Development of a novel assay for the characterization of germination responses in microsporidian parasites: An investigation into the biology of *Spraguea americanus*

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners. I understand that my thesis may be made electronically available to the public.

Statement of Contributions

The work presented in this thesis was done by Noah Peter Rogozynski under the supervision of Dr. Brian Dixon. This work was supported through a Canada Research Council Research Chair (950–232105) and Natural Sciences and Engineering Research Council of Canada Discovery Grant (NSERC DG; Grant # RGPIN-2018-04116) to Brian Dixon, as well as FishCAST funding provided to Noah P. Rogozynski. Contributions made to this work by collaborators are listed below:

Spore counts for germination experiments involving different ion treatments were conducted by summer student Sophie. A.R. Dyke.

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Abstract

The microsporidia are a widespread group of intracellular parasites which infect a broad range of hosts across the animal kingdom. In particular, the microsporidian *Spraguea americanus* has received considerable attention in recent years due to its role as an endemic pathogen in American anglerfish (*Lophius americanus*); a species of highly valued finfish currently at risk due to overfishing. However, like many species of microsporidia, the germination responses of *S. americanus* remain poorly described. This study outlines a novel *in vitro* germination assay for microsporidia, which is then used to comprehensively survey the germination responses of *S. americanus* under a variety of conditions. The results of this investigation indicate that *S. americanus* is responsive to mechanical pressure, hydrogen peroxide, sodium carbonate/bicarbonate and divalent cations, but not to mucin proteins as seen in closely related species. These observations provide evidence to refute the predominant hypothesis that members of *Spraguea* enter their hosts via the subcutaneous mucosal glands. In addition to providing much needed insight into the transmission of *Spraguea spp*., this study is among the first to extensively assess the germination responses of a single species of microsporidia; data which may lend itself to a more complete understanding of the mechanisms underlying the initiation of germination in microsporidia or support the establishment of new *in vitro* models for economically relevant species.

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Table of Contents

Table of Figures

Introduction

1.1 Introduction to microsporidia

The microsporidia are a group of unicellular, obligate, intracellular parasites which infect a broad range of animal hosts including insects, fish, rodents and humans (Fadhilah et al., 2024). Once thought to be a basal group to the Fungi, the microsporidia have undergone considerable secondary simplification in order to accommodate a parasitic lifestyle (Han and Weiss, 2017; Jespersen et al., 2022). To this end, microsporidia possess the smallest genomes of any eukaryote, lack functional mitochondria and peroxisomes, and rely on a highly reduced form of the Golgi apparatus for vesicular transport (Chen et al., 2023; Luo et al., 2022). However, despite their simplicity, the microsporidia possess a variety of distinct adaptations which enable them to effectively exploit their hosts (Han et al., 2020). One example unique to microsporidial cells is the presence of a polar filament: an organelle which functions as an extensible hypodermic needle to inject the parasite's cellular contents directly into susceptible cells (Han et al., 2020). Moreover, mature microsporidia exist as environmentally resistant spores which can remain viable for decades in even the harshest conditions (Han et al., 2020; Han and Weiss, 2017). Together, these adaptations facilitate a life history which shelters infectious microsporidia from the external environment almost entirely, thereby increasing transmission of infectious parasites within and between hosts (Han et al., 2020; Han and Weiss, 2017).

1.2 Life histories of microsporidia

Common among all known microsporidia species is the presence of an environmentally resistant spore stage which facilitates transmission of infectious material between hosts (Han et al., 2020; Han and Weiss, 2017). This infectious material, known as the sporoplasm, is surrounded by a thick spore coat composed of three layers: an electron-dense exospore composed of glycoproteins, an electron-lucent endospore coat made primarily of chitin, and an inner plasma membrane or plasmalemma (Han et al., 2021). Each spore also possesses an infection/extrusion apparatus; a unique complex of organelles which function to transport the sporoplasm into susceptible host cells upon stimulation by the correct environmental signals (Xu and Weiss, 2005). The extrusion apparatus consists of a long, coiled, proteinaceous filament (i.e. the polar filament), a system of stacked membranes called the polarplast, and a posterior vacuole (Xu and Weiss, 2005). After receiving the correct environmental signals, water is taken up into the spore via aquaporins, which results in swelling of the posterior vacuole and polarplast at the base of the spore (Han et al., 2020). The build up of hydraulic pressure eventually ruptures the spore coat at its tip/apex, which forces ejection of the polar filament (Han et al., 2020). However, since the polar filament is attached to the spore coat at the apex by an anchoring disc, the filament inverts, becoming a hollow tube (now known as the polar tube) (Han et al., 2020). As mannosylated proteins on the surface of the polar tube interact with mannose-binding proteins on the surface of the target cell, an invasion synapse forms (Han et al., 2020). This process ultimately results in endocytosis of the sporoplasm as it passes from the spore to the developing endosome through the hollow polar tube (Han et al., 2020).

After entering the host cell, the sporoplasm enters a proliferative stage which is marked by multiple rounds of replication by merogony (Han et al., 2020). Depending on the species of microsporidia, this process may occur within the host cytoplasm, in a parasitophorous vacuole made of host cell membrane, in parasite-secreted amorphous coat or surrounded by the host cell endoplasmic reticulum (Han et al., 2020). The resulting multinucleated plasmodia (meronts) typically differentiate into sporonts, which undergo schizogony to produce multiple immature sporoblasts (Fadhilah et al., 2024). Sporoblasts then develop into mature spores, and the cycle continues (Fadhilah et al., 2024).

In many species, replicating microsporidia transform individual host cells into hypertrophic, spore-filled growths called xenomas, which, upon rupture, disseminate infectious spores throughout the host organism and/or into the environment (Rodriguez-Tovar et al., 2011). To this end, it is thought that the majority of species exit host cells via inducing host-cell lysis (necrosis) or apoptosis (Han et al., 2020). However, some species have been observed to hijack host exocytosis pathways in order to re-enter the environment by fusing spore-filled compartments with the host's plasma membrane, and have even demonstrated the capacity for cell-to-cell spread *in vitro* (Han et al., 2020). To this end, microsporidial egress pathways remain poorly understood, but strategies to exit host cells after replication appear to be highly variable among species.

1.3 Diversity of germination stimuli in microsporidia

Perhaps the most crucial stage of the microsporidian life cycle is the initiation of germination, which enables spores to release infectious material only when in their ideal host environments. Although the specific pathways responsible for initiating germination remain unknown, changes in pH and or ion concentration appear to be among the most common inducers of germination in microsporidia *in vitro* (De Graaf et al., 1993; Han et al., 2020, 2019; Ishihara, 1967). For example multiple species of *Nosema*, *Encephalitozoon*, *Variphorma* and *Glugea* germinate in response to changes in cation and/or anion concentration if exposed during the appropriate shift in pH (De Graaf et al., 1993; Han et al., 2020; Ishihara, 1967; Malone, 1984; Undeen and Avery, 1988). Multiple species also exhibit pH dependent preferences for specific ions. For example, when exposed to 0.25 M NaCl or CsCl at pH 9.3, spores of *Glugea fumaniferae* exhibit 0 and 29% germination respectively, whereas at pH 10.8, germination rates of 71% and 68% (respectively) are observed (Ishihara, 1967). However, whether pH-dependent ion preferences are present across all ion-sensitive microsporidia remains to be seen. Microsporidia have also been observed to germinate in response to low doses UV-exposure (*Nosema algerae*), hydrogen peroxide (*Nosema bombycis*), desiccation/rehydration (*Gurleya* sp), sugars (*Ambylospora* sp.) and/or mechanical pressure (*Thelohania californica*)(Gibbs, 1953; Han et al., 2020; Kudo, 1918; Kudo and Daniels, 1963; Undeen and Avery, 1984; Undeen and Meer, 1990). It is unclear if these stimuli converge on a common mechanism for increasing intrasporal osmotic pressure, or if there are multiple independent mechanisms capable of inducing germination. To this end, vanishingly few studies have conducted exhaustive surveys of the germination conditions of a single species of microsporidia. Therefore, further research, particularly with respect to the range of conditions in which single species of microsporidia will germinate, will be necessary to better understand this process.

1.4 *Spraguea americanus*: microsporidian parasite of the American anglerfish

One genus of microsporidian parasites whose germination conditions remain particularly poorly described is *Spraguea*. Species of this genus infect neurons of the central nervous systems of anglerfishes (genus *Lophius*; commonly known as "monkfish"), resulting in the growth of large, hypertrophic cysts called xenomas (Freeman et al., 2004; Mansour et al., 2013). Within this genus, *Spraguea americanus* represents an endemic pathogen of the American anglerfish, infecting up to 100% of individuals in certain populations (Freeman et al., 2004). Although it has long been postulated that *Spraguea* infections are largely benign, observations from studies in Mediterranean lophiids (anglerfishes of the family Lophiidae) have suggested that infection with these parasites may result in severe neurological effects such as blindness (Colmenero et al., 2015; Freeman et al., 2011). However, since monkfish are notoriously difficult to keep in captivity, the life cycle, transmission and pathogenesis of *S. americanus* remains largely undescribed (Richards et al., 2011). With that said, research has demonstrated that the cutaneous mucosal glands of *Lophius* are the most common site for discharge of the sporoplasm of *Spraguea sp.* (Freeman et al., 2004). Since supramedullary cells (SMCs) of the brain often possess peripheral axons which innervate the cutaneous mucosal glands, it is thought that these glands may serve as the initial site of infection when spores come in contact with the cutaneous mucosal surfaces of susceptible lophiids (Freeman et al., 2004). In further support of this theory, SMCs also innervate the vagal and trimingeal nerves where xenomas are commonly observed (Freeman et al., 2004). With this considered, it is possible that infections are initially established in the nerves of the cutaneous mucosal glands (Freeman et al., 2004). How the parasite is able to migrate throughout the nervous system remains unknown, and it is unclear how it can pass from host to host. Some studies have suggested that heavy infections of the vagal nerve may contribute to parasite dissemination via the urinary bladder, but further research is required to support this theory (Freeman et al., 2004). Alternatively, since cannibalism plays a large role in the ecology of lophiids, it is possible that cannibalism and germination in the gut are another primary route of transmission (Freeman et al., 2004). This would explain the high prevalence of *Spraguea* infection and high rates of reinfection in natural populations (Campbell et al., 2013; Freeman et al., 2004). However, these hypotheses have yet to be validated *in vivo*.

1.5 Germination of *Spraguea spp.*

Although very few germination conditions have been explored using *Spraguea* species, studies with these organisms have indicated that mucin likely plays important role in stimulating germination of this genus *in vivo* (Pleshinger and Weidner, 1985; Weidner, 1982; Weidner et al., 1984). Indeed, exposure of *Spraguea* spores to pork mucin or human mucin at pH 9-9.5 in concentrations ranging from 0.2-2% (following incubation at neutral pH) results maximal germination (approximately 80%) (Pleshinger and Weidner, 1985; Weidner, 1982; Weidner et al., 1984). Similarly, the polyanion poly-D-glutamate, which is highly similar in structure and charge to mucin, induces comparable levels of germination at concentrations of 0.5 M when introduced to spores under the same pH conditions (Pleshinger and Weidner, 1985). These observations support the theory that *Spraguea* may enter their hosts by way of the subcutaneous mucosal glands, which has long been speculated (Freeman et al., 2004).

In addition to mucin, the movement and presence of calcium ions has been shown to play a significant role in the germination of *Spraguea*. To this end, incubation with the calcium ionophore A23187 at concentrations ranging from 1-20 μM following a shift from neutral to alkaline pH (7 to 9-9.5) has been shown to induce considerable germination in the spores of *Spraguea lophii* (Campbell et al., 2013; Pleshinger and Weidner, 1985). However, this effect is completely abolished following pretreatment with 0.15 mM EGTA (ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid), a calcium chelating agent (Campbell et al., 2013; Pleshinger and Weidner, 1985). Similarly, pretreatment of *S. lophii* with calmodulin antagonists trifluoperazine or chlorpromazine prior to mucin exposure results in dose dependent decreases in germination (Pleshinger and Weidner, 1985). To this end, radiochromatography analysis has shown that *Spraguea lophii* spores exhibit rapid uptake of calcium chloride present in the germination medium following an alkaline pH shift and exposure to A23187 or mucin (Pleshinger and Weidner, 1985). These results strongly implicate the involvement of calcium signalling in the germination of *Spraguea* species, although, it remains unclear whether calcium interacts with germination machinery directly or indirectly (Pleshinger and Weidner 1985; Weidner et al., 1984).

1.6 Objectives of this study

Thus far, only a very narrow range of germination conditions has been investigated in the *Spraguea* genus, namely mucin, poly-D-glutamate and the ionophore A23187 (Campbell et al., 2013; Pleshinger and Weidner, 1985; Weidner et al., 1984). Consequently, it is difficult to make sense of whether these species display similarities or differences to other species of microsporidians with respect to germination stimuli and mechanisms. Moreover, although calcium has been implicated in the germination of *Spraguea* spp., results across different studies are inconsistent, with some researchers inducing germination in the absence of external calcium supplementation (Campbell et al., 2013; Pleshinger and Weidner, 1985; Weidner et al., 1984). To this end, the primary objectives of this study are: 1) to develop a standardized assay to assess the germination responses of microsporidian species under different conditions *in vitro*, 2) to survey the responses of *S. americanus* to a range of stimulatory and inhibitory stimuli (especially those previous tested in other *Spraguea* species), and 3) to investigate treatments which may artificially facilitate infection in a nonhost species. An improved understanding of if/how *S. americanus* responds to different types of germination stimuli will provide valuable insight into the mechanism(s) underlying initiation of germination, a process which remains poorly understood in microsporidians. Moreover, with American anglerfish populations in serious decline due to overfishing (one of the most valuable finfish in the American northeast), an improved understanding of the biology of *S. americanus* will enable more effective conservation of the American anglerfish in the face of this endemic pathogen (Charbonneau et al., 2020; NOAA fisheries, 2024. Monkfish. Retrieved from https://www.fisheries.noaa.gov/species/monkfish).

Materials and Methods

2.1. Extraction and purification of *S. americanus* spores from *Lophius americanus*

American anglerfish (*L. americanus*) were acquired from NewCity Supermarket in Waterloo, Ontario, Canada. Fish were caught and euthanized (decapitation) by members of a third-party fisher-supplier in Boston Massachusetts, USA, at which point fish carcasses were placed in individual plastic bags and shipped/kept on ice until the time of experimentation. Spores were isolated and purified from xenomas as described by Sitjà-Bobadilla et al. (2021), with some minor modifications. Briefly, sterilized dissecting tools were used to extract xenomas from the spinal cords and vagal nerves of infected fish (Fig. 1A). The surfaces of fish were sprayed thoroughly with 70% ethanol (diluted from 100% stock: Commercial Alcohols Cat# P006EAAN Canada) prior to dissection to minimize cross-contamination and left until ethanol was completely evaporated. Individual xenomas were then washed in sterile 35 x 10 mm Petri dishes(Falcon Cat# 351008, USA) containing 100 µg/ml of fluconazole (Sigma Aldrich Cat# PHR1160-1G, USA), 100 µg/ml of amphotericin B (Cytiva Cat# SV30078.01, USA), 500 µg/ml of gentamicin (Sigma Aldrich Cat# G1264-250MG) and 1000 I.U. of penicillin/streptomycin (Corning Cat# 30-002-Cl, USA) in 1X PBS (Corning Cat# 21-040-CV) (hereby known as PURGE solution) in order to dispatch microbial contaminants (Fig. 1B). Xenomas from each fish were placed in separate 5 mL microfuge tubes (Axygen Inc. Cat# MCT500CS, USA), topped up to a final volume of 5 mL using PURGE solution, and then stored overnight at 4°C to enable further decontamination. The following day, individual xenomas were transferred to 5 mL microfuge tubes containing 1-2 mL of fresh PURGE solution and homogenized. Homogenates were then centrifuged at 350 *g* for 5 min at 4°C and supernatants were aspirated. Pellets were then passed through 40 µm cell strainers (Fisher Scientific Cat# 22363547, USA) fitted to 50 mL Falcon tubes (Falcon^{TM} Cat# 352070)via resuspending and rinsing with sterile 1X PBS. Falcon tubes were subsequently centrifuged at 4°C for 5 min at 350 *g* and supernatants were aspirated and discarded. This sequence was repeated 1-3 more times, with one exception. Prior to the final wash, pellets were resuspended in 1X PBS and solutions were transferred to sterile 15 mL Falcon tubes (Falcon^{TM} Cat# 352196) for further purification. Next, 100-200 µL of TritonX100 (BioShop Cat# TRX777.500, Canada) were added to pellets, together with an equal volume of sterile 1X PBS, and resuspended using a P1000 micropipette until a homogenous solution was formed. Detergent solutions where then vortexed on high for 90 s, and tubes were topped up with 1X PBS. Tubes were subsequently centrifuged at 4°C for 15 min at 700 *g*. Supernatants were discarded and this wash was repeated 2-3 more times. Final pellets were resuspended in 3 mL of fresh PURGE solution and stored at 4°C until further use (Fig. 2A).

