Experimental exposure of rainbow trout eggs to water mould, *Saprolegnia parasitica* (Oomycota): intraspecific differences in pathogenicity

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RESEARCH ARTICLE

ABSTRACT

In *in vivo* infection trials, rainbow trout eyed eggs were exposed to three *Saprolegnia parasitica* (Oomycota) isolates, which differed in biological and genetic characteristics. Infection prevalence, hatching rate, hatching dynamics of fish eggs were quantified, and the study was complemented with histopathology and phylogenetic analyses. We experimentally detected intraspecific differences in the pathogenicity of *S. parasitica* on rainbow trout eggs. The isolate from rainbow trout eggs was the most virulent to eggs of the same host, whereas isolates from carp skin and fry did not cause as much damage to the eggs. Comparing the outcome of two experimental settings, we confirmed that invasion of fish eggs is more effective by hyphae growth than by the actively moving zoospores. In addition, our findings highlighted that *S. parasitica* isolates with 100% identical ITS DNA sequences, could differ significantly in virulence. These isolates can be clearly distinguished based on a 650-bp DNA fragment of the DNA-directed RNA polymerase II subunit (*RPB2*) gene.

INTRODUCTION

The water mould, *Saprolegnia* spp. (Oomycota: Saprolegniales) is endemic in most freshwater habitats worldwide (van West, 2006; Eiras et al., 2008; Wuensch et al., 2018). Although *Saprolegnia* spp. are mainly saprotrophic, parasitic species may cause severe losses in fish farms and natural waters (Eiras et al., 2008). All freshwater fish are considered susceptible. Preventive measures include the use of disinfectants, but a selective breeding programme for Atlantic salmon (*Salmo salar*) is already underway to develop genetic lines less susceptible/ resistant to saprolegniosis (Misk et al., 2022).

Saprolegniosis is a well-known disease in rainbow trout (*Oncorhynchus mykiss*) farming, whereas the disease can also affect populations in natural waters (van West, 2006; Shin et al., 2017; Engblom et al., 2023). A recent study on cases of saprolegniosis in Finland revealed that one specific clone of *Saprolegnia parasitica* was associated with the majority of disease outbreaks and only weak associations with fish species were observed (Engblom et al., 2023). The authors, however, focused their study on affected adult and fry fish, rather than fish eggs. Rainbow trout have also been used as a laboratory model fish to study water mould for various purposes (Howe and Stehly, 1998; Pottinger and Day, 1999), among others to test the effectivity of plant-derived substances or other treatment options against saprolegniosis (Khosravi et al., 2012; Heikkinen et al., 2013; Eszterbauer et al., 2018; Özçelik et al., 2020; Özdemir et al., 2022).

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On eggs, the disease is manifested by mycelial growth on the surface and inside of the cells, which often induces the death of affected eggs. *Saprolegnia* species seem to employ different mechanisms to infect fish eggs. Whereas *Saprolegnia diclina* infection results in chorion destruction, *S. parasitica* is able to penetrate an intact chorion of Atlantic salmon eggs (Songe et al., 2016).

Some studies on fish-pathogenic *Saprolegnia* strains have reported that certain phenotypic factors (hooks on cysts, growth rate etc.) were associated with pathogenicity (Stueland et al., 2005; Thoen et al., 2011). A study on *S. parasitica* has shown that the abundance of gemmae may have a potential to predict pathogenicity (Yuasa and Hatai, 1995). These, probably indirect correlations with the likelihood of high pathogenicity are either not consistent (Stueland et al., 2005), or have not been confirmed by others until now. Nevertheless, these findings have drawn attention to biological (including virulence) differences that may be reflected in the phenotypic characteristics of *S. parasitica* (or even genotypic ones). When comparing nineteen *S. parasitica* isolates, we have confirmed (Erdei et al., in press) that the form and amount of durable vegetative and asexual reproductive structures of *S. parasitica* differ by culture nutrient, temperature and the presence of a potential host under *in vitro* laboratory conditions. We have distinguished three morphotypes of *S. parasitica* based on the number of zoosporangia, gemmae, the formation of gemma chains, and the induction of zoospore release. Furthermore, we have concluded that the commonly used genetic markers (mainly ITS regions) are not suitable for distinguishing the morphotypes of *S. parasitica*.

In the present study, we intended to compare the pathogenicity of *S. parasitica* isolates. The isolates were selected considering various parameters: their geographical origin, the source of isolation and the fish host, moreover the morphological characteristics, which have been examined in a previous study (Erdei et al., in press). The hatching rate and dynamics of exposed rainbow trout eggs were quantified in *in vivo* infection trials. Furthermore, the relevance of physical contact between eggs on the severity of infection was examined.

MATERIALS AND METHODS

Origin of *S. parasitica* isolates and fish eggs

The four *Saprolegnia* isolates examined in the present study were previously collected in different fish farms in Hungary (in a trout hatchery at Lillafüred, and in carp hatcheries at Dinnyés and Varsád). They were cryopreserved using the technique developed in our laboratory (Eszterbauer et al., 2020), and were stored at −80 °C after isolation in 2020 and 2021, respectively (Table 1). The isolates were selected from our water mould collection considering various parameters. Besides geographical origin, sample source and fish host, phenotypic characteristics were also taken into account. The selected isolates differed in the development of vegetative

Table 1. Saprolegnia spp. isolates used for in vivo infection trials on rainbow trout eyed eggs in the present study

Sample ID (species)	Geographic origin (in Hungary)	Year of isolation	Sample type	Host species	Morphological characteristics examined previously by Erdei et al. (in press)	ITS – NCBI Accession No.
SAP191 (*S. parasitica*)	Lillafüred	2020	eyed egg	rainbow trout (*Oncorhynchus mykiss*)	gemma and gemma chains of 5–7 usually; zoospore production preferred at RT; oogonium absent	OQ236393
SAP198 (*S. parasitica*)	Varsád	2020	fish skin scrapings	common carp (*Cyprinus carpio*)	gemma and gemma chains of 2–3 usually; intense zoospore production both at RT and cold-induced; oogonium absent	OQ236398
SAP203T (*S. parasitica*)	Dinnyés	2020	fish fry	common carp (*Cyprinus carpio*)	gemma moderate; some gemma chains of 2–3; in the absence of host moderate number of zoospores both at RT and with cold-induction; some oogonia	OQ236391
SAP213B (*S. ferax*)	Dinnyés	2021	egg	common carp (*Cyprinus carpio*)	gemma rare; no gemma chain; in the absence of host low zoospore production at RT or with cold-induction; oogonium absent	OQ236402

