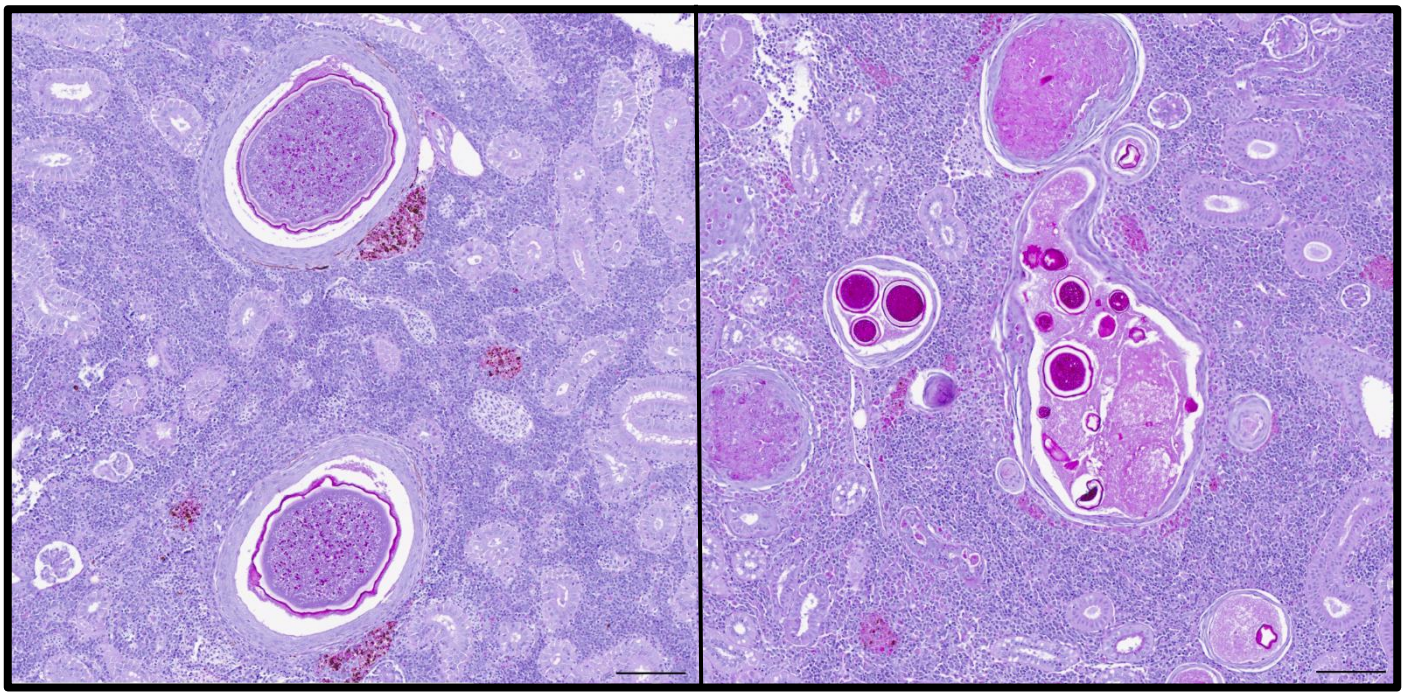
The fungus-like parasite *Ichthyophonus hoferi* infecting herring and mackerel in the Northeast Atlantic: is it really one species?

Caroline da Silva Nylund



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Front cover motive: PAS coloured kidney tissue: right for Atlantic mackerel (*Scomber scombrus*) and left for Atlantic herring (*Clupea harengus*). Scale bars are 100 µm.

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Abstract

Atlantic mackerel and Atlantic herring are two important commercial fish species in the North-eastern (NE) Atlantic. Since the early 1900s, the parasite *Ichthyophonus hoferi* has affected these and other marine fish species in the area. When causing heavy infections in herring, *I. hoferi* can cause mass mortality, usually followed by stock declines. In mackerel, no mortality events have been recorded. The parasite also varies in size in the two hosts, and recent molecular studies suggest that *I. hoferi* actually is a species complex, where different genotypes have been detected. Today, it is unclear if mackerel and herring in the NE Atlantic is infected with one species, *I. hoferi*, and hence are epizootiologically linked, or by two genotypes that may be epizoologically independent.

The aim of the present study was to clarify whether the typical *Ichthyophonus 'hoferi'* found in mackerel is the same species as found in herring by comparing the morphology using both histology and in vitro culture, genetic marker genes and prevalence. A total of 475 mackerel and 2416 herring were caught throughout three different cruises in the NE Atlantic. All the fish were examined for infection, and samples for histology, in vitro culture and molecular studies where taken.

The *Ichthyophonus* sp. prevalence in the two fish species based on catches from three different cruises clearly differed, where the overall prevalence for mackerel was 79% and herring was 2.5%. When comparing the *Ichthyophonus* sp. found in the two fish species, both histological and in vitro culture measurements showed significant differences between resting spores (significantly larger in mackerel), cytoplasm-filled hyphal width (broader in mackerel) and evacuated hyphal width (broader in mackerel). Further, the sequencing of 18S and 28S rRNA genes from *Ichthyophonus* sp. from both species showed a difference between them (98.5% and 99.7% identical respectively), and the phylogenetic analyses indicated that they grouped together in two separate clades: M-clade and H-clade.

In conclusion, Atlantic mackerel and herring in the NE Atlantic appears to be infected with different *Ichthyophonus* spp., so ichthyophonosis outbreaks in the two species appears not to be connected. Characters (morphological and molecular) that can be used to distinguish the two species are provided. However, the identity of *I. hoferi* sensu stricto, originally described from salmonids in freshwater in Germany, is at present unclear, prohibiting unambiguous identification.

Glossary - alphabetical order

Bifurcate	In this study ‘bifurcate’ refers to the cytoplasm-filled hyphal ends branching into two.
Corticosteroid	Steroid hormone with an anti-inflammatory effect on the host.
Endospore	A dormant, rough, and non-productive structure produced typically by bacteria, but in this study refers to budding produced by germinated hyphae.
Exospore	One of the new resting spores separated from the original cell, in this study the resting spore.
Focal	the centre of an area, here in tissues.
Granuloma	a small inflamated area encapsuled with connective tissue. Inside the capsule there are often lymphocytes, neutrophils, eosinophils, multinucleated giant cells, fibroblasts and collagen. Inside the granuloma can be different pathogens, such as viruses, bacteria, fungi and parasites.
Ichthyophpnosis	Infection with the parasite *Ichthyophonus* sp.
Resting spore	A spherical, thick-walled and multinucleated stage of *Ichthyophonus* sp.
Stamina	The ability to sustain prolonged physical effort, in this study referring to swimming performance.
Thallus	In this study ‘Thallus’ refers to the hyphal mass that ranges in size from a unicellular structure to a complex larger form.
Ulcer	Sores on an external or internal surface, caused by a break in the skin or mucous membrane which fails to heal. In this study referring to an effect on the host species of *Ichthyophonus* sp.

Abbreviations - alphabetical order

FW	Freshwater
IMR	Institute of Marine Research
ITS	Internal transcribed spacer
MEM-medium	«Minimum Essential Media», commonly used for cell culture media
NE	Northeast
NEA	Northeast Atlantic
NS	North Sea
NSS	Norwegian Spring Spawning
nt	nucleotide
PCR	Polymerase chain reaction, a method used to rapidly make millions of copies of the specific DNA sample given

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1 Introduction

1.1 Historical background

In 1893, a new mortality causing disease was observed in brown trout and rainbow trout farmed in Germany (Hofer, 1893). The affected fish left the shoals and resided at the edges of the ponds. They appeared lethargic, displayed tumbling or disorganised swimming ('Taumelkrankheit'), and severely infected fish tended to rest at the bottom. At time of autopsy, numerous granulomas were seen in internal organs such as the liver, kidney, heart, muscles and brain. When examined in a microscope, these were seen to contain 'cysts' with a rigid, often multiple striped wall and granulated, protoplasmic body inside (Hofer, 1893, 1904). The brain infection was assumed to be the cause of the characteristic tumbling swimming seen in diseased fish.

Subsequently, what appeared to be the same disease and infectious organism, was observed by Laveran & Pettit (1910) in farmed rainbow trout in France. In 1910, two dead rainbow trout were incidentally observed by Plehn and Mulsow (Plehn and Mulsow, 1911), apparently dead from the same infection. Affected fish was reported to show the irregular tumbling swimming behaviour, as observed earlier by Hofer (1893). However, in both cases infected fish were found with little or no brain infections (Lavéran and Pettit, 1910; Plehn and Mulsow, 1911). Instead, the infection appeared most notable in the liver, which was enlarged, of a coarse consistence, and with a brown pale colour and with numerous granulomas ('cysts'). They also observed these granulomas in the mesenteries and other organs. Pathological examinations showed active inflammatory formations with granuloma formation and necrosis (Plehn and Mulsow, 1911).

The parasite was first described and named *Ichthyophonus hoferi* by Plehn & Mulsow (1911). The type host is therefore rainbow trout, and the type locality is in freshwater in southern Germany (Munich) (Plehn and Mulsow, 1911). This described *I. hoferi* is looked at as the sensu lato.

1.2 Geographic distribution

The parasite has been a problem in marine fish, as well as in aquaculture in both freshwater and saltwater. For marine fish populations, most of the older records are from the North Atlantic and Japan (Mcvicar, 2011), such as in the Skagerrakk and Kattegatt area where it was reported to cause mass mortality of the North Sea (NS) herring in the early 1990s (Rahimian and Thulin, 1996), but the parasite has been detected in various other areas as well. For instance, extensive

mortalities of infected spring spawning herring in the Gulf of Saint Lawrence (1954-1955) has been recorded, and *I. hoferi* have been associated with mortalities in that area as early as 1930-1931 (Sindermann, 1957). In the Australian waters, it was detected in marine mullet (Sclombe, 1980), while in the North Pacific Ocean a widespread infection has been demonstrated in the region. It has also been detected in fish hatcheries in Russia (Gavryuseva, 2007), and in brackish water in South Africa (Paperna, 1986; Mcvicar, 2011).

For freshwater-aquaculture, *I. hoferi* has been proven to infect fish through feed consisting of raw, infected marine fish tissue (Sindermann, 1990), and *Ichthyophonus* sp. has globally been recognized as a risk in marine aquaculture for a long time. For instance, in early salmonid aquaculture, especially in the western United States (Sindermann, 1990), but also in aquaculture of yellowtail and ayu in Japan (Miyazaki and Jo, 1985). Recently, a genetic variant of the parasite identified as *Ichthyophonus* sp. has had a negative impact on the Peruvian rainbow trout aquaculture industry, where it is known as the 'silent killer of fish' (Castro *et al.*, 2021). The granulomatous reactions originating from infection with *Ichthyophonus* sp., especially in the organs such as the heart, brain, kidney and liver, are severe. This results in mortality before reaching commercial size, causing severe economic losses (high mortality rate and higher feed-consume needed) (Castro *et al.*, 2021).

The vast geographical distribution of *Ichthyophonus* sp. in both salt- and freshwater raises the question whether it is all caused by the same *Ichthyophonus* sp., or if there are several species involved.

1.3 Host range and diversity

Ichthyophonus sp. infections, where the parasite has mostly been identified as *I. hoferi*, has been detected in 147 species of fish, belonging to 107 genera and 48 families (Gregg *et al.*, 2016). This may indicate that the parasite has low host specificity (Gregg *et al.*, 2016). To look further into this, Gregg et. al. (2016) did a study that constructed a phylogeny based on the structural alignment of the internal transcribed spaces (ITS) region (Gregg *et al.*, 2016). The study included isolates of *Ichthyophonus* sp. from different fish in the Atlantic and Pacific oceans, rivers and aquaculture sites in North America, Europe and Japan. Based on this marker, 6 distinct clades were seen within genus *Ichthyophonus*. A major clade contained 71 of 98 isolates, representing 13 anadromous and marine hosts. Another clade contained isolates from freshwater aquaculture, despite major geographical separations (different continents). The other

four clades contained isolates from single host species. The clades illustrates the broad hosts specificity that *Ichthyophonus* sp. has, where in Clade C, both Puget Sound rockfish and rainbow trout from aquaculture clustered together, and in Clade D where both Pacific herring and chinook salmon clustered together (Gregg *et al.*, 2016). While this was the first broad study on the genetic differences within *Ichthyophonus*, only around 10% of nearly 150 reported host species were sampled at the time. The study also focused around North America, which indicates the need for more sampling, both in marine and freshwater environments to get a better understanding of the parasites' global diversity (Gregg *et al.*, 2016).

1.4 Morphology

«Cysts» is a term used by the early discoverers of *Ichthyophonus* sp. referring to the encapsulated parasite present in fish tissues, observed macroscopically or by microscope (Hofer, 1893; Plehn and Mulsow, 1911). In more recent studies, this stage of the parasite has been referred to in many ways: 'thick walled multinucleate spherical bodies', 'spherical terminal bodies', 'shizonts' and 'resting spores' (N. Okamoto *et al.*, 1985; Spanggaard, Huss and Bresciani, 1995; White *et al.*, 2013). In this study this stage will be referred to as «resting spore», which has been one of the most common designation, although, as pointed out by Kocan (2013) these stages are not true spores (Kocan, 2013).

The resting spores in tissues of infected fish are spherical, thick-walled and multinucleated cells (Rahimian, 1998), mostly less than 250 µm in diameter. The cytoplasm of the resting spore colour strongly red with PAS in histology, due to the high glycogen content (Kocan, Lapatra and Hershberger, 2013).

When developing, the resting spore grows rapidly in size, and may lack the surrounding capsule due to the speed of growth. When undergoing this growth-phase, the nuclei are peripherally scattered in the resting spore (Rahimian, 1998). When germinating, cytoplasm-filled hyphal ends penetrate the spore wall and grow out into the fish tissue, where they in time bifurcate to create more hyphae, and eventually become endospores or amoeboid cells. These endospores or amoeboid cells have 1-3 nuclei and spread in the host through the vascular system. These resting spores subsequently develops into new thick walled spherical multinucleate resting spores (N. Okamoto *et al.*, 1985).

Rahimian (1998) referred to the hyphae that appear during germination as a 'locomotive apparatus' (Rahimian, 1998). They translocate the parasite within a lesion or to a nearby healthy

tissue, and also represent a multiplication of a single resting spore to many (Mcvicar, 2011). Two different types of hyphae have also been distinguished: pre-mortem and post-mortem (Rahimian, 1998). Pre-mortem hyphae originated from the resting spore, but only one or two thin and elongated branches are formed. These do not bifurcate as fast as the post-morten hyphae, that develop thick hyphae that bifurcate shortly after the resting spore (Rahimian, 1998).

Different development patterns are seen at different pH. In acid pH (2.5-5.0), germination of resting spores lead to formation of aseptate bifurcating hyphae, that eventually form a new resting spore in the hyphal tips (N. Okamoto *et al.*, 1985; Franco-Sierra and Alvarez-Pellitero, 1999). At a neutral pH (7.0), internal cleavage and release of endospores through short hyphae has been observed (Franco-Sierra and Alvarez-Pellitero, 1999). At a more alkaline pH (8.8-9.0), the development appeared as a yeast-like budding. Even though the resting spores developed differently at different pH, all the spores eventually end up creating new resting spores that start a new cycle (Franco-Sierra and Alvarez-Pellitero, 1999).

1.4 Effect on the host

Ichthyophonus sp. can cause acute or chronic infection. In acute infections of herring there is degeneration and necrosis of the body muscles, particularly the lateral line musculature. (Sindermann, 1958). In addition to ulcerations, it has also been presented to have a «sandpaper effect», where the host may develop a roughened surface texture skin which is a result of epidermis elevating and superficial dermis overlying granulomas disseminated through the deeper layers of dermis (Powell and Yousaf, 2017). It has also been reported that an acute infection can result in mortality within 1 month of exposure, compared to 18 months with chronic infection (Mcvicar, 2011).

During a chronic infection, an inflammatory response is seen. Here, depending on the fish species, lymphocytes, macrophages, epithelioid cells, giant cells, fibrocytes, and eosinophilic granular cells surrounds the parasite (McVicar and McLay, 1985). This results in a granuloma, around individual resting spores or obliterative around groups of more resting spores. There may also be pigment deposition around the resting spores in the body muscles, seen both in chronic and subacute infections (Sindermann, 1958).

The parasite also affects the stamina of fish, where a negative correlation has been found between intensity of *Ichthyophonus* sp. infection and swimming performance in rainbow trout

(Kocan *et al.*, 2006). The mean heart weight of infected fish was 40% greater than in uninfected individuals, which was probably a result of the parasite biomass and infiltration of immune cells and fibrotic (granuloma) tissue around the parasite (Kocan *et al.*, 2006). This compromised heart performance under conditions that require severe exercise, such as climbing a river, and may increase the chances of cardiac failure (Kocan *et al.*, 2006). Kvalsvik (1995) also illustrated that fish caught in trawl have a higher prevalence of *Ichthyophonus* sp. than the ones caught with purse seine, and that the late arriving herring to spawning aeras had a higher prevalence than the early arrivers. This might point to a reduced swimming performance for the fish with ichthyophonosis (Kvalsvik and Skagen, 1995).

1.5 Parasite development and transmission

Corticosteroids are immunosuppressive hormones released during stress. Perry et. al., (2004) administered a corticosteroid to *Ichthyophonus* sp. infected starry flounder (*Platichthys stellatus*) which resulted in a predisposition to clinical ichthyophonosis and a progression from latent to patent *Ichthyophonus* sp. infection (Perry *et al.*, 2004).

The exact mechanism of *Ichthyophonus* sp. infection development in a piscine host is somewhat unclear (Kocan, 2019). Figure 1 illustrates (semi-schematiccally) what parasite development can look like when a host is experiencing stress. Here, *Ichthyphonus* sp. is enclosed in a granuloma developed by an immune response by the host. When the host is experiencing stress, the pH will drop and cause germination of the resting spore (Perry *et al.*, 2004). When the host eventually overcomes the stress, the pH will become normal again and the germination stop, leading to the development of more (usually smaller in size than to begin with) resting spores close to the original one. The new resting spores will develop gradually until a new stress-reaction is introduced.

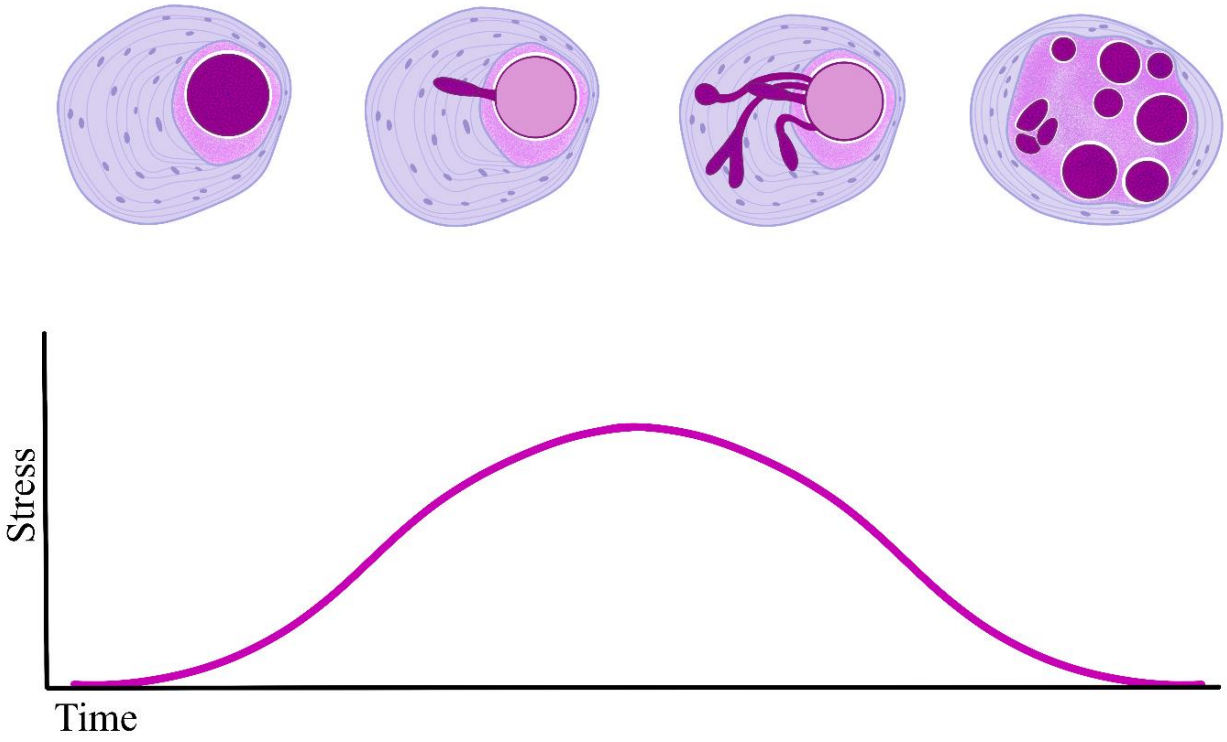

Figure 1. Encapsuled *Ichthyophonus* sp. resting spore germinating when host fish is experiencing stress, and how the germinating will pause when host is returned to normal state. Y-axe = stress level, X-axe = time. This illustration is semi-schematic.