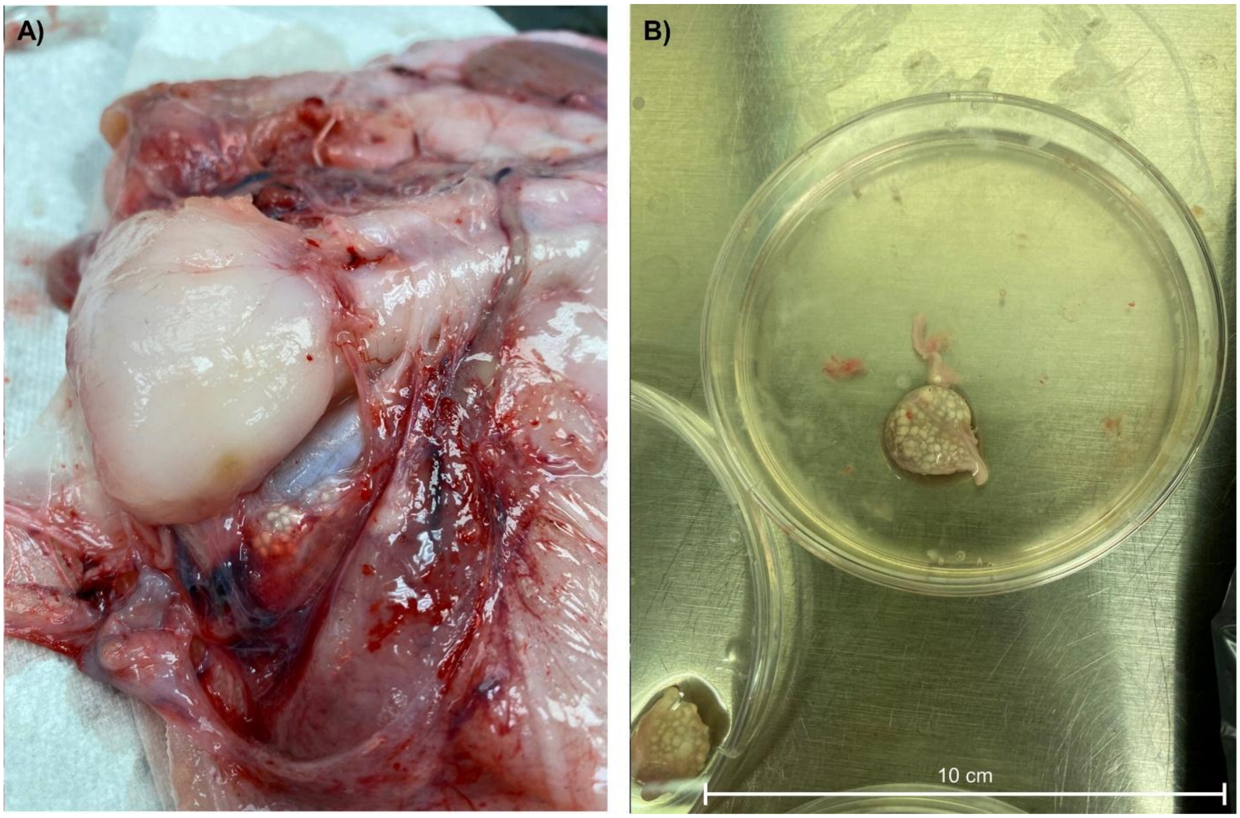


Figure. 1. Xenomas of *Spraguea americanus* isolated from *Lophius americanus*. (A) A cluster of xenomas found in nerves innervating the kidney in a *Lophius americanus* carcass. (B) An excised xenoma soaking in a Petri dish filled with 1X PBS. Representative images are shown.

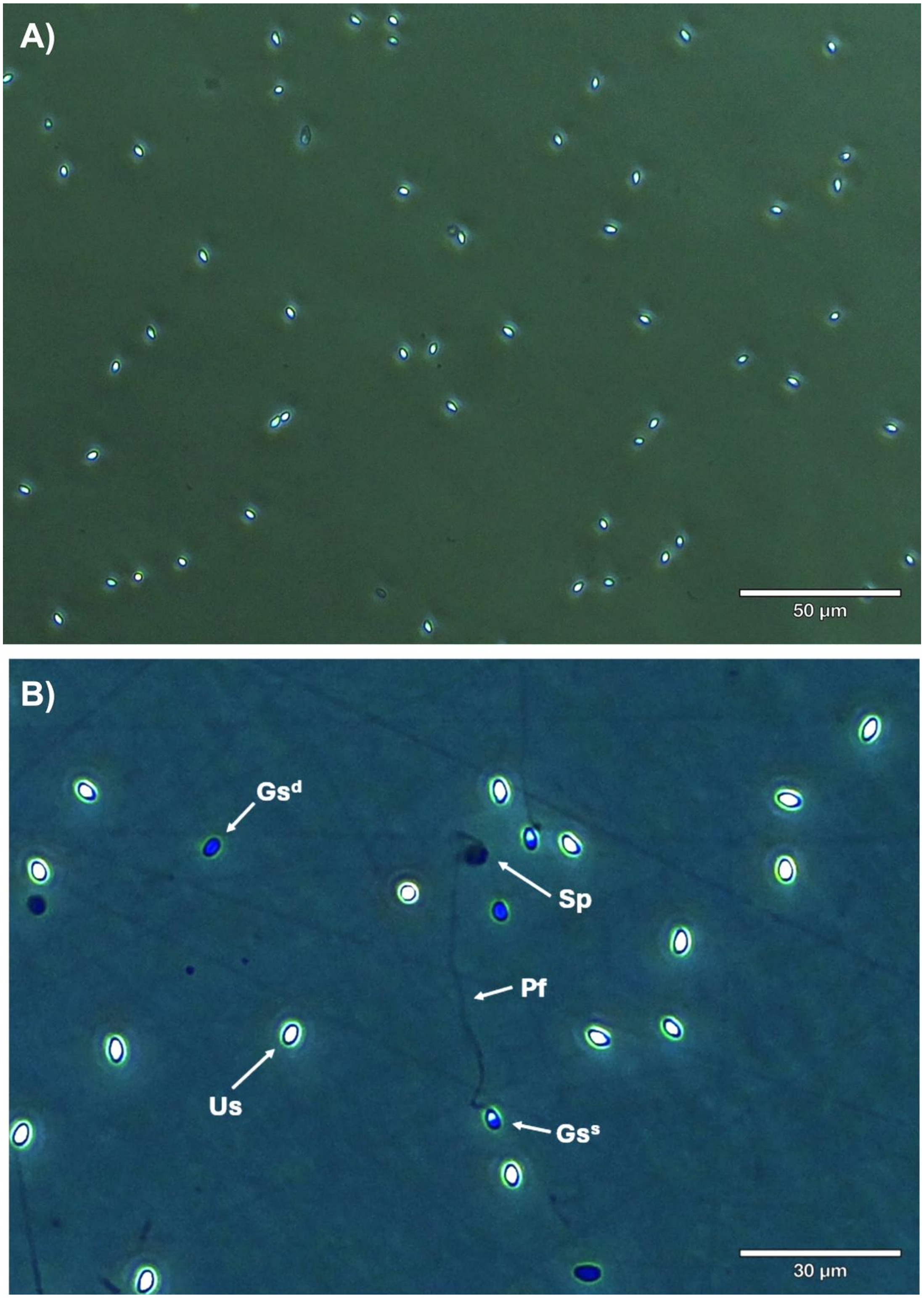


Figure. 2. Morphological characteristics of *Spraguea americanus* spores. (A) Final spore suspension (diluted in 1X PBS) visualized at 200X magnification. (B) Morphological characteristics of germinated and ungerminated spores after treatment with 15% hydrogen peroxide (Us, ungerminated spore; Gs^d, germinated spore, phase dark; Sp, sporoplasm; Gs^s, germinated spore, semi-phase dark; Pf, polar filament. Representative images are shown.

2.2. Speciation of spore isolates by transmission electron microscopy

Xenomas were surgically removed from monkfish as described in section 2.1. Xenomas from each fish were gently cut into small pieces and placed in 1.5 mL microfuge tubes (Axygen Inc. Cat# MCT150CS) containing 1 mL of 0.2 M Sodium Cacodylate Buffer (NaCac) pH 7.2 (VWR Cat# 100504-840, USA) with 2.5% glutaraldehyde (Polysciences Inc. Cat# BLI1909-10, USA) for fixation. Samples were fixed/stored in the fridge until further processing. Individual granulomas were excised from small tissue pieces and washed with 0.1 M (NaCac) buffer, pH 7.2 (Sigma Aldrich Cat# C-0125) and then postfixed in 1% OsO4 (Fisher Scientific Cat# A-235) in 0.1 M NaCac buffer for 1.5 h at RT (room temperature: 22°C). After washing with 0.1M NaCac three times and distilled water once, samples were dehydrated in a graded series of ethanol and en bloc stained with a saturated solution of Uranyl acetate (Fisher Scientific Cat# U-4 79850) (in 70% ethanol) for 1 h. After replacing ethanol with propylene oxide (Fisher Scientific Cat# AC220160010), tissues were infiltrated with Spurr's resin (Electron Microscopy Sciences Cat# 14300, USA) step-wise, at ratios of propylene oxide:Spurr's resin, 2:1, 1:1, and then 1:2, and finally left in pure Spurr’s resin overnight. The following day, samples were transferred to fresh Spurr’s resin in embedding molds, soaked for 2 h, and subsequently baked for 24 h at 60°C. Blocks were cut to 90 nm thickness and placed on Formvar-coated Cu 200 mesh grids (Formvar: Tedpall Cat# 19222, USA; Cu grids: Electron Microscopy Sciences Cat# G200TH-Cu) prior to 10 min of staining with Raynold’s lead citrate (Fisher Scientific Cat# L-62). Samples were imaged on a Hitachi HT7700 transmission electron microscope (Hitachi High-Technologies Corporation, Japan), and spores were speciated using ultrastructural characteristics.

2.3. Enumeration of *S. americanus* spores using a hemocytometer

Briefly, spore suspensions were aggressively vortexed, and 10 µL of suspension were diluted in L15 (Cytiva, Cat No. SH30525.01). Dilution factors varied per suspension. However, before enumeration, 10 µL of diluted spore suspension were loaded on to a hemocytometer and assessed qualitatively to ensure an average of 3-25 spores was present in each 1x1 mm square at 400x magnification. In this case, spores were enumerated, and the concentration of the original suspension was extrapolated. In cases where the number of spores fell below the specified threshold, a new subsample was made using a lower dilution factor. In cases where spores were too abundant, subsamples were diluted 1:2 until the ideal concentration was achieved. Each subsample was quantified a total of two times, and concentrations were averaged across both replicates.

2.4. Polar filament extrusion assay for germination of *S. americanus* spores

Spore aliquots were enumerated as described above, and 10 µL of original suspension were diluted in sterile L15 to produce a concentration of spores between 10,000,000-20,000,000 spores/mL. These L15 aliquots were then enumerated twice, and replicates averaged, to determine a final concentration. Media representing the germination condition of interest (1.5 mL) was then loaded into the wells of a 6-well cell culture plate (Thermo Fisher Scientific Cat# 140675, Denmark), followed by 300,000-450,000 spores. Depending on the conditions tested, spores were either incubated under specific conditions and then centrifuged, or immediately centrifuged at 4700 *g* for 3 min at RT. The fraction of germinated versus total spores was then assessed in 3-4 representative fields of view per well at 400x magnification using an OPTIKA$^{\text{TM}}$, IM-3 trinocular Inverted Microscope (OPTIKA$^{\text{TM}}$, Italy) (unless otherwise specified) and averaged to produce a single "germination ratio" for each well. To ensure consistency and accurate enumeration, only fields of view with 30-80 spores were examined, and three-dimensional aggregates of spores were disregarded. Spores demonstrating polar filament extrusion, or conversion to a phase dark or "bullseye/semi-phase dark" morphology (posterior pole is phase dark, anterior pole is phase bright) morphology, were considered germinated (Fig. 2B).

2.5. Treatment of *S. americanus* spores with hydrogen peroxide

Hydrogen peroxide experiments were conducted using the polar filament extrusion assay outlined above, with solutions prepared in and diluted using sterile MilliQ water. Wells of 6-well plates were loaded with 1.5 mL of hydrogen peroxide solution at a concentration of 0%, 0.1%, 0.3%, 1%, 3%, 5%, 10%, 15%, 20%, 25% and 30% (diluted from 30% stock solution, Fisher Chemical Cat#H325-500, USA), followed by 300,000 *S. americanus* spores (aliquoted in L15). Plates were then centrifuged at 4700 *g* for 3 min at RT and germination ratios were assessed as described above. For experiments examining the effects of different hydrogen peroxide concentrations, plates were centrifuged immediately following the addition of spores. Spores in wells loaded with 15% hydrogen peroxide were imaged using an ECHO Revolve microscope (ECHO, USA) at 200X magnification in the inverted configuration. For duration experiments, plates were centrifuged once, immediately prior to time zero. Germination ratios were then assessed in the same wells at 0, 24, 48 and 72 h post-centrifugation. For temperature experiments, spore aliquots were prepared in L15 and allowed to equilibrate to 4°C, 14°C or 22°C overnight. Plates were loaded with 15% hydrogen peroxide and allowed to equilibrate under the same conditions. After overnight incubation at each respective temperature, spores of each temperature were added to the corresponding plate and centrifuged immediately under the specified temperature for 3 min at 4700 *g* prior to observation.

2.6. Treatment of *S. americanus* spores with mechanical pressure

For pressure experiments, 50 µL of original spore suspension (concentration pre-determined) were diluted with sterile L15 to produce aliquots of approximately 500,000,000 spores/mL. For control treatments, two pieces of 2.5 cm green labeling tape (UW Chem Stores Cat# HTAP31, Canada) were affixed to the surface of a standard glass microscope slide, leaving a small rectangular space between them (slightly narrower than the width of a coverslip). Next, 1 µL of spore suspension, in addition to 9 µL of sterile L15, was placed in a single droplet between both pieces of tape. A glass coverslip was gently placed with either end resting on a piece of tape, thus preventing the coverslip from exerting pressure on the spores. For coverslip and coverslip + rolling treatments, 7 µL of suspension were placed in the center of the slide, and a coverslip was gently placed on top. Pressure treatments were prepared similarly, with one minor deviation. Briefly, an empty 500 mL glass bottle (347.47 g) was used to apply strong, even mechanical pressure to the coverslip for 5 s. In all cases, slides were incubated for 5 min at RT to allow complete germination. At this time, slides were examined under 200X magnification using a Nikon^{TM} Eclipse TS100 microscope (Nikon^{TM}, Japan) outfitted with a AmScope^{TM} MD500 microscope camera (AmScope^{TM}, USA), and four representative fields of view were imaged. The proportion of germinated/total spores was then counted in images using the same criteria described above, with the assistance of an ImageJ cell counting application (https://imagej.net/ij/plugins/cell-counter.html) and averaged across the four images for each slide.