RT: room temperature

and reproductive structures, besides in the intensity of zoospore production as described by Erdei et al. (in press). All four isolates were collected from infected fish or fish eggs, showing clinical signs of saprolegniosis (Table 1). Prior to *in vivo* infection trials, cryopreserved *Saprolegnia* isolates were thawed at room temperature and cultured on GY agar medium (containing 10 g L^{-1} glucose, 2.5 g L^{-1} yeast extract, 15 g L^{-1} agar, 500 μg mL^{-1} penicillin G, and 500 μg mL^{-1} streptomycin-sulphate).

Four-day-old, eyed eggs of golden rainbow trout (*Oncorhynchus mykiss aquabonita*) originated from the trout hatchery in Lillafüred, Hungary were used for the infection experiments. Prior to the experiments, fish eggs were disinfected with 0.01 V/V% Divosan Forte (Diversey Ltd., Hungary) twice for 40 min, as described previously (Eszterbauer et al., 2018), and white/dead eggs were removed.

Experimental infection of fish eggs

The experiments were set in two different layouts. *Experiment C*: eggs were attached to each other (in contact), and *Experiment NC*: eggs of the same group were separated from each other (no contact) (Fig. 1). For both experimental setups (Exp. C and NC), four *Saprolegnia* isolates (SAP191, SAP198, SAP203T and SAP213B) were used for egg exposure in 3 replicates (Table 1). For the negative controls (in 3 replicates per setup), eggs were handled the same way as for the exposed groups, except the exposure to *Saprolegnia* zoospores. The infection was achieved by zoospores released from mycelia plugs ($d = 4$ mm) placed in the water of egg trays. Prior to exposure, the plugs were incubated in GY + P + S liquid medium (containing 10 g L^{-1} glucose, 2.5 g L^{-1} yeast extract, 500 μg mL^{-1} P and 500 μg mL^{-1} S) in 24-well tissue culture plates at 21 °C for 3 days. Sporulation was induced by washing the mycelia plugs three times with sterile distilled water (SDW) to remove the liquid medium, incubating in sterile filtered, chlorine-free tap water (STW) at 21 °C for 3 days, and then washing three times again with SDW. Prior to setting up the experiments, the number of zoospores was counted in Bürker chamber, and the initial zoospore amount was set to 10^4–10^5 zoospores per replicate. As an agar plug colonised by the respective *Saprolegnia*

isolate was added to the plates, zoospore production was continuous during the experiment. At the end of the experiment (day 5 for Exp. C and day 6 for Exp. NC), we checked and re-estimated the amount of zoospores and found a similar amount of zoospores in each group.

For Exp. C, 35 eyed eggs per replicate were in direct contact in a small Petri dish (35 mm in diameter × 10 mm height), which was placed in a plastic box (75 × 110 × 55 mm) filled with STW (10-mm layer of water) (Fig. 1A). For Exp. NC, 23 eyed eggs per replicate were placed approximately 10 mm apart from each other in partially segmented racks (77 × 112 × 18 mm) with 24 compartments permeable for zoospores, and placed in a custom-made plastic box (105 × 170 × 70 mm) filled with sterile-filtered tap water (STW) (10 mm in depth) (Fig. 1B). The water temperature varied between 11.5 and 13.5 °C during the experiment. The experiments were terminated when fish larvae hatched or when all eggs died in a group. All hatched larvae and dead eggs were placed on GY + P + S agar for culturing *Saprolegnia* to evaluate infection and calculate infection prevalence. *Saprolegnia* isolates cultured by this way were identified by partial ITS DNA sequences to rule out contamination. Besides infection prevalence, the hatching rate and the timing of egg hatching (i.e. hatching dynamics) were estimated and statistically analysed.

Histological examination

On day 3 (prior to hatching), three fish eggs from all replicates were fixed in 8% NBF for histological examination (with few exceptions, when the eggs were not intact and could not be used for histology). Only visibly intact, live eggs were sampled. After fixation, the eggs were embedded in paraffin wax, cut into 4–5 μm sections, and then stained with haematoxylin and eosin.

Molecular and phylogenetic methods

To confirm infection after fish egg exposure, ITS-based molecular identification of *Saprolegnia* isolates was performed following the optimized protocol used in our laboratory (Eszterbauer et al., 2020). Shortly, Quick-DNA Fungal/Bacterial Miniprep Kit (Zymo Research, USA) was
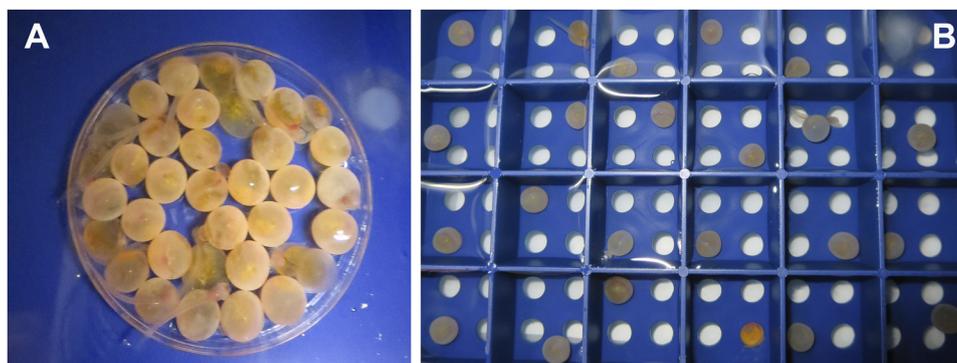