Kocan et. al. (2013) observed indications of an amoeba-like stage of *Ichthyophonus* sp. The small 'amoeboid cells' are believed to be the infectious stage of the parasite, observed in both the stomach contents and in the lamina propria of the stomach wall (Fig. 2) of Pacific staghorn sculpins and rainbow trout. This stage of the parasite was observed shortly after ingestion of *Ichthyophonus* sp. infected tissue containing resting spores (Kocan, Lapatra and Hershberger, 2013). Following infection, the development in the host is most likely quick. During an experimental infection, early stages (3-7 µm in diameter) with up to three nuclei were observed in the intertrabecular spaces of the heart ventricle only 26 hr after the fish was fed an infected meal (McVicar and McLay, 1985).

In nature, fish infected with *Ichthyophonus* sp. has been shown to have lower performance (Kocan *et al.*, 2006), most likely due to the reduced swimming performance, which presumably also makes them more vulnerable to predation. When infected fish is consumed by a bigger fish, the parasite may infect the new host. This has been shown in previous studies, where raw marine infected tissue were fed to fish in aquaculture (Sindermann, 1990).

Ichthyophonus sp. has experimentally been shown to transmit orally (by feeding or via waterborne cells) or experimentally by intraperitoneal inoculations (Gustafson and Rucker, 1956; McVicar and McLay, 1985; Kocan, 2019).

The direct life cycles have been demonstrated to use unidirectional transmission (Gustafson and Rucker, 1956; McVicar and McLay, 1985). This is considered the most recognisable transmission model for the parasite, since it uses the direct fish-to-fish method where one individual consumes infected prey and therefore becomes infected themselves (Fig. 2). This is also the one cycle that has been confirmed both in studies and in the field. For example, in the wild, *Ichthyophonus* sp. was found (stage of the parasite not specified) in the digestive tract of Atlantic cod (*Gadus morhua*) after feeding on infected herring (Sindermann and Chenoweth, 1993). Experimentally, it has been proven through intraperitoneal inoculation of fresh viscera, through feeding of fresh *Ichthyophonus* sp. infected viscera, and through indirect contact between infected an non-infected individuals (Gustafson and Rucker, 1956; McVicar and McLay, 1985; Franco-Sierra and Alvarez-Pellitero, 1999; Kocan, 2019). The intraperitoneal inoculation with homogenate infected tissue was looked at as the primary implantation of the pathogen from one tissue to another, while the feeding experiments and contact between infected and uninfected individuals were the ones closest to what happens in nature (Gustafson and Rucker, 1956)

Oral waterborne transmission, has also been demonstrated in freshwater trout and saltwater sculpins (Kocan, 2019). Consumption of free waterborne resting spores result in infected plantivores. Using this route, the parasite can sustain within a population of piscivores regardless of its size, ensuring a number of infected individuals. On the other hand, it has not been determined whether this mechanism functions among planktivores (Kocan, 2019). Exudates or shedding of granuloma from the gills are also a possible transmission method for *Ichthyophonus* spp. Granuloma has been observed in the primary lamella in Norwegian spring spawning (NSS) herring (Hodneland, Karlsbakk and Skagen, 1997), and these granulomas with resting spores can potentially shred into the water and infect a new host, possibly a filtrating species, such as Atlantic mackerel (*Scomber scrombus*, referred to as ‘mackerel’ in the text) and Atlantic herring (*Clupea harengus*, referred to as ‘herring’ in the text). Both species are planktivores that filtrate the water, which makes the gills a potentially suitable transmission-way.

An indirect lifecycle has been proposed in part based on observations of an *Ichthyophonus* like organism in copepods. It has been theorised that the indirect life cycle is started using a

copepod, more specific a calanoid copepod, but it was not presented with experimental evidence (Sindermann and Scattergood, 1954). Torgersen et. al. (2002) confirmed that it was not *Ichthyophonus* sp. found in the copepods. The belief that this transmission method is possible maybe because planktivores have no obvious direct transmission route. However, the role of other arthropods, annelids, and molluscs have not so far been looked at (Kocan, 2019).

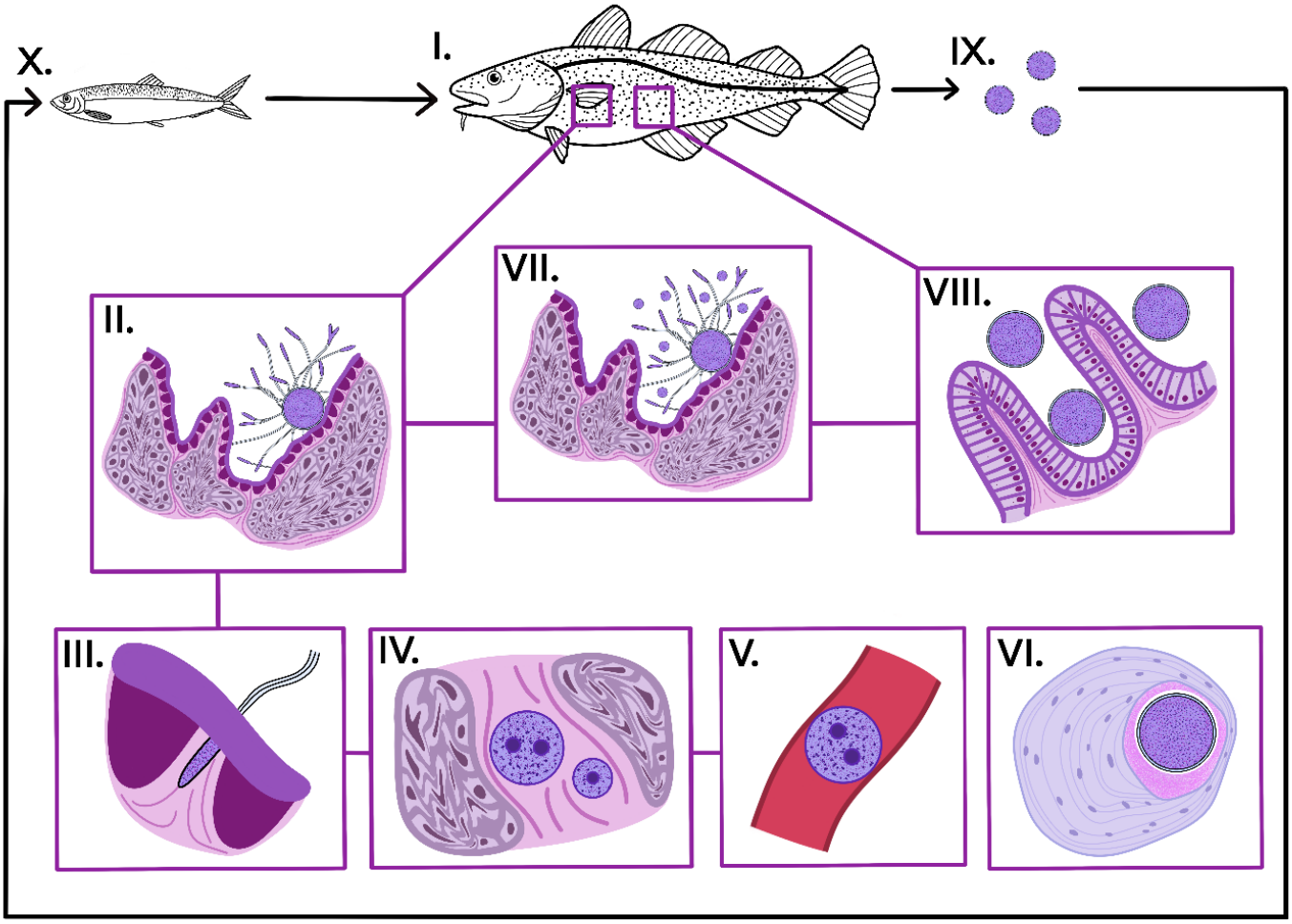

Figure 2. Illustrating how the different transmission-models for *Ichthyophonus* sp. One possible route is stage 1: piscivore consumes infected prey. Stage 2: resting spore germinates in the stomach due to acid environment. Stage 3: cytoplasm-filled hyphae penetrate the stomach-wall and enters lamina propria. Stage 4: hyphae produce 'amoeboid stages' with one or few nuclei. Stage 5: the amoeboid stage travels through the vascular system. Stage 6: an arrested vascular stage grows to a resting spore. Host reactions may form a capsule around. Another possible route for *Ichthyophonus* sp. after stage 2 is stage 7: germinated cytoplasm-filled hyphae are moved into the intestine, where the hyphal ends will produce new resting spores. Stage 8: the resting spores will not penetrate the cylindrical epithelium in the digestive track due to an alkaline environment and therefore travel right through. Stage 9: Resting spores leave the host through piscivore faeces and may be consumed by stage 10.

1.6.1 Epizootiology

I. hoferi has a wide range of fish host-species. Reports of *Ichthyophonus* sp. in marine fish started as early as in the early 1900s. Even though there were several reports of this fungal-like parasite, it was not discovered in the Western hemisphere until Cox in 1916 observed it in 'sea herring' (*Clupea harengus*) in the Gulf of Maine (Fish, 1934). Later, a widespread event of herring mortality was reported from the Gulf of Saint Lawrence in 1954, particularly of mature spring-spawning fish. Here shoals of dead fish were observed floating at the surface, and some were also washed up on the shores (Sindermann, 1958).

The parasite has attracted attention when causing several epizootics in the North-west Atlantic in a wide range of species (Hjeltnes and Skagen, 1992). This disease was not as common in the North-eastern Atlantic (NEA) at the time, so when infected NSS herring with reduced condition and skin ulcerations were noted during a trawl survey in the Northern Norwegian Sea in 1991, it caused concern (Hjeltnes and Skagen, 1992). Ichthyophonosis was subsequently found to impact a significant fraction (44%) of the population in the area. A systematic surveillance of the parasite in Norwegian waters was therefore started. At that time, the situation for the NSS herring was not alarmingly high, and stock estimates did not indicate an increase in the mortality. However, there were enough cases found for it to be a concern (Hjeltnes and Skagen, 1992). The first mass mortality event recorded on the eastern coast of the North Atlantic was not until 1991 (August-September), when thousands of floating North Sea herring were found dead or dying in Øresund. When examined, all dead or dying fish were positive for *I. hoferi*. (Rahimian and Thulin, 1996).

1.6.2 Mechanism behind epizootics

With episodes of mass mortality, there is a need to know the mechanisms behind to get a better understanding of the epizootics. After the massive mortality in the Northern Norwegian Sea, specifically in the third quarter of 1991, the prevalence of infection was at 11.3%, with no mortality being detected (Rahimian and Thulin, 1996). Rahimian and Thulin (1996) suggested that there may be a relationship between the shift in prevalence of ichthyophonosis and the migration pattern of herring. They posited that it might be linked to the migration of spring spawners from the Baltic Sea and the Sound of the Swedish west coast through Kattegat and Skagerrak to the Norwegian Seas followed by migration back to the Baltic areas and Skagerrak-Kattegat after spending the summer in there. The time varies yearly, but is usually around

September, and since the mass mortality was recorded in August-September, there is a possibility that they were infected in the Norwegian Sea (Rahimian and Thulin, 1996).

Sindermann (1990) proposed a transmission model which can explain the observed epizootic. He proposed that the infected reservoir hosts infect the not so resistant individuals in the population, and that this will in time result in a stressed population because of high parasite densities. As a result of the high host species population density, the spread of infections in the susceptible species will accelerate, which causes an epizootic. This can result in a 'spillover' effect where the infection spreads to less susceptible species because of the locally high infection pressure, with the occasional mortality within the naïve species population. Alternatively, the infection can spread to susceptible predators or scavengers feeding on dying or dead fish from the epizootic. These two possible effects will both end in declining epizootic levels, leaving the population temporarily less susceptible and therefore reducing infection pressure on other species (Sindermann, 1990).

Kocan (2019) also proposed a role for resident piscivores as reservoirs of the infection. Piscivores such as flatfish show limited migrations and are generally more long-lived, and once infected, they do not clear their infection (Kocan, 2019). If a planktivore, such as herring, migrates inshore and shed waterborne cells, the piscivores and demersal species (such as flatfish) might be exposed to the waterborne cells. This will, in turn, infect the demersal population, which in time can further infect piscivores via direct consumption (Kocan, 2019). Sindermann and Chenoweth (1993) also proposed that different demersal taxa, such as halibut (*Hippoglossus hippoglossus*) and flounder (*Limanda ferruginea*), act as reservoir hosts until a susceptible host appear (Sindermann and Chenoweth, 1993). This model is also well supported by other studies, such as Rahimian and Thulin (1996) and Holst et al. (1997). These studies report an increased prevalence of *Ichthyophonus* sp. in different herring populations at separate geographic locations during coastal migration (Rahimian and Thulin, 1996; Holst, Salvanes and Johansen, 1997).

1.6.3 Prevalence

For herring, the prevalence of *Ichthyophonus* sp. has been shown to fluctuate. The prevalence of *Ichthyophonus* sp. in Canadian spring-spawning herring fluctuated between 10-78% in the Gulf of St. Lawrence in 1954/56 (Sindermann, 1958) and an yearly average of 26.7% in Baltic herring in Skagerrak-Kattegat in 1996 (Rahimian and Thulin, 1996). Some years, the prevalence for herring has been reported at a stable 0%, but this varies temporally (Rahimian

and Thulin, 1996). In an Icelandic study going from 2008- 2014, the prevalence of heart lesions varied from 13%-43% for the Icelandic summer-spawning herring and 19%-46% in Breiðafjörður during the winter for the same stock. This study concluded that the ichthyophonosis disease was only causing massive mortality in the stock for the first three-year classes, raising the question of what the reason for this pattern might be. The authors suggested four possible causes: immune resistance, environmental conditions, multiple species of *Ichthyophonus* in the herring population, or a less invasive source of infection (Óskarsson, Pálsson and Gudmundsdottir, 2018). This may indicate that the prevalence might grow with the population stock before an eventual collapse, followed by a lower parasite prevalence.

For the mackerel, the prevalence appears to fluctuate, but with a generally higher prevalence than in herring. The first signs of high prevalence in mackerel were shown in 1941 when Sproston reported samples with up to 100% infection (Sproston, 1944). The prevalence varied throughout different catches in the North Sea (0%-100%) over three years, however, the yearly averages ranged between 38%-70%. Rahimian, on the other hand, in his survey of 1998, did not find any infected mackerel during his examinations in Skagerrak-Kattegat, where 108 Atlantic mackerel were examined (Rahimian, 1998). It is not clear whether these 108 mackerel were sampled at the same station or scattered over several stations. Storesund et. al. (2022) did a survey on the prevalence of *Ichthyophonus* sp. in mackerel in the NEA, and found that the majority of mackerel that displayed granulomatous tissue were infected with *Ichthyophonus* sp. (Storesund *et al.*, 2022)[1]. These indicate that the prevalence in mackerel can vary greatly, but more data is needed to understand the infection patterns observed in mackerel. Regardless of the high parasite prevalence, no mass mortality event has been reported for mackerel infected with ichthyophonosis, which raise the question whether mackerel react differently to *Ichthyophonus* than herring, or if this might be another *Ichthyophonus* sp. type than the one found in herring.

Several studies have associated large stock variations in herring and other species with *Ichthyophonus* sp., including high prevalence and elevated mortalities (Sproston, 1944; Sindermann, 1958; Rahimian and Thulin, 1996; Rahimian, 1998; Óskarsson, Pálsson and Gudmundsdottir, 2018; Storesund *et al.*, 2022). A very high prevalence in mackerel could therefore signify a risk for a population collapse. However, the organism infecting mackerel and herring does not necessarily represent the same species. For instance, Gregg et. al. (2016)

[1] This study by Storesund et al. (2022) included data collected for and used in this thesis and can be found in appendix 2.

showed that a sequence isolate from a Portuguese mackerel was not the same species that was found in Icelandic herring. If these important commercial fish species are infected with different *Ichthyophonus* spp., they could have different epizootiology. This problem motivates the aims of the present study.

1.7 Aims

The overall aim of this study was to clarify whether the typical *Ichthyophonus* sp. found in mackerel is the same as the one found in herring. The sub goals were:

i) Study the prevalence in the same geographical locations

ii) Compare the morphology using histology

iii) Compare the morphology using in vitro cultures

iv) Compare the genetics

2 Material and methods

2.1 Sampling

Mackerel and herring were caught at three different cruises in 2021 (Fig. 3, Table 1). Two of the cruises consisted of sampling from commercial catches onboard fishing vessel (M/S 'Kings Bay') as part of the project «Surveillance for biohazards of wild marine fish reflecting authentic fishing conditions», and one was a part of the annual ecosystem research cruise conducted by IMR (M/S 'Vendla'). Commercial fishing was done using a purse seine and pelagic trawl, whereas the research cruise employed only a pelagic trawl.

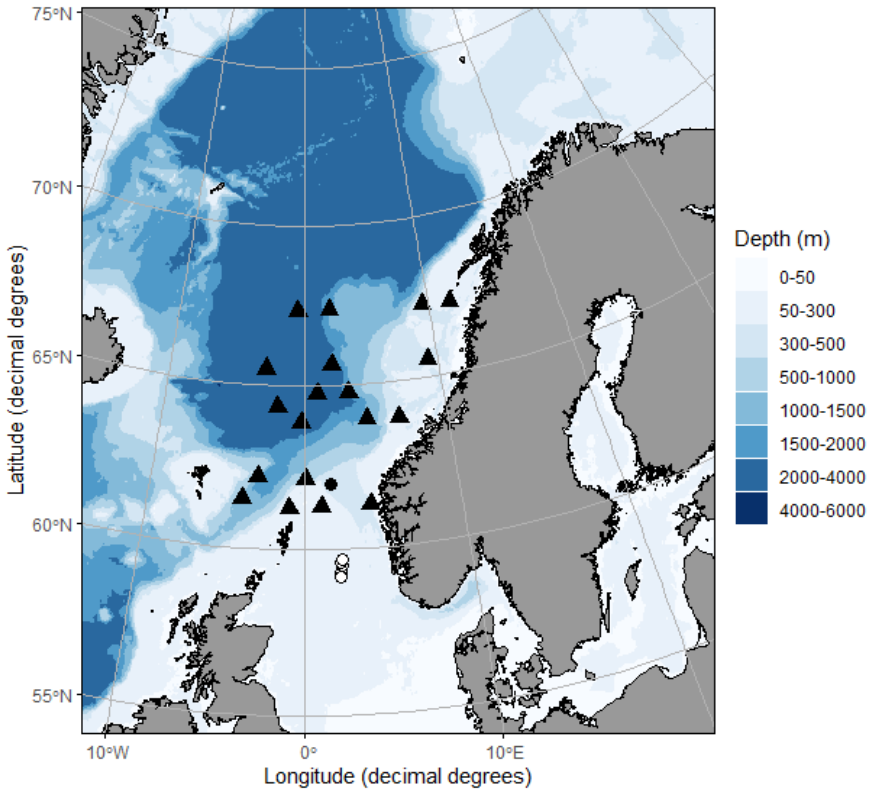

Figure 3. Overview over the different catches. White dots represent the catches done on M/S 'Kings Bay' in June, black triangles samples from M/S 'Vendla' in July, and black dot M/S 'Kings Bay' in August.

Table 1. Samples of herring (H) and mackerel (M) examined, all from 2021, from M/S 'Vendla' (V) and M/S 'Kings Bay' (KB). Positions are decimal coordinates for the trawls representing the start positions. Conversion of coordinates from degrees to decimal was done using the PGC Coordinate converter available from the University of Minnesota (https://www.pgc.umn.edu/apps/convert/).

Station/Catch	Date (Vessel)	Technique	Latitude (°N)	Longitude (°E)	Cruise type	Fish
Catch 1	2. June (KB)	Pelagic trawl	59.30	2.18	Commercial	H
Catch 2	2. June (KB)	Pelagic trawl	59.15	2.17	Commercial	H
Catch 3	3. June (KB)	Pelagic trawl	59.50	2.23	Commercial	H
Catch 4	4. June (KB)	Pelagic trawl	59.60	2.13	Commercial	H
Catch 5	4. June (KB)	Pelagic trawl	59.70	2.23	Commercial	H
37451	1. July (V)	Pelagic trawl	61.33	4.23	Research	H & M
37453	2. July (V)	Pelagic trawl	61.33	1.09	Research	H & M
37454	2. July (V)	Pelagic trawl	61.28	-0.97	Research	H & M
37457	3. July (V)	Pelagic trawl	61.52	-4.01	Research	H & M
37460	4. July (V)	Pelagic trawl	62.23	-3.05	Research	H & M
37462	4. July (V)	Pelagic trawl	62.18	0.09	Research	H & M
37465	5. July (V)	Pelagic trawl	62.23	4.32	Research	H & M
37467	6. July (V)	Pelagic trawl	63.93	6.63	Research	H & M
37469	6. July (V)	Pelagic trawl	63.95	4.38	Research	H & M
37473	7. July (V)	Pelagic trawl	63.90	-0.20	Research	H & M
37476	8. July (V)	Pelagic trawl	64.36	-1.90	Research	H & M
37479	9. July (V)	Pelagic trawl	64.77	0.91	Research	H & M
37480	9. July (V)	Pelagic trawl	64.79	3.19	Research	H & M
37487	11. July (V)	Pelagic trawl	65.58	9.26	Research	H & M
37491	12. July (V)	Pelagic trawl	65.67	2.04	Research	H & M
37493	13. July (V)	Pelagic trawl	65.55	-2.85	Research	H & M
37496	14. July (V)	Pelagic trawl	67.36	-0.55	Research	H & M
37498	14. July (V)	Pelagic trawl	67.40	2.01	Research	H & M
37500	16. July (V)	Pelagic trawl	67.28	9.53	Research	H & M
37501	16. July (V)	Pelagic trawl	67.22	11.74	Research	H & M
Catch 1	31. August (KB)	Purse seine	61.99	1.69	Commercial	M

2.2 Dissection and parasite examination

To humanely kill fish still alive after capture, the live fish were killed by breaking their necks. A total of 2921 fish was examined at three different sample rounds. At examination, all fish were measured (fork length mm), weighed (g) and inspected externally for macroscopic signs of infection (Hodneland, Karlsbakk and Skagen, 1997). Following this, the gills were examined, and the fishes were opened, the sex noted, and the viscera inspected for abnormalities as signs of infection (white spots). The following organs were systematically examined:

i) Pericardial cavity and heart
ii) Spleen
iii) Kidney
iv) Caudal lateral musculature

If white spots or other abnormalities (see Fig. 4) were seen in the heart, spleen, kidneys or caudal lateral musculature, the fish were selected for further sampling. The caudal musculature was inspected by cutting a slice off with a scalpel, searching for black discolouration, granulomas or ulcers.

