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Prevalence of *Kudoa thyrsites* (Myxozoa, Multivalvulida) in Atlantic Mackerel, *Scomber scombrus* L., in the Vicinity of the Faroe Islands

Dánjal Petur Højgaard, Eydna í Homrum and Ian Salter**

Faroe Marine Research Institute, Tórshavn, Faroe Islands

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***Correspondence:**
Ian Salter
ians@hav.fo
Dánjal Petur Højgaard
danjalpeturh@gmail.com

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Atlantic mackerel *Scomber scombrus* is an important migratory fish in Faroese waters and the Northeast Atlantic at large. Similar to other fish species from marine environments, mackerel can be infected by the myxozoan parasite *Kudoa thyrsites*, leading to myoliquefaction that renders the fish commercially unviable. Despite the ecological and economic significance of *K. thyrsites* as a parasite, little is known regarding its prevalence in the Faroese mackerel fishery. Prior to analysing field samples, we examined 104 samples of Atlantic mackerel selected from a Faroese processing plant on the basis of visible soft-tissue. Using microscope smears we observed *K. thyrsites* in 98% of the soft-tissue mackerel specimens and a direct comparison with qPCR demonstrated strong agreement between the two techniques ($\Phi = 0.429$, $p<0.01$; Fisher's exact test). We used qPCR to analyze a total of 594 Atlantic mackerel specimens, collected from Faroese fishing grounds during 2017 and 2018. Overall prevalence was 4.1% (95% confidence interval; 2.5-5.7%) and ranged from 0-14% at different locations. Infection by *K. thyrsites* was documented in fish as young as 2 years (195g) and up to 14 years (615g) of age. A logistic regression model indicated the odds of infection were statistically associated with sampling location and month, but not gender, age, weight or length, although a Chi-squared test on age categories showed the oldest fish (>10 years) had highest prevalence (10.8%, CI: 4.2-17.5%). In order to investigate potential infection pathways we performed qPCR analysis on eDNA in water samples collected from the Faroese Plateau. However, we found no molecular evidence for *K. thyrsites* spores in Faroese plankton assemblages. Our data support the prevailing hypothesis that both the alternating invertebrate host and infection of Atlantic mackerel by *K. thyrsites* occurs in more southern waters.

Keywords: *Kudoa thyrsites*, Atlantic mackerel, Faroe Islands, myoliquification, FAMEOS, Faroese Marine Ecosystem Observing Study, eDNA

INTRODUCTION

Fish infected with myxosporidia from the family *Kudoidae* (Meglitsch, 1947) show myoliquefaction, more commonly referred to as soft-tissue. The mechanism for this phenomenon has been linked to proteases, like cathepsin, which are released by the pre-sporogenic plasmodia into host muscle tissue (Henning et al., 2013). The post mortem drop in muscle pH boosts the activity of the cathepsin enzyme (Levsen, 2015) leading to further decomposition. Notably, the presence of parasite-derived proteases in fresh and frozen/thawed commercial fish species can render the filet unfit for consumption, leading to significant economic losses.

The number of documented species in the genus *Kudoa* has increased from 44 (Moran et al., 1999a) to close to 100 (Levsen, 2015). Myoliquefaction of numerous fish species occurs in association with the genus *Kudoa* across a broad range of environments. In particular, *K. thyrsite*s exhibits a global distribution (Whipps and Kent, 2006) and has been reported in 18 different fish families, comprising 9 orders (Henning et al., 2013), it is thus of major concern for both capture fisheries and aquaculture. It has been reported in northeast Pacific Hake (*Merluccius productus*) (King et al., 2012), Mediterranean silver scabbardfish (*Lepidopus caudatus*) (Kovaleva et al., 1979; Giulietti et al., 2019) and Atlantic mackerel (*Scomber scombrus*) (Levsen et al., 2008). Aquaculture of Atlantic salmon (*Salmo salar*) in British Columbia, Canada has suffered financial losses due to *K. thyrsites* infection (St-Hilaire et al., 1997a, St-Hilaire et al., 1998; Moran et al., 1999b). More recently, *K. islandica* has been identified in spotted wolfish (*Anarchicas minor*), Atlantic lumpfish (*Anarhichas lupus*) and lumpsucker (*Cyclopterus lumpus*) in the North Atlantic (Kristmundsson and Freeman, 2014). Additionally, *K. islandica* has been found in reared lumpsucker in Norway (Alarcon et al., 2015). Currently, there are no documented cases of *K. thyrsites* or *K. islandica* in Faroese aquaculture.

