (Table 2). The highest infection level was in the gill for non-reproductive *L. delineatus* and the kidney for reproductive fish.

DISCUSSION

Determining the infection level of intracellular parasites is particularly difficult and infection estimates are limited to arbitrary infection categories following examination of histological sections or impression smears. This study describes the first real time quantitative PCR developed for the detection and quantification of *S. destruens* infections. The real time PCR developed here was capable of detecting 1 pg of purified genomic *S. destruens* DNA both in the presence and absence of *C. carpio* genomic DNA. This was equivalent to the detection limit of the nested PCR developed for *S. destruens* by Mendonca and Arkush (2004). The lack of non-specific amplification when the qPCR was performed on 270 ng and 540 ng of common *C. carpio* genomic DNA and *D. salmonis* DNA supported the specificity of the reaction to *S. destruens* genomic DNA.

The qPCR developed here detected *S. destruens* in 6 of the 10 *L. delineatus* samples that were previously determined positive for *S. destruens* (by histology). The discrepancy between the histology and qPCR for 4 of the 10 *S. destruens*-positive samples can be explained by: (a) very little available fish tissue, as each organ was halved for histology and qPCR analysis, (b) unequal distribution of the parasite within the organ, and (c) the possible misidentification of other *Dermocystidium* spp. as *S. destruens* in

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Table 1. Percentage of *Sphaerothecum destruens* infected organs in non-reproductive and reproductive sunbleak *Leucaspius delineatus*

(*n*, Number of *L. delineatus* tested.)

	n	Gill	Kidney	Posterior intestine	Gonad	Liver	Overall prevalence
Non-reproductive	40	5	0	0	5	5	19
Reproductive	32	9	22	16	9	3	41

Table 2. Mean infection level (pg *Sphaerothecum destruens* DNA per 250 ng sunbleak *Leucaspius delineatus* DNA) in non-reproductive and reproductive *L. delineatus*' organs

(Standard deviation from the mean and number (*n*) of samples are provided in parentheses. * Only 1 positive liver sample was present therefore mean infection level and standard deviation could not be calculated.)

	Gill	Kidney	Posterior intestine	Gonad	Liver
Non-reproductive	52·9 (74·7; *n* = 2)	0	0	0·19 (0·05; *n* = 2)	0·13 (0·007; *n* = 2)
Reproductive	0·63 (0·6; *n* = 3)	166·8 (321·9; *n* = 7)	22·7 (50; *n* = 5)	0·26 (0·17; *n* = 3)	5·9*

histological sections (Mendonca and Arkush, 2004). Small tissue samples, in some cases below 5 mg, showed non-homogeneous distribution of the parasite in the organ as well as low extraction efficiencies. This could lead to very low parasite genomic DNA available for PCR. As a result, such samples may test negative by PCR (i.e. false negative).

Discrepancies between histology-positive samples and PCR analysis have also been reported in other studies involving aquatic organisms (Jones *et al*. 2003; Kozubikova *et al*. 2008; True *et al*. 2009). Jones *et al*. (2003) and True *et al*. (2009) failed to detect the parasite *Parvicapsula minibicornis* in *Oncorhynchus tshawytscha* with histological evidence of infection. This was attributed to either misidentification of the parasite in histological sections, mislabelling of samples or PCR false negatives due to the presence of PCR inhibitors. The difficulty of detecting low parasite numbers in histological sections (Mendonca and Arkush, 2004) is also evident in the detection of *S. destruens* in 4 of the 5 histologically negative samples. Discordance is typical for samples with low parasite quantities (True *et al*. 2009).

Only 15% of reproductive *L. delineatus* (*n* = 13) and 33% of non-reproductive *L. delineatus* (*n* = 6) that were detected as positive using qPCR were also found to be positive using light microscopy, reflecting the low agreement between these methods. In *O. tshawytscha* challenged with *S. destruens* by bath immersion, the percentage agreement between nested PCR (with 1 pg purified genomic *S. destruens* DNA detection limit) of the kidney and Gram-stained kidney smears was 33% (Mendonca and Arkush, 2004). More fish were positive by PCR perhaps because PCR has a lower detection limit than histology of kidney smears.

Reproductive *L. delineatus* experienced significantly higher prevalence and infection levels of *S. destruens* compared to non-reproductive *L. delineatus*. Although these results indicate that *L. delineatus*' reproductive state influences *S. destruens* prevalence and infection levels, other potentially explanatory factors also need to be considered. Firstly, the mean water temperature between the two experiments significantly differed (by 6 °C) and variation in temperature is known to affect fish metabolism (Wang *et al*. 2009). Moreover, seasonality in temperature has also been associated with variation in immunocompetence (Bowden, 2008).