When a fish with suspected infection was found, a complete sampling for histology, cultivation and samples for DNA analysis was done. All stages of the sampling were completed using sterile technique.

For histology, the tissue pieces (10 x 10 mm) were placed in a briquette and stored in 4% formalin until further examination for conservation. Tissues sampled for histology were heart (ventricle and atrium), liver (middle left), spleen, head kidney, mid-kidney and caudal lateral muscle tissue. The softer kidney pieces were enclosed in microscope lens paper inside the briquettes to prevent tissue leakage from the briquettes in the formalin.

Samples for DNA extraction was taken from the same tissues as for histology. These samples (10 x 10 mm) were placed in Eppendorf tubes with 80% ethanol and stored at – 20 °C.

Samples for *Ichthyophonus*-cultivation were taken from two tissues: heart and caudal lateral muscle. Tissue pieces (preferably with visible *Ichthyophonus*) were placed in a 15 mL falcon tubes containing 5 mL MEM medium (Gibco™) containing 5 % fetal bovine serum (One Shot™) and with the pH adjusted to 7.8 – 7.9. See point 7.9 following the cultivation media and procedures described by Okamoto et. al (1985) and Kocan (2013). See point 7.9 in appendix 1 for specifications. These enrichment cultures were initially stored in a cooler at 10 °C at the

research vessel, and later in the laboratory in Bergen in an incubator at 10 °C. If any individuals seemed especially infected with *Ichthyophonus*, the rest of the fish were placed in a sterile bag and stored frozen

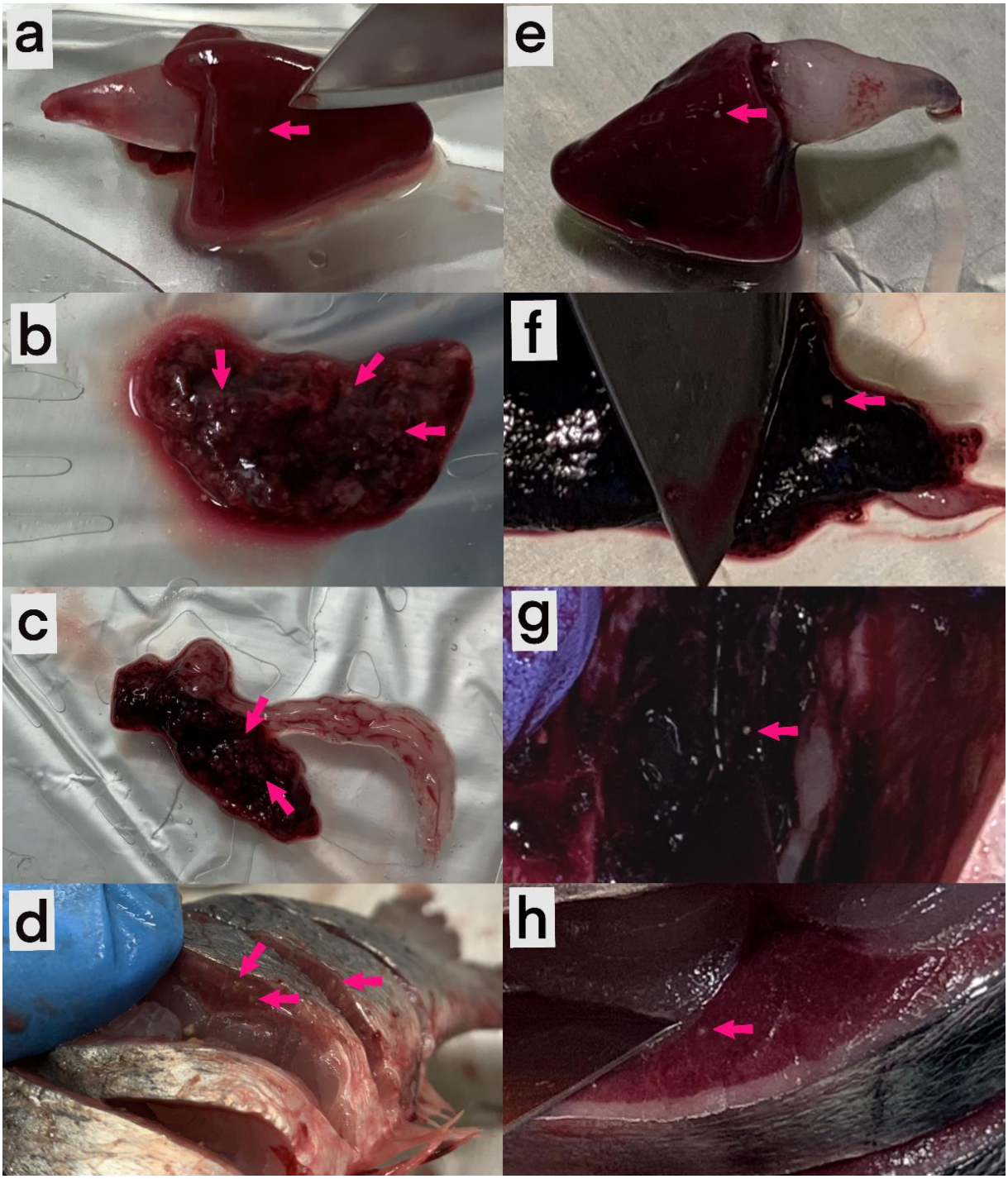

Figure 4. Showing *Ichthyophonus* sp. infection in different organs. a) Herring heart, b) Herring spleen, c) Herring kidney, d) Herring caudal lateral muscle, e) Mackerel heart, f) Mackerel spleen, g) Mackerel kidney, h) Mackerel caudal lateral muscle. Pink arrows points to granuloma with possible resting spore(s).

2.3 Preparations of samples for monitoring

Tissue samples were kept in 5 mL MEM medium in 15 mL falcon tubes and incubated at 15 °C (7-30 days). At examination of the falcon tubes, some showed growth, visible as resting spores attached to the tissue or free in the growth media. Some of the positive samples were then chosen for hyphal growth examination.

The chosen samples were transferred to sterile Petri dishes with sterile medium (pH 7.9), where some resting spores were separated from the tissue using sterile scalpels and tweezers.

The freed resting spores were then transferred to 2% saline (NaCl) washing medium in a new sterile petri dish using a 1000 µl pipette and sterile tips. The resting spores were cleaned by pipetting carefully up and down. Then they were transferred to a new sterile petri dish containing MEM medium (pH 3.5), washed again, and transferred to a second sterile Petri dish containing MEM at pH 3.5. From there, they were separated and transferred into wells in a Sterile Nunclon Delta Surface 96 well plate, with one resting spore in each well with 180 µl with MEM (pH 3.5) growth media per well. To record initial spore size, the individual wells and spores were examined and photographed using an inverted confocal microscope (Nikon confocal ECLIPSE Ti with a DC IN 12V EXT I/O light source) with NIS Elements Imaging Software (version 4.51.01). The samples were then incubated at 10 °C and examined daily for growth.

Some wells contained more than one resting spore. These were kept and examined for hyphal growth, but the resting spore sizes were not included in the final dataset. Some wells eventually showed contamination with a yeast (*Rhodotorula* sp.). In these cases, the spores were removed from the wells after a week and washed with a 2% saline solution and fresh growth medium to remove the contaminating yeast. After washing, single spores were transferred into new wells. Cleaning followed the same procedure as mentioned earlier (transferred to 2% saline (NaCl) washing medium, further transferred to MEM medium). When a hyphal mass from a germinating resting spore grew too large for a well, it was transferred to a bigger well containing 500 µl MEM media.

2.4 Monitoring and measuring resting spores

Resting spore germination and hyphal growth was monitored daily (24h intervals) using an inverted confocal microscope (Nikon confocal ECLIPSE Ti with a DC IN 12V EXT I/O light source) directly observing the wells. Digital pictures were then taken with the integrated NIS Elements Imaging Software (version 4.51.01).

Corresponding image of an object micrometre scale was obtained for the microscope-settings used. Measurements from the images were then obtained using the software ImageJ (1.53k), calibrated from the micrometre scale-image.

The following measurements were taken:

i) Resting spore diameter.
ii) Hyphal mass diameter (see Fig. 5).
iii) Width of cytoplasm-filled hyphal ends (see Fig. 6 and 8). These were measured only if there were no apical thickening indicative of branching, and if length exceeded width 3 times. The latter premise was included to avoid the measurement of hyphal ends that had initiated the process of producing spores (Fig. 7).
iv) Evacuated hyphal width (Fig. 8). These were measured only at a distance from bifurcations or the cytoplasm filled ends, where they could be slightly thicker.

As a rule, diameters were measured in two axes, and the average diameter recorded. When measuring the hyphal mass diameter (Fig. 5), if possible, the hyphal mass was captured in one single picture to get the diameter. However, the growth diameter could proceed to be greater than what the confocal could capture, making it necessary to take. This resulted in multiple pictures of the same hyphal mass, so that the pictures could be stitched together to get the real hyphal mass diameter.

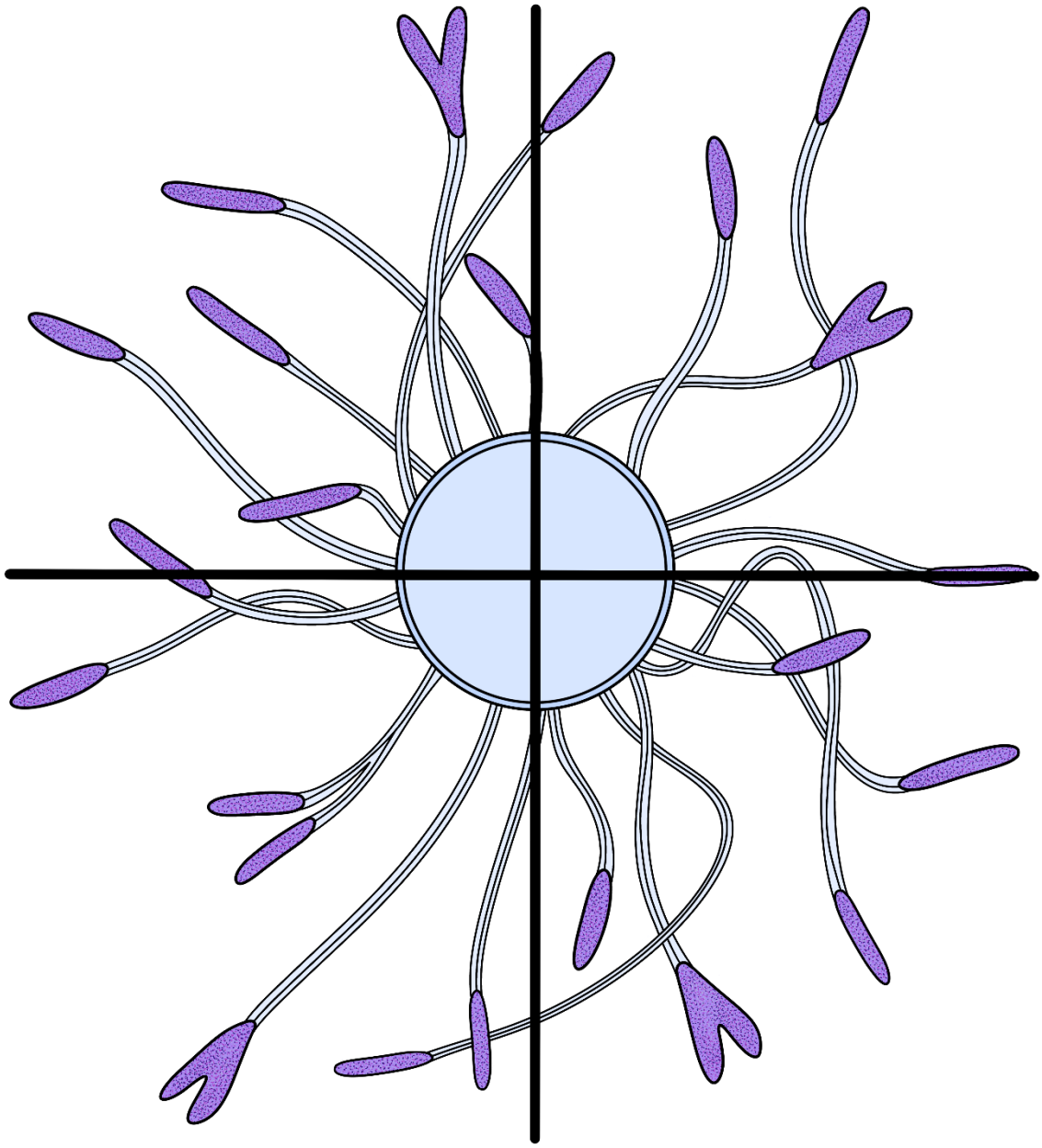

Figure 5. Illustrating how hyphal mass diameter was measured (black lines) for the calculation of diameter. The lines would follow the hyphae that wandered the furthest from the original empty resting spore.

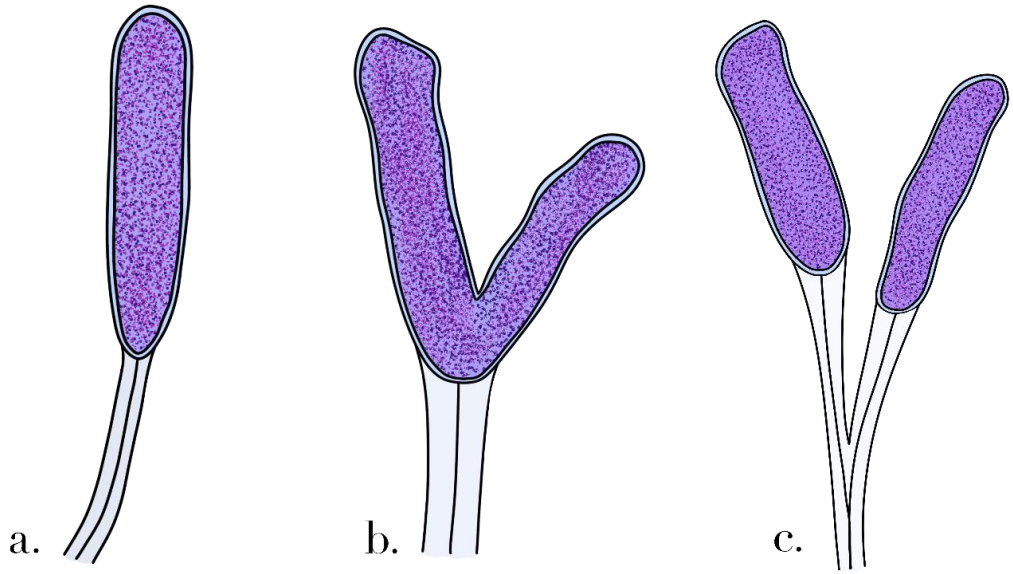

Figure 6. Illustrating qualified and not qualified cytoplasm-filled hyphal ends for measurement: a) a qualified hyphae (more than 3 times longer than it is broad), b) splitting hyphae, c) recently split hyphae. b and c are not qualified for measurement due to the uncertainty around development stage. They are also usually not more than 3 times longer than they are broad, and therefore do not meet the qualifications set for this study.

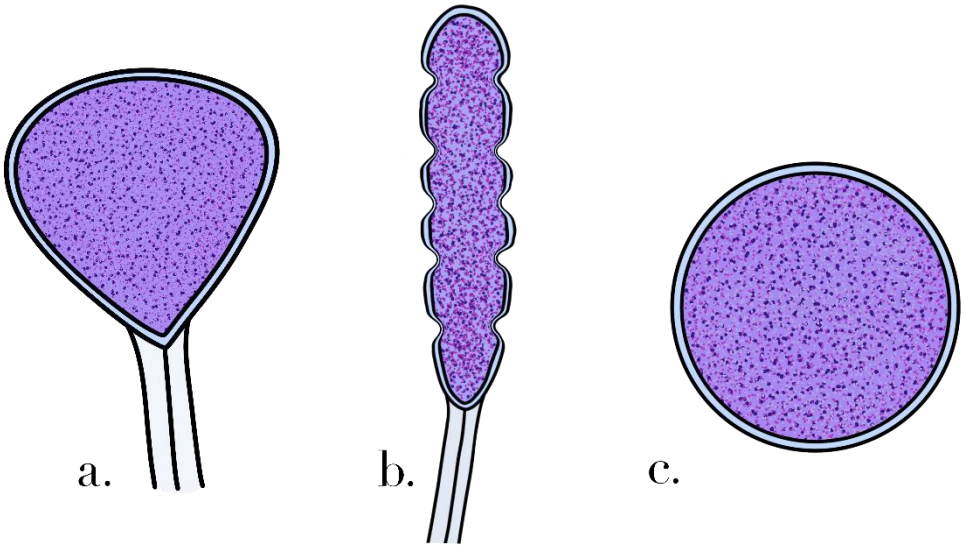

Figure 7. Resting spore formation: a) the hypha becomes more compact and resembles more of a resting spore, b) beginning of presumed exospore formation preparing for development of more than one resting spore, c) resting spore after development.

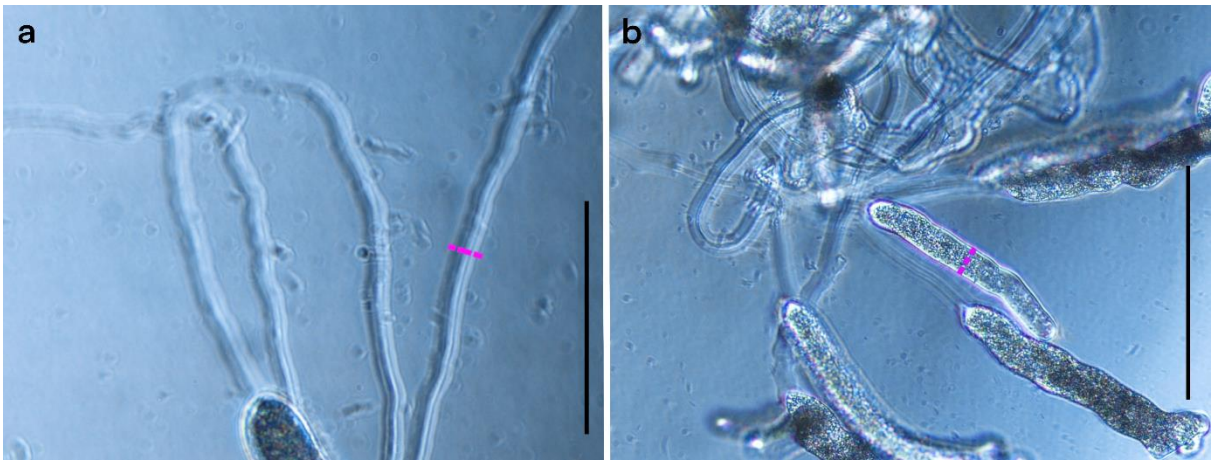

Figure 8. The images illustrate how measurements were conducted to measure width of evacuated hyphae and cytoplasm-filled hyphae: a) purple line across evacuated hypha, b) purple line across cytoplasm-filled hyphae show where hyphal widths were measured. Both scalebars are 25 µm.

As a backup, and for future studies, cultures where measurements were finished, or where fungal contamination was observed were samples into cryotubes containing 80% ethanol, and stored at – 20 °C.

Some of the original cultures were also transferred to a low pH medium as described by Franco-Sierra and Alvarez-Pellietro (1999) to trigger further growth (Franco-Sierra and Alvarez-Pellitero, 1999) for future studies.

2.5 Dehydration/Paraffin embedding and sectioning

Formalin-fixed cassettes containing the different tissues were transferred from the formalin to a LEICA TP 1020 histokinette. A detailed protocol is included in the appendix (Table 1, appendix 1). Further, the tissues were embedded with paraffin using a Kunz instruments FH-4 (following table 2, appendix 1). Before sectioning, each block with paraffin and tissue was kept in the freezer for a minimum of 15-20 minutes. For tissue-sectioning, a Thermo Scientific Rotary Microtome Microm HM355S was used. The samples were first sectioned at 10 µm, followed by 3 µm further in the block. Then, the blocks were sectioned at least 5-10 times before taking four sections on commercial Poly-L-lysine coated slides (Sigma Aldrich). Each slide was marked accordingly with the tissue sample, date, fish id and project number.

2.6 Histological staining

Poly-L-lysine slides with 3 µm sections were deparaffinised by heating in a heating cabinet (38-44°C) for approximately 30 minutes and then rehydrated in a descending alcohol series.