Atlantic mackerel is an economically important species for the Faroe Islands, and in the Northeast Atlantic at large. Previous work has developed our understanding of mackerel in regards to feeding preferences (Langøy et al., 2012; Bachiller et al., 2016), distribution of spatial biomass (Nøttestad et al., 2016) and recent patterns of geographical expansion (Ólafsdóttir et al., 2019). Earlier work in the northern North Sea found up to 8.9% of mackerel exhibiting "soft flesh" phenomenon in association with *K. thyrsites* spores in muscle tissue (Levsen et al., 2008). Annual surveys of mackerel have documented prevalence of post-mortem myoliquefaction at 0.6-3.1% (n=2500) and have observed significantly lower prevalence in medium (400-600g) compared to large-sized (>600g) mackerel (Levsen, 2015, Giulietti et al. 2022).

Kudoa thyrsites is classified as a multivalvulid myxosporidia (Myxozoa) (Lom and Dyková, 2006), an obligate parasite that alternates between invertebrate and vertebrate hosts (Kent et al., 2001; Americus et al., 2020). The general biology of Myxozoa displays bi-phasic life cycles that alternate between myxospores and actinospores and is well characterized in fresh water myxozoans (Yokoyama, 2003). Actinospores are released from an invertebrate host and may attach and enter a fish host, resulting in the development of a plasmodium (Lom and Dyková, 2006; Okamura et al., 2015). The skin, fins, gills and buccal cavity have all been demonstrated as portals of entry for actinospores (triactynomyxon) of *Myxobolus cerebralis* (El-Matbouli et al., 1995). The release of myxospores can occur when the fish host is alive, through the egestion of phagocytosed spores as observed in common Carp (*Cyprinus carpio*) (Ogawa et al., 1992). In *Kudoa* spp., it has been suggested that post-mortem myoliquefaction may serve to facilitate myxospore release (Langdon, 1991). Most actinosporeans have been reported to infect oligochaetes (Kent et al., 2001), although polychaetes, sipunculids, bryozoans and cephalopods have been described as invertebrate hosts for myxozoans (Yokoyama, 2003). The invertebrate host for *K. thyrsites* infection in mackerel is unknown. Infections in aquaculture are known from exposure to infected seawater (Moran et al., 1999c; Jones et al., 2012) and PCR-based analysis of raw seawater from infection hotspots detects *K. thyrsites* year round (Jones et al., 2016).

In recent years, quality control procedures used at land-based fish processing plants in the Faroes have identified soft-flesh in the mackerel fishery as an economically significant problem. However, the prevalence of *K. thyrsites* infection in Faroese fishing grounds remains currently uncharacterised. The specific objectives of the present study were (i) compare detection of *K. thyrsites* with microscopic and molecular techniques as the causative agent of post-mortem myoliquefaction, (ii) determine overall prevalence and spatio-temporal variability of *K. thyrsites* in the commercial Faroese fishery and (iii) examine the potential occurrence of *K. thyrsites* in water samples in Faroese waters.

MATERIAL AND METHODS

Sampling of Muscle Tissue

Selected Soft-Flesh Mackerel Specimens From Production Plant

A total of 104 mackerel were obtained from a land-based commercial production plant in the Faroe Islands (Varðin Pelagic, Tvøroyri). The samples originated from the production line in August-November 2015 and were selected on the basis of those specimens that exhibited evidence of post-mortem myoliquefaction (i.e. "soft mackerel"). All samples were stored at -20°C at the Faroe Marine Research Institute until further analysis.