Fig. 1. Experimental settings of rainbow trout eyed eggs exposed to *Saprolegnia* spp. isolates *in vivo*: A: eggs in contact; attached to each other (Exp. C); B: eggs with no contact; separated from each other (Exp. NC)

used for DNA extraction according to the manufacturer's manual. The internal transcribed spacer (ITS) regions, included in an approximately 710-bp PCR product containing the partial sequence of the 18S ribosomal RNA gene (rDNA), followed by ITS 1, the 5.8S rDNA, ITS 2 and the partial sequence of the 28S rDNA, were amplified using a primer pair universal for eukaryotes (forward primer ITS-1 5′-TCC GTA GGT GAA CCT GCGG-3′, reverse primer ITS-4 5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al., 1990) with a modified PCR protocol (Eszterbauer et al., 2020). To distinguish isolates with identical ITS DNA sequences, an approximately 650-bp DNA fragment from the gene of the DNA-directed RNA polymerase II subunit (RPB2) was amplified using the forward primer SAP-RPB2f: CGA CCG CGA TCA CTA TGG, and the reverse primer SAP-RPB2r: CGA CAC TTC GGC GTC AAT GT) according to Ravasi et al. (2018). All PCR products were purified using MEGAquick-spin Plus Total Fragment DNA Purification Kit (Intron Biotechnology, Korea) according to the manufacturer's manual. Sanger DNA sequencing was performed using BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, USA), and detected on Applied Biosystems Genetic Analyzer 3500 (Thermo Fisher Scientific, USA). The molecular species identification was based on ITS DNA sequence similarity search using Megablast. The DNA sequences of the examined isolates and experimental samples, in addition to the relevant (and available) *Saprolegnia* sequences retrieved from the NCBI GenBank, were aligned using the MAFFT alignment tool with default settings in Geneious Prime v2019.2.1. For phylogenetic analyses, maximum likelihood (RAxML) method using GTR + GAMMA + I model (with 1000 repeats) was applied with Geneious Prime v2019.2.1.

Statistical analysis

The hatching rate of the exposed groups was compared to that of the control groups using Welch's *t*-test. Statistical differences among groups were examined with ANOVA followed by Dunnett's *post hoc* test, whereas group-related differences were analysed with ANOVA followed by Tukey's *post hoc* test. A Generalized Linear Mixed Model (GLMM) was used for the statistical analysis of hatching dynamics. Here, the ratio of intact eggs to fish larvae (i.e. hatched eggs) was analysed as a function of time (from the start of the experiment to 144 h) and the experimental groups were compared.

RESULTS

The initial, ITS-based genetic identification showed that all isolates belonged to species *S. parasitica*. However, the final control check following the *in vitro* trials revealed that isolate SAP213B was identical to *Saprolegnia ferax* on the basis of ITS. It is likely that isolate SAP213B was originally contaminated with *S. ferax*, and *S. ferax* became the dominant species at the end of the experiment. Therefore, the infectivity of SAP213B on fish eggs could not be clearly linked to *S. parasitica* or *S. ferax* species.

When we compared the effect of saprolegniosis induced by three isolates of *S. parasitica* and one *S. ferax* isolate, isolate-related differences were detected in infection prevalence, hatching rate and hatching dynamics especially in Exp. C, when rainbow trout eyed eggs were attached to each other.

Prevalence of saprolegniosis

In the prevalence of *Saprolegnia* infection, notable difference was observed between the two experimental settings (Table 2). Whereas the infection prevalence was rather high (89.7–100%) among eggs attached to each other (in Exp. C), this value was lower (48.3–66.7%) for eggs not in contact with each other (in Exp. NC). The ITS-based, control PCR and DNA sequences confirmed *S. parasitica* infections of the group-related isolates (i.e. the ones used for exposure), except for SAP213B, which surprisingly had the same ITS sequence as *S. ferax* at the end of the experiment. Negative control groups remained uninfected until the end of the experiment.

Histological examination

Histological examination showed an intact chorion of eyed eggs in the unexposed, control groups (Fig. 2A). In groups exposed to isolate SAP198, scattered lesions were observed on the surface of the chorion (Fig. 2B). No differences in the histopathology of the eggs were detected among the groups exposed to SAP198 and SAP203T. Extended lesions were

Table 2. Prevalence of saprolegniosis in the *in vivo* trials performed on rainbow trout eyed eggs, using four *Saprolegnia* isolates. Three replicates per group type were applied in two different settings (Exp. NC vs Exp. C). Isolates SAP198, SAP203T, SAP191: *S. parasitica*; isolate SAP213B: *S. ferax*

Group IDs	Group type	Mean No. of eggs: Infected/Total*	Prevalence (%)
NC1 –NC3	negative control	0/63	0
NC4 – NC6	SAP198	40/60	66.7
NC7 – NC9	SAP213B	39/60	65.0
NC10 – NC12	SAP203T	29/60	48.3
NC13 – NC15	SAP191	31/59	52.5
C1 – C3	negative control	0/102	0
C4 – C6	SAP198	87/97	89.7
C7 – C9	SAP213B	91/96	94.8
C10 – C12	SAP203T	92/99	92.9
C13 – C15	SAP191	96/96	100