HES (Hemalun Eosine Safran) and PAS (Periodic acid Schiff) staining and slide assembly was done following established protocols in the IMR system with a few changes. Protocols and solutions can be found in table 3-5, appendix 1.

2.7 Histometry

In order to get as representative spore diameters as possible from histological sections, some selected infected tissues were serial sectioned. The purpose was to establish objective criteria for the selection of the resting spores with a reasonable central plane of section for diameter estimation. The spleen and kidney from one mackerel and one herring were serial sectioned for further analysis (Fig. 9-10).

Resting spore serial sectioning measurements were standardised as follows: a resting spore was qualified for measurements if it had not started germination. This standardisation was included to try to determine the centre of the resting spores. Once this was done, and the resting-spore centre could be determined with greater accuracy, the resting spores in the other histological sections were measured using the criteria established after the serial sectioning.

Illustrated by fig. 9 and 10, sections 9f and 10c-d were deemed to represent the centre of the resting spores in mackerel and herring respectively. Following the chosen visual criteria, only resting spores resembling Fig 9f were measured in mackerel samples, and only resting spores resembling Fig. 10c-d were measured in herring samples. In addition, the spores were only deemed qualified if there was no sign of abnormalities (germination development) that could lead to measurement errors and thus disqualified them from measurement.

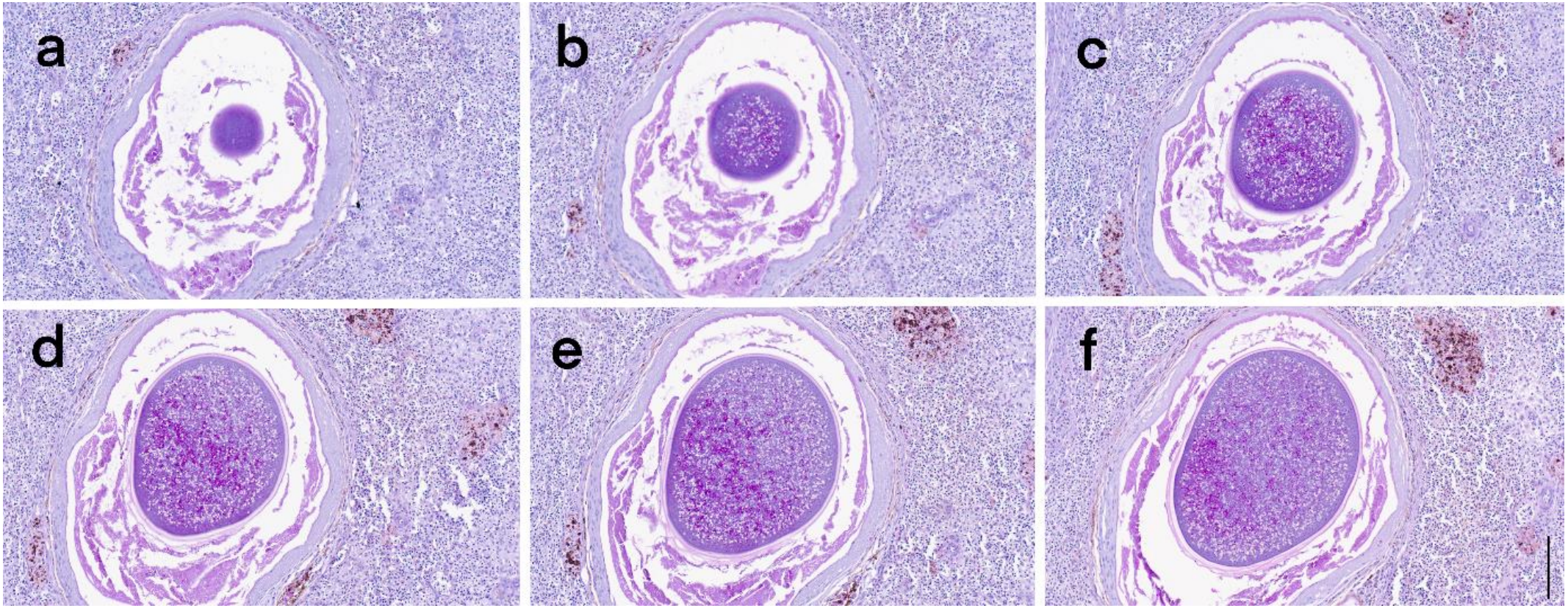

Figure 9. Shows PAS-stained serial sections for a mackerel in spleen. The center of the resting spore was determined from max. mean diameter (picture f). The scale bar is at 100 µm.

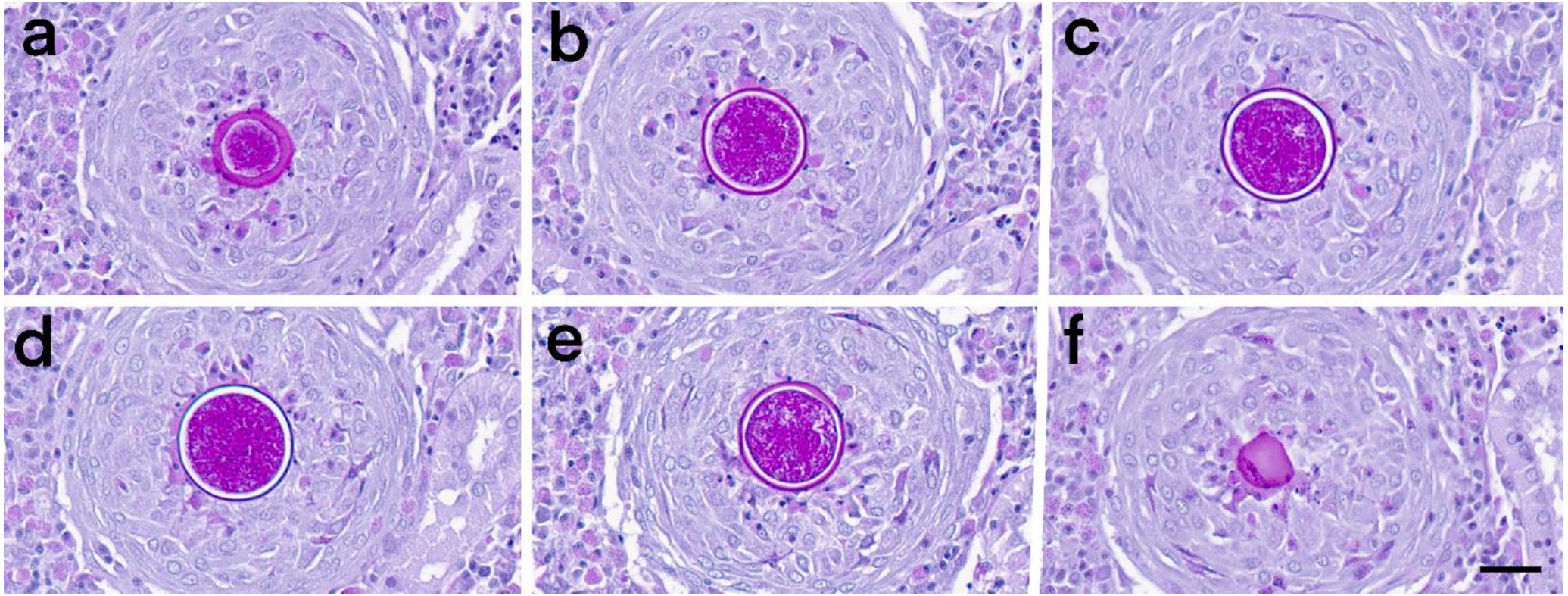

Figure 10. Shows PAS-stained serial sections for a herring in kidney. The section planes closest to the centre of the resting spore is seen in picture c and d. The scale bar is at 25 µm

Selected sections were also chosen for capsule-thickness measurements, as illustrated in fig. 11. When measuring the capsule thickness, only the connective tissue was measured. Areas with necrosis, inflammatory cells and leucocytes was not included.

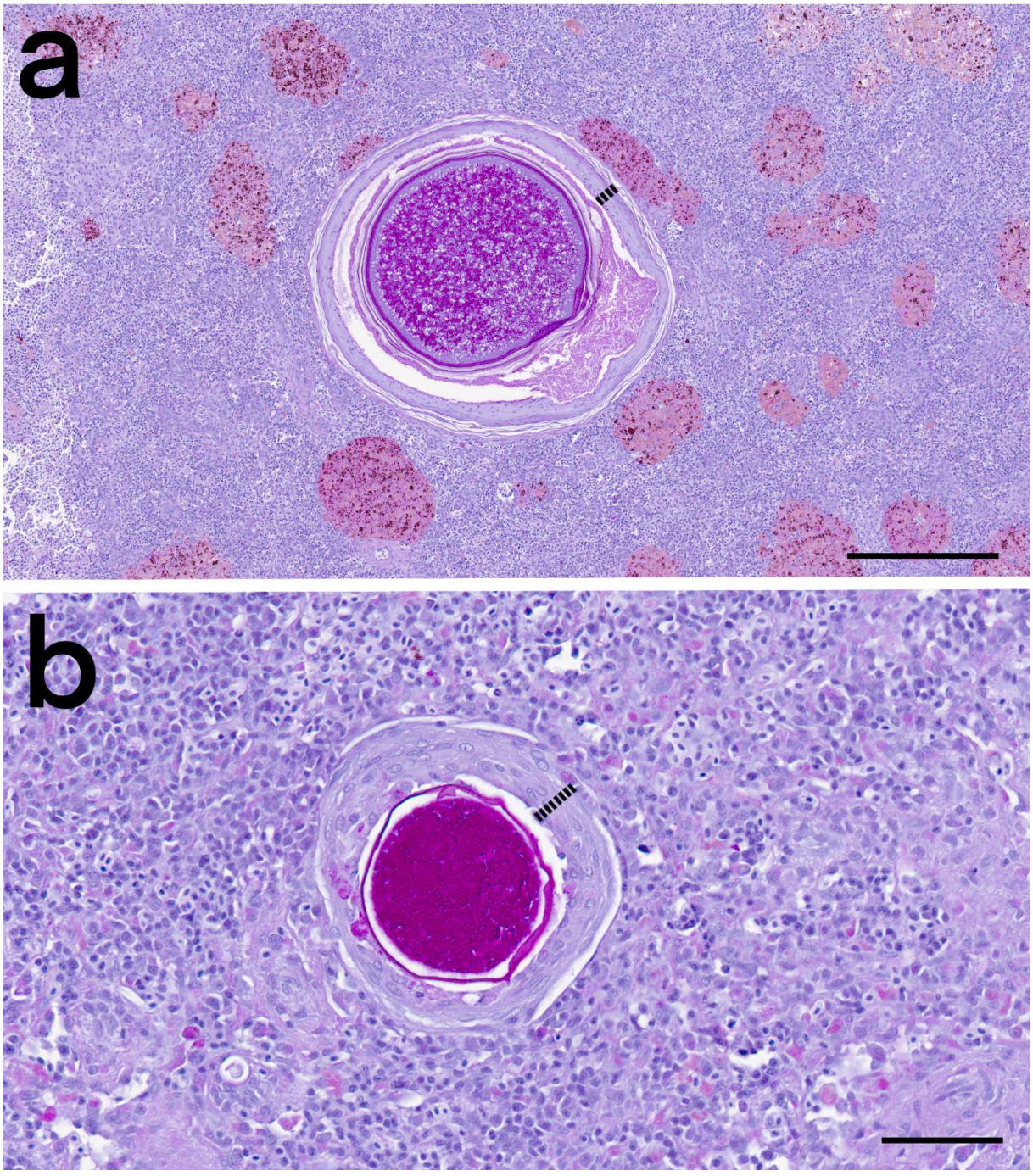

Figure 11. Sections of resting spores from mackerel (a) and herring (b) spleen showing how thickness of the connective tissue capsule surrounding the parasite was estimated (Stippled line show measurement) in sections considered close to the centre of the resting spore. Stained with PAS. The scale bar is at 250 µm (a) and 50 µm (b).

2.8 Extraction of DNA

To extract DNA from the different tissues, the QIAGEN DNeasy® Blood & Tissue Kit (50) was used according to instructions from the manufacturer. The protocol was followed as written for the tissue sample (1a) as described by the manufacturer, except for step 8 (lysis), where 100 µl Buffer AE was used instead of 200 µl. The Buffer AE was also preheated to 50 °C before use. For some large tissue pieces, the protocol was followed but with double or triple volumes of ATL lysis Buffer.

To check the concentration of DNA in the samples before proceeding to PCR, a Qubit 2.0 fluormeter was used according to instructions from the manufacturer.

2.9 PCR

Polymerase chain reaction (PCR), was used to amplify marker gene from *Ichthyophonus* sp. from mackerel and herring samples obtained in this study, and from some additional herring samples kindly provided by Egil Karlsbakk. The chosen markers were: the 18S rRNA gene and the 28S rRNA gene. In addition, a part of the ITS-region of *Ichthyophonus* sp. was also amplified. The primers pairs used were designed to specifically target either *Ichthyophonus* sp. or Mesomycetozoan genes (Table 2).

The master mix contents used are listed in table 3. For the different PCR runs, positive controls and blanks were included.

Three different PCR programs were used:

i) For Ich28-F1/R1: Stage 1: 5 minutes 95°. Stage 2: 35 x (30 seconds 95°, 1 minute 65°, 1 minute 72°). Stage 3: 7 minutes 72°.

ii) For IchEK-F1/MesR1: Stage 1: 5 minutes 95°. Stage 2: 35 x (30 seconds 95°, 1 minute 58°, 1 minute 72°). Stage 3: 7 minutes 72°.

iii) For Hers-Out-ITS1-F/ITS2-F: Stage 1: 5 minutes 95°. Stage 2: 35 x (45 seconds 95°, 45 seconds 65°, 1.5 minutes 72°). Stage 3: 7 minutes 72°.

For a more illustrative overview for the PCR programs, see fig. 1-3, appendix 1.

PCR products were examined on agarose gels for visible bands, and positive samples were sent to Eurofins (Germany) for purification and sequencing using Sanger® sequencing.

Table 2. Overview for the different primers used in this study.

Primer name	Primer sequence (5'-3')	Target DNA	Ref.
Ich28-1F	ACA GGC CAA CAT CAG TTC G	28S	JES (this study)
Ich28-1R	TTG GCA CTT TAA CTT CGC GTT	28S	JES (this study)
IchEK-F1 (Mod Plch F1)	ACC CGA CTT CTG GAA GGG TTG T	18S	(White et al., 2013)
MesR1	GCT TAC TAG GAA TTC CTC GTT GAA GA	18S	(White et al., 2013)
Hers-Out-ITS1-F	GCG GAA GGA TCA TTA CCA AAT AAC G	ITS	(Gregg et al., 2016) (Hershyberger et al., 2010)
Hers-Out-ITS2-F	GCC TGA GTT GAG GTC AAA TTT	ITS	(Gregg et al., 2016) (Hershyberger et al., 2010)

*Modified Plch F1 primer of White et al. (2013).

Table 3. Master mix content for the 25 µL reaction.

Reagent	Concentration	Volume (µl) per 25 µL reaction
GoTaq® GT Colorless Master Mix		
Buffer		5
MgCl2		2
dNTP mix	10 µM	0.5
Forward primer	10 µM	0.5
Reverse primer	10 µM	0.5
BSA	10 %	1
DMSO	10 %	1
DNA template		0.125
Nuclease-free water		12.375
Templat		2
Total reaction volume		25

2.10 Sequence processing and phylogenetic analyses

The chromatograms were visualized and edited using two different programs: Chromas Pro (version 2.1.10) and Notepad++ (v8.1.9.2, 32-bit).

Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 11.0.10(Tamura, Stecher and Kumar, 2021). Alignments were made in MEGA using ClustalW aligner (Thompson, Higgins and Gibson, 1994), and the best substitution model was determined using jModelTest (Posada, 2008). Both programs are implemented in MEGA.

Further, a Maximum-Likelihood tree was constructed in Mega for each marker gene using the best-fit model and 100 bootstraps. In addition to sequences from this study, related sequences obtained from GenBank (Benson *et al.*, 2013) were included.

2.11 Statistics and maps

Statistics were conducted in R (version x64 4.1.2) through R Studio (version 2021.09.01 Build 372). The map of the sampling area was conducted using following packages: ggOceanMapsData and ggOceanMaps from https://cloud.r-project.org and https://mikkovihtakari.github.io/drat, respectively.

3 Results

3.1 Prevalence of *Ichthyophonus* spp. infection

A total of 475 mackerel and 382 herring were examined during the July cruise with M/S 'Vendla'. The overall prevalence of *Ichthyophonus* sp. was 79% in mackerel and 2.5% in herring, whereas the prevalence in individual catches varied between 0-15% and 56-100% for herring and mackerel, respectively (Table 4-5). Of the confirmed infected microscopic samples, they were also confirmed by histological, genetical and in vitro culture samples (18/26 mackerel and 7/9 herring) (see appendix 1, section 7.10).

From the MS 'Kings Bay' cruise in August/September, 26/30 mackerel were infected.

None of the 2034 North Sea herring caught in June 2021 by M/S 'Kings Bay' were found to be infected.

Table 4. Prevalence (P, %) of *Ichthyophonus* sp. in Atlantic mackerel from different samples based on macroscopic internal examination. Prevalence of lesions (granules) in different organs are also listed: H: heart tissue, S: spleen, K: kidney, M: muscle tissue.

Mackerel								
Date	**Station**	**N**	**H**	**S**	**K**	**M**	**N infected**	**P, %**
July 1	37451	25	11	20	20	15	20	80
2	37453	25	6	13	13	10	17	68
2	37454	25	7	16	16	9	20	80
3	37457	25	17	22	23	18	25	100
4	37460	25	12	17	19	11	21	84
4	37462	25	15	18	19	10	22	88
6	37467	25	10	14	17	6	18	72
6	37469	25	5	16	14	4	17	68
7	37473	25	6	14	15	7	18	72
8	37476	25	9	15	17	5	20	80
9	37479	25	5	12	17	4	18	72
9	37480	25	4	16	15	6	18	72
11	37487	25	6	14	19	6	21	84
12	37491	25	1	9	11	1	14	56
13	37493	25	17	21	22	16	23	92
14	37496	25	5	16	17	6	22	88
16	37498	25	6	16	18	6	23	92
16	37500	25	7	21	18	10	24	96
16	37501	25	2	12	16	7	20	80
August 31	-	30	15	22	26	14	26	86
ALL							379/475	79

Table 5. Prevalence (P, %) of *Ichthyophonus* sp. in Atlantic herring from different samples based on macroscopic internal examination. Prevalence of lesions (granules) in different organs are also listed: H: heart tissue, S: spleen, K: kidney, M: muscle tissue.

Herring								
Date	**Station**	**N**	**H**	**S**	**K**	**M**	**N infected**	**P, %**
June 2-4	-	2034	0	0	0	0	0	0
July 1	37451	25	0	0	0	0	0	0
2	37453	25	0	0	0	0	0	0
2	37454	25	0	0	0	0	0	0
3	37457	25	1	1	0	1	1	4
4	37460	25	1	0	0	0	1	4
4	37462	25	3	1	1	1	3	12
5	37465	2	0	0	0	0	0	0
6	37467	2	0	0	0	0	0	0
6	37469	1	0	0	0	0	0	0
7	37473	25	0	0	0	0	0	0
8	37476	14	0	0	0	0	0	0
9	37479	13	1	1	0	0	2	15
9	37480	25	0	0	0	0	0	0
12	37491	25	1	1	1	1	1	4
13	37493	25	1	1	1	0	1	4
14	37496	25	0	0	0	0	0	0
16	37498	25	0	0	0	0	0	0
16	37500	25	0	0	0	0	0	0
16	37501	25	0	0	0	0	0	0
ALL							9/382	2,50

A statistical comparison of prevalence between mackerel and herring was done for the 14 stations where a full sample of both species was obtained. In all stations, the prevalence of *Ichthyophonus* sp. was significantly higher in mackerel than in herring (Table 6).

Table 6. Comparison of *Ichthyophonus* sp. prevalence in mackerel and herring from 14 stations, selected due to full set of individuals (N=25) of each species was obtained. In all cases, the prevalence was higher in mackerel. Uninf. = uninfected, Inf. = infected, FET = Fisher's Exact test.

	Mackerel		Herring		FET
Station	**Uninf.**	**Inf.**	**Uninf.**	**Inf.**	**P-value**
37451	5	20	25	0	<0.001
37453	8	17	25	0	<0.001
37454	5	20	25	0	<0.001
37457	0	25	24	1	<0.001
37460	4	21	24	1	<0.001
37462	3	22	22	3	<0.001
37473	7	18	25	0	<0.001
37480	7	18	25	0	<0.001
37491	11	14	24	1	<0.001
37493	2	23	24	1	<0.001
37496	3	22	25	0	<0.001
37498	2	23	25	0	<0.001
37500	1	24	25	0	<0.001
37501	5	20	25	0	<0.001

3.2 Morphology and growth in culture

The most frequently observed stage of *Ichthyophonus* spp. in both mackerel and herring was spherical thick-walled multinucleate 'resting spores'.

Resting spores that were transferred to low pH media for the growth experiment were measured. The resting spores that germinated in in vitro culture did so within a week. The mean diameter of the resting spores that germinated varied greatly in both species, ranging between 130-415 µm in mackerel and 83-232 µm in herring (Table 7). Only one resting spore was included for herring in the size measurement comparisons, due to the fact that most of the herring samples consisted of a cluster of smaller resting spores, which made them unqualified for measuring. This makes the mean diameter for herring in the in vitro culture based on only one resting spore.