2.7. Treatment of *S. americanus* spores with sodium carbonate-bicarbonate buffer

To assess the effects of sodium carbonate-bicarbonate buffer, 0.2 M stock solutions of sodium carbonate (Fisher Scientific, Cat# S263-1) and sodium bicarbonate (Fisher Scientific, Cat# S233-3) were prepared and mixed in the appropriate ratios to produce 0.1 M solutions (total concentration) at pH 9.3, 9.8, 10.3 and 10.8. Following validation by an accument® AB200 pH meter (Fisher Scientific), buffers were further diluted to 10 mM and 2 mM. In cases where the pH required adjustment, 0.1 or 0.01 M NaOH (BioShop Cat# SHY700.500) and/or HCl (Fisher Scientific Cat# 351280-212) were used when appropriate. Negative controls were produced via the addition of 0.1 or 0.01 M NaOH to sterile MilliQ water. Next, 6-well plates were filled with the various test solutions (1.5 mL/well) and inoculated with 300,000 spores/well. Plates were then centrifuged at 4700 *g* for 3 min, and incubated at RT for 80 min. Following incubation, germination ratios were examined as described above. To capture phenotypic differences in germination responses, spores germinated with 15% hydrogen peroxide (as described in section 2.5) or 10 mM sodium-carbonate bicarbonate were imaged on an ECHO Revolve microscope at 200X magnification following centrifugation and 80 min incubation at RT. Individual stock solutions were prepared independently for each replicate in all experiments to account for variation in preparations.

2.8. Treatment of *S. americanus* spores with glycylglycine

Glycylglycine experiments were conducted using the same protocol as sodium carbonate-bicarbonate experiments, with minor deviations. Briefly, stock solutions of 0.1 M glycylglycine (Fisher Scientific Cat# AAA1052314) (henceforth used interchangeably with GlyGly or GlyGly buffer) were diluted to concentrations of 2 and 10 mM using sterile MilliQ water and adjusted to pH 9 using a pH meter and 0.1 M and/or 0.01 M NaOH. Spores were then incubated with GlyGly solutions for 80 min at RT and analyzed as described in section 2.7.

2.9. Treatment of *S. americanus* spores with group-I and group-II metal salts and halides

To determine the impact of different ions on germination, spores were incubated in the presence of different salt solutions. To assess the impact of monovalent cations, spores were exposed to 2, 50, and 250 mM concentrations of Li, Na, K, Rb and Cs chloride solutions (made in 2 mM GlyGly) adjusted to pH 9 as described above (LiCl: BioShop Cat# LIT704.100; NaCl: Bio Basic Inc. Cat# DB0483, Canada; KCl: Fisher Scientific Cat# BP366-1; RbCl: BioShop Cat# RUB123.10, CsCl: BioShop Cat# CES001.25). Wells of a 6-well plate were loaded with 1.5 mL of each solution and seeded with 300,000 spores. Following 80 min of incubation at RT, germination was assessed. To examine the influence of halogen anions and divalent cations, spores were incubated in solutions composed of sodium-halides (NaBr, NaCl, NaI, NaF) and chloride of divalent metals ($CaCl_2$, $BaCl_2$, $MgCl_2$) prepared using the same protocol. Negative controls consisted of 2 mM GlyGly buffer adjusted to pH 9 (NaBr: BioShop Cat# SBR222.100; NaI: BioShop Cat# SOD333.100; NaF: BioShop Cat# SFL001.100; $CaCl_2$: Fisher Scientific Cat# C79-500; $BaCl_2$: Fisher Scientific Cat# B34; $MgCl_2$: Fisher Scientific Cat# BP214-500)

2.10. Treatment of *S. americanus* spores with calcium ionophore A23187

Calcium ionophore A23187 was prepared as described by Pleshinger and Weidner, (1985). Briefly, A23187 powder obtained from Sigma (Sigma Aldrich Cat# C7522-10MG) was dissolved in pure DMSO (VWR Cat# 0231-500ML) to produce a 1 mM stock solution. Stock was aliquoted and stored at -20°C and protected from light until further use. At the time of experimentation, aliquots were thawed and diluted with 2 mM GlyGly pH 9 to produce solutions of 1, 5, 10 and 20 µM. Next, 300,000 spores were used to seed wells of a 6-well plate filled with 1.5 mL of sterile 2 mM GlyGly pH 7 and were incubated for 30 min at RT. Plates were then centrifuged at 4700 *g* for 3 min, and media were gently aspirated and replaced with either 2 mM GlyGly pH 7 (NTC: no transfer control), 2 mM GlyGly pH 9 (TC: transfer control), or GlyGly with A23187. Plates were then centrifuged again and germination evaluated.

2.11. Treatment of *S. americanus* spores with pork mucin type III

The effects of mucin on germination were examined according to Pleshinger and Weidner (1985) with slight modifications. Briefly, pork mucin type III (Sigma Aldrich Cat# M1778-10G) was dissolved in 2 mM of glycylglycine (GlyGly) adjusted to produce 0.2% and 0.5% mucin solutions, which were subsequently adjusted to pH 9 (verified by a pH meter) using 0.1 or 0.01 M NaOH. Next, 300,000 spores were incubated in 1.5 mL of either 2 mM GlyGly pH 7 (adjusted as described above) or 2 mM GlyGly pH 7 + 50 nM $CaCl_2$ in a 6-well plate and centrifuged at 4700 *g* for 3 min. Following 30 min of incubation at RT, media were gently aspirated and replaced with mucin-adjusted GlyGly pH 9. Following a final centrifugation, spores were incubated at RT for an additional 80 min and germination was examined. NTCs were resuspended in GlyGly Ph 7, while TCs were moved to pH 9 GlyGly without mucin.

2.12. Treatment of *S. americanus* spores with biological solutions from rainbow trout (*Oncorhynchus mykiss*)

Fish were sourced from the Alma Aquaculture Research Station, Canada, and kept in a recirculating aquaculture system containing dechlorinated city water. Fish were screened for external signs of disease prior to sampling. Prior to the collection of blood and serum, five rainbow trout (*O. mykiss*) (~1.5 kg) were lightly anesthetized (stage 3) using benzocaine according to the University of Waterloo, Canada standard operating procedure CAF AQUA-FIELD 003. Upon loss of equilibrium, fish were placed on a sterile sheet of plastic (previously disinfected using 70% ethanol) and cutaneous mucus was gently liberated from the skin between the operculum and tail fin using sterile microscope slides. Mucus was then aspirated from the microscope slides and plastic sheet using a P1000 micropipette, dispensed into 15 mL microfuge tubes and stored on ice. Next, blood was drawn from the caudle peduncle using sterile 3 mL syringes (BD REF# 309657, USA) fitted with 23 ½ gauge needles (BD REF# 305194) rinsed with 100 IU/mL of heparin (Sigma Aldrich, H3149-100KU). Blood was then dispensed into 15 mL Falcon tubes containing 300 μL of 100 IU/mL of heparin (100 μL per 1 mL of blood) and stored on ice.

Upon completion of sampling, fish were returned to their respective troughs and blood samples were centrifuged at 2500 *g* for 5 min at 4 °C. Following centrifugation, serum was aspirated from blood samples and combined in a single 15 mL Falcon tube. Serum was then filtered using a 3 mL syringe fitted with a 0.22 μM filter (Pall Corporation REF# 4612, USA), and subsequently diluted to 2%, 10% and 20% using sterile MilliQ water to preserve the initial physiological pH of the solution. Similarly, mucus samples were pooled, diluted to 1%, 10% and 50% using sterile MilliQ water, and filtered using syringes fitted with 0.22 μM filters). Wells of a 6-well plate were subsequently filled with 1.5 mL of the respective serum and mucus dilutions, seeded with 300,000 spores per well and centrifuged at 4700 *g* for 3 min at RT prior to examination.

2.13. Comparing propidium iodide exclusion and hydrogen-peroxide-induced polar filament extrusion as measurements for spore viability in *S. americanus*

To produce ethanol-inactivated spores, 90,000,000 *S. americanus* spores were added to sterile 1.5 mL tubes and centrifuged at 1600 *g* for 5 min. Supernatants were aspirated and replaced with 1 mL of 100% molecular biology grade ethanol (Commercial Alcohols, Cat# P006EAAN). Following 1 h of incubation at RT, spores were centrifuged (at 1600 *g* for 5 min), ethanol was aspirated, and spores were washed with sterile 1X PBS. Wash steps were repeated three times, after which spores were resuspended in 1 mL of 1X PBS, and renumerated. These samples (hereby known as “inactivated” or “100% inactivated” samples) were then used to produce a sample containing 50% inactivated spores by mixing 45,000,000 untreated spores with 45,000,000 ethanol inactivated spores (also re-counted to ensure accurate loading during experimentation).

Spore viability was assessed by propidium iodide (PI) (Sigma Aldrich Cat# P4864) staining as described by Elsheikha and Mansfeild (2004), with minor modifications. First, a 1 mg/mL stock solution of PI was prepared (pH = 7.2) and stored in aliquots below 0°C in the dark. Upon use, 900 µL of staining solution were mixed with 30,000,000 spores (either live, 50% inactivated or 100% inactivated) resuspended in 300 µL of PBS in a 15 mL Falcon tube. Mixtures were then incubated in the dark for 25 min at RT and washed 3X with 1X PBS (~10 mL of 1X PBS, centrifuged at 1600 *g* for 5 min at 4 °C). Following the final centrifugation, 1X PBS was aspirated and spores were resuspended in approximately 100 µL of 1X PBS. Next, 10-20 µl of this suspension were spotted onto a glass slide, on which a coverslip was fixed with clear nail polish. Slides were viewed at 400X magnification on an EVOS™ M5000 Imaging System (Thermo Fisher Scientific, USA) fluorescence microscope using the Texas Red channel. To quantify viability, three fields of view were imaged per sample, and the number of live spores was deduced as those remaining unstained following merging with brightfield microscopy. The ratio of dead versus total spores was then counted for each image using ImageJ cell counting software (https://imagej.net/ij/plugins/cell-counter.html) and averaged across the three images/slide. To compare the results of fluorescence and germination assays, 300,000 spores from live, 50% inactivated or 100% inactivated samples were added to wells of a 6-well plate containing 15% hydrogen peroxide. Plates were centrifuged (4700 *g* for 3 min) and germination ratios were assessed as described above.

2.14. Inactivation of *S. americanus* spores by various chemical and mechanical treatments.

To assess the effects of different chemical and mechanical treatments on spore germination, the germination assay described in section 2.13 was adjusted to examine rates of spore inactivation. Briefly, untreated spores were exposed to 15% hydrogen peroxide as a negative control, demonstrating maximum germination. Treated spores were then exposed to the same concentration of hydrogen peroxide and the proportion of germination to total spores was quantified in 3-4 fields of view per well and averaged to produce a single germination ratio for each well.

For heating experiments, 1,500,000 spores were added to sterile PCR tubes (Axygen Inc. Cat# PCR02B5), and incubated at RT or in a thermocycler (T100 Thermal Cycler (BioRad Cat# 1861096, USA) and PTC Tempo Thermal Cycler (BioRad Cat# 12015392) at 22°C (RT), 37°C, 60°C, 100°C (lid set to same temperature) for 1 h, followed by an indefinite hold at RT. Plates were then loaded with 15% hydrogen peroxide as described above, and 450,000 spores were added to each well. Plates were centrifuged (4700 *g* for 3 min at RT), and germination was assessed. Similarly, for freezing experiments, 1,500,000 spores were added to sterile PCR tubes, and stored at 4°C, -20°C and -80°C for 24 h. The following day, tubes were gently thawed at RT, and spores were added to wells containing 15% hydrogen peroxide (450,000 spores/well). Plates were then centrifuged (4700 *g* for 3 min at RT), and germination was assessed. To examine susceptibility to microwaving, 1,500,000 spores (taken from original spore aliquots made in L15) were added to 5 mL sterile glass test tubes fitted with lids, and microwaved for 0, 5, 15 or 30 s (GoldStar model No. MS-102MC, 850 W, USA). After cooling to RT, 300,000 spores were transferred from each glass test tube to the well of a 6-well plate containing 1.5 mL of 15% hydrogen peroxide. Plates were then centrifuged, and germination ratios were examined as described above. To examine the effects of ethanol treatment, 100% molecular biology grade ethanol was diluted to the required concentrations using sterile MilliQ water. Next, 450,000 spores were added to wells each containing 1.5 mL of each respective concentration of ethanol and incubated for 15 min at RT. Plates were then gently washed with sterile MilliQ water, which was subsequently aspirated, and

1.5 mL of 15% hydrogen peroxide was added. Following a second centrifugation (4700 *g* for 3 min), germination was assessed.

In UV exposure experiments, 1.5 mL of sterile MilliQ water was used to fill each of the wells of a 6-well plate. Wells were subsequently seeded with 450,000 spores each and incubated under the UV light of a biosafety hood at RT (lid off) for 0, 10, 30 or 60 min (UVC, ~125 $\mu W/cm^2$). Following incubation, plates were centrifuged at 4700 *g* for 3 min, and water was gently aspirated in order to not disturb spores. Spores were then submerged in 1.5 mL of 15% hydrogen peroxide and centrifuged at 4700 g for 3 min prior to examination by phase contrast microscopy (400x magnification). For desiccation experiments, wells were filled with 1.5 mL of sterile MilliQ water, and 450,000 spores were added/well. Following centrifugation, water was gently aspirated such that wells were left completely dry (no traces of water). Plates were then incubated for the desired durations (with the lids removed) inside the biosafety cabinet with the blower on. Following incubation, 1.5 mL of 15% hydrogen peroxide was added to each well, and plates were centrifuged again at 4700 x g for 3 min prior to examination. Finally, to examine the effects of aging on germination, spores of different ages were exposed to 15% hydrogen peroxide and assessed for germination. Briefly, spores freshly isolated from monkfish (suspended in PURGE), as well as spores stored in PURGE solution for 5 and 10 months, were used to produce aliquots in L15 in concentrations ranging from 10,000,000-20,000,000 spores/mL. Spores of each age were added to 6-well plates (300,000 spores/well) containing either 1.5 mL of sterile MilliQ water or 15% hydrogen peroxide, centrifuged and assessed for germination.

2.15. Statistical analysis

All data are shown as mean ± S.D. In germination assays, ratios of germinated to total spores were averaged across three fields of view per well (technical replicates), and experiments were repeated with at least three different spore isolates (i.e. isolates from different anglerfish). Germination ratios for each treatment were compared using either one-way or two-way ANOVAs ($\alpha = 0.05$) with a post-hoc Tukey's test. All graphing and statistical analysis were preformed using GraphPad Prism 10 (GraphPad Software, www.graphpad.com). Groups which do not share letters in the figures below exhibit differences which are statistically significant. Letters denoting statistical significance were assigned to groups from highest to lowest mean.

Results

3.1. Germination of *S. americanus* spores following exposure to hydrogen peroxide under various conditions

To determine if *S. americanus* was capable of germinating in response to hydrogen peroxide, purified spores were exposed to a range of hydrogen peroxide concentrations from 0-30% and examined using a polar filament extrusion assay. To this end, the greatest mean germination ratio was observed when spores were exposed to 15% hydrogen peroxide (Fig. 3A). However, differences in germination following exposure to 0.3%, 15% and 20% hydrogen peroxide were statistically insignificant (Fig. 3A). Although germination in 0.1% and 1% hydrogen peroxide was near maximal, and statistically indistinguishable from 0.3% and 20%, these doses produced significantly less germination than 15% hydrogen peroxide (Fig. 3A). Together, these five doses (0.1, 1, 0.3, 15 and 20% hydrogen peroxide) represented two distinct peaks in germination, producing a bimodal dose response. To this end, significant increases in germination were observed in a stepwise manner as doses were increased from 0 to 0.1%, 5 to 10% and 10 to 15%, respectively (Fig. 3A). Similarly, decreases in germination where statistically meaningful when doses were increased from 1 to 3%, 3 to 5%, 20 to 25% and 25 to 30% (Fig. 3A). To evaluate how external factors may influence the efficiency of germination, spores were exposed to 15% hydrogen peroxide at different temperatures, and for varying durations. Although rates of germination decreased as temperatures moved from 22°C to 14°C, 14°C to 4°C and 22°C to 4°C, no statistically significant differences were detected among any treatment groups (Fig. 3B). Similarly, although 15% hydrogen peroxide induced statistically more germination than 30% hydrogen peroxide at all time points (and both treatments were significantly greater than 0%

hydrogen peroxide at all time points), no meaningful differences were observed within treatments over time (Fig. 3C).