NC: eggs without contact; C: eggs attached to each other; * total number of eggs per group (3 replicates per group were counted together; the total number of eggs was lower at the end of the experiment than at the start, because 3 eggs per replicate were used for histology in average).
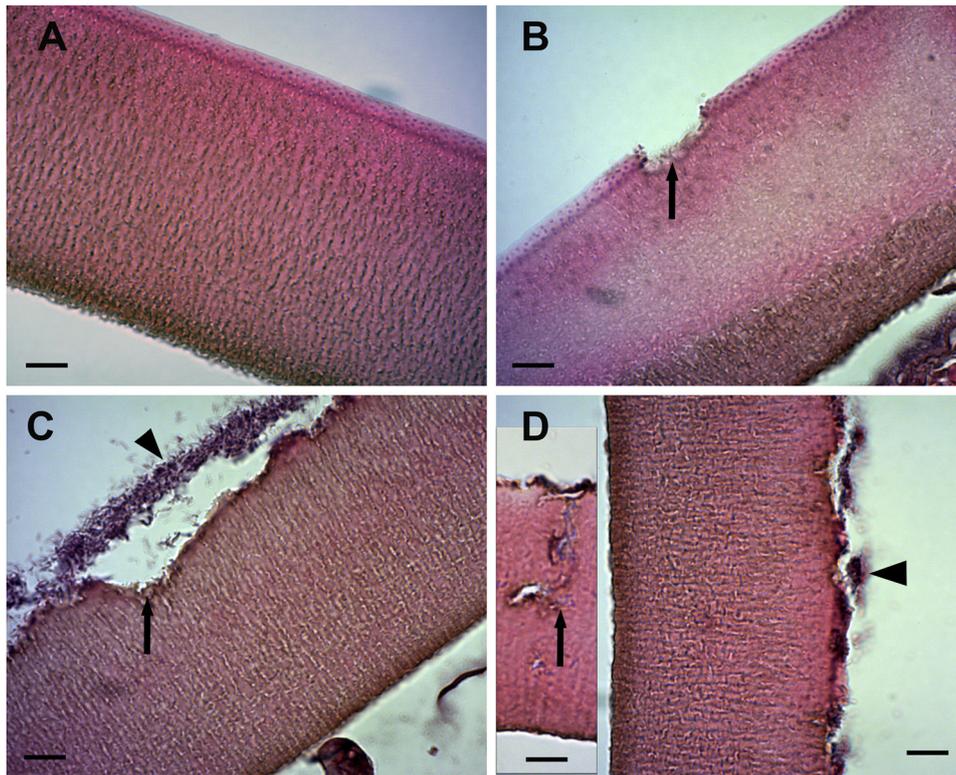
Fig. 2. Histological images of rainbow trout eggs 3 days after exposure to isolates of *Saprolegnia* spp. *in vivo*. The eggs were attached to each other during the experiment (Exp. C). A, negative control: intact egg chorion; B, group exposed to isolate SAP198: lesions on the surface of the chorion (arrow); C, group exposed to isolate SAP191: extensive lesions on the surface of the chorion (arrow) and a clump of water mould hyphae attached to the surface of the egg (arrowhead); D, group exposed to isolate 213B: lesions on the surface of the chorion and an extensive mass of hyphae around the egg (arrowhead); D inset: water mould hyphae affecting the inner region of the chorion (arrow). H&E staining. Scale bar: 10 µm

observed in groups exposed to SAP191 (Fig. 2C), and water mould hyphae were attached to the surface of eggs, or they were seen at the necrotic surface of egg chorion (Fig. 2C). In eggs exposed to isolate SAP213B (*S. ferax*), the chorion of affected eggs showed similar picture as those infected with SAP191 (Fig. 2D). Occasionally, however, hyphal penetration was observed not only on the surface, but also in the deeper area of the chorion (Fig. 2D inset).

Phylogenetic analysis

The ITS-based RAxML phylogenetic tree reconstruction confirmed that DNA sequences of all examined egg and larva samples exposed to SAP213B, collected during (labelled 'exp') or after the experiment (labelled 'post-exp') are identical with the ITS sequence of *S. ferax* (Fig. 3), whereas the samples of original isolates (labelled 'pre-exp') clustered with *S. parasitica*. *S. parasitica* isolates grouped together with the GenBank reference sequences proposed by Sandoval-Sierra et al. (2014). The DNA sequences of the experimental samples clustered on the tree with the *Saprolegnia* isolates. Samples of group SAP203T were 99.9–100% identical to each other, and 96.9% identical to the *S. ferax* clade. However, the sequences of eggs exposed to isolates SAP198 and SAP191 were 100% identical (despite being

only 99.1–99.2% identical to the SAP203T subclade), thus the samples of the two isolates could not be distinguished on the basis of ITS sequences. Therefore, we used *RPB2* gene-based differentiation, which was able to distinguish all *S. parasitica* isolates (Fig. 4). The *RPB2* sequence identities between isolates SAP191 and SAP198 ranged from 97.6 to 98.2%, whereas the identities within isolates were 98.7–99.1% for SAP198 and 98.6–99.8 for SAP191.

Hatching rate

Eggs in contact with each other (in Exp. C) hatched at day 5, one day earlier than those separated from each other (in Exp. NC). In Exp. NC, the hatching rate was rather high (97–100%), and no significant differences were detected compared to negative control and among the exposed groups (Fig. 5).