After germination, the expansion of the rounded hyphal mass around the resting spore for mackerel and herring measured daily showed a vast difference between the two. Hyphal mass diameter ranged between for mackerel 230-1448 µm, and for herring 60-2295 µm. Only two resting spores from mackerel germinated, but the hyphal mass developed the same (One hyphal

mass developed from 230-1285 µm diameter in three days, and the other hyphal mass developed from 570-1155 µm diameter in three days). For the herring hyphal mass, the developments were also similar to each other, but some of them expanded further than others (ranging from 1254-2294 µm diameter hyphal mass after three days). Number of hypha that emerged first from each germinating resting spore was 2-5 in those from mackerel and 1-6 in spores from herring. The advancing hyphae bifurcated into more hyphae in samples from both fish species (Fig. 12). The cytoplasm-filled advancing hyphal ends left behind an evacuated hyphae with a clear string in the middle (Fig. 12a). Eventually, the cytoplasm-filled hyphal ends thickened and rounded up into ball-like structures. There was a significant difference in the width of evacuated as well as cytoplasm-filled hyphae between mackerel and herring ($p < 0.005$, T-tests) (Table 7).

Table 7. Comparing *Ichthyophonus* sp. from different host species in µm (min-max). M = mackerel, H = herring.

Feature	***Ichthyophonus* sp. (M)**	***Ichthyophonus* sp. (H)**
Resting spore diameter	181 (83-232)	284 (130-415)
Hyphal mass morphology on heart	Circular raised/convex	Circular raised
Hyphal width	18 (11-30)	33 (22-43)
Evacuated hypha	13 (8-22)	21 (16-27)

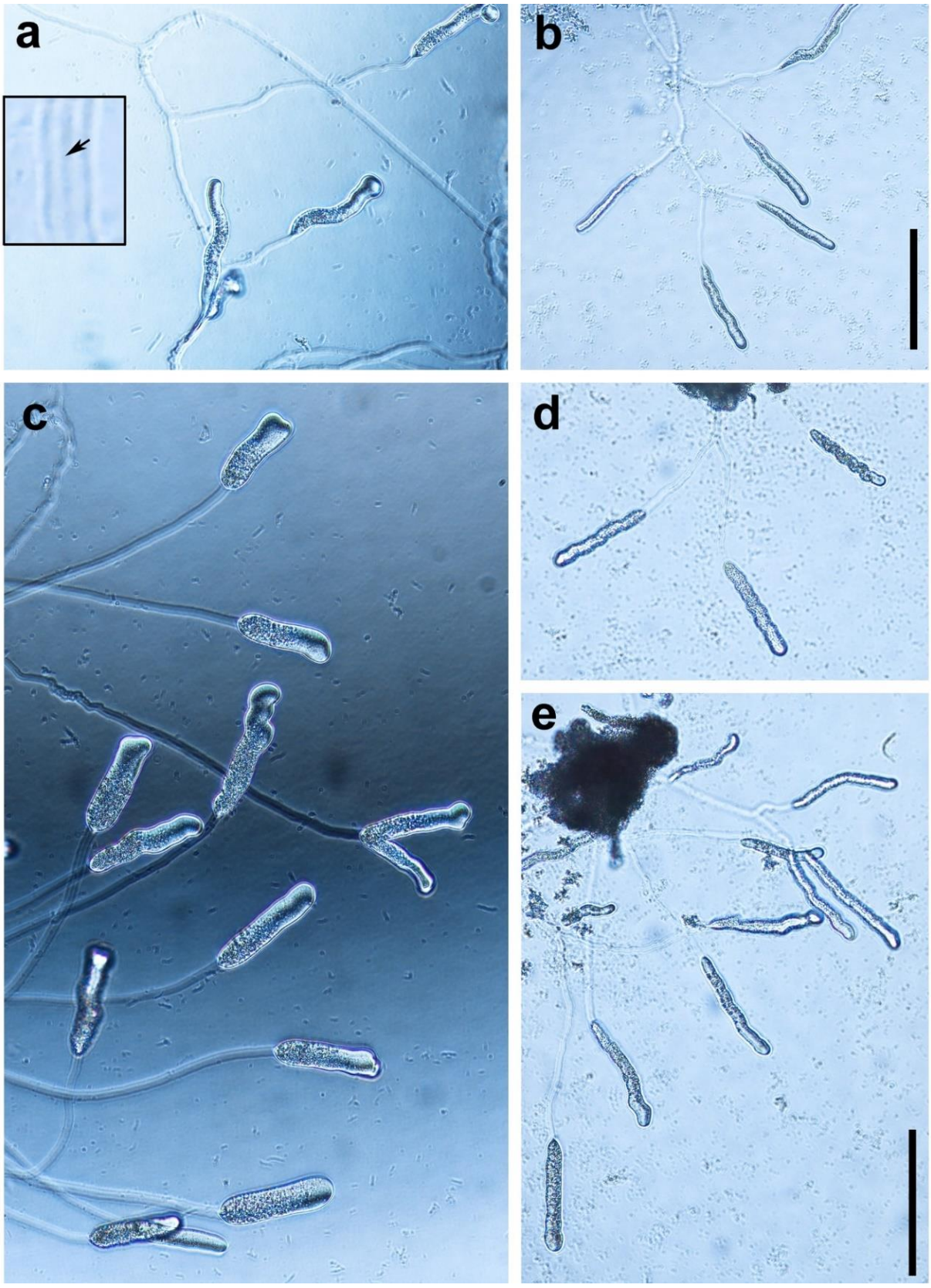

Figure 12. *Ichthyophonus* sp. from mackerel (a,c) and herring (b, d, e) grown in culture, showing comparative hyphal morphology. In a, the inserted image illustrates the thin thread left behind by the advancing cytoplasmic end. Comparing *Ichthyophonus* sp. from mackerel with that from herring (a and b same scale; c-e same scale), it is evident that both evacuated and cytoplasm-filled hyphae of the mackerel originating *Ichthyophonus* sp. is thicker. Both scale bars are 100 µm.

3.3 Series sectioning to determine spore midpoint

Resting spores from one mackerel and one herring were serial sectioned to reveal characteristics that aid the discrimination of near-central vs peripheral sagittal sections. For the outer part of the resting spores, a heavily stained part is found where there is no real structure observed (lacks vacuoles and nuclei in the centre). When sectioning further into the resting spore, more of the structures were observed, but only in the true central sections large amounts of vacuoles and nuclei are visible in addition to a well formed wall-structure.

Further, some of the included resting spores did not qualify for measuring because the sections showed hyphal germination. Two serial sections, one from each fish species, were deemed of good quality and used as standards (Fig. 9f and 10c-d). The one for mackerel did not have complete sectioning through the spore due to less tissue material being available. However, the mid-point of the spore was still determined since the sectioning slides showed that the resting spore was decreasing in size in the last sections.

The final visual criteria used to identify near-central sections were:

i) Observing clear internal structures (vacuoles and nuclei)
ii) Even cell wall thickness
iii) Circular form of the resting spore (no abnormalities in form)

Based on this serial sectioning, resting spores identified as being from sagittal sections were not included in the measuring procedure, and only resting spores that showed typical equatorial sections were measured.

3.4 Resting spore size distribution

During histological examination *Ichthyophonus* sp. was found in different tissues, usually enclosed in a granuloma. The resting spores were PAS-positive (resting spores from mackerel somewhat less than herring) and therefore easy to identify.

In mackerel, the resting spores were fewer but larger in size than in herring (Fig. 13), usually observed as singles spores and never in a cluster (a-d mackerel, e-h herring, Fig. 14). In addition, there were generally larger resting spores in heavily infected tissues surrounded by smaller spores in the herring samples (e and g, Fig. 14). There was a vast size difference between the two species (Fig. 13). The largest resting spore measured from mackerel was up to 1002 µm om diameter, but for herring the largest resting spore only measured 138 µm in diameter.

Emerging hyphae, and empty evacuated spores could also be seen (e-h, Fig. 14). Formation of amoeboid cells as those observed from hyphae in herring (exospores) was not observed in mackerel. The connective tissue capsule thickness in the granuloma surrounding the resting spores varied from 14-40 µm in mackerel (average 29 µm, N 15) and 11-25 µm in herring (average 20 µm, N 15), when measured at the average thickness of the granuloma ($p < 0.005$, T-test).

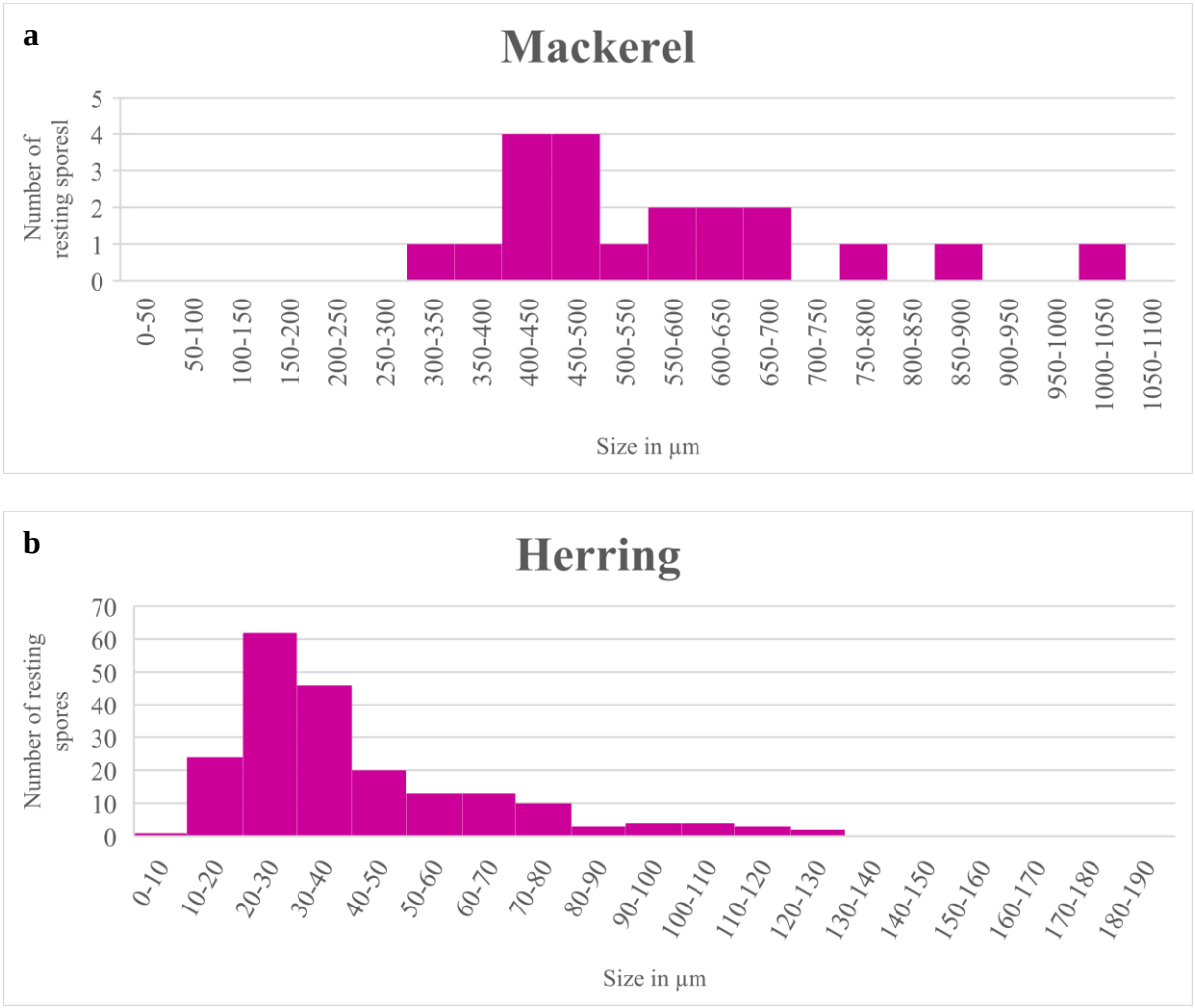

Fig. 13. Frequency distribution of *Ichthyophonus* sp. resting spore diameters, as measured in histological sections from mackerel and herring. A non-overlapping size distribution is evident, those from mackerel being much larger. a) resting spores from mackerel, which varied from 316-1002 µm in diameter. b) resting spores from herring, which varied from 9-138 µm in diameter. Note different scales on the axes.

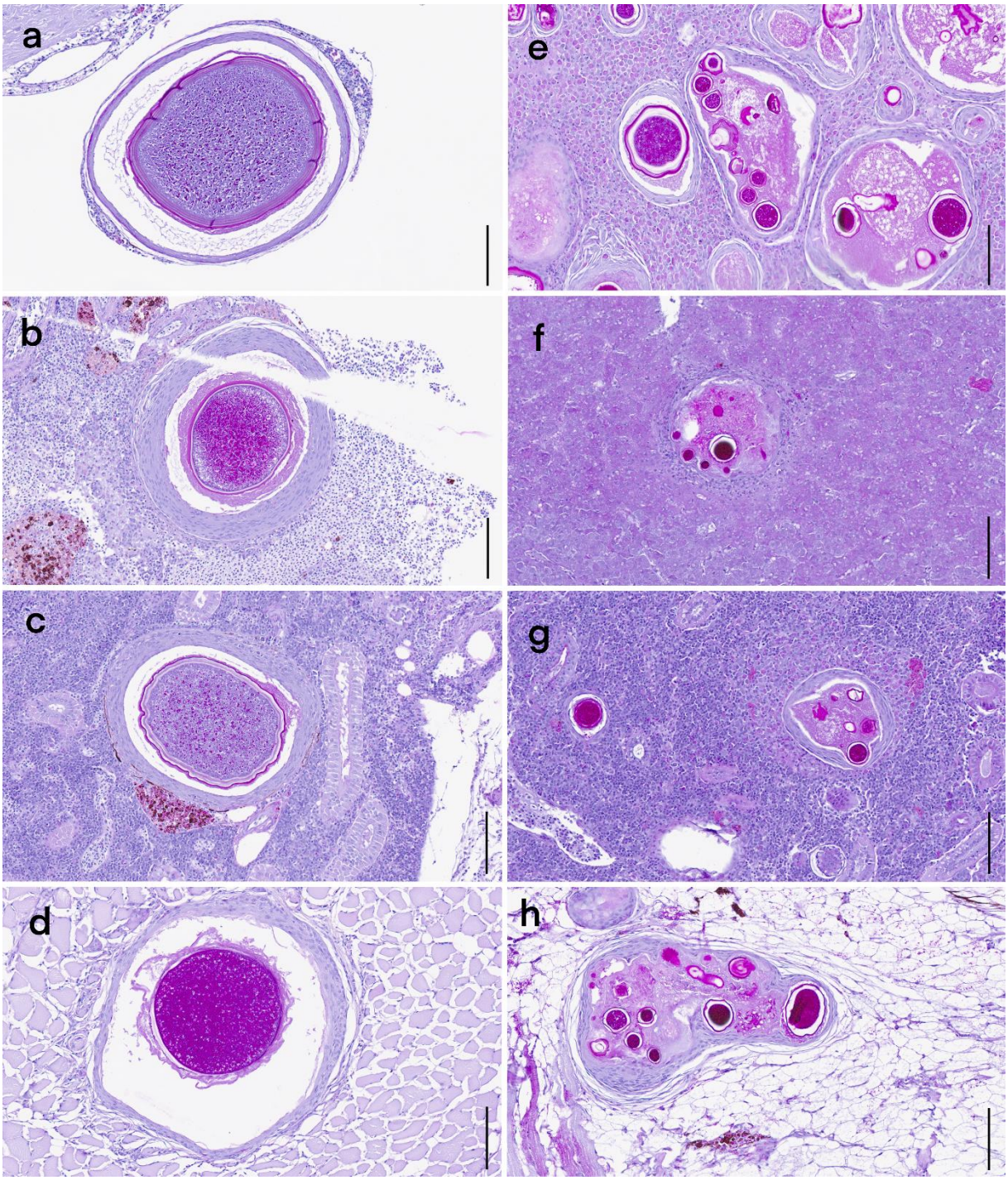

Figure 14. Shows histological PAS-stained sections for mackerel (A-D) and herring (E-H) side by side. A & E is the heart, B & F is the spleen, C & G is the kidney, D is the lateral muscle and H in adipose septum. Scale bars are 100 µm.

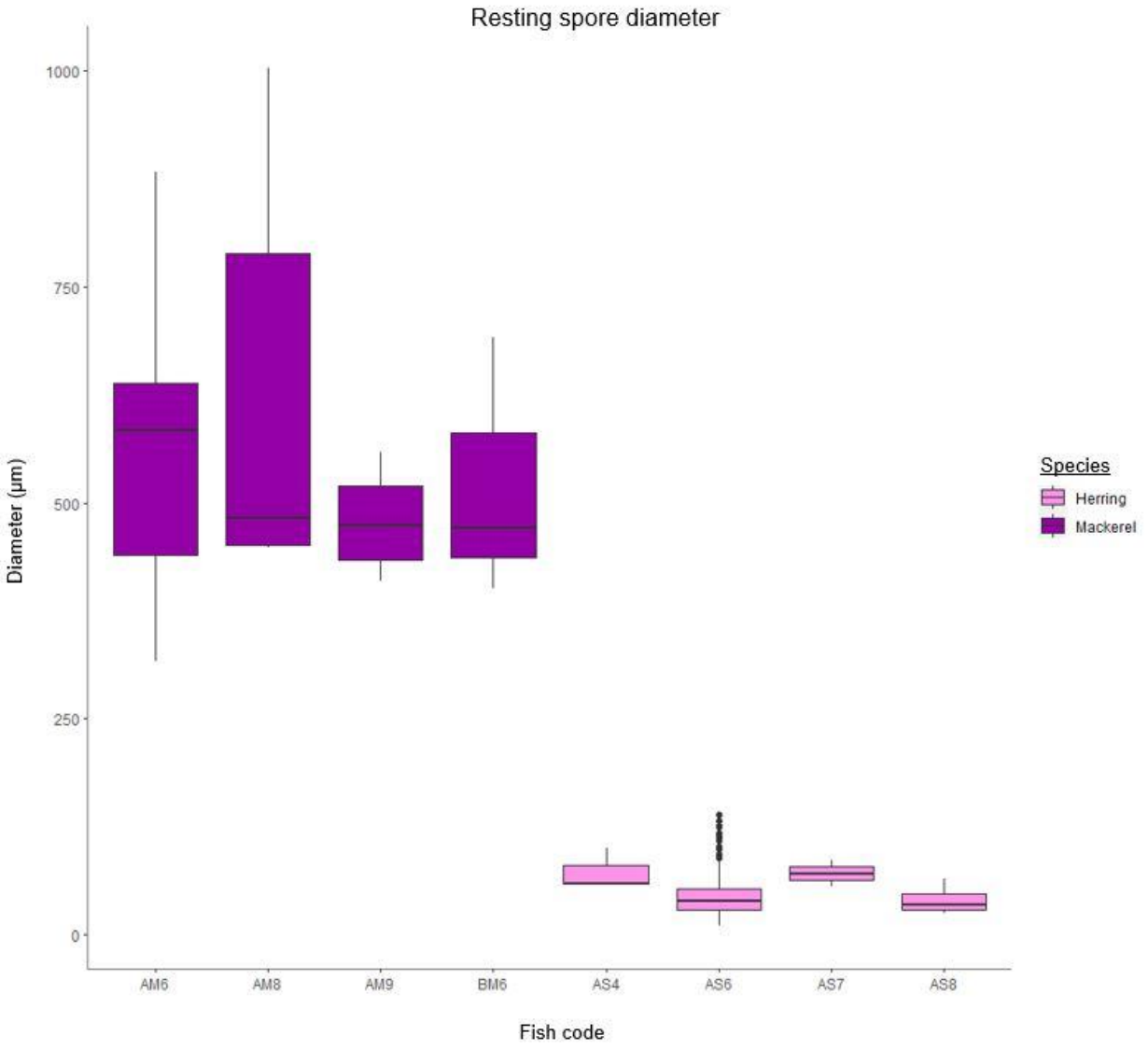

Figure 15. Illustrates the distribution of resting spores sizes between different individuals withing the two species. Here, AM6, AM8, AM9 and BM6 represent four different mackerels, and AS4, AS6, AS7 and AS8 represent four different herring. The boxes contain the spore sizes between the 25th and 75th percentile of the data from the individual fish, and the line inside the boxes indicate the median value. The whiskers indicate minimum and maximum values. Dots indicate outliers. There was no overlap between the sizes of resting spores from mackerel and herring.

3.5 Molecular analyses

28S rRNA gene sequences were obtained from 13 of 19 samples from mackerel and 5 of 7 samples from herring. The 13 samples from mackerel (835-912 nucleotide long) had some ambiguous sites, while the 5 partial 28S rRNA gene sequences from herring (911-912 nt long) were identical. The sequence type from mackerel and from herring differed in 7-8 substitutions (98.5% identical). Best hit for the 28S rRNA gene sequences for mackerel (98.6%) and for herring (98.9%), 781 and 783 positions respectively, was with an *Ichthyophonus* sp. 28S gene sequence (KT595186) obtained from rainbow trout from freshwater in Idaho, USA.