Parasites have a range of permissive temperatures at which they complete their life cycle with some temperatures being more permissive than others (Fels and Kaltz, 2006; Kerans *et al*. 2005; Wolinska and King, 2009). For example, experimental infection with proliferative kidney disease *Tetracapsuloides bryosalmonae* in rainbow trout *O. mykiss* was more severe at 18 °C compared to 12 °C possibly due to greater proliferation of the parasite in the host at higher temperatures (Bettge *et al*. 2009). Temperature dependence in *S. destruens* spore survival and production of zoospores has been observed (Andreou *et al*. 2009).

The influence of temperature on *S. destruens* prevalence and infection level cannot be excluded and it would be interesting for future work to control for its effect. However, it should be noted that the observed *S. destruens* prevalence in reproductive *L. delineatus* was quite similar to that reported by Gozlan *et al*. (2005), in reproductive *L. delineatus* co-habited in the presence of *S. destruens* at a mean temperature of 20 °C. It is also important to note that higher temperatures are naturally experienced by

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wild populations of *L. delineatus* during their reproductive season between spring and summer (Pinder and Gozlan, 2004).

A natural infection method was used, with co-habitation of test fish with individuals originating from an infected natural population. Although the difference in time between the two treatments (4 months) could have resulted in differences in the initial dose of *S. destruens* it is unlikely that such differences could have confounded the results. Specifically, the prevalence of *S. destruens* in the population of origin was determined at the beginning of both treatments and was not found to significantly differ (2% and 1% for reproductive and non-reproductive *L. delineatus*, respectively). However, since higher doses of an infectious agent are more likely to cause infection and disease, future work should control for initial dose of *S. destruens*.

Some parasites have been reported to increase in prevalence during their host's reproductive season (Simkova *et al*. 2005), whilst others have been shown to synchronize the shedding of infectious stages with reproduction (Browne *et al*. 2006). *S. destruens* spores have been reported from seminal fluids of reproductive *O. tshawytscha* and it has been hypothesized that release through seminal fluids is one possible route of the parasite's dissemination (Arkush *et al*. 1998, 2003). The hypothesis that *S. destruens,* a freshwater parasite, synchronizes its release with *O. tshawytscha* reproduction is likely as the host's return to freshwater is marked with spawning followed by mortality (Quinn, 2004). Accordingly, the possibility that the observed increase in prevalence and infection level of *S. destruens* during *L. delineatus*'s reproduction is the result of the parasite's interaction with hormonal changes during reproduction cannot be excluded.

The high reproductive investment experienced by both sexes in *L. delineatus* has led to the hypothesis that male and female *L. delineatus* would not differ in the level of parasitism by *S. destruens*. In line with this prediction, sex-biased parasitism has not been observed here as reproductive female and male *L. delineatus* did not differ in the prevalence and infection level of *S. destruens*. This suggests that the species high reproductive investment has resulted in an immunocompromised state.

The reproductive state of *L. delineatus* appeared to influence fish condition. In particular, reproductive *L. delineatus* had significantly lower condition factors compared to non-reproductive *L. delineatus*. In the reproductive state, parasitized male and female *L. delineatus* did not have significantly different condition factors suggesting that there was no apparent cost of parasitism, at least as measured by fish condition, between the sexes. Poor fish condition, specifically emaciation was reported for dead and moribund *L. delineatus* that were infected with *S. destruens* during *L. delineatus*' reproductive season (Gozlan *et al*. 2005). Emaciation was also observed in *L. delineatus* mortalities during the reproductive period experiment. However, when non-moribund parasitized fish were compared to their non-parasitized counterparts, there was no significant difference in their condition factors. Nevertheless, the possibility that advanced *S. destruens* infections could result in decreases in fish condition during spawning cannot be excluded. Emaciation was not observed in the non-reproductive period experiment.

DNA-based technologies for detecting parasites are becoming commonplace and the use of real time quantitative PCR can provide additional information such as infection levels. The development of a quantitative PCR for *S. destruens* provides an improved method for detecting the parasite and quantifying infection levels and can be used as a tool to address questions on the parasite's life-history traits. The present work indicates that *S. destruens* infection in *L. delineatus* can be influenced by the latter's reproductive state or temperature. Future work should include the monitoring of *S. destruens* infection level in pre-spawning, spawning and post-spawning wild *L. delineatus* while measuring reproductive hormone levels in each group.

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REFERENCES

Andreou, D., Gozlan, R. E. and Paley, R. (2009). Temperature influence on production and longevity of *Sphaerothecum destruens*' zoospores. *Journal of Parasitology* **95**, 1539–1541.

Arkush, K. D., Frasca, S. and Hedrick, R. P. (1998). Pathology associated with the rosette agent, a systemic protist infecting salmonid fishes. *Journal of Aquatic Animal Health* **10**, 1–11.