In Exp. C, the hatching rate varied remarkably (2–83% among exposed groups), and it was significantly lower in exposed groups compared to negative control ($P = 0.00118$). The highest mortality was measured for trout eggs exposed to SAP191, the isolate that was originally isolated from trout eggs. By analysing the differences among groups, the hatching rate compared to negative control was significantly lower in SAP191 ($P < 0.001$) and in SAP213B ($P < 0.001$) (both isolates originated from fish eggs). Besides, the differences were
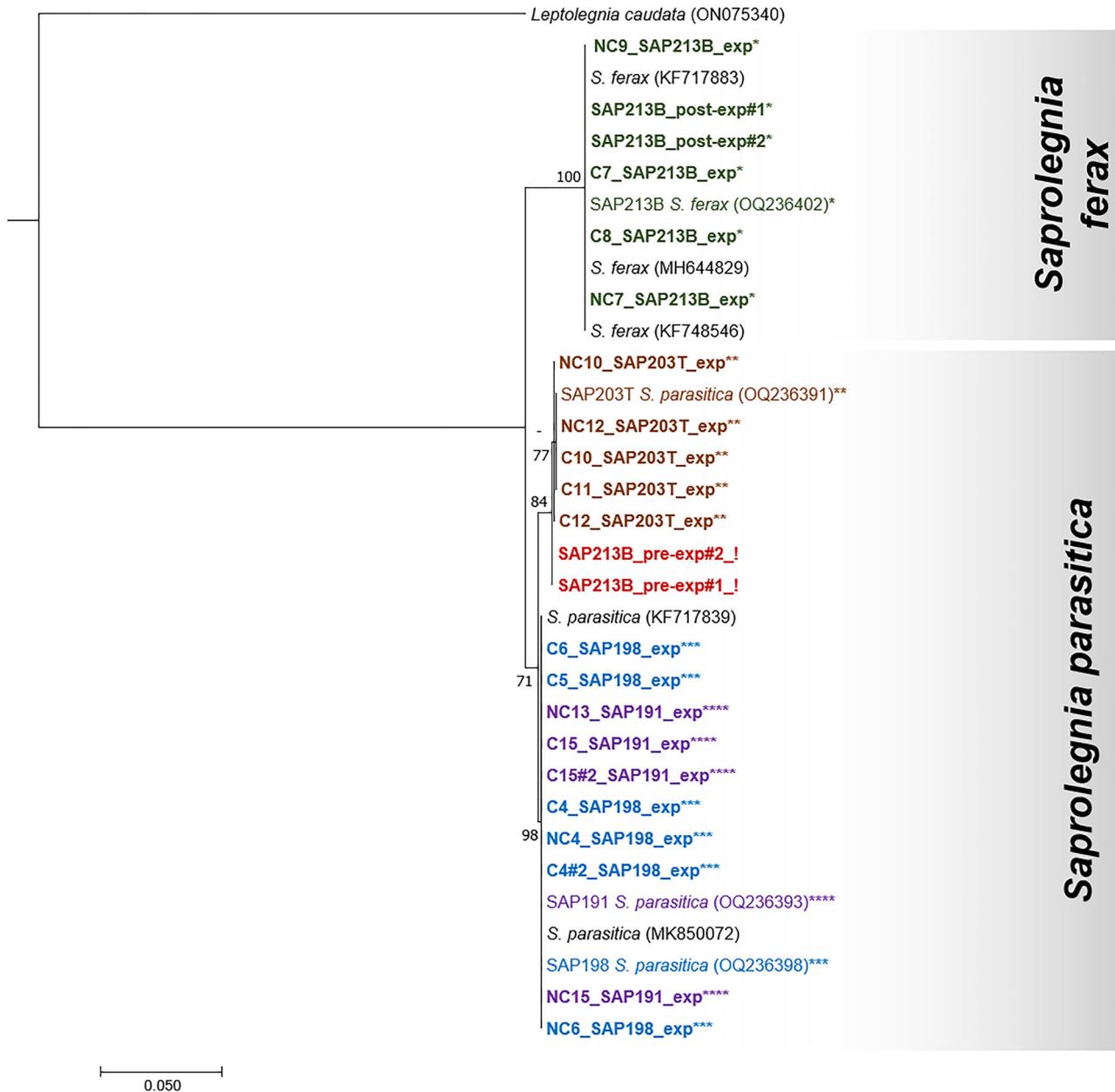
Fig. 3. Maximum likelihood (RAxML) tree reconstruction of *S. parasitica* and *S. ferax* isolates examined, and related sequences from the NCBI database, based on a 684-bp sequence alignment of ITS 1& 2 regions. *Leptolegnia caudata* (ON075340) was chosen as outgroup. Numbers at nodes indicate the bootstrap values in percentage (shown above 50%). Scale bar shows the estimation of the number of nucleotide substitutions per site. NCBI accession numbers are in parenthesis. DNA sequences obtained in the present study are labelled bold. Pre-exp: sampling prior to experiment; exp: sampling during the experiment, 5 d p.e.; post-exp: control sampling after the experiment. */green: group exposed to isolate SAP213B (*S. ferax*); **/brown: group exposed to isolate SAP203T (*S. parasitica*); ***/blue: group exposed to isolate SAP198; ****/purple: group exposed to isolate SAP191 (*S. parasitica*); !/red: isolate SAP213B sampled prior to the *in vivo* experiment (*S. parasitica*)

significant between groups SAP191 and SAP198 ($P = 0.00164$); SAP191 and SAP203T ($P = 0.00173$), SAP203T and SAP213B ($P = 0.0099$), SAP198 and SAP213B ($P = 0.01045$) (Fig. 5).