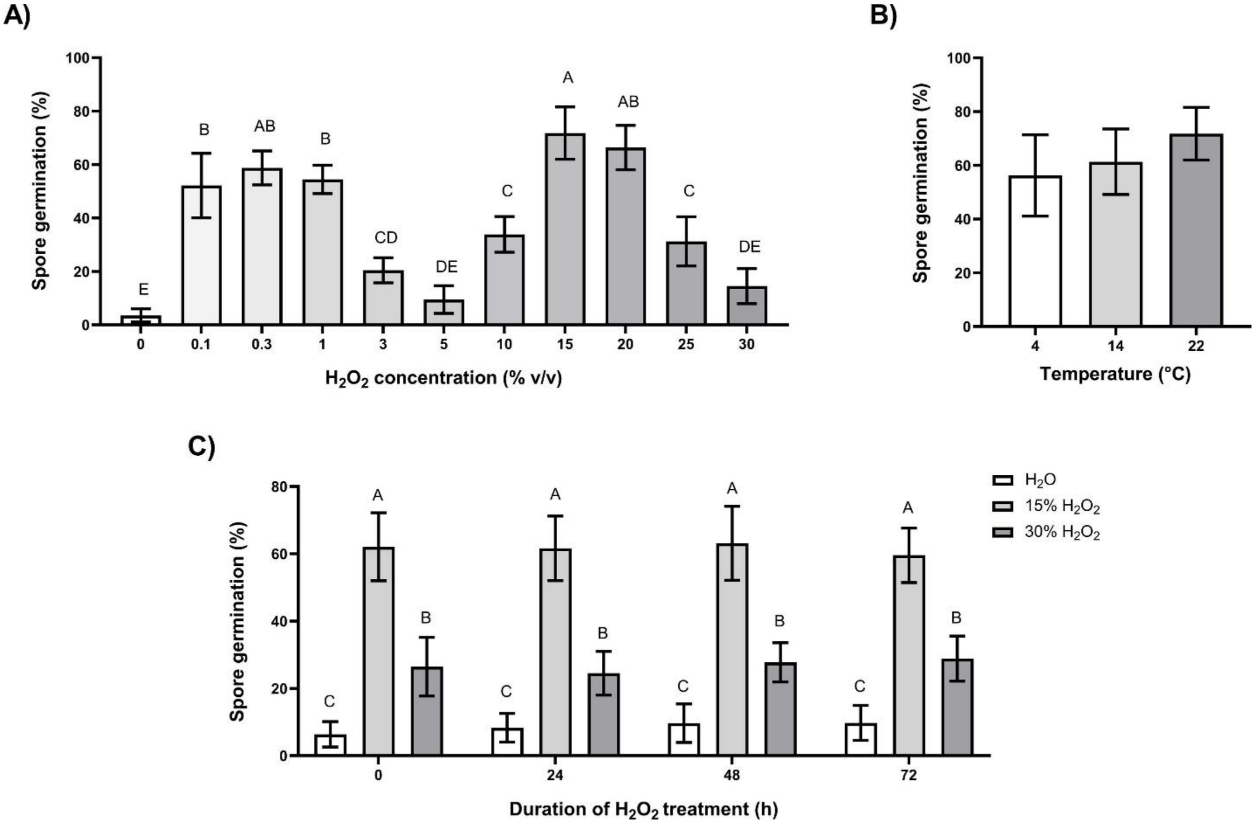


Figure. 3. Effects of concentration, temperature and duration of exposure on the hydrogen peroxide-induced germination of *Spraguea americanus* spores. (A) Percentage of germinated *S. americanus* spores observed following exposure to hydrogen peroxide concentrations ranging from 0-30%. (B) Percentage of germinated *S. americanus* spores observed following exposure to 15% hydrogen peroxide solutions at 4 °C, 14 °C, 22 °C. (C) Germination of *S. americanus* spores observed following incubation in 0%, 15% and 30% hydrogen peroxide solutions for 0, 24 h, 48 h and 72 h at 22 °C. Values are shown as mean ± S.D. $n = 6$ replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by either a one-way ANOVA (A, B) or two-way ANOVA (C) and post-hoc Tukey's test.

3.2. Germination of *S. americanus* spores in response to coverslipping and manual pressure

To examine the reactivity of *S. americanus* to mechanical pressure, spores were loaded onto microscope slides, after which manual pressure was applied to slides by pressing on coverslips. To account for the pressure imparted by coverslips themselves, spore suspensions were placed between two pieces of laboratory tape such that coverslips were suspended slightly above the surface of the microscope slide, producing a “hanging drop” preparation. Indeed, little to no phase dark spores were observed in these “pressure-free” negative controls (Fig. 4A). However, when coverslips were gently placed on spore suspensions, a small number of spores appeared phase dark after 5 min of incubation at RT, adopting both phase dark and semi-phase dark morphologies (Fig. 4B). Conversely, in samples subjected to 5 s of even manual pressure, nearly all spores appeared phase dark after 5 min, with the vast majority adopting a phase dark morphology (Fig. 4C). To this end, when the germination ratios of each sample and treatment were quantified, germination was significantly greater in samples exposed to manual pressure when compared with coverslip or “pressure-free” samples (Fig. 4D). While more germinated spores were observed in samples incubated with a coverslip compared with “pressure-free” samples, these differences were statistically insignificant (Fig. 4D).

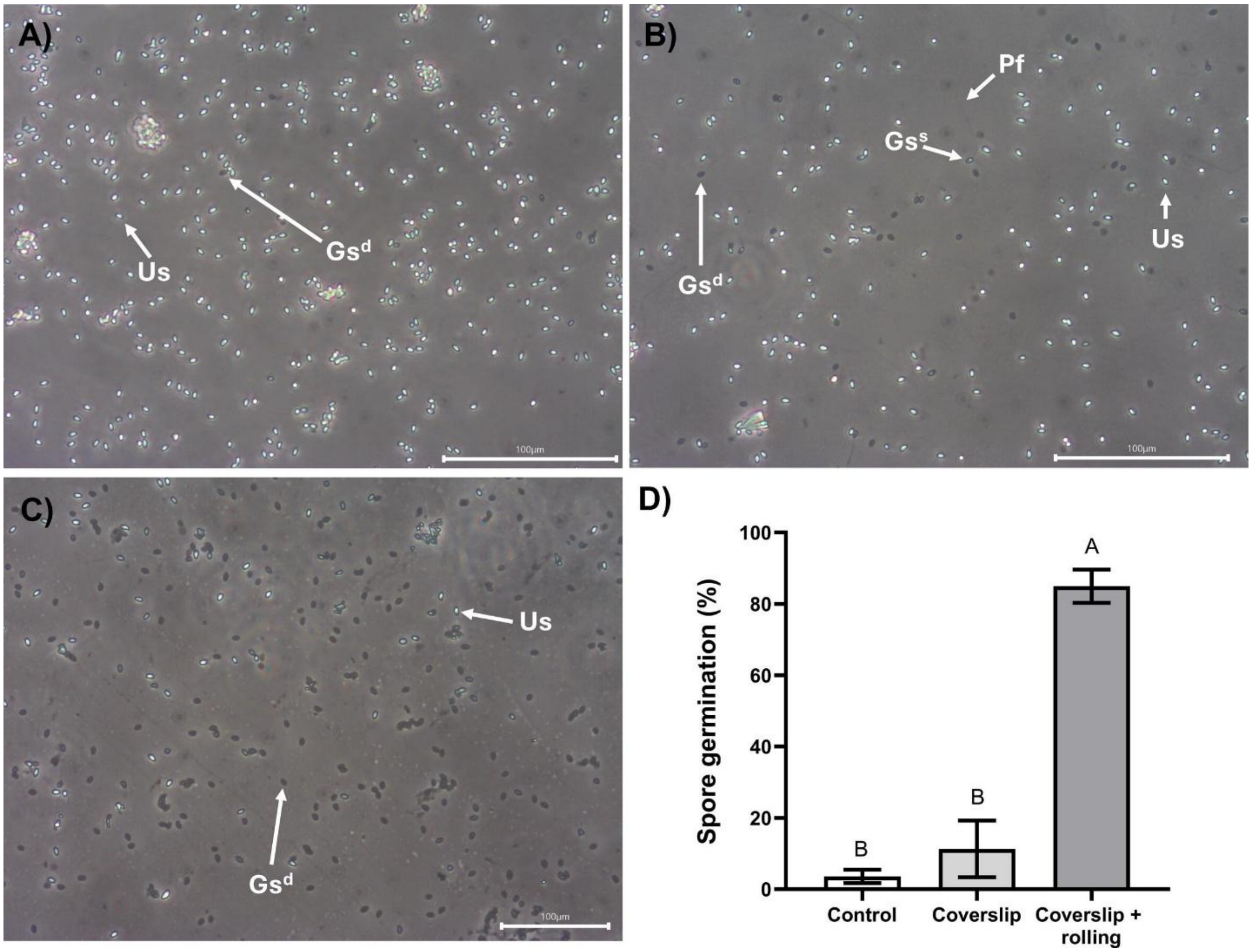
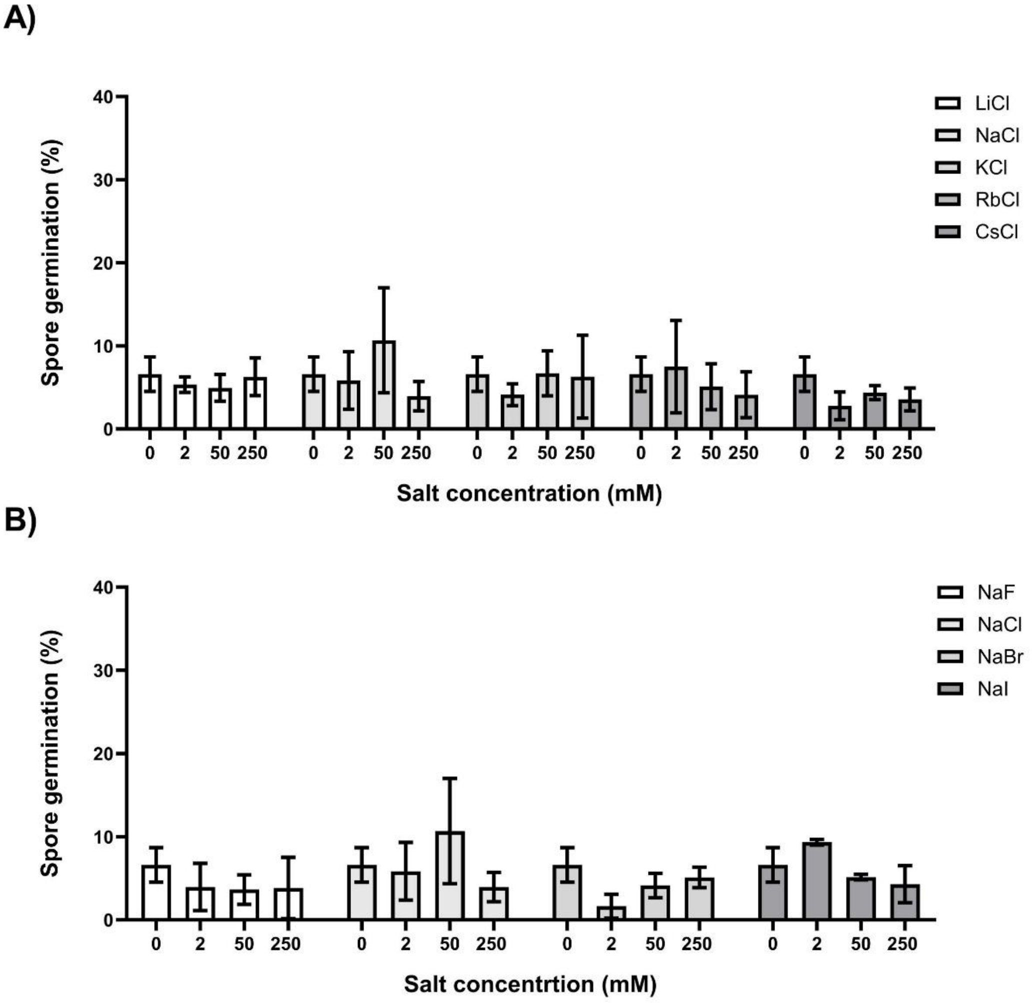


Fig. 4. Germination of *Spraguea americanus* spores in response to mechanical pressure. (A) *Spraguea americanus* spores after 5 min of incubation at room temperature (RT: 22 °C) in a "hanging drop" preparation in the absence of pressure. (B) *Spraguea americanus* spores following 5 min of exposure to pressure from a coverslip at RT. (C). Representative image of *S. americanus* spores following coverslipping, manual rolling with a 500 mL glass bottle for 5 s and subsequently incubation at RT for 5 min. Images were taken at 200x magnification (representative images shown; Gs^{d}, germinated spore, phase dark; Us, ungerminated spore; Gs^{s}, germinated spore, semi-phase dark; Pf, polar filament). (D) Percentage of germinated *S. americanus* spores observed in treatments (A-C). Spore counts were determined using ImageJ cell counting software and averaged across four fields of view/slide at 200X magnification. Values are shown as mean ± S.D. $n = 3$ replicates per experiment using spores isolated from separate anglerfish. Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a one-way ANOVA and post-hoc Tukey's test.

3.3. Germination of S. americanus spores in response to monovalent cations, divalent cations and halogen anions

To determine if specific ions play a role in inducing the germination of *S. americanus*, spores were exposed to solutions of various salts over a range of different concentrations. No significant differences in germination were observed between control treatments and solutions containing any of the salts tested in this study (Fig. 5A-C). However, it is noteworthy that 2 mM solutions of calcium chloride induced considerable germination, although these observations were statistically indistinguishable from control treatments (Fig. 5C).



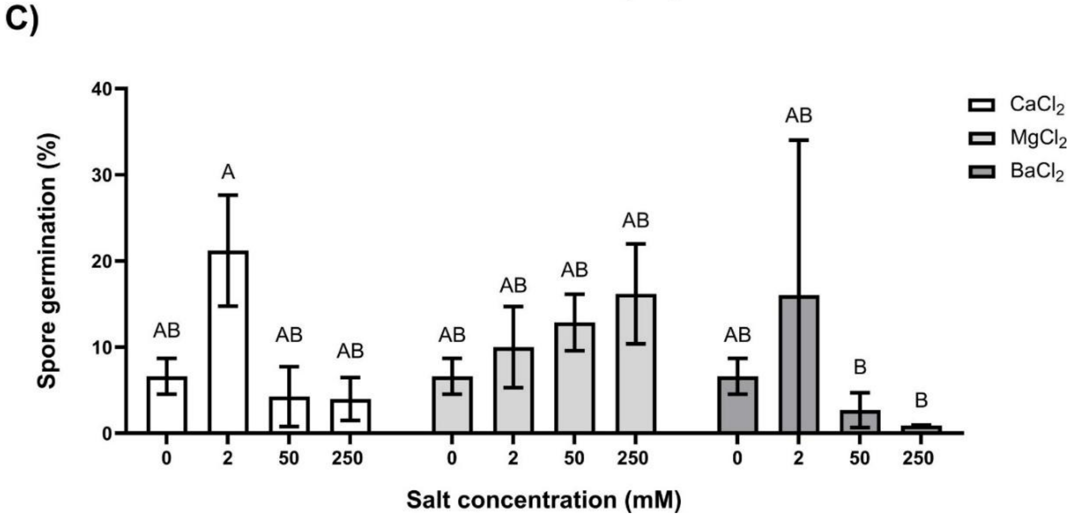


Fig. 5. Effects of various cations and anions on the germination of *Spraguea americanus* spores. (A) Percentage of germinated *S. americanus* spores observed following exposure to 2 mM, 50 mM or 250 mM solutions of NaCl, KCl, LiCl, RbCl or CsCl (pH 9, prepared in 2 mM glycylglycine) at room temperature (RT: 22 °C) for 80 min. (B-C) Experiments were conducted as in (A), except sodium halides (NaCl, NaF, NaBr, NaI) and group-2 chlorides ($BaCl_2$, $MgCl_2$, $CaCl_2$) were used in B and C, respectively. Values are shown as mean ± S.D. $n = 3$ replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a two-way ANOVA and post-hoc Tukey's test

3.4. Germination of *S. americanus* spores in response to biologically active solutions

To investigate the potential for *in vivo* infection of *Oncorhynchus mykiss* in future studies, *S. americanus* spores were exposed to dilute solutions of fresh trout mucus and serum. While concentrations of 1% and 10% mucus were sufficient to induce germination above control levels, germination in response to 50% trout mucus was statistically indistinguishable from controls (Fig. 6C). Conversely, the concentrations of trout serum tested in this study were insufficient to induce germination above control levels (Fig. 6D). Similarly, no statistically significant differences in germination were observed when *S. americanus* spores were exposed to calcium ionophore A23187 or 0.2-0.5% pork mucin at pH 9 (with and without calcium pretreatment), despite these treatments being well established inducers of germination in *Spraguea lophii* (Fig. 6 A,B).