18S rRNA gene sequences were obtained from 5 of 13 mackerel and 2 of 6 herring. For mackerel, the 5 samples (1150-1214 nt long) had some ambiguous sites, while the partial rRNA gene sequences obtained from herring (1152-1214 nt long) were identical. The sequences from mackerel and herring differed in 3-7 substitutions (99.7% identical). Best hit (99.6% for mackerel and 99.8% for herring, 1187-1189 positions compared respectively) was with an *Ichthyophonus* sp. 18S rRNA gene clone sequence (JX509909) from an Alaska pollock (*Theragra chalcogramma*) from Seattle, USA.

The primer pair used to amplifying the ITS-region of *Ichthyophonus* spp. only gave products from the samples from herring, not mackerel. ITS-region sequences obtained from 3 of 4 herring (417-424 nt long) showed 99.8% identity, but with ambiguous sites.

Phylogenetic analyses based on the obtained *Ichthyophonus* sp. 28S rRNA gene sequences from mackerel and herring in Norway and similar sequences obtained from GenBank, revealed that the present sequences grouped in two well supported clades (Fig. 16). These clades represented *Ichthyophonus* sp. from mackerel (M-clade) and herring (H-clade) respectively. The sister group to these sequences from Norwegian marine fishes was a sequence from *Ichthyophonus* sp. infecting rainbow trout in freshwater in Idaho, USA.

The 18S rRNA sequences from *Ichthyophonus* sp. mackerel also grouped in a well supported clade (Fig. 17). However, the relationship between the M-clade and the *Ichthyophonus* sp. sequences from herring and other *Ichthyophonus* sp. sequence isolates that belong (or likely belong) to the 28S H-clade were not well resolved.

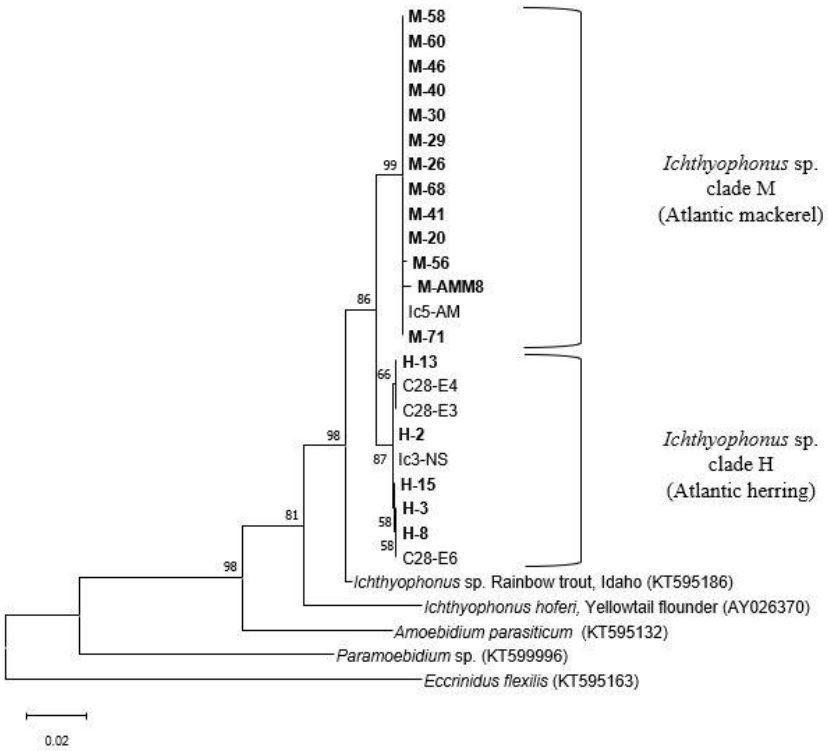

Figure 16. Maximum-likelihood tree (K2 + G substitution model) showing 28S rRNA gene distribution. M-x represent mackerel samples, H-x represent herring samples. Bootstrap values > 50 are indicated at the branch points, bar represents 0.02 substitutions.

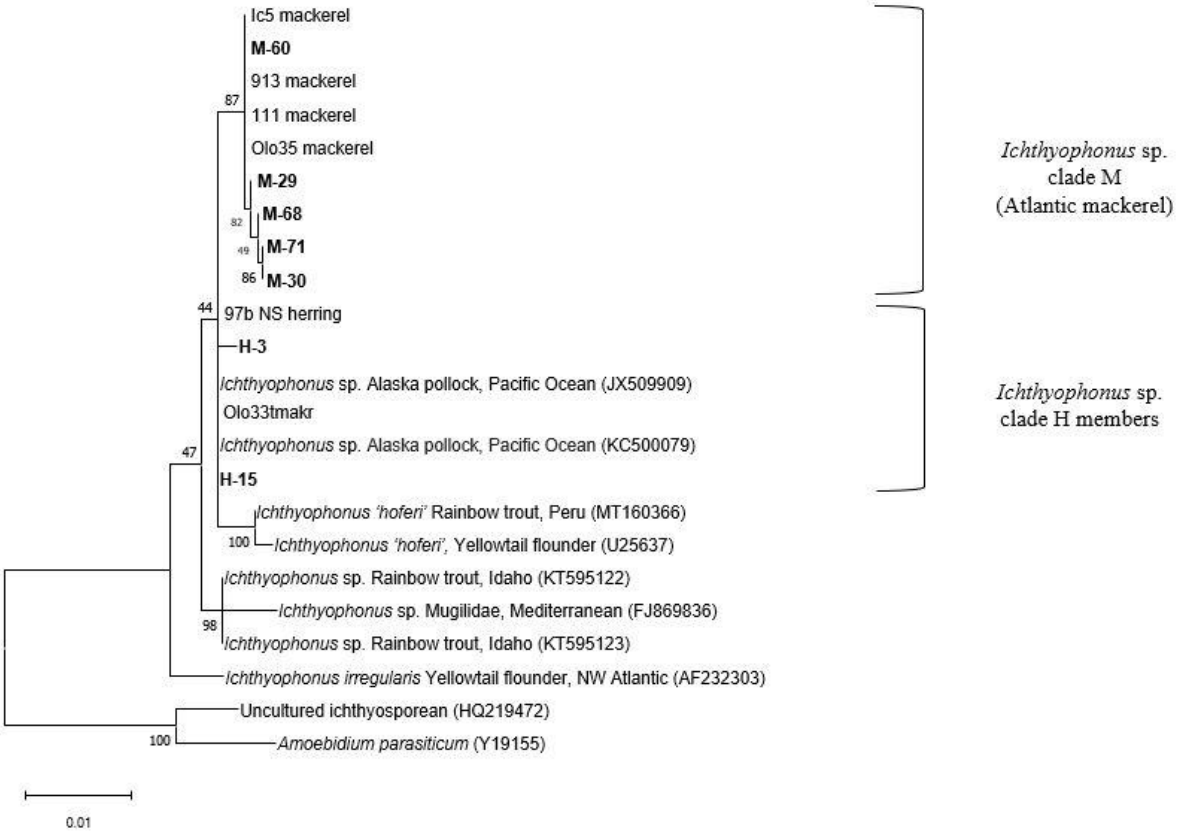

Figure 17. Maximum-likelihood tree (T92 substitution model) showing 18S rRNA gene distribution. M-x represent mackerel samples, clade H members include sequences from herring and other hosts that group together. Bootstrap values are indicated at the branch points, bar represents 0.01 substitutions.

4 Discussion

4.1 Methodological considerations

Sampling of the fish was done as thoroughly and quick as possible in order to get the best possible quality of the tissue samples for the different methods. The primary focus was to sample for histology, due to the fact that tissues degenerate quickly after death, and *Ichthyophonus* is known to quickly germinate in fish tissues post-mortem (Rahimian, 1998). IMR personnel also sampled the fish, and collected otoliths for determining age. However, due to the time-pressure, individual information for each fish was not noted. Information about which individual each sample came from was therefore unfortunately lost.

For histological sectioning, five mackerel and four herring were selected. These were the fish showing extensive macroscopic changes (visible white 'cysts'). Typical resting spores from PAS stained *Ichthyophonus* sp. were observed in all the examined tissues, except for mackerel, where no resting spores were found in the liver.

One mackerel and one herring were also chosen for serial sectioning in order to reveal characteristics objectively identifying the eccentric sagittal sections. The definite central section of the resting spores could be approximated, however it was not possible to distinguish them with absolute certainty from slightly eccentric sections, so all sizes are slightly estimated. This should have affected mackerel and herring *Ichthyophonus* resting spore dimensions in a similar way.

Another challenge was that rather few resting spores fit the criteria set prior to measurements, some showed signs suggesting onset of germination (bulbs in a thinner part of the wall, or partial cytoplasmic evacuation). Because of this, several spores were excluded and not measured.

The primer targeting 28S and 18S rRNA genes worked well for both species (M and H clade types). However, the primers targeting the ITS region worked only for the herring samples (Rasmussen *et al.*, 2010; Gregg *et al.*, 2016), not for the samples from mackerel. No product appeared on the agarose gels for mackerel. For herring, the samples that were sent for sequencing resulted in sequences with poor quality.

4.2 Discussion of results

Ichthyophonus sp. has been observed in fish since before the 1900s (Hofer, 1893, 1904; Lavéran and Pettit, 1910; Plehn and Mulsow, 1911; Sproston, 1944). Signs of infection included oval or round 'cysts' found in the internal organs (specifically the liver, kidney, heart, muscles and brain), skin ulcers, lateral swimming behaviour, low weight and in some cases mortality. This makes the parasite an important disease for wild fish, as well as a risk in aquaculture, especially when raw marine fish is part of the feed regime (Sindermann, 1990).

Even though *Ichthyophonus* sp. has a widespread global impact on both wild and farmed fish, little is known about the diversity withing the clade. Gregg et al. (2016) did a study on the ITS rDNA sequences on different hosts and ended up with 6 distinct clades. However, this study included only 10% of the approximately 150 reported host species (Gregg *et al.*, 2016).

The present study examined the difference in prevalence, histological morphology, in vitro culture growth, morphology, and sequence variation (rRNA marker genes) for *Ichthyophonus* sp. in mackerel and herring. The samples were gathered through different cruises, and a total of 475 mackerel and 2416 herring were inspected.

4.2.1 Prevalence

The different samplings conducted during this study show a significant difference in prevalence of *Ichthyophonus* infections between mackerel and herring (Table 6). For mackerel, the overall prevalence was 79% (ranging from 56-100%), and for herring it was 2.5% (ranging from 2.5-15%).

The prevalence of *Ichthyophonus* sp. in mackerel has been examined in different studies, but to a lesser extent than in herring. Sproston (1944) observed samples with prevalence up to 100% in mackerel, however the prevalence varied between the different catches examined (0-100%) over the three years she conducted the study in the North Sea (Sproston, 1944). The averages were reported to range from 38-70%, but she did not specify sample size (N). This makes it difficult to compare the results of the present study to the results of Sprostons' study, but the overall prevalence observed by Sproston seems to correspond well with the results seen in the present study. Muchelano et. al. (1986) observed histological samples of 23 lesions from 16 mackerel from the NE Atlantic, but only 4 of these showed *Ichthyophonus* sp. infection. However, it was not stated where these fish were caught (somewhere between the German Bight and Spain), and the true prevalence may have been higher. Would they have examined a higher number fish, the prevalence could have been different. This makes the knowledge on the

prevalence of *Ichthyophonus* sp. in mackerel low, which makes it difficult to come to an overall conclusion about the general prevalence of infection. However, the results from the present studies seem to correlate well with the previous studies done.

For *Ichthyophonus* sp. in herring, several studies have been conducted. The prevalence appears to vary temporally (Rahimian and Thulin, 1996; Kramer-Schadt, Holst and Skagen, 2010), and the parasite is known to have caused several mass mortality events, as seen in Øresund (1991) and in the North-Western Atlantic (1992), where large stocks of herring were lost (Hjeltnes and Skagen, 1992; Rahimian and Thulin, 1996). In the Gulf of St. Lawrence in 1954/56, it fluctuated between 10-78% with a yearly average of 27% in 1954 (Sindermann, 1958), whereas in Skagerrak-Kattegat the prevalence was at 11.3% with no mortality detected in 1991 (Rahimian and Thulin, 1996). Kramer-Schadt et. al. (2010) also showed that the prevalence in the catches from the Norwegian and Baltic Sea varied seasonally within the different years, with peaks during the summer and winter-months. The prevalence in the NSS herring in the Norwegian and Baltic Sea would vary from year to year, where stock prevalences levels of 10% in 1992 went down to almost extinction in later years, but increased again to a major peak in 1999 (Kramer-Schadt, Holst and Skagen, 2010). Russian researches reported prevalences of *Ichthyophonus* sp. in NSS herring Norewgian Sea and the Baltic Sea close to 100% (Hodneland, Karlsbakk and Skagen, 1997). IMR (1997) set up an evaluation of examination methods for *Ichthyophonus* sp. and found results very different from those from the Russian research, with prevalences below 6% in the wintering area in Tysfjorden-Ofoten (2.9%) and from commercial catches from the spawning area off Møre (2.8%) in Norway. This research showed nothing close to 100% as reported in 1993-1995 by Karaseva (Karaseva *et al.*, 1993; Hodneland, Karlsbakk and Skagen, 1997). The prevalence for NSS herring found in this study (4-15% prevalence in different catches) show great similarity to prevalence found in previous studies.

4.2.2 Distribution in different tissues

An indication of the distribution of *Ichthyophonus* spp. in different tissues in mackerel and herring was obtained from the prevalence of resting spores in the visceral organs and muscle tissue. In general, the prevalence of the parasite in the visceral organs and muscle tissue appears to depend on the type of *Ichthyophonus* sp., host species, total prevalence and the intensity of the infection. Table 8 gives an overview of the differences in prevalence shown in different tissues in different species obtained from this study and previous studies. When looking at the distribution of the resting spores, mackerel seems to have a higher intensity in kidney than herring. For mackerel, the organ with lowest prevalence in resting spores observed were the

heart. However, this is not the case for herring, where heart is the tissue with the highest prevalence. These results indicates a difference between the two species of fish, and could also indicate differences in the parasites infecting the two species.

Comparing the results from this study with previous studies, there is some similarities. The *Ichthyophonus* sp. type found in rainbow trout (Castro *et al.*, 2021) have a similar distribution of resting spores found in the kidney. For Castro et. al. (2011), 97% of the individuals were found to have resting spores in the heart, where in this study 74.1% where found. The different studies have different sample sizes, but the distribution seems to have a similarity. For other studies done for sprat, flounder and yellowtail flounder, there is no correlation for the distribution in mackerel.

When comparing the results for herring form this study to previous studies, there is also a correlation. Rahimian (1998) had the highest prevalence of resting spores in the heart (4.5 %) in herring, which is also found in this study (2.2 %). From the same study, flounder had a high prevalence in the heart (14.2 %) aswell. The overall prevalence for Rahimians study are generally higher, but the distribution seems to be the same, with the exception of lateral muscle. In this present study, only 3 (0.8 %) fish were found with resting spores in the lateral muscle tissue, while Rahimian had 12 (4.2 %) in herring. Huntsberger et. al. (2017) did also show a higher prevalence of resting spores in the heart (78.1 %) in yellowtail flounder than in other tissues examined, which correlates well with the findings in this study for herring.

Table 8. Shows the number examined and prevalence (%) of *Ichthyophonus* sp. resting spores observed in visceral organs and muscle tissue during the macroscopical examination and microscopical examination for the present study other studies (*).

Fish species	**No. Examined (Prevalence, %)**	**Heart**	**Spleen**	**Kidney**	**Lateral muscle**	**References**
Atlantic mackerel	475 (79)	166 (34.9)	324 (68.2)	352 (74.1)	172 (36.2)	This study
Atlantic herring	357 (2.5)	8 (2.2)	5 (1.4)	3 (0.8)	3 (0.8)	This study
Herring*	287 (13)	13 (4.5)	7 (2.4)	9 (3.1)	12 (4.2)	(Rahimian, 1998)
Sprat*	77 (9)	5 (6.5)	6 (7.8)	6 (7.8)	3 (3.9)	(Rahimian, 1998)
Flounder*	120 (17)	17 (14.2)	17 (14.2)	15 (12.5)	4 (3.3)	(Rahimian, 1998)
Rainbow trout*	33	27 (81.8)	18 (54.5)	32 (97.0)	ns	(Castro *et al.*, 2021)
Yellowtail flounder*	32 (78)	25 (78.1)	ns	12 (37.5)	ns	(Huntsberger *et al.*, 2017)

4.2.3 Parasite morphology in histological sections

Histological sections of *Ichthyophonus* sp. from mackerel and herring from the present study showed high similarity to observations from yellowtail flounder (Huntsberger *et al.*, 2017) and rainbow trout (Castro *et al.*, 2021). In yellowtail flounder the sections showed large granulomas with multiple small resting spores surrounded by necrotic tissue. The sections from rainbow trout also showed these large granulomas, but less resting spores. However, there were still more than one resting spores in the granuloma, surrounded by necrotic tissue. In our results, the herring endospores were present with hypha extending through ruptured cell walls, followed by inflamed and necrotic cells within the granuloma. The same was observed by Castro et. al. (2021), where the resting spores in heart tissue were surrounded by smaller resting spores, inside a developing granuloma (Castro *et al.*, 2021). In sections from mackerel there were no signs of this budding, and the sections usually showed one single resting spore surrounded by connective tissue and few necrotic cells. The same was observed for rainbow trout in the study by Castro et. al., where the resting spore in the skeletal muscle tissue were surrounded by connective tissue and not necrotic cells (Castro *et al.*, 2021). In general, the spores from mackerel were larger in size than those observed in herring (in this study) and in other fish species (Table 9).

4.2.4 *Ichthyophonus* spp. in vitro cultures

Microscopic observations of the in vitro cultures made from mackerel and herring showed that resting spore size clearly differed between mackerel and herring, being much larger in the former, even compared to the largest seen in herring. In mackerel, the median was 284 µm (ranging from 130-415 µm), compared to 181 µm in herring (ranging from 83-232 µm), making the mackerel spores significantly larger than those observed in other fish.

The hyphal width also varied between the two species examined in this study, and measurements from previous studies. The hypha width observed in *Ichthyophonus* from herring (this study) was 11-30 µm, 22-43 µm for mackerel, compared to 10-20 µm observed in rainbow trout (N. Okamoto *et al.*, 1985). The mean hyphal width was about double the size for mackerel (33 µm) than for herring (18 µm). Mean evacuated hyphal width was 21 µm in the *Ichthyophonus* sp. from mackerel and 13 µm in *Ichthyophonus* sp. from herring, where the broadest ranged from 22 to 27 µm in mackerel and 11 to 30 µm in herring. Few previous studies have investigated the differences in hyphal width, making it difficult to make an overall comparison between *Ichthyophonus* hyphae in different fish species. However, these results show that there is a significant difference between the resting spore size ($p<0.005$, T-test) and

hyphal width ($p<0.005$, T-test) found in the clades of *Ichthyophonus* from herring and mackerel and supports the suspicion of a different type of *Ichthyophonus* sp. in mackerel.

Results from this present study compared to the results of other studies (Table 9), the resting spore diameter varied: 83-232 (*I. hoferi*, this study), 130-415 (*Ichthyophonus* sp. this study), 11,7-250,3 (*I. hoferi*, Plehn & Mulsow, 1911), 90-130 (N. Okamoto *et al.*, 1985) and 6-199 (*Ichtyophonus* sp. (Franco-Sierra and Alvarez-Pellitero, 1999). Here, one can see the great difference in size between the resting spores for mackerel and herring. Comparing the results for mackerel and herring from this study, one can see that the resting spores are almost double the size in mackerel (130-415 µm) compared to herring (160-181 µm) ($p<0.005$, T-test).

Comparing the different measurements of resting spore diameter, hypha width and evacuated hypha, illustrated a difference between the results in this study and Plehn & Mulsows' original measurements in 1911 (Table 9). For *I. hoferi* in 1911, the median for the resting spores were 106 µm in trout and 181 µm in this study. However, for the present study, only one resting spore from herring was present as a single spore, whereas all other spores were accompanied by other smaller resting spores, making measurements less accurate. To make certain all measurements were comparable, only data from the one single resting spore from herring was included in the table (Table 9).

Table 9. Comparing *Ichthyophonus* sp. from different host species in µm (min-max).

			In vitro morphology				**Histology measurements**
Study	Location	Host Species	Thallus morphology	Resting spore diameter	Hypha width	Evacuated hypha	Resting spore diameter
Ichthyophonus sp. in Atlantic herring	NE Atlantic	Atlantic Herring	Circular raised/convex	181 (83-232)	18 (11-30)	13 (8-22)	45 (11-130)
Ichthyophonus in Atlantic mackerel	NE Atlantic	Atlantic Mackerel	Circular raised	284 (130-415)	33 (22-43)	21 (16-27)	557 (316-1002)
Ichthyophonus sp. of Sproston, 1944	NE Atlantic	Atlantic mackerel	ns	(250-570)	ns	ns	498*
I. hoferi sensu Plehn & Mulsow, 1911	NE Atlantic	Rainbow trout	ns	106 (11.7-250.3)	ns	ns	ns
Ichthyophonus sp. of Castro et. al., 2021	FW river (Peru)	Rainbow trout	ns	ns	ns	ns	93 (51-147)
Ichthyophonus hoferi, of Okamoto et. al., 1985	_	Rainbow trout	ns	(90-130)	(10-20)	ns	ns
I. irregularis of Rand, 1994	NW Atlantic	Yellowtail founder	Colaroid	ns	ns	ns	ns
Ichthyophonus. spp. of White et. al., 2013	NW Atlantic	Walleye pollock	ns	ns	ns	ns	(5-246)
Ichthyophonus sp. of Sierra, 1999	Mediterranian	Mullet	ns	(6-199)	ns	ns	ns

*Measurments taken from Sprostons study was collected from illustration fig. 19

4.2.5 Diversity

Phylogenetic analyses performed using the 28S and 18S rRNA genes indicated some genetic differences between *Ichthyophonus* sp. found in mackerel and herring.