Hatching dynamics

Overall, the egg hatching time was slower in the exposed groups than in the unexposed, negative control groups (Fig. 6). In Exp. NC, only eggs of group SAP191 hatched significantly slower ($P = 0.0087$), the hatching time of eggs in the other groups was also longer than that for the controls, but the difference was not significant. In Exp. C, eggs in exposed groups hatched significantly slower compared to control; $P < 0.01$ (except for SAP203T, where the difference was slightly above the limit of significance; $P = 0.05108$).
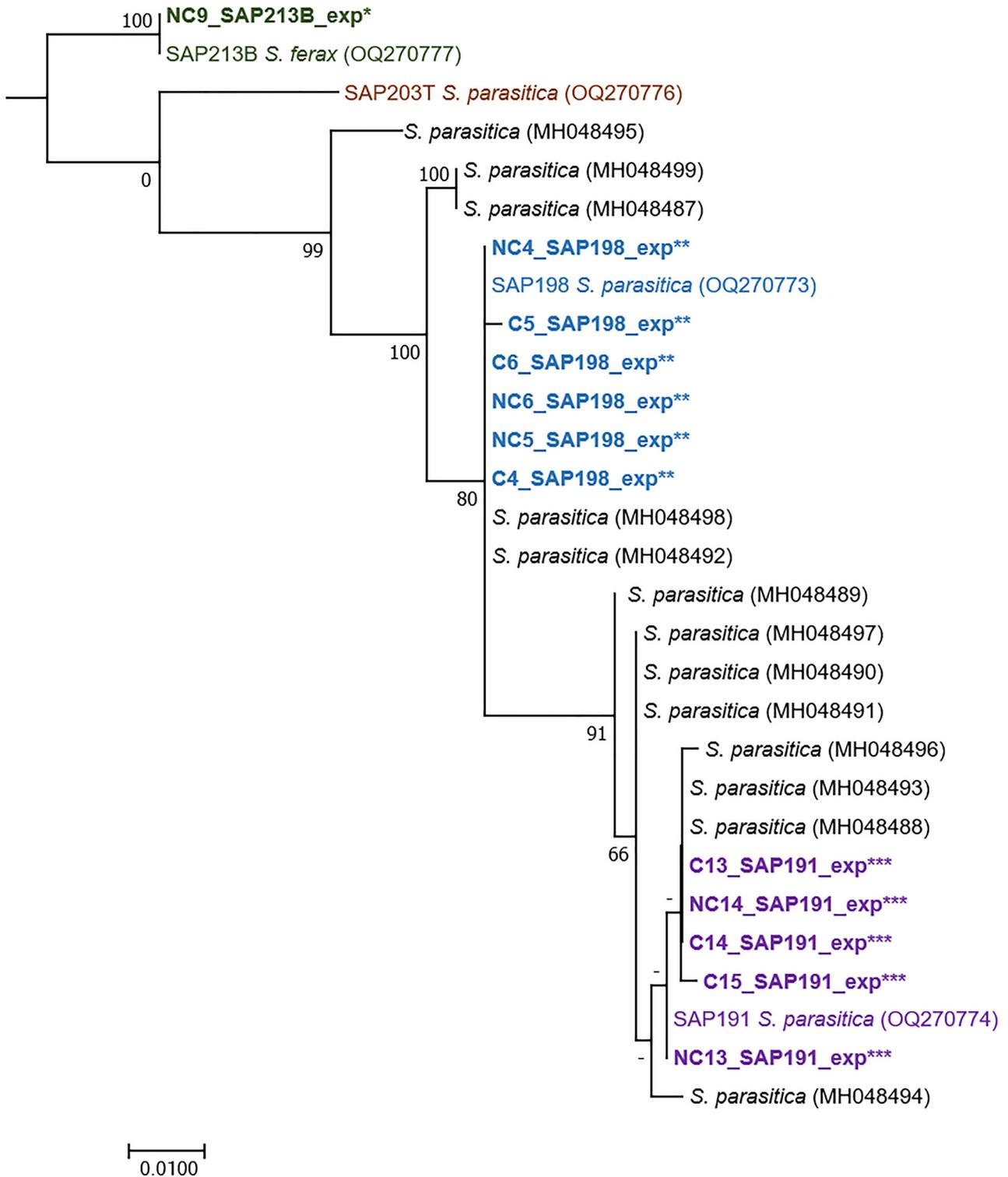
Fig. 4. Maximum likelihood (RAxML) tree reconstruction of *S. parasitica* and *S. ferax* isolates examined, and related sequences from NCBI database, based on a 506-bp sequence alignment of *RPB2* gene. The tree was rooted on the midpoint. Numbers at nodes indicate the bootstrap values in percentage (shown above 50%). Scale bar shows the estimation of the number of nucleotide substitutions per site. NCBI accession numbers are in parenthesis. DNA sequences obtained in the present study are labelled bold. */green: group exposed to isolate SAP213B (*S. ferax*); **/blue: group exposed to isolate SAP198 (*S. parasitica*); ***/purple: group exposed to isolate SAP191 (*S. parasitica*)
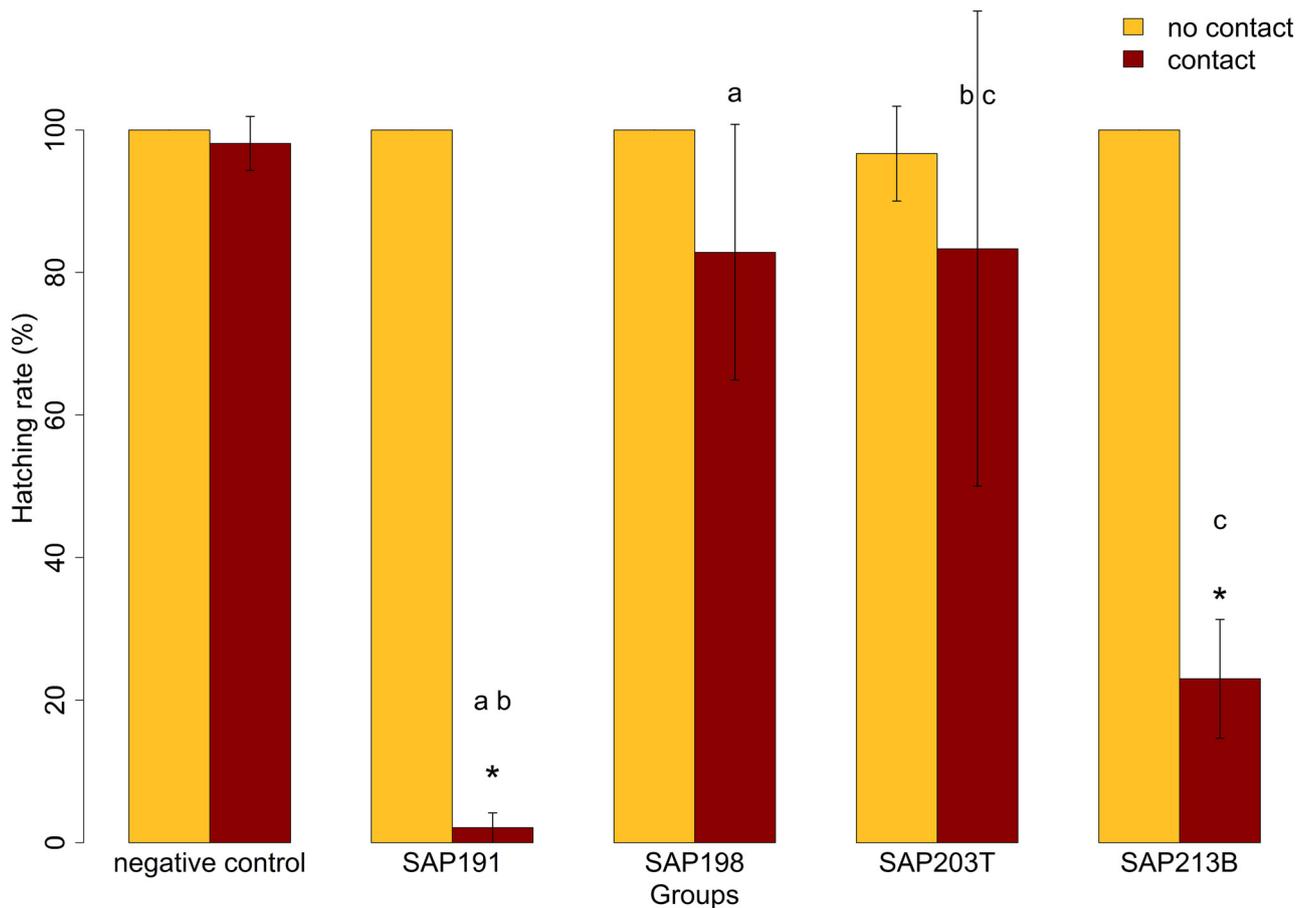
Fig. 5. Bar plot of the hatching rate (%) of rainbow trout eyed eggs exposed to *Saprolegnia parasitica* and *S. ferax* in the five experimental groups with two settings (pale grey/yellow bars: Exp. NC; fish eggs separated from each other; dark grey/red bars: Exp. C; fish eggs attached to each other). Standard deviations are shown. Significant differences between groups are indicated with lower-case letters (*P* < 0.01)