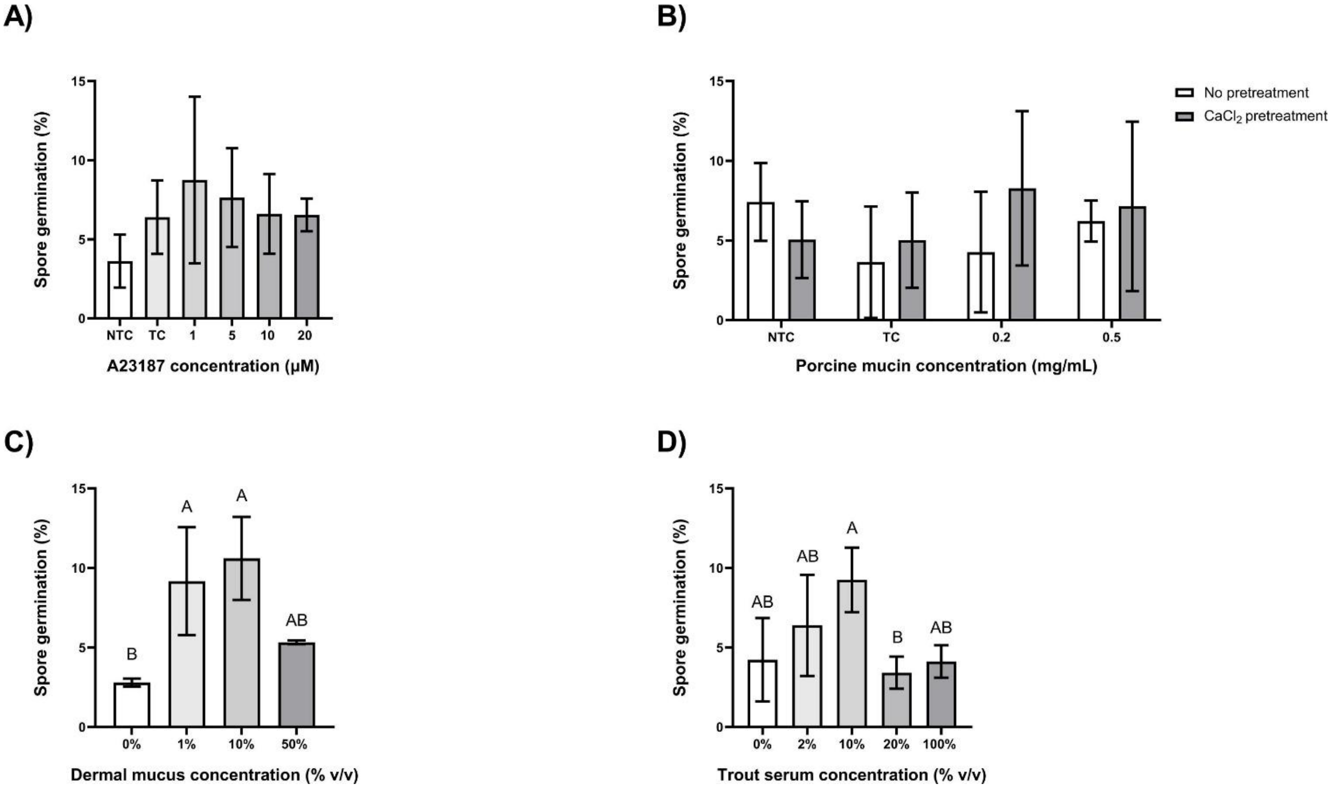


Fig. 6. Effects of various biological solutions on the germination of *Spraguea americanus* spores. (A) Percentage of germinated *S. americanus* spores observed following pretreatment with 2 mM GlyGly pH 7 for 30 min at room temperature (RT: 22 °C) and subsequent incubation in A23187 ionophore solution (1–20 µM in 2 mM GlyGly pH 9) for 80 min at RT. (B) Percentage of germinated *S. americanus* spores observed following pretreatment with 2 mM GlyGly pH 7, with or without 50 nM of $CaCl_2$, for 30 min at RT, and subsequent treatment with mucin solution (0.2% or 0.5%, w/v, in 2 mM GlyGly pH 9). No-transfer controls (NTC) remained in GlyGly pH 7 for the duration of the experiment, while transfer controls (TC) were transferred to GlyGly pH 9. (C-D) Percentage of germinated *S. americanus* spores observed following exposure to dilute solutions of dermal mucus (0%, 2%, 10% and 20%) or serum (0%, 1%, 10%, 50%) from *Oncorhynchus mykiss*. Values are shown as mean ± S.D. $n = 3$ replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a one-way ANOVA (A, C, D), two-way ANOVA (B) and post-hoc Tukey's test.

3.5 Comparison of hydrogen-peroxide induced polar filament extrusion and propidium iodide exclusion assay readouts

To quantify differences in capacity of the outlined germination assay and well-established propidium iodide viability assays, the proportions of germinated/total spores in live, 50%

inactivated and 100% inactivated samples exposed to 15% peroxide were compared to the proportions of PI^+/total spores from the same samples following PI staining. After staining, live spore samples exhibited no fluorescence when excited by 561-594 nm laser, while approximately half of the spores in 50% inactivated samples stained red (Fig. 7A,B). Conversely, almost all spores in fully inactivated samples fluoresced red when excited under this wavelength (Fig. 7C). Within treatments, both assays detected significant reductions in viability between the live and 50% inactivated, live and 100% inactivated, and 50% and 100% inactivated samples respectively (Fig. 7D). However, the proportion of PI^+ spores was significantly greater than the fraction of germinated spores in live spore samples (Fig. 7D). No significant differences were observed between PI exclusion and germination assay readouts in 100% inactivated or 50% inactivated samples (Fig. 7D).

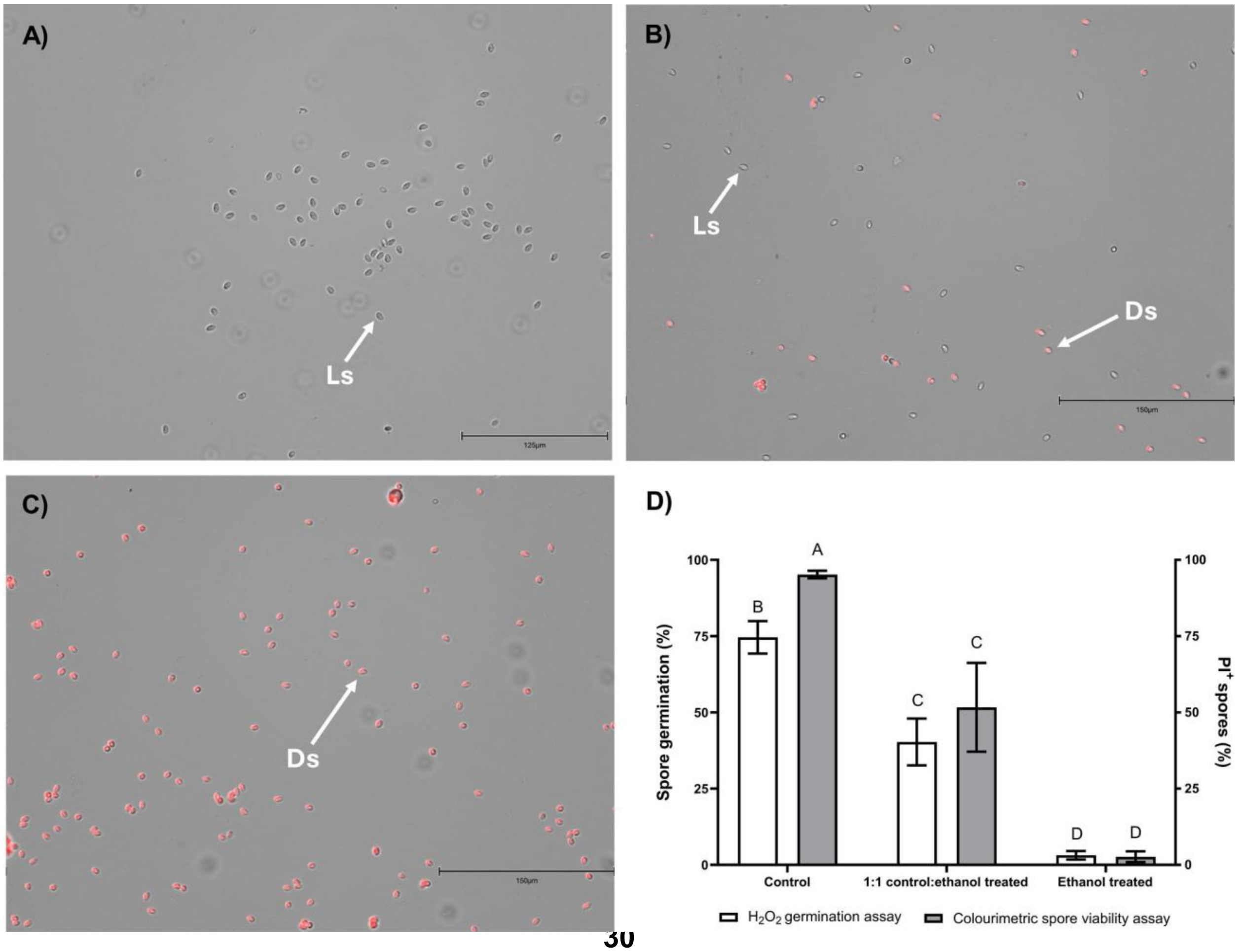


Fig. 7. Comparison of propidium iodide (PI) exclusion and polar filament extrusion in 15% hydrogen peroxide as measurements for viability and infectivity in *Spraguea americanus*. Representative images of live (A), 50% inactivated (B) and 100% inactivated (C) *S. americanus* spores stained with PI at 400x magnification following ethanol inactivation treatments. Final images were produced by merging images taken on differential interference contrast (DIC) and Texas Red channels of an EVOS M5000 Imaging System (Ls, live spore; Ds, dead spore). (D) Mean percentage of germinated and PI + *S. americanus* spores in live, 50% inactivated and 100% inactivated samples following exposure to 15% hydrogen peroxide, or staining with PI, respectively. Values are shown as mean ± S.D. *n* = 3 replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment in germination experiments, and 3-4 fields of view per sample per treatment in colorimetric assay experiments (both at 400X magnification). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a two-way ANOVA and post-hoc Tukey's test.

3.6. Inactivation of *S. americanus* germination responses by temperature, ethanol, microwaving, UV light, desiccation and age.

To examine the effectiveness of different physical treatments in inactivating the germination responses of *S. americanus*, spores were exposed to a variety of harmful chemical and physical stimuli and subsequently stimulated with 15% hydrogen peroxide. With respect to heating, spores incubated at 37°C, 60°C and 100°C for 1 h exhibited significantly less germination than control spores held at room temperature (22°C) (Fig. 8A). Furthermore, while germination was significantly reduced in 60°C and 100°C treatment groups compared with the 37°C treatment group, no differences were observed among spores held at 60°C or 100°C (Fig. 8A). Similarly, spores frozen at -20°C and -80°C for 24 h demonstrated considerable reductions in germination compared with spores at 4°C (Fig. 8B). However, no significant differences were observed between spores frozen at -20°C and -80°C (Fig. 8B). Upon restimulation with hydrogen peroxide, spores exposed to microwaves for 5, 15 or 30 s displayed significantly less polar filament extrusion compared with control spores (Fig. 8C). Nevertheless, spores microwaved for 5 s were only moderately inactivated in comparison to spores microwaved for 15 or 30 s which exhibited significantly greater inactivation (Fig. 8C). Following exposure to 10% ethanol for 15 min, *S.*

americanus spores exhibited a small but statistically significant reduction in germination (Fig. 8D). Conversely, concentrations equal to or exceeding 30% produced considerable inactivation with respect to the control, 1% and 10% treatments (Fig. 8D). However, germination among spores treated with 30%, 50%, 70% and 100% ethanol treatments were comparable. Moreover, no statistically significant differences were observed between control and 1%, and 1% and 10% ethanol treatments (Fig. 8D).

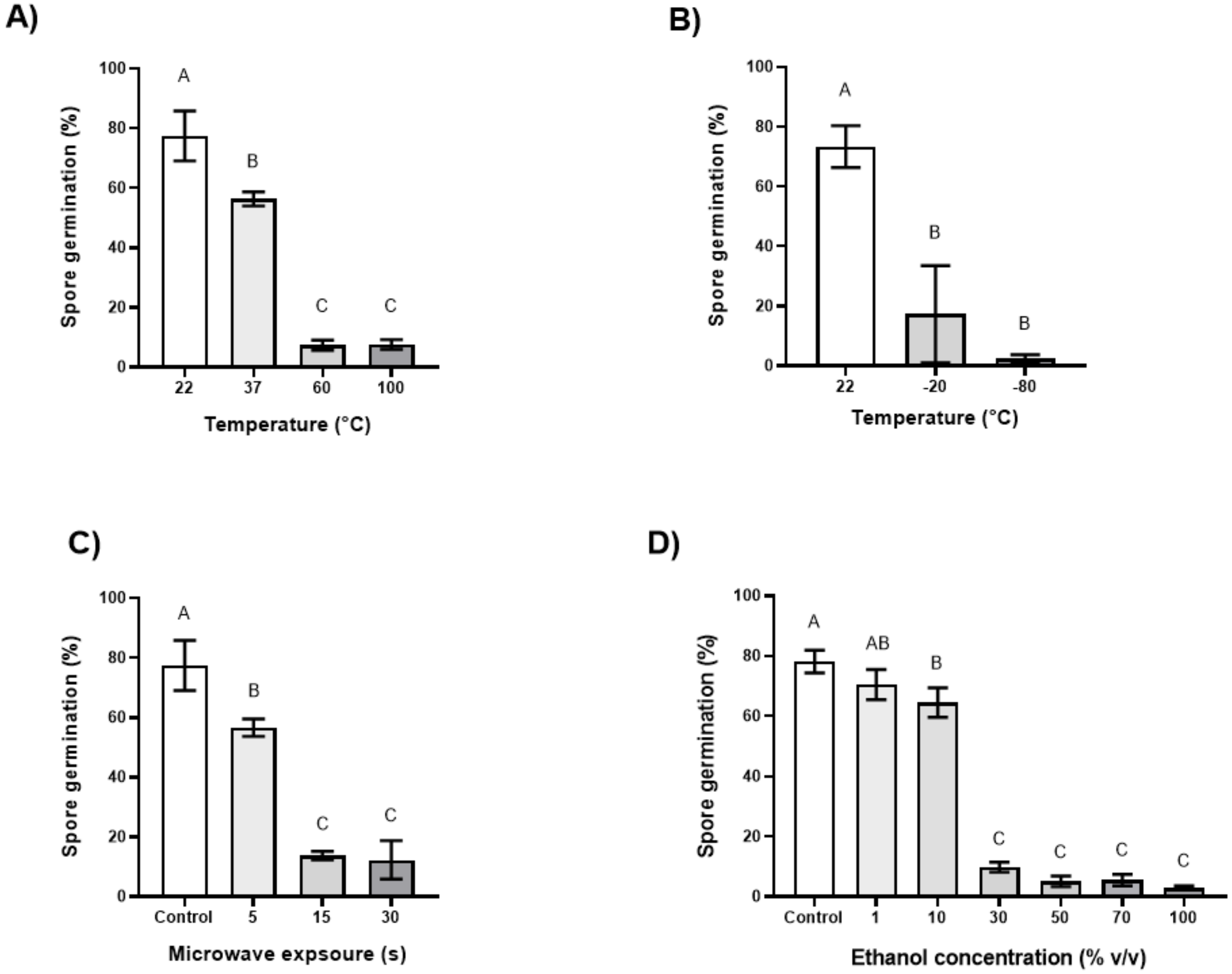


Fig. 8. Inactivation of *Spraguea americanus* germination responses by temperature, ethanol and microwaving. (A) Percentage of germinated *S. americanus* spores after incubation at 22 °C, 37 °C, 60 °C or 100 °C for 1 h and subsequent exposure to 15% hydrogen peroxide. (B) Percentage of germinated *S. americanus* spores observed following incubation at 4 °C, −20 °C or −80 °C for 24 h and subsequent exposure to 15% hydrogen peroxide. (C) Percentage of germinated *S. americanus* spores observed following 0, 5, 15 or 30 s of microwaving (850 W) and subsequent exposure to 15% hydrogen peroxide. (D) Percentage of germinated *S. americanus* spores after exposure to ethanol (0–100% for 15 min at room temperature (RT: 22 °C) and subsequent treatment with 15% hydrogen peroxide. Values are shown as mean ± S.D. *n* = 3 replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a one-way ANOVA and post-hoc Tukey's test.