Arkush, K. D., Mendoza, L., Adkison, M. A. and Hedrick, R. P. (2003). Observations on the life stages of *Sphaerothecum destruens* N. G., N. Sp., a mesomycetozoean fish pathogen formally referred to as the rosette agent. *Journal of Eukaryotic Microbiology* **50**, 430–438.

Arnold, K. E., Adam, A., Orr, K. J., Griffiths, R. and Barber, I. (2003). Sex-specific survival and parasitism in three-spined sticklebacks: seasonal patterns revealed by molecular analysis. *Journal of Fish Biology* **63**, 1046–1050.

Bettge, K., Wahli, T., Segner, H. and Schmidt-Posthaus, H. (2009). Proliferative kidney disease in rainbow trout: time- and temperature-related renal pathology and parasite distribution. *Diseases of Aquatic Organisms* **83**, 67–76.

https://doi.org/10.1017/S0031182010000983 Published online by Cambridge University Press

Bowden, T. J. (2008). Modulation of the immune system of fish by their environment. *Fish & Shellfish Immunology* **25**, 373–383.

Browne, R. K., Li, H. and Vaughan, M. (2006). Sexually mediated shedding of *Myxobolus fallax* spores during spermiation of *Litoria fallax* (Anura). *Diseases of Aquatic Organisms* **72**, 71–75.

Buchmann, K. (1997). Population increase of *Gyrodactylus derjavini* on rainbow trout induced by testosterone treatment of the host. *Diseases of Aquatic Organisms* **30**, 145–150.

Connors, V. A. and Nickol, B. B. (1991). Effects of *Plagiorhynchus cylindraceus* (Acanthocephala) on the energy-metabolism of adult starlings, *Sturnus vulgaris*. *Parasitology* **103**, 395–402.

Fange, R. and Nilsson, S. (1985). The fish spleen – structure and function. *Experientia* **41**, 152–158.

Fels, D. and Kaltz, O. (2006). Temperature-dependent transmission and latency of *Holospora undulata*, a micronucleus-specific parasite of the ciliate *Paramecium caudatum*. *Proceedings of the Royal Society of London, B* **273**, 1031–1038.

Funk, V. A., Raap, M., Sojonky, K., Jones, S., Robinson, J., Falkenberg, C. and Miller, K. M. (2007). Development and validation of an RNA- and DNA-based quantitative PCR assay for determination of *Kudoa thyrsites* infection levels in Atlantic salmon *Salmo salar*. *Diseases of Aquatic Organisms* **75**, 239–249.

Gozlan, R. E., St-Hilaire, S., Feist, S. W., Martin, P. and Kent, M. L. (2005). Biodiversity – Disease threat to European fish. *Nature, London* **435**, 1046.

Gozlan, R. E., St-Hilaire, S., Feist, S. W., Longshaw, M. and Peeler, E. J. (2006). The effect of microbial pathogens on the diversity of aquatic populations, notably in Europe. *Microbes & infections* **8**, 1358–1364.

Gozlan, R. E., Whipps, C., Andreou, D. and Arkush, K. (2009). Characterisation and geographical isolation of *Sphaerothecum destruens* in Europe. *International Journal for Parasitology* **39**, 1055–1058.

Hallett, S. L. and Bartholomew, J. L. (2006). Application of a real-time PCR assay to detect and quantify the Myxozoan parasite *Ceratomyxa shasta* in river water samples. *Diseases of Aquatic Organisms* **71**, 109–118.

Hamilton, W. D. and Zuk, M. (1982). Heritable true fitness and bright birds – a role for parasites. *Science* **218**, 384–387.

Jones, S. R. M., Prosperi-Porta, G., Dawe, S. C. and Barnes, D. P. (2003). Distribution, prevalence and severity of *Parvicapsula minibicornis* infections among anadromous salmonids in the Fraser River, British Columbia, Canada. *Diseases of Aquatic Organisms* **54**, 49–54.

Kerans, B. L., Stevens, R. I. and Lemmon, J. C. (2005). Water temperature affects a host-parasite interaction: *Tubifex tubifex* and *Myxobolus cerebralis*. *Journal of Aquatic Animal Health* **17**, 216–221.

Kortet, R., Taskinen, J., Sinisalo, T. and Jokinen, I. (2003). Breeding-related seasonal changes in immunocompetence, health state and condition of the cyprinid fish, *Rutilus rutilus*, L. *Biological Journal of the Linnean Society* **78**, 117–127.

Kozubikova, E., Petrusek, A., Duris, Z., Martin, M. P., Dieguez-Uribeondo, J. and Oidtmann, B. (2008). The old menace is back: recent crayfish plague outbreaks in the Czech Republic. *Aquaculture* **274**, 208–217.

Kurtz, J., Kalbe, M., Langefors, Å., Mayer, I., Milinski, M. and Hasselquist, D. (2007). An experimental test of the immunocompetence handicap hypothesis in a teleost fish: 11-ketotestosterone suppresses innate immunity in three-spined sticklebacks. *The American Naturalist* **170**, 509–519.