DISCUSSION

In the present study, the virulence of four *Saprolegnia* isolates was studied on rainbow trout eggs to examine whether the biological and genetic differences are reflected in pathogenicity.

The *Saprolegnia* isolates examined were cryopreserved using the protocol developed in our laboratory (Eszterbauer et al., 2020). It is a common opinion that *in vitro* sub-culturing or ultrafreezing may alter the behaviour of microorganisms, and pathogen strains could even lose their virulence (Fierer et al., 2017). This concern legitimate and valid for most prokaryotes, and it has also been raised for oomycetes in relation to subculturing. Songe et al. (2014) have reported that sequential *in vitro* subculturing of *S. parasitica* might reduce the virulence of strains, however the change in virulence was strain-dependent, and significant decline was detected at passage 15 only. They have also found that the strains, the virulence of which declined due to subculture could regain their virulence in Atlantic salmon host. The effect of cryopreservation on the virulence of water mould strains/isolates has not yet been experimentally validated up to now. Our results suggest that the

pathogenicity of cryopreserved *Saprolegnia* isolates can be retained after revival. We hypothesize that appropriate culture conditions may reactivate oomycetes, as it was the case for the *Saprolegnia* isolates examined here.

We observed hatching rate differences between the two experimental layouts (Exp. NC vs. Exp. C). When fish eggs were placed a few cm apart from each other (Exp. NC), the hatching rate of eggs was higher (i.e. mortality was lower) compared to the layout when eggs were attached (Exp. C). Although the motile, secondary zoospores are considered the main dispersion of water moulds, in the case of saprolegniosis in fish eggs, it is well known that water moulds spread to the neighbouring egg by the growing and proliferating hyphae (van West, 2006). Zoospores, which are capable of active movement seem to be responsible for "long-distance" propagation, whereas hyphae growth results in colony enlargement and the invasion of attached eggs (even living ones). Our results gave an experimental confirmation for that the invasion of fish eggs is more effective by hyphal growth than by zoospore spreading (for *Saprolegnia* isolates SAP191 and SAP213B even significantly higher). It should be added, however, that in the experimental setups, we purposefully minimized the water flow
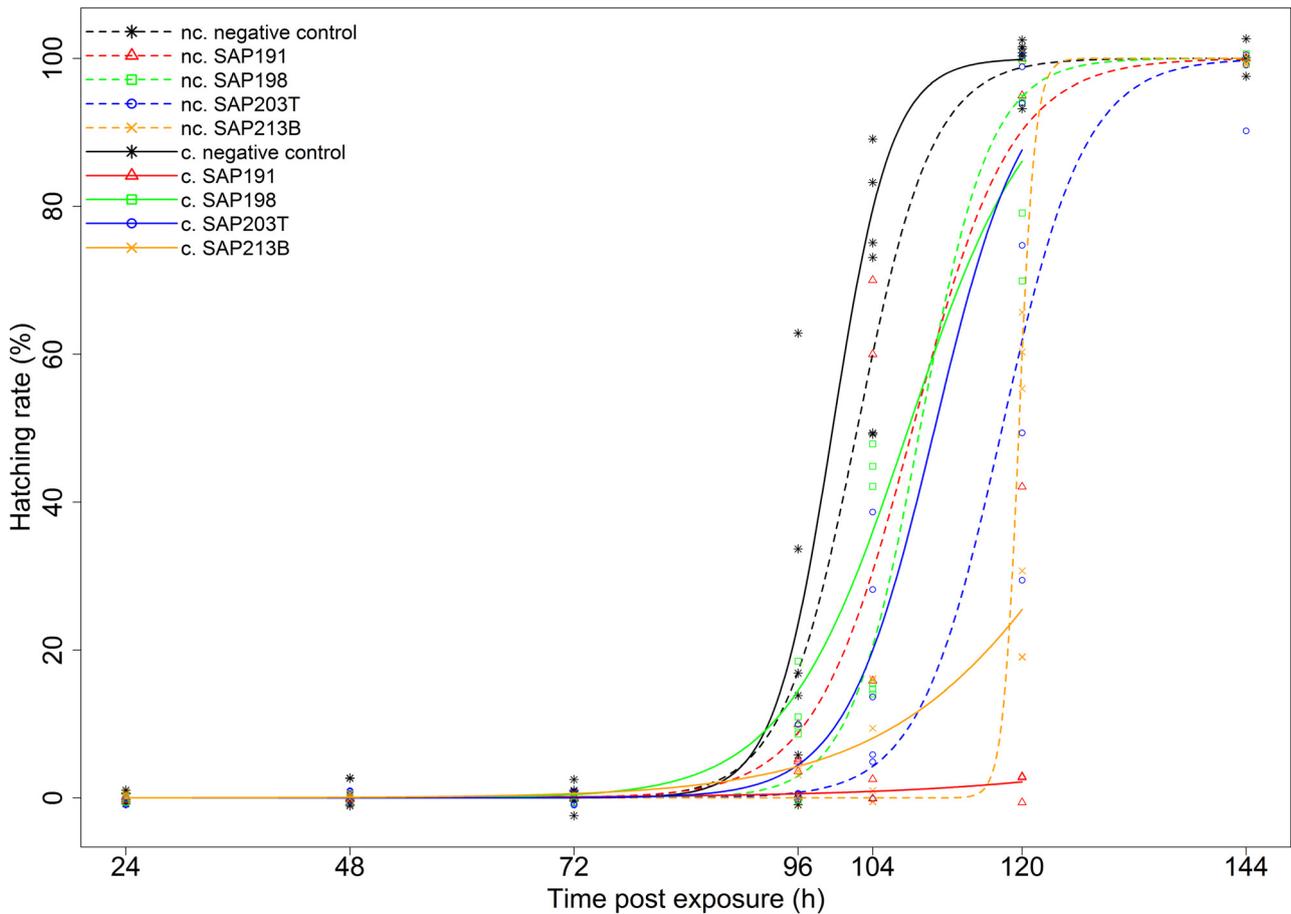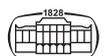
Fig. 6. Hatching dynamics (i.e. hatching rate over time) of rainbow trout eyed eggs exposed to four different *Saprolegnia* isolates up to 144 h p.e., and negative (unexposed) group. *In vivo* exposures were performed in two settings: dashed line: Exp. NC, fish eggs were separated from each other, continuous line: Exp. C, eggs were attached to each other