Germination of *S. americanus* spores exposed to UV light for 30 or 60 min was significantly reduced compared to untreated spores or spores exposed to light for 10 min upon restimulation (Fig. 9A). However, inactivation was considerably more prominent in the 60-min treatment group, which demonstrated significantly less germination than the 30-min treatment group (Fig. 9A). No significant differences were observed between control spores and spores treated with UV light for 10 min (Fig. 9A). Moreover, no significant differences were observed between 3, 5, 10, 15 and 30-min treatments (Fig. 9B). With respect to age, fresh and 5-month old spores exposed to 15% peroxide demonstrated no differences in germination, while both considerably exceeded the germination of 10-month-old spores (Fig. 9B). To this end, germination of 10-month old spores was comparable to ungerminated controls of all three ages (Fig. 9B). While desiccation of *S. americanus* spores for 1 min was insufficient to reduce germination below control levels, desiccation for 3 min or more resulted in significant inactivation compared to control and 1-minute treatments (Fig. 9C).

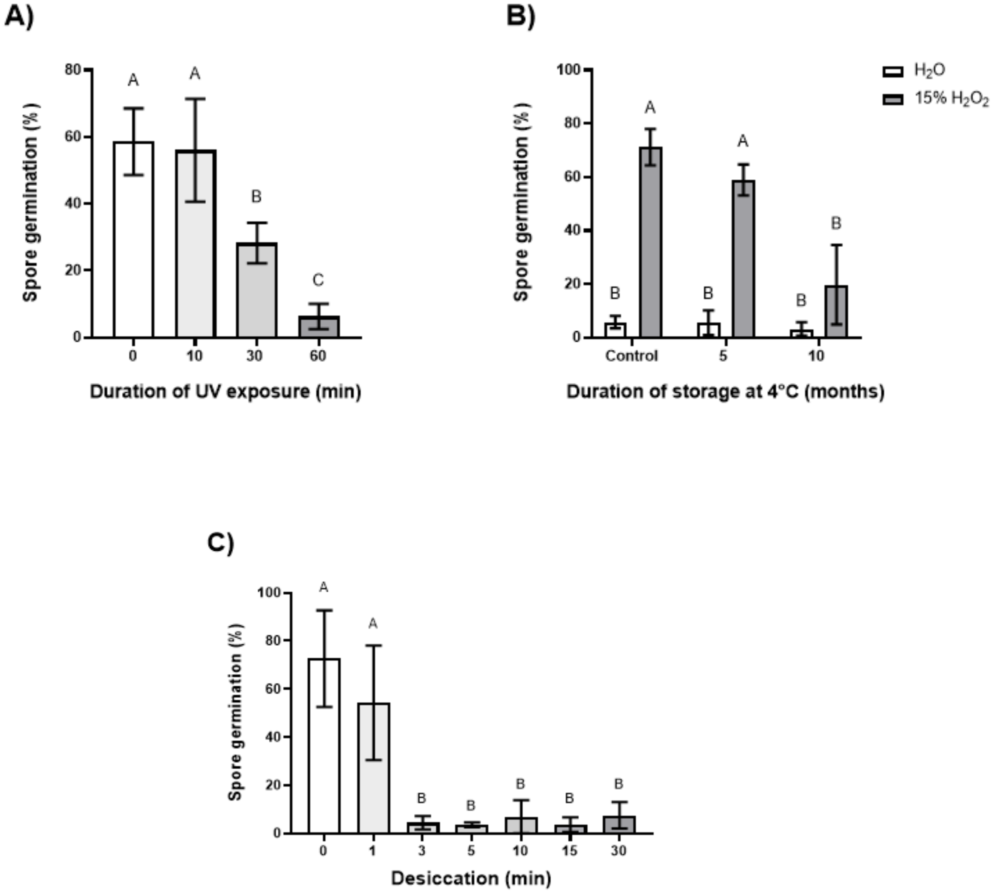


Fig. 9. Inactivation of *S. americanus* germination responses by UV light, aging and desiccation. (A) Percentage of germinated *S. americanus* spores observed after exposure to UV light (~125 µW/cm^2) for 0, 10, 30 or 60 min and subsequent treatment with 15% hydrogen peroxide. (B) Percentage of germinated *S. americanus* spores observed after 0, 5 or 10 months of storage in PURGE solution at 4°C and subsequent stimulation with MilliQ water or 15% hydrogen peroxide. (C) Percentage of germinated *S. americanus* spores observed after 0-30 min of desiccation in air and subsequent stimulation 15% hydrogen peroxide. Values are shown as mean ± S.D. $n = 3$ replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three independent fields of view per sample per treatment at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a one-way ANOVA and post-hoc Tukey's test.

Discussion

4.1 Spore ultrastructural elements identify isolate as *S. americanus*

Due to high similarity between the ssrRNA (small subunit ribosomal RNA) sequences of *Spraguea spp*. (the only published gene for many members of this genus), traditional molecular techniques were insufficient to speciate the spore isolates used in this study (Appendix A: Fig. A1). Consequently, spore isolates were identified as *S. americanus* using ultrastructural features. When xenomas from infected fish were examined by transmission electron microscopy, all spores imaged possessed surface ornamentation, 6–9 polar filament coils, and a single nucleus (Appendix A: Fig. A2) (Freeman et al., 2004). Since these traits are specific to *S. americanus*, and the only species of *Lophius* native to the region sampled is *L. americanus*, spores were concluded to be *S. americanus* (Fariña et al., 2008; Freeman et al., 2004). Additionally, preliminary genomic sequencing data identified the spores and their host fish as *S. americanus* and *L. americanus*, respectively (data not shown).

4.2 Spores of *S. americanus* are highly responsive to hydrogen peroxide and display a bimodal dose response

Despite previously having never been investigated as a germination stimulus in *Spraguea spp.*, hydrogen peroxide treatment induced the highest levels of germination of any stimulus examined in this study. This data suggests that *S. americanus* may be capable of germinating in response to the environment of the phagosome, which often contains an abundance of reactive oxygen species (Kotsias et al., 2013). Indeed, species of *Encephalitozoon* have shown the capacity to germinate in response to phagocytosis (Cox et al., 1979; Franzen, 2005). With that said, the specific mechanisms underlying the bimodal dose response of *S. americanus* observed in this study remain a mystery. It is possible that an enzymatic process may be involved, such that there is an ideal ratio of substrate which can be accommodated. Indeed, it has been postulated that single enzyme cascades responsible for signal transduction in eukaryotic cells are capable of bimodal dose responses (Parundekar et al., 2019). However, whether this phenomenon is implicated in the *in vivo* pathogenesis of *S. americanus* remains to be seen.

When exposed to hydrogen peroxide at decreasing temperatures, *S. americanus* spores exhibited no significant changes in germination. This contrasts with observations in many other species of microsporidians such as *Trachipleistophora sp.* and *Parathelohania iranica*, which exhibit dramatic reductions in *in vitro* germination as temperatures decrease from 25 °C to 4 °C, (Imura et al., 2023; Omrani et al., 2023). However, since lophiids can be found in waters ranging from 0-24 °C, it is possible that *S. americanus* may possess unique adaptations to preserve germination machinery over a broader range of temperatures (Campbell et al., 2013; Siemann et al., 2018). Similarly, germination of *S. americanus* in response to hydrogen peroxide did not exhibit a duration-dependent effect. This differs from *Glugea fumaniferae*, a closely related species, which exhibits maximal responses at 40 min post exposure to a germination stimulus (Ishihara, 1967). Therefore, it is possible that *S. americanus* may simply respond to hydrogen peroxide in an “all or nothing” fashion which is not universal to all germination mechanisms. To this end, *S. americanus* spores appear to demonstrate a time-dependent response to pressure, but an instantaneous response to hydrogen peroxide (Appendix C: Video C1 and C2, respectively). Although this evidence may point to the existence of multiple mechanisms for germination, more comprehensive surveys of the germination conditions of various species are necessary to evaluate these hypotheses.

4.3 Manual pressure is sufficient to induce germination in *S. americanus*

Among one of the first germination conditions examined in microsporidians was the application of mechanical pressure. For example, in the early-to-mid 1900s, several publications document the use of coverslips to apply pressure to spores of different species on microscope slides in order to induce germination, including *Nosema helminthorum*, *Thelohania californica* and *Thelohania magna* (Dissanaike, 1955; Kudo, 1920, 1918; Kudo and Daniels, 1963). Therefore, it is surprising that few scientists have followed up on these observations in the following decades, especially considering that slides and coverslips are routinely used to assess responses of microsporidians to other germination stimuli. Although placement of a coverslip alone was not sufficient to increase germination of *S. americanus* above control levels in this study, some germination in response to a coverslip alone was observed. This indicates that current germination studies in microsporidians may be subject to a slight bias as a result of auto-germination in response to the small amounts of pressure exerted by coverslips. Upon applying full, consistent pressure to the coverslip for 5 s, the majority of spores appeared phase dark. These observations indicate that *S. americanus* is likely capable of responding to changes in pressure. Considering that virtually nothing is known about the transmission of *Spraguea spp.*, it is possible that transmission may occur in shallow waters, and germination occurs following a rapid change in depth (Freeman et al., 2011). Indeed, *Lophius spp*. can be found from inshore areas to depths exceeding 900 m; the equivalent of approximately 90 atmospheres of pressure (Siemann et al., 2018). To this end, it has been hypothesized that monkfish may migrate to greater depths during the summer months to avoid increasing water temperatures (Siemann et al., 2018). In further support of this theory, research suggests that *Lophius* species are highly resistant to acute barotrauma (Weissman et al., 2021). However, further research is necessary to determine if pressure plays a significant role in transmission or the natural lifecycle of *S. americanus*.

4.4 Spores of *S. americanus* germinate in response to sodium carbonate-bicarbonate, but not group-I or group-II metal halides

Prior to this study, the effects of different cations and anions on the germination of *Spraguea spp.* were largely undescribed. However, differential responses of *G. fumaniferae* have been well characterized in response to different metal cations at different pH levels (Ishihara, 1967). In an attempt to emulate these experiments, the effects of sodium carbonate-bicarbonate buffer (the buffer used in the study) were first examined in order to validate that the buffer did not induce sufficient germination (Ishihara, 1967). Surprisingly, sodium carbonate-bicarbonate buffer alone was sufficient to induce germination above control levels at several pH levels, even at extremely low concentrations (Appendix B: Fig. B1). Spores germinated by the presence of bicarbonate also exhibited markedly different morphologies from those germinated using hydrogen peroxide. Most notably, bicarbonate-stimulated spores appear to have germinated less forcefully than their hydrogen peroxide-stimulated counterparts (Appendix B: Fig. B1 CD). Considering that spores of microsporidian species have been observed to “misfire” in related studies, resulting in incomplete germination, our observations may suggest differences in velocity or force with which the sporoplasm is ejected following stimulation with hydrogen peroxide and sodium bicarbonate (Sharma et al., 2024). With this in mind, 2 mM glycylglycine buffer, which was insufficient to induce germination above control levels at pH 9, was used for future experiments.

When *S. americanus* spores were exposed to different salt solutions at pH 9, none of the treatments tested were able to significantly increase the number of germinated spores above control levels. However, it is noteworthy that at concentrations of 2 mM, calcium chloride was able to induce >20% germination. These results are interesting given that a number of studies have supported the involvement of calcium in the germination of *S. lophii* (Pleshinger and Weidner, 1985). To this end, the gastric secretions of teleosts have been shown to contain concentrations of bicarbonate ranging from 40-130 mM, which functions to regulate the balance of bivalent cations such as calcium (Wilson et al., 2002). Considering that the guts of teleosts generally possess a pH of 9, which is within the optimal pH of germination of *S. lophii*, and germination of *S. americanus* was observed in response to bicarbonate or calcium at pH 9, it is possible that the initial focus of infection for *Spraguea spp.* is the gut (Pleshinger and Weidner, 1985; Wilson et al., 2002). Indeed, the high prevalence of *Spraguea* in monkfish populations has long been attributed to their cannibalistic nature, and this evidence may help to bridge the gap between these two observations (Freeman et al., 2011). However, further studies of monkfish physiology and *Spraguea* germination *in vivo* will be necessary to confirm these hypotheses.

4.5 Spores of *Spraguea americanus* fail to germinate in conditions under which *Spraguea lophii* is highly responsive (A23187 and mucin)

The germination stimuli most well studied for species of *Spraguea* are mucin and the calcium ionophore A23187. Indeed, numerous studies have used mucin (pork, fish, human) to induce germination in *S. lophii* (Pleshinger and Weidner, 1985; Weidner et al., 1995, 1984). However, while some studies include a preincubation with calcium prior to mucin exposure, others do not (Pleshinger and Weidner, 1985; Weidner et al., 1995; Weidner and Findley, 1999). Consequently, *S. americanus* spores were exposed to the polyanion mucin with and without calcium chloride pretreatment. Surprisingly, despite the overwhelming consensus in the literature that mucin was capable of inducing germination, it remained at control levels in both the presence and absence of calcium when mucin was introduced. Similarly, while the ionophore A23187 has been well established to induce germination in *Spraguea spp*., in this study no germination was observed in response to this ionophore, even 80 min post-incubation (Campbell et al., 2013; Pleshinger and Weidner, 1985). It is unclear why these well-established germination methods were ineffective in this study. It is possible that *S. americanus* may possess different germination mechanisms from that of *S. lophii* and thus is unresponsive to specific stimuli. Such divergence could reasonably occur as a consequence of co-evolution with their respective hosts, which remain highly isolated from one another geographically (Fariña et al., 2008). Regardless, replication of this experiment with different types/brands of mucin or A23187 may provide more insight into whether the observed lack of germination is of biological significance, or due to slight differences in procedure/reagents.

4.6 Spores of *S. americanus* germinate in response to mucus but not serum taken from *Oncorhynchus mykiss*; a nonhost species.

To further investigate the potential use of *S. americanus* in future *in vivo* experiments using a non-host species, spores were exposed to solutions of trout mucus and serum to determine if they were capable of inducing germination. Indeed, fish mucus exposure experiments conducted using buffers adjusted to pH 9 have shown some success in the past (Weidner and Findley, 1999). However, for this study solutions of mucus and serum were diluted in MilliQ water to best preserve their original pH levels and best represent the host environment without the addition of extra ions. Although germination was observed above control levels at some concentrations, germination levels were considerably lower than what was observed in hydrogen peroxide experiments. It is possible that 70% germination is not reflective of what occurs in the host, and that very few spores are required to germinate to propagate an infection. However, based on our observations, we believe it is unlikely that bath emersion or intravenous inoculation of *Spraguea* into rainbow trout (*Oncorhynchus mykiss*) would be sufficient to induce infection and, in light of these observations, that alternative avenues such as oral gavage should be explored for such ventures.

4.7 Hydrogen-peroxide-induced polar filament extrusion underrepresents viability in untreated spores of *S. americanus*

Although polar filament extrusion is often used to measure viability in microsporidia, it is evident that spore infectivity and viability are two different metrics (McGowan, J, 2012. Viability Assessment and Cryopreservation of the Honey Bee (*Apis mellifera*) Parasite, *Nosema ceranae*. (Masters thesis). University of Guelph, Canada; McGowan et al., 2016). While infectivity typically refers to the percentage of spores which are capable of full evagination of the sporoplasm under appropriate environmental stimulation (and thus would result in infection), viability refers to the percentage of spores which possess an intact membrane and thus, do not stain with dyes such as propidium iodide and Sytox green (McGowan, 2012; McGowan et al., 2016). To this end, the relationship between viability and infectivity was examined in *Spraguea americanus* by comparing the novel germination assay described in this study to a common fluorescence-based viability using propidium iodide. Interestingly, when 100% or 50% of a spore sample was treated with 100% ethanol, germination and fluorescence assays provide germination and viability measurements which are statistically indistinguishable. However, in samples where spores were left untreated, viability was significantly greater than infectivity. This can be attributed to the fact that not all viable spores are capable of germination, and therefore, some spores may be viable, but incapable of infection (McGowan, 2012; McGowan et al., 2016). Therefore, if fluorescence-based methods are an overestimation of viability, then germination rates should serve as an effective proxy measurement for viability, while the inverse would not be true (McGowan, 2012; McGowan et al., 2016). With this in mind, future *in vitro* and *in vivo* studies involving microsporidia should determine infectious doses on the basis of infectivity, not viability, as this will result in a more accurate representation of the number of infectious individuals in a population.