Lamkova, K., Simkova, A., Palikova, M., Jurajda, P. and Lojek, A. (2007). Seasonal changes of immunocompetence and parasitism in Chub (*Leuciscus cephalus*), a freshwater cyprinid fish. *Parasitology Research* **101**, 775–789.

Lochmiller, R. L., Vestey, M. R. and Boren, J. C. (1993). Relationship between protein nutritional-status and immunocompetence in northern bobwhite chicks. *Auk* **110**, 503–510.

Mendonca, H. L. and Arkush, K. D. (2004). Development of PCR-based methods for detection of *Sphaerothecum destruens* in fish tissues. *Diseases of Aquatic Organisms* **61**, 187–197.

Nelson, R. J. and Demas, G. E. (1996). Seasonal changes in immune function. *Quarterly Review of Biology* **71**, 511–548.

Nordling, D., Andersson, M., Zohari, S. and Gustafsson, L. (1998). Reproductive effort reduces specific immune response and parasite resistance. *Proceedings of the Royal Society of London, B* **265**, 1291–1298.

Ostlund-Nilsson, S., Curtis, L., Nilsson, G. E. and Grutter, A. S. (2005). Parasitic isopod *Anilocra apogonae*, a drag for the cardinal fish *Cheilodipterus quinquelineatus*. *Marine Ecology-Progress Series* **287**, 209–216.

Phelps, N. B. D. and Goodwin, A. E. (2007). Validation of a quantitative PCR diagnostic method for detection of the Microsporidian *Ovipleistophora ovariae* in the cyprinid fish *Notemigonus crysoleucas*. *Diseases of Aquatic Organisms* **76**, 215–221.

Pinder, A. C. and Gozlan, R. E. (2004). Early ontogeny of sunbleak. *Journal of Fish Biology* **64**, 762–775.

Quinn, T. P. (2004). *The Behavior and Ecology of Pacific Salmon and Trout*. University of Washington Press, Washington, WA, USA.

Reimchen, T. E. and Nosil, P. (2001). Ecological causes of sex-biased parasitism in threespine stickleback. *Biological Journal of the Linnean Society* **73**, 51–63.

Sanz, J. J., Arriero, E., Moreno, J. and Merino, S. (2001). Interactions between hemoparasite status and female age in the primary reproductive output of pied flycatchers. *Oecologia* **126**, 339–344.

Sheldon, B. C. and Verhulst, S. (1996). Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends in Ecology & Evolution* **11**, 317–321.

Simkova, A., Jarkovsky, J., Koubkova, B., Barus, V. and Prokes, M. (2005). Associations between fish reproductive cycle and the dynamics of Metazoan parasite infection. *Parasitology Research* **95**, 65–72.

Simkova, A., Lafond, T., Ondrackova, M., Jurajda, P., Ottova, E. and Morand, S. (2008). Parasitism, life

history traits and immune defence in cyprinid fish from central Europe. *BMC Evolutionary Biology* **8**.

Skarstein, F. and Folstad, I. (1996). Sexual dichromatism and the immunocompetence handicap: an observational approach using Arctic charr. *Oikos* **76**, 359–367.

Skarstein, F., Folstad, I. and Liljedal, S. (2001). Whether to reproduce or not: immune suppression and costs of parasites during reproduction in the Arctic charr. *Canadian Journal of Zoology-Revue Canadienne De Zoologie* **79**, 271–278.

Slater, C. H. and Schreck, C. B. (1993). Testosterone alters the immune response of Chinook salmon *Oncorhynchus tshawytscha*. *General and Comparative Endocrinology* **89**, 291–298.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. and Higgins, D. G. (1997). The Clustal_X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research* **25**, 4876–4882.

True, K., Purcell, M. K. and Foott, J. S. (2009). Development and validation of a quantitative PCR to detect *Parvicapsula minibicornis* and comparison to histologically ranked infection of juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), from the Klamath River, USA. *Journal of Fish Diseases* **32**, 183–192.

Vainikka, A., Jokinen, E. I., Kortet, R. and Taskinen, J. (2004). Gender- and season-dependent relationships between testosterone, oestradiol and immune functions in wild roach. *Journal of Fish Biology* **64**, 227–240.

Wang, N., Xu, X. L. and Kestemont, P. (2009). Effect of temperature and feeding frequency on growth performances, feed efficiency and body composition of pikeperch juveniles (*Sander lucioperca*). *Aquaculture* **289**, 70–73.

WCMC (1996). *Leucaspius delineatus*. In *IUCN 2007. 2007 IUCN Red List of Threatened Species*. www.iucnredlist.org.

Wolinska, J. and King, K. C. (2009). Environment can alter selection in host-parasite interactions. *Trends in Parasitology* **25**, 236–244.

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