and thus the chance of passive movement of zoospores, so that we can measure the infectivity of actively moving zoospores. Thoen et al. (2011) have found that live eggs of Atlantic salmon could not be killed by zoospore immersion. Here in Exp. NC, when the infection was induced only by zoospores, we detected mortality of rainbow trout eyed eggs, albeit at low levels (up to 3%). However, since we ended the experiment 6 days after exposure (p.e.), the duration of our study may be too short to clearly see the pathological effect of the zoospore-mediated infection.

It is likely that the experimental setting affected the hatching time and caused a day earlier hatching (on day 5 p.e. instead of day 6 p.e.) in the groups with attached eggs in Exp. C. We tried several different settings in preliminary experiments, and this one was the best for keeping the eggs alive (i.e. maintaining the oxygen level required, even if it was not the optimal one) while the spread of zoospores was not disturbed by water flow, which could have altered the outcome of the experiments.

Comparing the virulence of isolates, we found that the ones from fish eggs were the most pathogenic to rainbow trout eggs, and the isolates from fish did not cause as much damage to the eggs. Moreover, the SAP191 originally isolated from rainbow trout eggs was the most virulent one to the eggs

of the same fish species in our experiment. Experts have suggested that water mould strains proven to be pathogenic to fish would be the most virulent ones to fish eggs (Kitan-charoen and Hatai, 1997). This view has been questioned by Thoen et al. (2011), who challenged eyed eggs of Atlantic salmon to eight *Saprolegnia* isolates of various species. They have found that strains pathogenic to salmon parr were not particularly infective towards eggs, and the saprotrophic species (e.g. *S. diclina*) gave the highest infection rates to eggs. It is consistent with the findings of others (Engblom et al., 2023) and also our results, and two kinds of conclusions can be drawn. First, it confirms that egg pathogens may cause more severe damage and mortality to fish eggs, and second, the invasion strategy of egg pathogens is probably similar to that of the saprotrophs, namely the hyphae growth around a "core spot" of water mould. Probably the invasion strategy also differs between species considered saprotroph (e.g. *S. diclina*, *S. ferax*) and parasitic (e.g. *S. parasitica*). This may be due to the mechanical and biochemical processes during hyphae penetration, including chymotrypsin-like enzyme activity, as it has been suggested for oomycetes (Peduzzi and Bizzozero, 1977) or even for other fish parasites (Eszterbauer et al., 2021). The morphological changes in eyed eggs of Atlantic salmon infected with *Saprolegnia* spp. have

been studied earlier (Songe et al., 2016), where the authors described two infection mechanisms, a necrotrophic strategy (represented by *S. diclina*) and a facultative biotrophic one by *S. parasitica*. In our study, the virulence was not consistent among the *S. parasitica* isolates, and it is likely that the infection mechanism behind was not the same either; which would be worth investigating further. Nevertheless, the histopathological alterations in the egg chorion, observed in our study, highlighted that the hyphae of the saprotrophic *S. ferax* (isolate SAP213B) managed to damage the deeper regions of the egg chorion, whereas the three *S. parasitica* isolates caused lesion on the surface of the chorion only, on day 3 p.e.

The observed intraspecific differences in the virulence of *S. parasitica* also raised host specificity issues. The *in vivo* experimental observations by Matthews et al. (2021) on Atlantic salmon and sea trout indicated that while *S. parasitica* could be considered true generalists, some isolates might cause more severe infection in hosts to which they have been adapted to. Our findings are in accordance with their conclusions, as the most virulent *S. parasitica* isolate, SAP191, was originated from rainbow trout eggs, whereas SAP198 and SAP203T were from the skin of adult and fry common carp, respectively. Considering the ITS-based genetic similarities among *S. parasitica* isolates, the situation is even more unique. Isolates SAP191 and SAP198 were 100% identical over the 710-bp ITS–rDNA region, but showed rather different pathogenicity, further confirming that ITS is not sufficient for the intraspecific differentiation of *S. parasitica*. In contrast, the two isolates could be clearly distinguished based on the 650-bp DNA fragment of gene *RPB2*.

Besides the overall negative impact of *Saprolegnia* infection on the hatching rate and hatching dynamics of rainbow trout eggs, we detected intraspecific differences in the virulence of *S. parasitica*. To the best of our knowledge, this was the first study to experimentally confirm the phenomenon in rainbow trout eggs. In addition, our findings highlighted that *S. parasitica* isolates with 100% identical ITS DNA sequences, could differ in virulence, even significantly. However, the cause could not be explained yet. We intend to perform high throughput transcriptome analyses that are expected to shed light on the underlying processes.

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