4.8 *S. americanus* spore germination is readily inactivated by numerous chemical and mechanical stimuli

Thus far, only one study has extensively characterized the effects of different chemical and mechanical treatments on the viability of *Spraguea spp.* (Leiro et al., 2012). This research, conducted using *S. lophii* spores, used germination rates as a proxy measurement for viability in order to characterize the effects of ethanol, microwaving, freezing and heating on spore viability (Leiro et al., 2012). Although viability and polar filament extrusion represent different metrics, comparisons of PI colorimetric viability assays and the polar filament extrusion assay outlined above have indicated germination can provide a reasonable estimate for viability under inactivation conditions (McGowan, 2012; McGowan et al., 2016). Therefore, to examine the relative resistance of *S. americanus* and *S. lophii* to such conditions, the polar filament extrusion assay described in this study was used to assess the responses of *S. americanus* to the same stimuli. Interestingly, both species exhibit similar response to ethanol, reaching nearly 0% viability after 15 min of treatment with concentrations equal to or exceeding 30% (Leiro et al., 2012). However, while microwaving for 15 s decreased the viability of *S. lophii* to approximately 5%, *S. americanus* samples remain 15% viable 15 and 30 s post microwaving (Leiro et al., 2012). Similarly, freezing at −20 °C for 24 h decreased *S. lophii* viability to less than 5%, while *S. americanus* samples retained nearly 20% viability following the same treatment (Leiro et al., 2012). This data may suggest that *S. americanus* is slightly more resistant to adverse environmental conditions. However, it is possible other factors such as differences in spore age maybe be responsible for these discrepancies. Therefore, such experiments should be repeated using spores of both species under the same conditions to enable direct comparison.

Although age-dependent declines in the germination response had previously not been described in *Spraguea*, such observations have been made in a number of other fish-infecting species, including *Loma* and *Glugea* (Amigó et al., 1996; Shaw et al., 2000). To this end, the germination of *Spraguea americanus* spores remained at consistent levels 5 months post harvesting, but decreases considerably after 10 months of storage at 4 °C. These results are puzzling considering that *Loma salmonae* spores have been shown to exhibit 0% germination (3% peroxide exposure) after 100 days of storage under similar conditions (Shaw et al., 2000). Despite, different concentrations of peroxide being used in these experiments, *Loma* germination decreased from 55 to 0 % in 100 days, while *S. americanus* decreased from 80% to 20% 304 days (Shaw et al., 2000). Although not directly comparable, it is apparent that *S. americanus* is more resistant to age-dependent decreases in function of germination machinery. Conversely, spores *Glugea stephanie*, a more closely related species, exhibit a germination half-life of approximately 17 months, indicating a greater resistance to the effects of aging (Amigó et al., 1996). Regardless of these observations, it is evident that different species are more resistant to the effects of aging, demonstrating a greater need for the characterization of mechanisms underly resistance to this phenomenon.

The responses of different microsporidia to both UV and desiccation appear to vary dramatically depending on the species. To this end, while some species, namely *Nosema algerae*, germination in response to low doses of UV radiation, three species of *Encephalitozoon* have been shown to inactivate following exposure to similar wavelengths (Marshall et al., 2003; Miao et al., 2024). Consequently, in this study, *S. americanus* spores were exposed to low doses of UV radiation for varying durations and then exposed to 15% peroxide to examine differences in germination responses. Considering that germination decreased significantly following 30 min of UV exposure, it is likely that *Spraguea* species do not germinate in response to UV light but are instead inactivated. However, even *Nosema algerae* spores are inactivated by UV exposure at nonoptimal doses, indicating a need for further research into the UV-dose response relationships of *Spraguea americanus* to confirm these findings (Miao et al., 2024). Similarly, while some species of *Nosema* are highly resistant to desiccation and may even germinate upon rehydration, *Spraguea americanus* appears to be highly susceptible to desiccation (Fenoy et al., 2009; Han et al., 2020). Indeed, while *Nosema ceranea* may remain viable 12-months post desiccation, *S. americanus* spores are inactivated by as little as 3 min of drying in air (Fenoy et al., 2009). This may be reflective of the different life histories of these genera, since *Spraguea* infect deep-sea lophiids, while *Nosema* primarily infect terrestrial insects (Freeman et al., 2004; Solter et al., 2012). However, whether high sensitivity to desiccation is a common trait among fish-infecting microsporidia remains to be seen.

Conclusions

As the first comprehensive characterization of germination responses in *S. americanus*, this work characterizes a crucial aspect of the pathogenesis of a neglected pathogen affecting the American anglerfish: a highly prized finfish stock facing serious decline due to overfishing (Charbonneau et al., 2020). In establishing considerable evidence to support an ingestion-driven path of transmission for *S. americanus*, this work refutes many of the common hypotheses associated with the transmission of *Spraguea spp*. and provides new insight into the relationships between this pathogen and its host in the absence of effective *in vivo* or *in vitro* models (Freeman et al., 2011). For example, the responses of *S. americanus* to pressure and hydrogen peroxide presented in this study suggest novel routes of transmission by way of fish depth migration or phagocytosis − two phenomena not yet examined in this genus. Such observations can be used to exploit the germination pathways of these species to facilitating *in vitro* infections in nonhost species; work which would facilitate research countless new avenues of research in the field of microsporidia infection biology. Furthermore, results of this study indicate that *S. americanus* and *S. lophii* respond to different stimuli, with the latter displaying a preference for mucin which the former lacks. Ultimately, understanding the mechanisms and conditions underlying the germination of microsporidians will support the development of novel treatments or methods to block germination in species of economic relevance or environmental concern, thus improving fish health and protecting finfish stocks from the detrimental effects of microsporidians.

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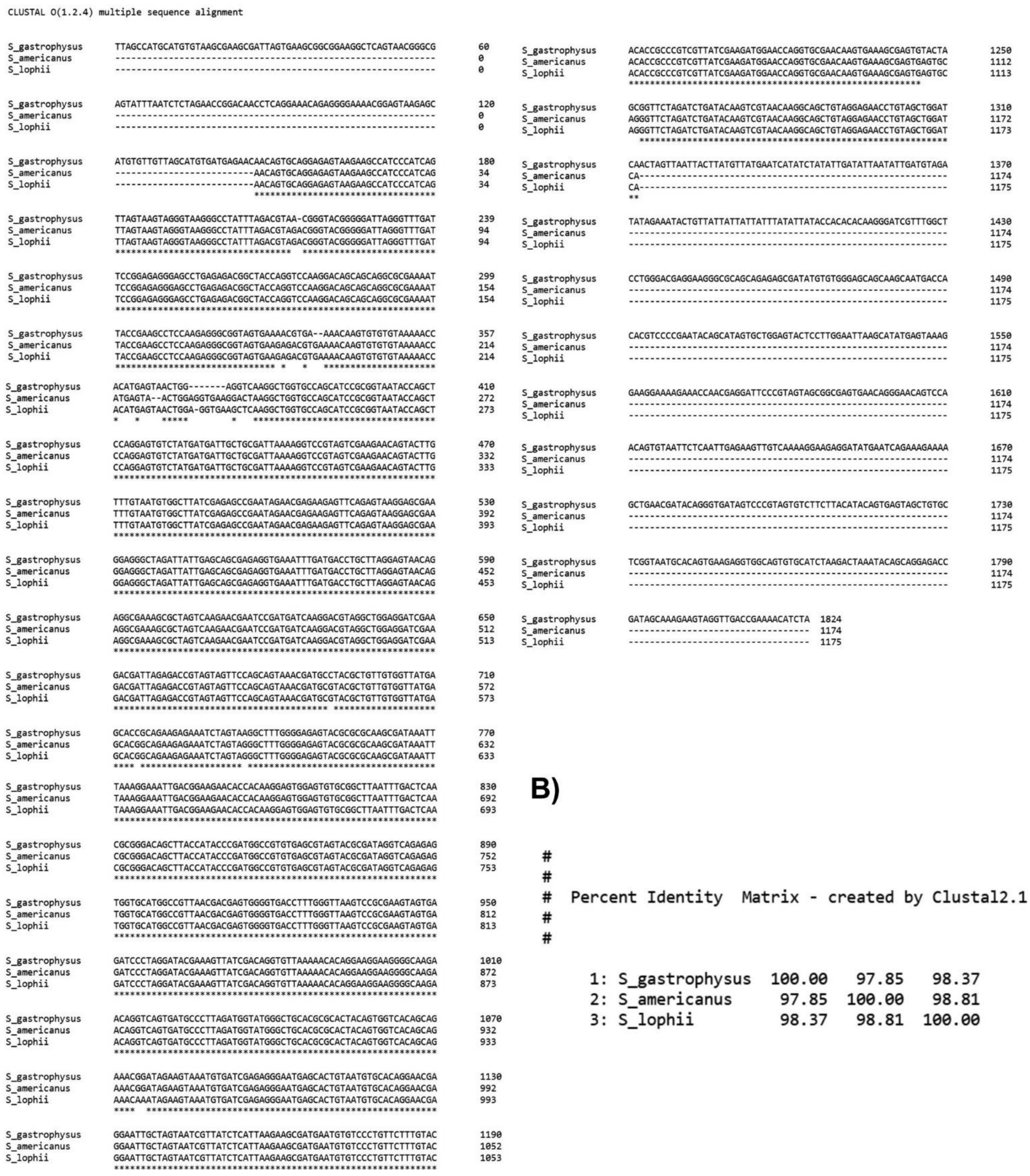
Appendices

Appendix A: Genetic and morphological data used in the speciation of spore isolates

A)



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CLUSTAL O(1.2.4) multiple sequence alignment


S_gastrophysus      TTAGCCATGCATGTGTAAGCGAAGCGATTAGTGAAGCGGCGGAAGGCTCAGTAACGGGCG      60
S_americanus        ------------------------------------------------------------      0
S_lophii            ------------------------------------------------------------      0


S_gastrophysus      AGTATTTAATCTCTAGAACCGGACAACCTCAGGAAACAGAGGGGAAAACGGAGTAAGAGC      120
S_americanus        ------------------------------------------------------------      0
S_lophii            ------------------------------------------------------------      0


S_gastrophysus      ATGTGTTGTTAGCATGTGATGAGAACAACAGTGCAGGAGAGTAAGAAGCCATCCCATCAG      180
S_americanus        --------------------------AACAGTGCAGGAGAGTAAGAAGCCATCCCATCAG      34
S_lophii            --------------------------AACAGTGCAGGAGAGTAAGAAGCCATCCCATCAG      34
                                              **********************************

S_gastrophysus      TTAGTAAGTAGGGTAAGGGCCTATTTAGACGTAA-CGGGTACGGGGGATTAGGGTTTGAT      239
S_americanus        TTAGTAAGTAGGGTAAGGGCCTATTTAGACGTAGACGGGTACGGGGGATTAGGGTTTGAT      94
S_lophii            TTAGTAAGTAGGGTAAGGGCCTATTTAGACGTAGACGGGTACGGGGGATTAGGGTTTGAT      94
                    *********************************  *************************

S_gastrophysus      TCCGGAGAGGGAGCCTGAGAGACGGCTACCAGGTCCAAGGACAGCAGCAGGCGCGAAAAT      299
S_americanus        TCCGGAGAGGGAGCCTGAGAGACGGCTACCAGGTCCAAGGACAGCAGCAGGCGCGAAAAT      154
S_lophii            TCCGGAGAGGGAGCCTGAGAGACGGCTACCAGGTCCAAGGACAGCAGCAGGCGCGAAAAT      154
                    ************************************************************

S_gastrophysus      TACCGAAGCCTCCAAGAGGGCGGTAGTGAAAACGTGA--AAACAAGTGTGTGTAAAAACC      357
S_americanus        TACCGAAGCCTCCAAGAGGGCGGTAGTGAAGAGACGTGAAAACAAGTGTGTGTAAAAACC      214
S_lophii            TACCGAAGCCTCCAAGAGGGCGGTAGTGAAGAGACGTGAAAACAAGTGTGTGTAAAAACC      214
                    ****************************** *   *   *********************

S_gastrophysus      ACATGAGTAACTGG-------AGGTCAAGGCTGGTGCCAGCATCCGCGGTAATACCAGCT      410
S_americanus        ATGAGTA--ACTGGAGGTGAAGGACTAAGGCTGGTGCCAGCATCCGCGGTAATACCAGCT      272
S_lophii            ACATGAGTAACTGGA-GGTGAAGCTCAAGGCTGGTGCCAGCATCCGCGGTAATACCAGCT      273
                    *   *    *****        *   **********************************

S_gastrophysus      CCAGGAGTGTCTATGATGATTGCTGCGATTAAAAGGTCCGTAGTCGAAGAACAGTACTTG      470
S_americanus        CCAGGAGTGTCTATGATGATTGCTGCGATTAAAAGGTCCGTAGTCGAAGAACAGTACTTG      332
S_lophii            CCAGGAGTGTCTATGATGATTGCTGCGATTAAAAGGTCCGTAGTCGAAGAACAGTACTTG      333
                    ************************************************************

S_gastrophysus      TTTGTAATGTGGCTTATCGAGAGCCGAATAGAACGAGAAGAGTTCAGAGTAAGGAGCGAA      530
S_americanus        TTTGTAATGTGGCTTATCGAGAGCCGAATAGAACGAGAAGAGTTCAGAGTAAGGAGCGAA      392
S_lophii            TTTGTAATGTGGCTTATCGAGAGCCGAATAGAACGAGAAGAGTTCAGAGTAAGGAGCGAA      393
                    ************************************************************

S_gastrophysus      GGAGGGCTAGATTATTGAGCAGCGAGAGGTGAAATTTGATGACCTGCTTAGGAGTAACAG      590
S_americanus        GGAGGGCTAGATTATTGAGCAGCGAGAGGTGAAATTTGATGACCTGCTTAGGAGTAACAG      452
S_lophii            GGAGGGCTAGATTATTGAGCAGCGAGAGGTGAAATTTGATGACCTGCTTAGGAGTAACAG      453
                    ************************************************************

S_gastrophysus      AGGCGAAAGCGCTAGTCAAGAACGAATCCGATGATCAAGGACGTAGGCTGGAGGATCGAA      650
S_americanus        AGGCGAAAGCGCTAGTCAAGAACGAATCCGATGATCAAGGACGTAGGCTGGAGGATCGAA      512
S_lophii            AGGCGAAAGCGCTAGTCAAGAACGAATCCGATGATCAAGGACGTAGGCTGGAGGATCGAA      513
                    ************************************************************

S_gastrophysus      GACGATTAGAGACCGTAGTAGTTCCAGCAGTAAACGATGCCTACGCTGTTGTGGTTATGA      710
S_americanus        GACGATTAGAGACCGTAGTAGTTCCAGCAGTAAACGATGCGTACGCTGTTGTGGTTATGA      572
S_lophii            GACGATTAGAGACCGTAGTAGTTCCAGCAGTAAACGATGCGTACGCTGTTGTGGTTATGA      573
                    **************************************** *******************

S_gastrophysus      GCACCGCAGAAGAGAAATCTAGTAAGGCTTTGGGGAGAGTACGCGCGCAAGCGATAAATT      770
S_americanus        GCACGGCAGAAGAGAAATCTAGTAGGGCTTTGGGGAGAGTACGCGCGCAAGCGATAAATT      632
S_lophii            GCACGGCAGAAGAGAAATCTAGTAGGGCTTTGGGGAGAGTACGCGCGCAAGCGATAAATT      633
                    **** ******************* ***********************************

S_gastrophysus      TAAAGGAAATTGACGGAAGAACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAA      830
S_americanus        TAAAGGAAATTGACGGAAGAACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAA      692
S_lophii            TAAAGGAAATTGACGGAAGAACACCACAAGGAGTGGAGTGTGCGGCTTAATTTGACTCAA      693
                    ************************************************************