Mackerel Specimens From Faroese Fishery

A total of 584 mackerel were sampled from commercial trawlers and during two research trips of the Faroe Marine Research Institute during 2017 (#184) and 2018 (#400). Samples were collected from different locations from the Faroese fishery, both in Faroese and British territorial waters (**Figure 1**). Full details of the region and time of year samples were collected are documented in **Tables 1**, **2**. The biological parameters sampled were pinched-tail-length (Hansen et al., 2018), ungutted weight, sex, sexual maturity and age (as inferred from annual growth

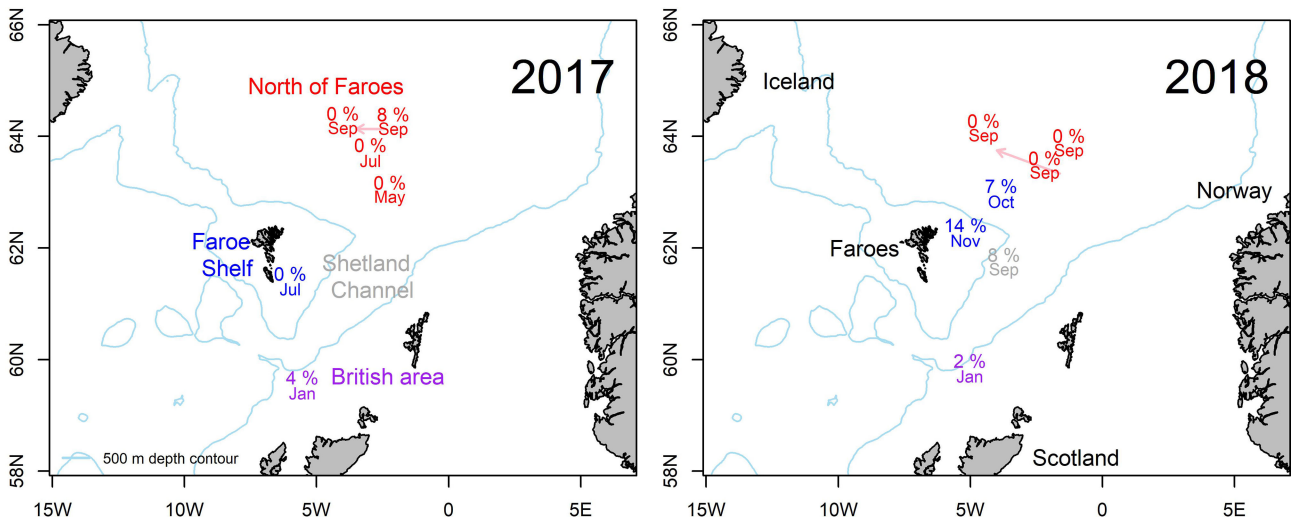

FIGURE 1 | Map of the study area documenting sample locations. Prevalence of *Kudoa* thyrsites in Atlantic mackerel in 2017 (left) and 2018 (right). Month of capture and prevalence of infection is shown. Arrows indicate displacement of text label for plotting clarity. Text colours correspond to different sampling regions: North of Faroes (Red), Faroe Shelf (Blue), Shetland Channel (grey) and British Area (purple).

TABLE 1 | Prevalence of *Kudoa thyrsites* infection in Atlantic mackerel samples collected during 2017 and 2018.