Only two other 28S rRNA gene sequences from *Ichthyophonus* sp. are available in GenBank, one from rainbow trout in Idaho, USA (KT595186) and one from a yellowtail flounder from the Northeast Atlantic (AY026370). These M and H-clade sequences showed an identity of 98.9% and 95.6% respectively with KT595186 and AY026370. Although this dataset is limited, the available 28S rRNA data indicates that *Ichthyophonus* sp. found in mackerel is phylogenetically distinct.

The 18S rRNA gene sequences from mackerel were also unique. The sequences from herring grouped with Pacific Ocean gadids, which includes the same genotype that infects Pacific and Icelandic herring. Gregg et. al. (2016) also found different clades of *Ichthyophonus*, where 4 distinct clades stuck out: *I. irregularis*, a rockfish type, shad type of Hershberger and a clade C (Gregg *et al.*, 2016).

Sequence similarity for 18S rRNA gene obtained from GenBank and sequences from this study ranged from 96.1-99.7%, together with some sequences from *Ichthyophonus* sp. from other species. When grouped together with the M-clade and H-clade obtained, two clades grouped together in the phylogenetic tree (Fig. 17). In the M-clade, only samples from this study grouped together, while for the H-clade two clone samples from Alaska pollock grouped together with the two herring samples. Even though the dataset is limited, these results indicate a genetic difference between the clades of *Ichthyophonus* sp.

Overall, this study shows that there is a genetic difference between the M-clade and the H-clade of *Ichthyophonus* sp. found in mackerel and herring.

Several non-identical sequences in GenBank obtained from multiple fish species have been referred to as *I. hoferi*. *Ichthyophonus hoferi* was originally described from farmed salmonids in freshwater in Munich area, Germany. This species may be a separate freshwater species infecting native salmonids, or possibly be of marine origin, as marine fish was used as feed. Our ability to describe and name new species, is today hampered by the ambiguous identity of *I. hoferi* sensu stricto. It is therefore very important to obtain and characterize *I. hoferi* from German freshwater salmonids.

5 Conclusion

The prevalence of *Ichthyophonus* sp. infection was significantly higher in mackerel than in herring. The resting spores and hyphae of the parasite in mackerel were significantly larger and broader than the ones found in herring. rRNA gene sequences of the parasites from mackerel and herring clearly differed. *Ichthyophonus* sp. that infect mackerel and herring hence show different pheno- and genotypes, and are therefore likely separate species that are epizootiologically independent.

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7 Appendix I

7.1 Dehydration and Paraffin infiltration

To dehydrate the sections, they must undergo dehydration in the histokinette carousel. Table 1 lists the different stages in the program.

Table 1. Histokinette carousel program 2, dehydration and paraffin infiltration

Bath	Solution	Duration
1	buffer/4% phosphate buffered formalin	1h
2	50% ethanol	1h
3	70% ethanol	1h
4	80% etahanol	1h
5	96% ethanol	2h
6	96% ethanol	2h
7	100% ethanol	2h
8	100% ethanol	2h
9	xylen, hist	2h
10	xylen, hist	2h
11	paraffin 56-58℃	2h
12	paraffin 56-58℃	2h

In this study, paraffin casting was used for the different sections. Table 2 explains how to cast a tissue in paraffin.

Table 2. Standardized paraffin casting protocol description.

Step	Process description
1	When the histokinette carousel is complete, the samples are covered with paraffin from the bath in step 12.
2	The carousel is lifted, and the casting cassettes are exposed. Next, they are transferred to the heating block of the Kunz instruments FH4. Try to minimize the spilling of paraffin during this transfer and close the histokinette after.
3	An already warm stainless casting mould is filled with a bit of liquid paraffin (Histowax 56-58℃) from the dispenser on the machine.
4	The tissues are placed in the mould with paraffin.

5	The mould is placed on a cooling ice block that has been stored in the freezer in advance. With pre-heated tweezers, push the tissue towards the bottom of the mould until well attached. The tweezer will cool and stick to the tissue, so it is essential to work quickly.
6	The casting cassette from the infiltration process is now used to cover the mould with the tissue sample. Here it is essential to keep the marking of the cassette visible. The mould now needs to be filled up with liquid paraffin. This is to avoid any tearing during sectioning later on. It is also important to remember that delamination and breakpoints may appear if it takes too long before adding the second paraffin layer.
7	The stainless mould gets put on a tray containing freezing elements and then placed in a freezer (-20℃) for at least 15 to 20 minutes. This makes the paraffin block detach easily from the stainless mould.

7.2 HES staining

HES staining is a well-established protocol in the IMR system, but in this study, some changes have been made in Table 3. In stage 8, 2.5 minutes were shortened to 1.5 due to a highly concentrated Haematoxylin. In stage 14, 20 seconds were shortened to 10 seconds due to a highly concentrated Alcoholic saffron. Stage 19 has also been added as a safety margin.

Table 3. Standardized HES staining protocol for histological sections. Bath 8 is usually 2,5 minutes but 1,5 minutes with a new haematoxylin mix.

Bath	Solution	Time (minutes)
1	Xylene - hist	10
2	100% ethanol	5
3	100% ethanol	5
4	96% ethanol	5
5	80% ethanol	5
6	50% ethanol	5
7	Running tap water	5
8	Filtrated Haematoxylin	1,5
9	Running tap water	4

10	1% Erythrosin, aqueous, pH 6.5	1,5
11	Running tap water	1
12	96% ethanol	1
13	100% ethanol	1
14	Alcoholic saffron	10 sec
15	100% ethanol	1
16	100% ethanol	1
17	Xylene – hist	5
18	Xylene – hist	5
19	Xylene – hist	5

7.3 PAS staining

PAS staining is also a well-established protocol at IMR, but some changes have been made in Table 4. At stage 15, 2.5 minutes has been shortened to 1.5 minutes due to highly concentrated Haematoxylin. Stage 19 has also been added as a safety margin.

Table 4. Standardized PAS staining protocol for histological sections.

Bath	Solution	Time (minutes)
1	Xylene - hist	10
2	100% ethanol	5
3	100% ethanol	5
4	96% ethanol	5
5	80% ethanol	5
6	50% ethanol	5
7	1% periodic acid	10
8	Running tap water	10
9	Schiff' reagents	Minimum 20
10	Running tap water	10
11	Filtrated Haematoxylin	2
12	Running tap water	4
13	70% ethanol	5
14	96% ethanol	5
15	100% ethanol	5

16	100% ethanol	5
17	Xylene - hist	5
18	Xylene - hist	5
19	Xylene - hist	5

7.4 Slide assembly

Slide assembly protocol is a known protocol in the IMR system, and Table 5 explains the different details.

Table 5. Standardized slide assembly protocol description for histological sections.

Step	Process description
1	The slides are lifted with a tweezer and placed on a clean paper with the tissue side up.
2	Use one drop of the Histokitt glue on the coverslips. Place the coverslip with the adhesive facing down on the tissue.
3	Press lightly with the tweezer so all the air can escape.
4	Place the slide on a slide table without the lid.
5	Place a brass solder, 20 to 50 g, on the coverslip. The slide needs to stay leveled.
6	After two to three days of evaporation (xylene), the slide is ready to be studied.
7	Store the slide in an archive system for slides (for example, a marked slide cassette or a metal archive).

7.5 PCR programs

There was used three different PCR programs. Figure 1 illustrates the program regarding the 28S rRNA gene, figure 2 for the 18S rRNA gene and figure 3 for the ITS-region of *Ichthyophonus* sp.

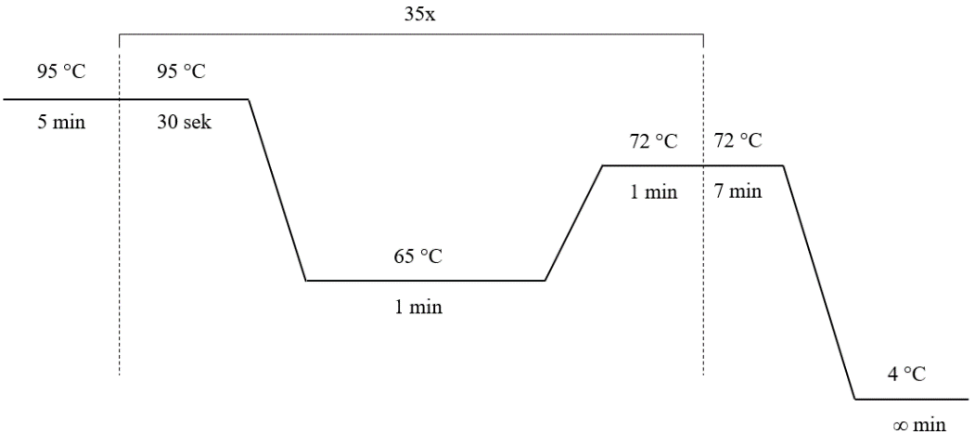

Figure 1. Illustration of the PCR thermoprofile for 28S. Stage 1: 5 minutes of denaturation at 95°. Stage 2: 35 cycles of denaturation at 95° for 30 seconds, annealing at 65° for 1 minute and one minute of extension at 72° . Stage 3: Final extension for 7 minutes.

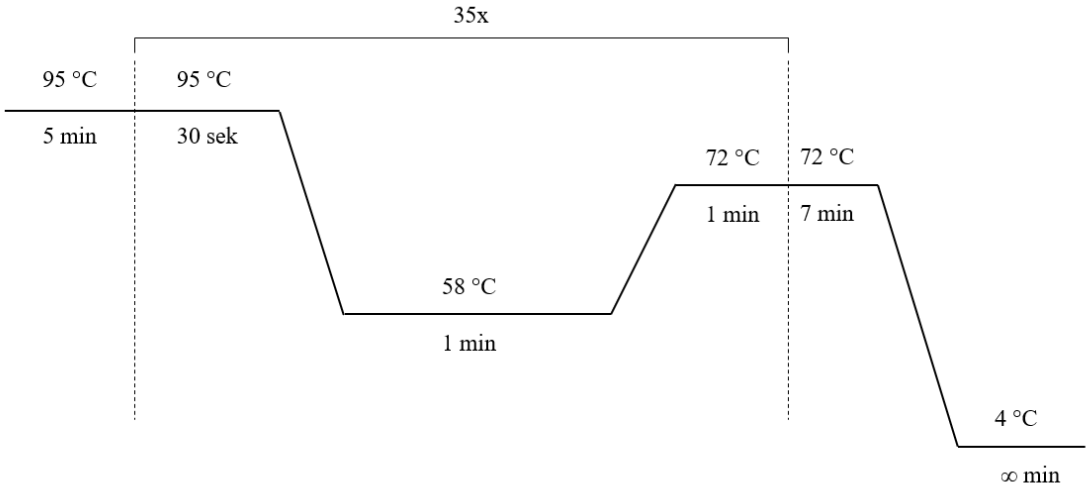

Figure 2. Illustration of the PCR thermoprofile for 18S. Stage 1: 5 minutes of denaturation at 95°. Stage 2: 35 cycles of denaturation at 95° for 30 seconds, annealing at 58° for 1 minute and 1 minute of extension at 72° . Stage 3: Final extension for 7 minutes.

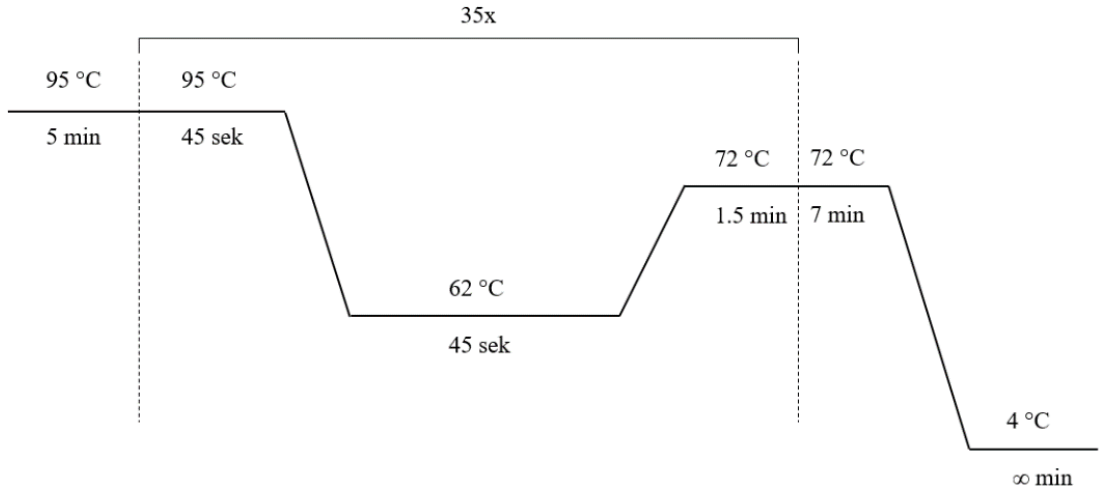

Figure 3. Illustration of the PCR thermoprofile for ITS. Stage 1: 5 minutes of denaturation at 95°. Stage 2: 35 cycles of denaturation at 95° for 45 seconds, annealing at 62° for 45 seconds and 1.5 minutes of extension at 72° . Stage 3: Final extension for 7 min.

7.6 Buffers and media

Buffer used is based on different literature, and is not identical to neither of the ones referred due to a different antibiotical mix (Nobuaki Okamoto *et al.*, 1985; Kocan, Hershberger and Winton, 2004; Kocan, Dolan and Hershberger, 2011)

7.7 Tris-HCl buffer – for high pH media

Required components:

- Tris base
- 5M HCl-solution

To prepare a 1M stock solution of Tris-Cl:

i) Dissolve 121 g Tris base in 800 ml H2O
ii) Adjust to desired pH with concentrated HCl. Approximately 70 ml HCl is needed to achieve a pH 7.4 solution, and 42 ml for a pH 8.0 solution
iii) Adjust volume to 1 liter with H2O
iv) Filter sterilize if necessary (and it is necessary)
v) Store up to 6 months at 4°C or room temperature

7.8 Glycine-HCl buffer – for low pH media

Table 6. Required components for making the glycine-HCl buffer.

Component	Amount	Concentration
Glycine (mw: 75.07 g/mol)	7.5 g	0.1 M
Hydrochloric acid (mw: 36.46 g/mol)	0.832g	0.02M

i) Prepare 800 mL of distilled water in a suitable container.
ii) Add 7.5 g of Glycine to the solution.
iii) Add 0.832 g of Hydrochloric acid to the solution.
iv) Adjust solution to final desired pH using HCl or NaOH
v) Add distilled water until the volume is 1 L.

7.9 MEM media

Required components:

i) Gibco™ MEM (with or without L-glutamine and phenol red)
ii) Fetal Bovine Serum (FBS) certified, One Shot™ format, United States
iii) Penicillin (5mg/mL)-Streptomycin (5mg/mL)-Neomycin (10mg/mL) Antibiotic Mixture (100x PSN antibiotic mixture)

Working sterile, the pH of Gibco™ MEM was adjusted to 7.8-8 using Tris-HCl buffer or to pH 2.5-3 using glycine-HCl buffer.

For high pH media, FBS was added to a final concentration of 5%. PSN antibiotic mixture was added to a final concentration of 0.1, 0.1 and 0.2 of the respective antibiotics in the mixture.

Low pH media was prepared like the high pH media, but was in addition added glucose to a final concentration of 1%.

7.10 Detection methods

An overview over what method confirmed the positive infection of Ichthyophonus sp. in the tissues can be seen in table 7.

Table 7. Overview over the different detection methods used on the suspected infected tissues. Mackerel (M) and herring (H) samples listed showing what detection method gave positive results.

			Method	
Sample	**Species**	**Tissue**	**In vitro culture**	**PCR**
AMH2	M	Heart	X	X
AMM5	M	Muscle	_	X
AMM6	M	Muscle	X	X
AMM7	M	Muscle	X	X
AMH8	M	Heart	X	X
AMM9	M	Muscle	X	
AMH11	M	Heart	X	X
AMH12	M	Heart	_	X
AMM13	M	Muscle	X	_
BMM1	M	Muscle	X	_
BMM3	M	Muscle	X	_
BMH4	M	Heart	X	_
BMH6	M	Heart	X	X
BMM7	M	Muscle	X	X
BMM8	M	Muscle	X	X
BMH9	M	Heart	X	_
BMH10	M	Heart	X	X
BMH11	M	Heart	X	_
ASM1	H	Muscle	X	_
ASH2	H	Heart	_	X
ASH3	H	Heart	X	X
ASM4	H	Muscle	X	X
ASM6	H	Muscle	_	X
ASH8	H	Heart	X	X
ASH9	H	Heart	X	_

Appendix II – short communication

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SHORT COMMUNICATION

High prevalence of *Ichthyophonus* sp. infections in Northeast Atlantic mackerel (*Scomber scombrus*)

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Ichthyophonus spp. are cosmopolitan parasites causing proliferative, systemic disease in a number of marine and freshwater fish, including several commercially important species such as Atlantic and Pacific herring (*Clupea harengus*, *C. pallasii*), rainbow trout (*Oncorhynchus mykiss*), Chinook salmon (*O. tshawytscha*) and sockeye salmon (*O. nerka*) (Gregg et al., 2016; Kocan et al., 2006; Rahimian & Thulin, 1996; Tierney & Farrell, 2004; Zuray et al., 2012). There is some uncertainty regarding both species diversity and host specificity within the *Ichthyophonus* genus, and at present only two species have been formally described, *I. hoferi* Plehn and Mulsow (1911) from rainbow trout and *I. irregularis* Rand et al., 2000 from yellowtail flounder (*Limanda ferruginea*). There are, however, strong genetic indications that the genus comprises more species than the two described so far (Hershberger et al., 2016; Rasmussen et al., 2010).

Atlantic mackerel (*Scomber scombrus*) is known to be susceptible to *Ichthyophonus* infections (Gregg et al., 2016; Johnstone, 1913; Murchelano et al., 1986; Sproston, 1944), but the prevalence has not been extensively monitored. A few studies indicate differences between geographic areas, seasons and individual shoals of mackerel. Sproston (1944) observed varying *Ichthyophonus* sp. (as *I. hoferi*) prevalence across different catches in the North Sea, ranging between 0% and 100% over a 3-year period with annual means of 38–70%, whereas Rahimian (1998) did not find any infected mackerel during a survey in the adjacent Skagerrak and Kattegat. Murchelano et al. (1986) observed infected individuals both in the eastern and western North Atlantic, but the general prevalence could not be determined due to low sample sizes. The diverging results of these studies indicate large differences in the prevalence of *Ichthyophonus* infections in Atlantic mackerel, possibly due to temporal fluctuations or variations in infection pressure in different geographic regions. Recent studies indicate frequent intermixing between the different spawning components within the Northeast Atlantic (NEA) mackerel stocks (Henriksen, Nøttestad, Olafsdottir, Slotte, & Sánchez, 2020; Jansen & Gislason, 2013), and infected individuals in some components may thus potentially spread parasites to other spawning components. NEA mackerel is also found increasingly further north and west, most likely due to changes in the migration pattern following climate change (Nøttestad et al., 2016; Nøttestad et al., 2020). Parasites infecting the mackerel, such as *Ichthyophonus* spp., can thus potentially spread and infect new fish host species with little or no inherent resistance to them, which could have great ecological and commercial ramifications. The diversity and prevalence of *Ichthyophonus* sp. in NEA mackerel should be monitored closely. The present study details our observations of the prevalence of *Ichthyophonus* infections in mackerel obtained from the Northeast Atlantic.