S_gastrophysus      CGCGGGACAGCTTACCATACCCGATGGCCGTGTGAGCGTAGTACGCGATAGGTCAGAGAG      890
S_americanus        CGCGGGACAGCTTACCATACCCGATGGCCGTGTGAGCGTAGTACGCGATAGGTCAGAGAG      752
S_lophii            CGCGGGACAGCTTACCATACCCGATGGCCGTGTGAGCGTAGTACGCGATAGGTCAGAGAG      753
                    ************************************************************

S_gastrophysus      TGGTGCATGGCCGTTAACGACGAGTGGGGTGACCTTTGGGTTAAGTCCGCGAAGTAGTGA      950
S_americanus        TGGTGCATGGCCGTTAACGACGAGTGGGGTGACCTTTGGGTTAAGTCCGCGAAGTAGTGA      812
S_lophii            TGGTGCATGGCCGTTAACGACGAGTGGGGTGACCTTTGGGTTAAGTCCGCGAAGTAGTGA      813
                    ************************************************************

S_gastrophysus      GATCCCTAGGATACGAAAGTTATCGACAGGTGTTAAAAACACAGGAAGGAAGGGGCAAGA      1010
S_americanus        GATCCCTAGGATACGAAAGTTATCGACAGGTGTTAAAAACACAGGAAGGAAGGGGCAAGA      872
S_lophii            GATCCCTAGGATACGAAAGTTATCGACAGGTGTTAAAAACACAGGAAGGAAGGGGCAAGA      873
                    ************************************************************

S_gastrophysus      ACAGGTCAGTGATGCCCTTAGATGGTATGGGCTGCACGCGCACTACAGTGGTCACAGCAG      1070
S_americanus        ACAGGTCAGTGATGCCCTTAGATGGTATGGGCTGCACGCGCACTACAGTGGTCACAGCAG      932
S_lophii            ACAGGTCAGTGATGCCCTTAGATGGTATGGGCTGCACGCGCACTACAGTGGTCACAGCAG      933
                    ************************************************************

S_gastrophysus      AAACGGATAGAAGTAAATGTGATCGAGAGGGAATGAGCACTGTAATGTGCACAGGAACGA      1130
S_americanus        AAACGGATAGAAGTAAATGTGATCGAGAGGGAATGAGCACTGTAATGTGCACAGGAACGA      992
S_lophii            AAACAAATAGAAGTAAATGTGATCGAGAGGGAATGAGCACTGTAATGTGCACAGGAACGA      993
                    ****  ******************************************************

S_gastrophysus      GGAATTGCTAGTAATCGTTATCTCATTAAGAAGCGATGAATGTGTCCCTGTTCTTTGTAC      1190
S_americanus        GGAATTGCTAGTAATCGTTATCTCATTAAGAAGCGATGAATGTGTCCCTGTTCTTTGTAC      1052
S_lophii            GGAATTGCTAGTAATCGTTATCTCATTAAGAAGCGATGAATGTGTCCCTGTTCTTTGTAC      1053
                    ************************************************************

S_gastrophysus      ACACCGCCCGTCGTTATCGAAGATGGAACCAGGTGCGAACAAGTGAAAGCGAGTGTACTA      1250
S_americanus        ACACCGCCCGTCGTTATCGAAGATGGAACCAGGTGCGAACAAGTGAAAGCGAGTGAGTGC      1112
S_lophii            ACACCGCCCGTCGTTATCGAAGATGGAACCAGGTGCGAACAAGTGAAAGCGAGTGAGTGC      1113
                    *******************************************************

S_gastrophysus      GCGGTTCTAGATCTGATACAAGTCGTAACAAGGCAGCTGTAGGAGAACCTGTAGCTGGAT      1310
S_americanus        AGGGTTCTAGATCTGATACAAGTCGTAACAAGGCAGCTGTAGGAGAACCTGTAGCTGGAT      1172
S_lophii            AGGGTTCTAGATCTGATACAAGTCGTAACAAGGCAGCTGTAGGAGAACCTGTAGCTGGAT      1173
                      **********************************************************

S_gastrophysus      CAACTAGTTAATTACTTATGTTATGAATCATATCTATATTGATATTAATATTGATGTAGA      1370
S_americanus        CA----------------------------------------------------------      1174
S_lophii            CA----------------------------------------------------------      1175
                    **

S_gastrophysus      TATAGAAATACTGTTATTATTATTATTTATATTATACCACACACAAGGGATCGTTTGGCT      1430
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      CCTGGGACGAGGAAGGGCGCAGCAGAGAGCGATATGTGTGGGAGCAGCAAGCAATGACCA      1490
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      CACGTCCCCGAATACAGCATAGTGCTGGAGTACTCCTTGGAATTAAGCATATGAGTAAAG      1550
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      GAAGGAAAAGAAACCAACGAGGATTCCCGTAGTAGCGGCGAGTGAACAGGGAACAGTCCA      1610
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      ACAGTGTAATTCTCAATTGAGAAGTTGTCAAAAGGAAGAGGATATGAATCAGAAAGAAAA      1670
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      GCTGAACGATACAGGGTGATAGTCCCGTAGTGTCTTCTTACATACAGTGAGTAGCTGTGC      1730
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      TCGGTAATGCACAGTGAAGAGGTGGCAGTGTGCATCTAAGACTAAATACAGCAGGAGACC      1790
S_americanus        ------------------------------------------------------------      1174
S_lophii            ------------------------------------------------------------      1175


S_gastrophysus      GATAGCAAAGAAGTAGGTTGACCGAAAACATCTA 1824
S_americanus        ---------------------------------- 1174
S_lophii            ---------------------------------- 1175
```

B)

```
#
#
#  Percent Identity  Matrix - created by Clustal2.1
#
#

     1: S_gastrophysus  100.00   97.85   98.37
     2: S_americanus     97.85  100.00   98.81
     3: S_lophii         98.37   98.81  100.00
```

Fig. A1. Alignment and pairwise identity matrix of published ssrRNA sequences from *Spraguea americanus*, *Spraguea lophii* and *Spraguea gastrophysus*. (A) Alignments and (B) identity matrices were preformed/calculated using Clustal Omega (Madeira et al., 2024). Dashes represent sequence gaps, while conserved residues are highlighted with an asterisk. GenBank accession numbers: AF056014.1, AF056013.1, GQ868443, respectively.

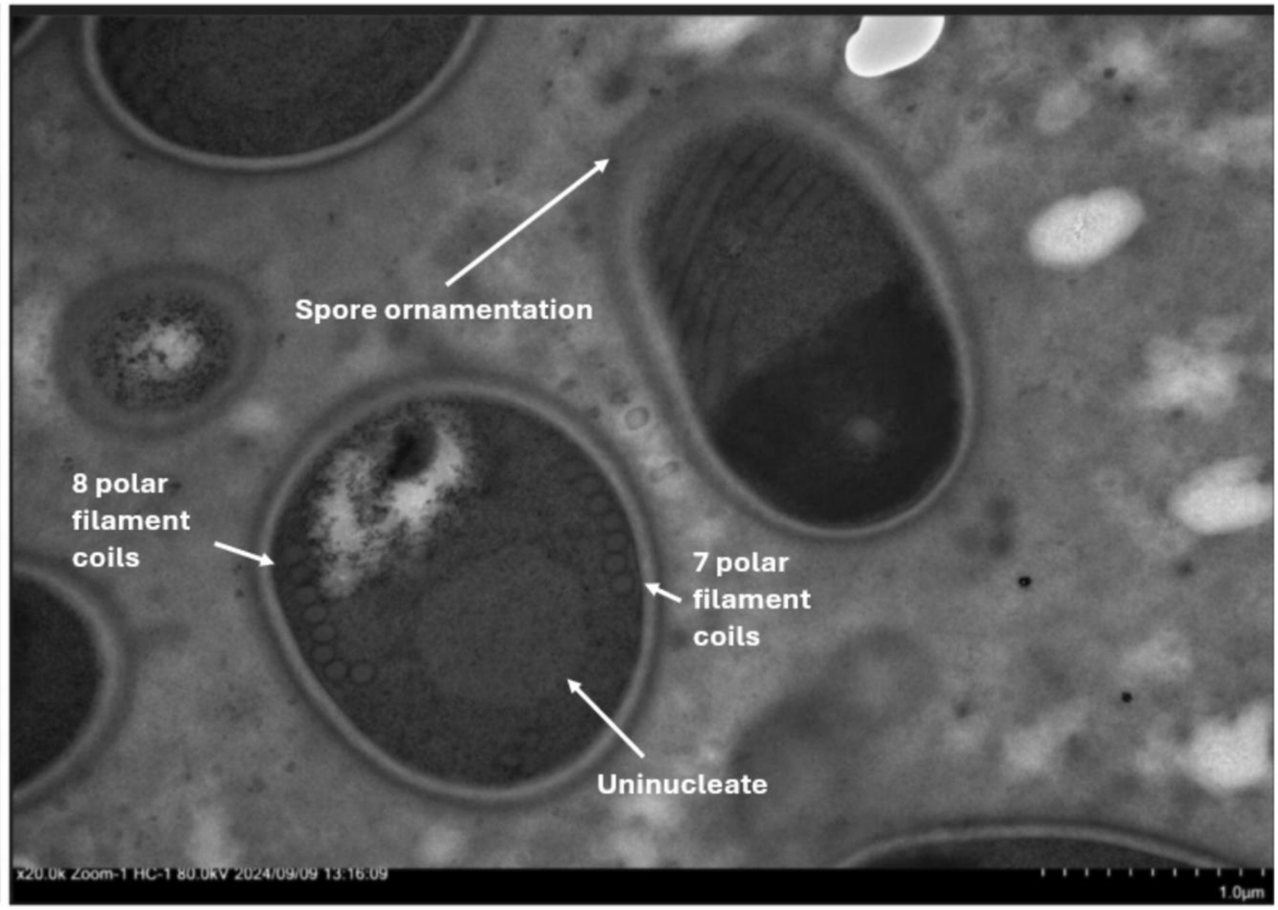

Fig. A2. Transmission electron micrograph of *Spraguea americanus* spores. A representative transmission micrograph of xenomas used to isolate spores for this study. Ultrastructural characteristics specific to *S. americanus* are indicated.

Appendix B: Germination of *S. americanus* spores following exposure to sodium carbonate-bicarbonate and glycylglycine buffers

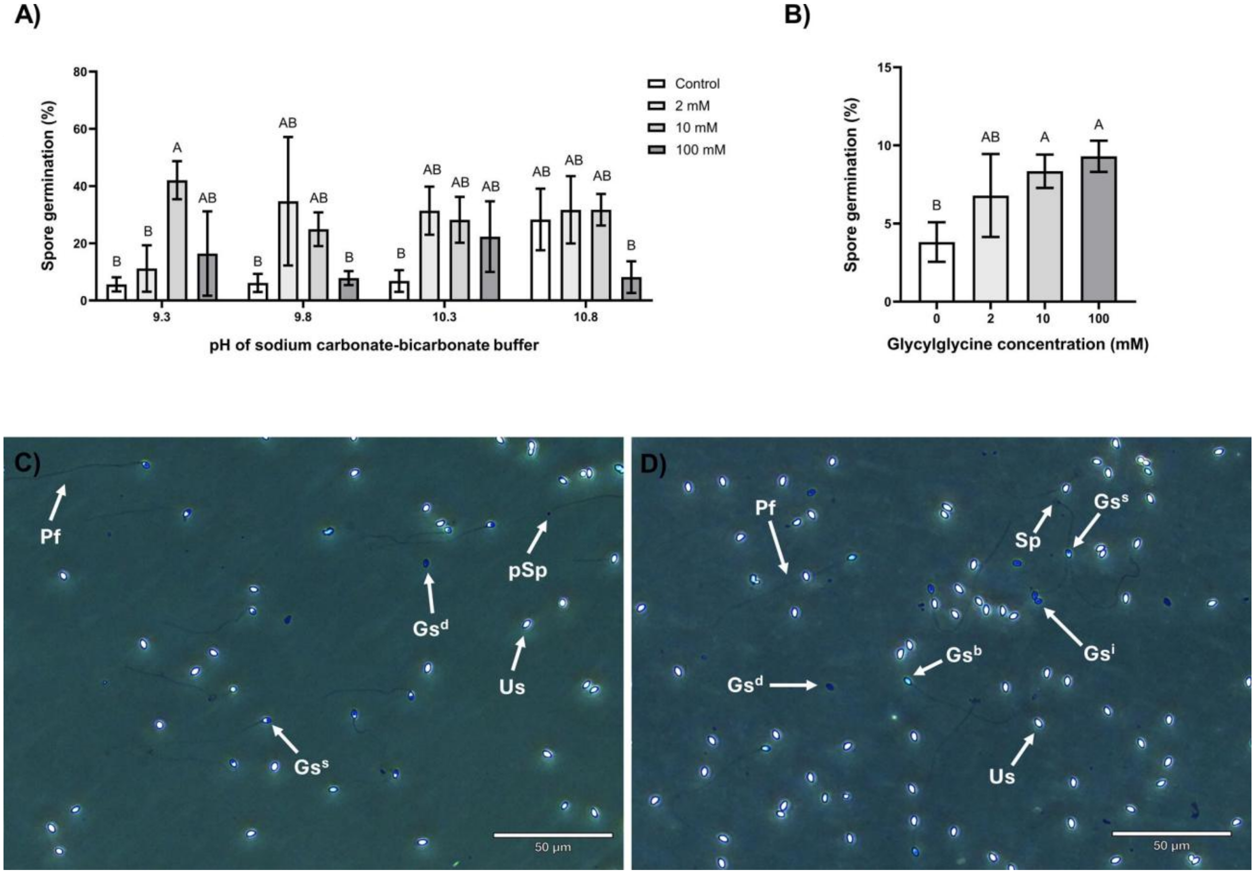

Fig. B1. Germination of *Spraguea americanus* spores in response to sodium carbonate-bicarbonate and glycylglycine buffers. (A) Percentage of germinated *S. americanus* spores observed following exposure to 2 mM, 10 mM or 100 mM sodium carbonate-bicarbonate buffer at pH 9.3, 9.8, 10.3 or 10.8 for 80 min at room temperature (RT: 22 °C). (B) Percentage of germinated *S. americanus* spores following exposure to 2 mM, 10 mM or 100 mM glycylglycine at pH 9 for 80 min at RT. (C-D) Representative images of *S. americanus* spores following (C) exposure to 15% hydrogen peroxide or (D) 10 mM sodium carbonate-bicarbonate buffer at pH 9.3 for 80 min at RT. Images taken at 200X magnification (representative images shown; Pf, polar filament; Gs^{s}, germinated spore, semi-phase dark; Gs^{d}, germinated spore, phase dark; Us, ungerminated spore; pSp, partial sporoplasm; Gs^{i}, germinated spore, phase-intermediate; Gs^{b}, germinated spore, phase-bright; Sp: sporoplasm). Values are shown as mean ± S.D. $n = 3$ replicates per experiment using spores isolated from separate anglerfish. Spore counts were averaged across three representative fields of view per treatment per sample at 400X magnification (phase contrast microscopy). Groups that do not share letters exhibit statistically significant differences ($\alpha = 0.05$) as determined by a one-way ANOVA (B), two-way ANOVA (A) and post-hoc Tukey's test.

Appendix C: video footage of *S. americanus* spore germination

This appendix is comprised of two videos illustrating *Spraguea americanus* spore germination in response to 15% hydrogen peroxide (Video C1) and mechanical pressure (Video C2). Videos can be obtained by accessing the open access publication: https://doi.org/10.1016/j.ijpara.2025.02.002 (Rogozynski et al., 2025). Video captions are as follows:

Video C1. Germination of *Spraguea americanus* spores after exposure to 15% hydrogen peroxide. Real-time video footage (1X speed) of *S. americanus* spores (suspended in L15 medium) before, during and after exposure to 15% hydrogen peroxide. Footage was taken using a Nikon^{TM} Eclipse TS100 microscope outfitted with a AmScope^{TM} MD500 microscope camera at 200X magnification.

Video C2. Germination of *Spraguea americanus* following exposure to mechanical pressure. Video footage (3.5X speed) of *S. americanus* spores (suspended in L15 medium) on a microscope slide (with a coverslip) before, during and after exposure to 5 s of even mechanical pressure, followed by 3 min incubation at room temperature (RT: 22 °C). Footage was taken using a Nikon^{TM} Eclipse TS100 microscope outfitted with a AmScope^{TM} MD500 microscope camera at 200X magnification.