A total of 960 NEA mackerel were sampled during research cruises and from commercial catches in the North, Norwegian and Greenland Seas in 2019–2021. To assess the prevalence of *Ichthyophonus* sp., freshly caught or defrosted fish were examined macroscopically for visible signs of infection in the form of granulomas in the heart, kidney, spleen or red muscle tissue (Hodneland

TABLE 1 Prevalence of *Ichthyophonus* infections in catches of NEA mackerel from 2019–2021, tentatively observed macroscopically or microscopically as resting spores or hyphae in the heart, spleen, kidney or red muscle tissue

Cruise no./catch no.	Date	Geographical area	Coordinates (Decimal)		Fishing method	Research/commercial catch	Notes on examination	#of tentatively positive fish/#fish examined (percentage infected %)	
			Latitude °N	Longitude °E				Macroscopic[a]	Microscopic[b]
2019856/1[e]	08.06.19	North Sea	58.25	3.34	Purse seine	Commercial	Bycatch of mackerel from herring fishing	11/22 (50%)	11/11[c]
2019853/1[e]	02.10.19	North Sea	59.31	−0.63	Purse seine	Commercial	Examined fresh	16/22 (73%)	14/16[c]
2019854/1[e]	11.10.19	North Sea	59.02	−0.47	Purse seine	Commercial	Stored cold in tank (~2°C), examined 13.10.2019	25/30 (83%)	ND
2020811/1	04.06.20	North Sea	59.80	2.68	Purse seine	Commercial	Bycatch from herring fishing, stored cold in tank (~2°C), examined 06.06.2020	19/36 (52%)	ND
2020814/37582	30.07.20	Greenland Sea	74.92	5.51	Trawl	Research	Stored frozen until examination	63/100 (63%)	31/50 (62%)[d]
2020808/1	09.10.20	North Sea	58.98	−0.82	Purse seine	Commercial	Macroscopic examination on fresh fish, microscopic examinations on tissue stored frozen	51/100 (51%)	21/30 (70%)[d]
2020816/37451	01.07.21	Norwegian Sea	61.33	4.23	Trawl	Research	Examined fresh	20/25 (80%)	ND
2020816/37453	02.07.21	Norwegian Sea	61.33	1.09	Trawl	Research	Examined fresh	17/25 (68%)	ND
2020816/37454	02.07.21	Norwegian Sea	61.28	−0.97	Trawl	Research	Examined fresh	20/25 (80%)	ND
2020816/37457	03.07.21	Norwegian Sea	61.52	−4.01	Trawl	Research	Examined fresh	25/25 (100%)	ND
2020816/37460	04.07.21	Norwegian Sea	62.23	−3.05	Trawl	Research	Examined fresh	21/25 (84%)	ND
2020816/37462	04.07.21	Norwegian Sea	62.18	0.09	Trawl	Research	Examined fresh	22/25 (88%)	ND
2020816/ 37465	05.07.21	Norwegian Sea	63.93	6.63	Trawl	Research	Examined fresh	18/25 (72%)	ND
2020816/37467	06.07.21	Norwegian Sea	63.95	4.38	Trawl	Research	Examined fresh	17/25 (68%)	ND
2020816/37469	06.07.21	Norwegian Sea	63.90	−0.20	Trawl	Research	Examined fresh	18/25 (72%)	ND
2020816/37473	07.07.21	Norwegian Sea	64.36	−1.90	Trawl	Research	Examined fresh	20/25 (80%)	ND
2020816/37476	08.07.21	Norwegian Sea	64.77	0.91	Trawl	Research	Examined fresh	18/25 (72%)	ND
2020816/37479	09.07.21	Norwegian Sea	64.79	3.19	Trawl	Research	Examined fresh	18/25 (72%)	ND
2020816/37480	09.07.21	Norwegian Sea	65.58	9.26	Trawl	Research	Examined fresh	21/25 (84%)	ND
2020816/37491	11.07.21	Norwegian Sea	65.67	2.04	Trawl	Research	Examined fresh	14/25 (56%)	ND
2020816/37493	12.07.21	Norwegian Sea	65.55	−2.85	Trawl	Research	Examined fresh	23/25 (92%)	ND

TABLE 1 (Continued)

Cruise no./catch no.	Date	Geographical area	Coordinates (Decimal)		Fishing method	Research/commercial catch	Notes on examination	#of tentatively positive fish/#fish examined (percentage infected %)	
			Latitude °N	Longitude °E				Macroscopic[a]	Microscopic[b]
2020816/37496[e]	14.07.21	Norwegian Sea	67.36	−0.55	Trawl	Research	Examined fresh	22/25 (88%)	ND
2020816/37498	14.07.21	Norwegian Sea	67.40	2.01	Trawl	Research	Examined fresh	23/25 (92%)	ND
2020816/37500	16.07.21	Norwegian Sea	67.28	9.53	Trawl	Research	Examined fresh	24/25 (96%)	ND
2020816/37501	16.07.21	Norwegian Sea	67.22	11.74	Trawl	Research	Examined fresh	20/25 (80%)	ND
2021810/1	28.08.21	Norwegian Sea	63.72	2.93	Purse seine	Commercial	Examined after storage in tank (~2°C) appr. 48 h, followed by storage on deck (~10°C) appr. 24 h	8/25 (32%)	13/25 (52%)
2021810/2[e]	31.08.21	Norwegian Sea	61.99	1.69	Purse seine	Commercial	Fresh, examined after storage on deck (~10°C) for appr. 24 h	32/50 (64%)	34/50 (68%)
2021810/3	02.09.21	Norwegian Sea	63.36	1.42	Purse seine	Commercial	Fresh, examined immediately (1–8 h) after catch	34/50 (68%)	32/50 (64%)
2021810/3	02.09.21	Norwegian Sea	63.36	1.42	Purse seine	Commercial	Fresh, examined 18–21 h after catch	15/25 (60%)	19/25 (76%)
2021811/1	26.10.21	Norwegian Sea	63.01	4.54	Purse seine	Commercial	Stored frozen immediately after catch, thawed on deck (~10°C) for 48 h prior to examination	24/25 (96%)	24/25 (96%)

Note: Samples were obtained either from dedicated research cruises, or from research studies investigating fish obtained from commercial catches.

Abbreviation: ND, not determined.

[a]Visible granuloma containing resting spores in heart, spleen or kidney.

[b]Resting spores and/or hyphae observed microscopically in heart, spleen or kidney.

[c]Only samples positive for macroscopic signs were examined with microscopy.

[d]Random selection of samples examined regardless of macroscopic results.

[e]Tissue transplant cultures from these catches were used for genetic identification.

et al., 1997; Sproston, 1944). Moreover, the spleen and kidney of either all fish or a random sub-sample of fish from selected catches (see Table 1 for details) were examined microscopically for the presence of granulomas with thick-walled multinuclear bodies in the granulomas, generally called 'resting spores' (Okamoto et al., 1985) or schizonts (Kocan, 2013), and hyphae described by Meyers et al., (Meyers et al., 2019). Macroscopic observations of resting spores (Figure 1a–d, Table 1) indicated 32%–100% *Ichthyophonus* sp. prevalence in individual batches (Table 1). Further microscopic observations of resting spores and hyphae in spleen and kidney confirmed this finding (Figure 1e–g, Table 1).

In July 2021, samples for histology were prepared from internal organs and muscle tissue from selected NEA mackerel showing macroscopic signs of infections. The tissues were preserved on 4% formaldehyde (pH 6.9) and stained with haematoxylin-erythrosine saffron (HES) or periodic acid-Schiff (PAS).

Resting spores and hyphae observed microscopically were similar in size and appearance to those observed in NEA mackerel by Sproston (1944), and the histological sections showed the presence of typical *Ichthyophonus* sp. resting spores in the examined tissues (Figure 2). There were some differences in the microscopic observations depending on the time between catching and analysing the fish, and between fresh fish and fish that was stored frozen. In fresh fish examined within 1–8 h post catch, resting spores were visible while hyphae were not seen. However, some resting spores showed early signs of germination (Figure 1e–f). Hyphae, observed either as hyphal tips protruding from resting spores or as free hyphae in the tissues (Figure 1g), were mainly found in fresh fish examined >18 hours post mortem (Figure 3). In many instances, evacuated hyphae remained attached to the parental resting spore by a hyphal thread (Figure 1g). In fish examined 18–30 h post catch, hyphae were observed in most tissues harbouring resting spores (Figure 3). The observed lag in post mortem hyphal growth is consistent with the findings of Rahimian (1998) on infected herring, and may suggest that biochemical processes or changes in pH in the tissues trigger germination. Still, some fish examined >18 h post mortem contained resting spores only, with no hyphae or signs of germination being observed. It is unclear if these tissues contained predominantly ungerminated or dead spores.

Several bacteria and parasites can induce granuloma formation in fish that superficially resemble *Ichthyophonus* infections (Kocan et al., 2004; Murchelano et al., 1986), for example, *Mycobacterium* spp. which commonly occur in Atlantic mackerel (Murchelano et al., 1986). A study of Northwest Atlantic mackerel from New Jersey coastal waters found that 39% of the mackerel (N = 91) contained granulomas in the kidneys, but only 5% contained identifiable *Ichthyophonus* sp. stages (Murchelano et al., 1986). The currently most accurate method for confirming *Ichthyophonus* infections is through cultivation of infected tissues in selective growth media (Richard Kocan et al., 2011) or using PCR- or

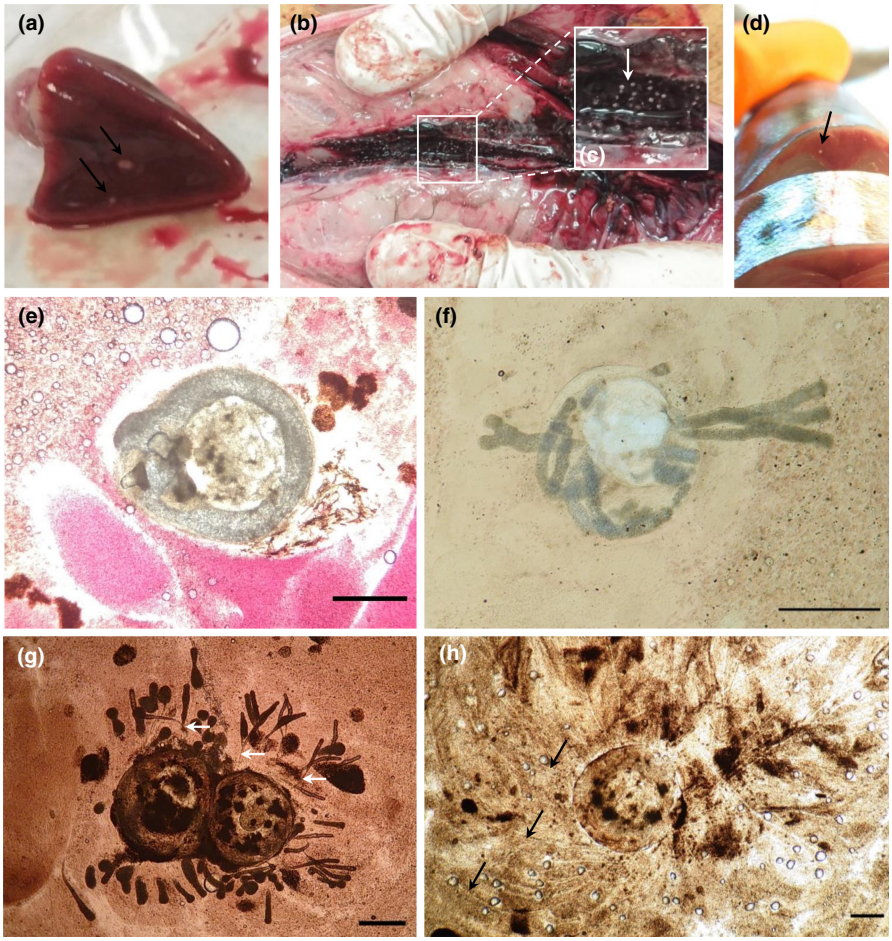

FIGURE 1 Macroscopic and microscopic signs of *Ichthyophonus* infection in NEA mackerel. Macroscopic signs seen as granulomas (arrows) on the heart (a), kidney (b,c) and in the red muscle (d). Microscopic signs of infection seen as granulomas containing resting spores, germinating resting spores and short hyphae in the kidney of fresh fish (e–g). (e–f) The first signs of germinating resting spores, approximately 8 h post mortem. (g) More advanced germination approximately 20 h post mortem. Arrows indicate evacuated hyphal tubes connecting the hyphae to the resting spores. (h) Kidney after freezing, with resting spore and connected hyphal tubes (arrow) and degraded hyphae. All scalebars are 250 μM

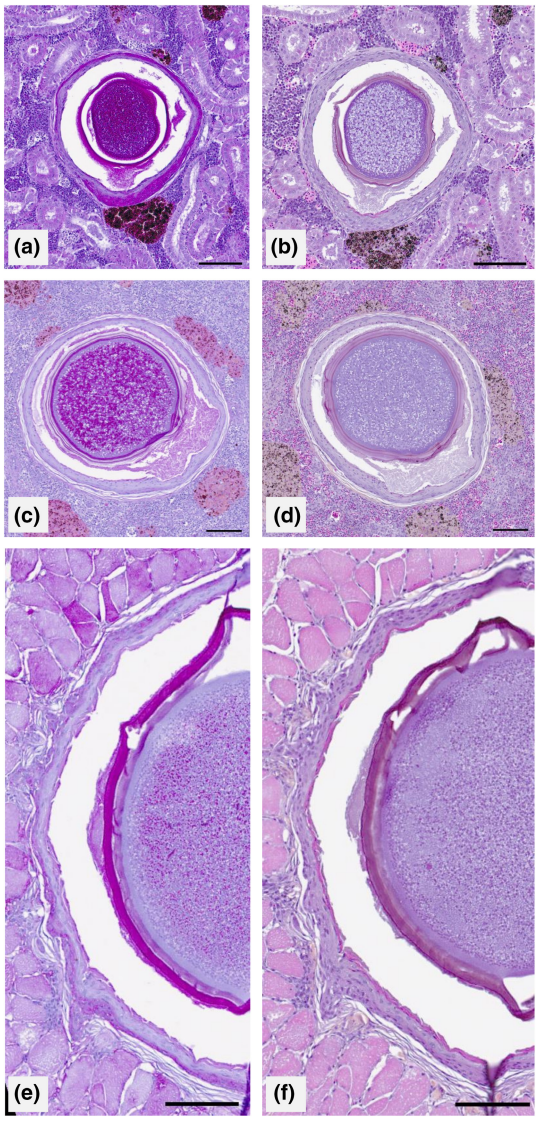

FIGURE 2 *Ichthyophonus* resting spores in NEA mackerel tissues. PAS- (a) and HES-stained (b) resting spore in kidney, spleen (c, d) and muscle tissue (e,f). All scalebars are 100 μM

qPCR-based assays (White et al., 2013). However, these methods can be very time-consuming and are not always feasible during routine examinations of large numbers of fish. In addition, PCR-based methods do not separate between living and dead parasites. An alternative method for detecting infections in NEA mackerel is to examine small pieces of the kidney and spleen for the presence of hyphal growth. No other histozoic marine fish parasites or fungi produce the hyphal growth seen in *Ichthyophonus* sp. The presence of aseptate hyphae in combination with resting spores in NEA mackerel tissues is therefore highly indicative of infection with live *Ichthyophonus* sp.

In August/September 2021, 50 and 75 fresh fish were examined microscopically at 1–8 and 18–30 h intervals post mortem (Table 1, Figure 2). Of 50 fish examined at 1–8 h, 33 displayed resting spores only, with a single mackerel showing signs of early hyphal growth (Figure 2). In the fish examined 18–30 h post mortem, resting spores were observed in 47 and 71 fish macroscopically and microscopically, respectively, whereas *Ichthyophonus* hyphae were observed in 43 of the fishes. Thus, 91% of mackerel displaying macroscopic signs of infection (granulomas with resting spores) also contained hyphae in the spleen and/or kidney, indicating that the majority of fish displaying granulomatous tissues were infected with *Ichthyophonus* sp.

Ichthyophonus resting spores do not survive prolonged freezing at −20°C (Athanassopoulou, 1992), and mackerel stored frozen in the present study only displayed hyphae in a few cases, most likely where freezing was delayed, allowing germination prior to freezing. Thus, hyphae were almost exclusively observed in fresh fish, that is, not previously frozen. Those hyphae seen in frozen mackerel often appeared evacuated, seemingly having lost their typical shape (Figure 1g). Such hyphae may easily be overlooked if not connected to a resting spore, making the observations less accurate. Granulomas, on the other hand, are readily observable in frozen fish, but can be confused with other parasitic or bacterial infections. Therefore, we considered microscopic detection of hyphal growth in the kidney and spleen of fresh fish, approximately 18–30 hours post catch, to be most reliable for detecting *Ichthyophonus* infections in the mackerel.

To confirm *Ichthyophonus* sp. presence and reveal the genotype, samples for tissue transplant cultures were taken from heart or muscle tissue of randomly selected, freshly caught mackerel (see Table 1). Samples were cultured in Tris-buffered MEM-media (Gibco™) contaning 5% fetal bovine serum at pH 7–9 or 2–3.5 as described by Okamoto et al. (1985) and Kocan et al., (2004), and kept at 15°C for 7–14 days prior to examination. Cultures that showed growth resembling *Ichthyophonus* sp. with spherical, multinucleate bodies growing from the tissue-samples, were confirmed by PCR and sequencing to be *Ichthyophonus* sp. based on their 18S ribosomal genes. Primers IchEK-F1 5′-ACCCGACTTCTGGAAGGGTTGT-3′ (a modified PIchF1 primer (White et al., 2013) and MesR1 5′-GCTTACT AGGAATTCCTCGTTGAAGA-3′ designed by EK were used with PCR settings: 5 min at 95°C, followed by 35 amplification cycles at 30s-95°C, 1 min-58°C, 1 min-72°C, followed by 7 min at 72°C. Sanger sequencing was done by Eurofins Genomics (Cologne, Germany). The resulting five sequences were identical and showed >99% similarity with *Ichthyophonus* sp. 18S rRNA gene sequences in GenBank® originating from rainbow trout and Alaska pollock (*Gadus chalcogramma*). Sequences obtained in this study are available in GenBank under accession no. OM869424-OM869428.

Melanomacrophage assemblies were observed in close association with granulomas in the kidney and spleen indicating an immune response by the fish (Figure 1f–g), but overall, infected fish did not differ significantly in Fulton's condition factor K ($K = 100 \times \text{Length}^3(\text{cm})/\text{Weight (g)}$; T-test, $p = .45$) from uninfected fish (Table 2). Hence, we found no signs of *Ichthyophonus* sp. being particularly pathogenic to NEA mackerel. A study on *Ichthyophonus* in Pacific halibut in North America found a similar pattern, with high prevalence but seemingly low pathogenic infections (Hershberger et al., 2018). In contrast, studies of *Ichthyophonus* in Pacific herring, Atlantic herring and American

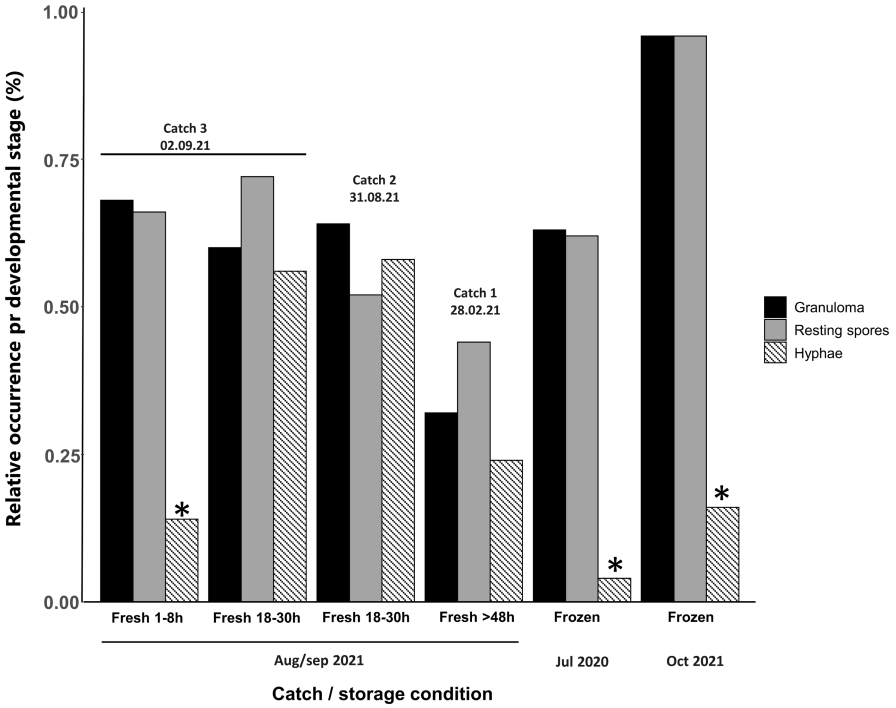

FIGURE 3 Macroscopic observations of granuloma and microscopic observations of resting spores and hyphae examined in fresh or previously frozen spleen and kidney of NEA mackerel at different time intervals post catch. * indicates that most hyphae observed were at an early germination stage, as seen in Figure 1e

TABLE 2 Descriptive statistics of mackerel length (cm) and weight (g) used to calculate Fulton's K. Results included are from microscopic examinations

	Length (cm) and weight (g)			
Variable	Mean	Minimum	Maximum	Std.dev.
Uninfected fish $N = 95$				
Length	35	21	40	2.7
Weight	340	206	645	93.5
Infected fish $N = 197$				
Length	36	30	55	2.9
Weight	440	193	692	97.3

shad (*Alosa sapidissima*) indicated that *Ichthyophonus* infections can be detrimental to host health (Richard Kocan et al., 2006; Marty et al., 1998) or may cause high mortalities (Rahimian & Thulin, 1996). This could be due to different immune responses in different fish species, with some hosts having higher level of tolerance to *Ichthyophonus* infections. Another possibility is that different strains or species of *Ichthyophonus* infect different fish species, and that *Ichthyophonus* sp. in NEA mackerel is less pathogenic than *Ichthyophonus* species infecting other fish hosts.

Future work should explore the species diversity and differences in host specificity between *Ichthyophonus* sp. infecting NEA mackerel and the strains or species that infect other fish. The migration pattern of NEA mackerel is changing rapidly, being found increasingly further north and west (Nøttestad et al., 2016; Nøttestad et al., 2020). Therefore, parasites such as *Ichthyophonus* spp. can be transported further north, potentially spreading to new naïve fish host species with little or no resistance to them. As the Arctic Ocean continues to warm up, *Ichthyophonus* should be monitored closely.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study will be made openly available in the Norwegian Marine Data Centre (https://nmdc.no/) upon publication, and a DOI will then be attached to the final version of the document.

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