Date	Sampling area	No. Of fish	Infected Mackerel	Prevalence %
29/01/2017	British Area	25	1	4
05/05/2017	North of Faroes	22	0	0
06/07/2017	The Faroe Shelf	20	0	0
11/07/2017	North of Faroes	18	0	0
15/09/2017	North of Faroes	50	0	0
18/09/2017	North of Faroes	50	4	8
16/01/2018	British Area	50	1	2
16/09/2018	North of Faroes	50	0	0
20/09/2018	North of Faroes	50	0	0
23/09/2018	North of Faroes	50	0	0
28/09/2018	Shetland Channel	50	4	0
09/10/2018	The Faroe Shelf	50	3	8
09/10/2018	The Faroe Shelf	50	4	6
08/11/2018	The Faroe Shelf	50	7	8

TABLE 2 | Contingency table of methodological comparison between microscope smears and qPCR.

	qPCR - positive	qPCR - negative
Microscope - positive	94	8
Microscope - negative	0	2

increments of the otoliths). All samples were stored at -20°C prior to further analysis.

Analysis of *K. thyrsites* Infection

The 104 soft-flesh samples obtained from the factory processing plant were analysed using both microscope smears and qPCR detection. The 594 field samples of mackerel from the wild fishery were analysed with qPCR. Positive molecular detections were verified by microscope smears of archived muscle samples.

Microscope Smears

Specimens were defrosted at room temperature and muscle samples extracted (approx. 1.5 cm deep) from both the left and right side of the anterior dorsal fin. Muscle smears were prepared and examined by light microscopy (Optica DM-20) at 400x magnification. An approximately 1mm x 1mm piece was extracted from each muscle sample with a scalpel (chopping of muscle fibres) and forceps and used to prepare microscope wet-smears (St-Hilaire et al., 1997a; Giulietti et al., 2022). Muscle samples were subsequently stored at -20°C for molecular analysis. The left side was used for sample preparation and the right-side as a sample archive. One-hour searches were conducted on each smear for the detection of *K. thyrsites* spores, which were recorded photographically (**Figures 2**, **3**). The selected smears were preserved by mounting in glycerol-gelatin (Berland, 2005). Samples were classified as infected or not-infected depending on the detection of *K. thyrsites* spores. *K. thyrsites* were clearly identified by their morphology, specifically the presence of one enlarged polar capsule (**Figure 3**), which are evenly sized in the recently documented *K. islandica* (Kristmundsson and Freeman, 2014).

qPCR Detection

The three muscle tissue replicates (approx. 200 mm^3) were extracted separately using a HotSHOT DNA preparation based on a previously published method (Truett et al., 2000). In brief, 50 µL of alkaline lysis buffer (25 mM NaOH, 0.2 mM disodium EDTA, pH 12) was added to a sterile 200 µL PCR tube. The muscle sub-samples were added to the tube and mashed with a sterile spatula. The lysis solution was incubated in a PCR

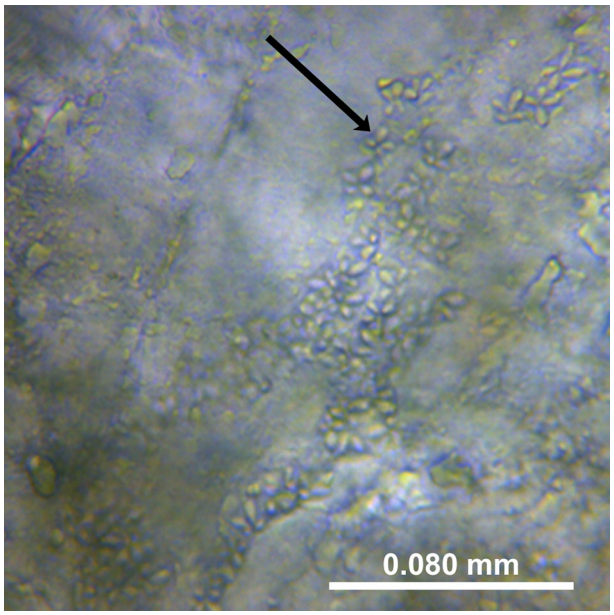

FIGURE 2 | A photo from a wet smear of Kudoa thyrsites spores from the soft mackerels examined. The muscle is partially degraded by the cathepsin enzyme; magnification 400x.

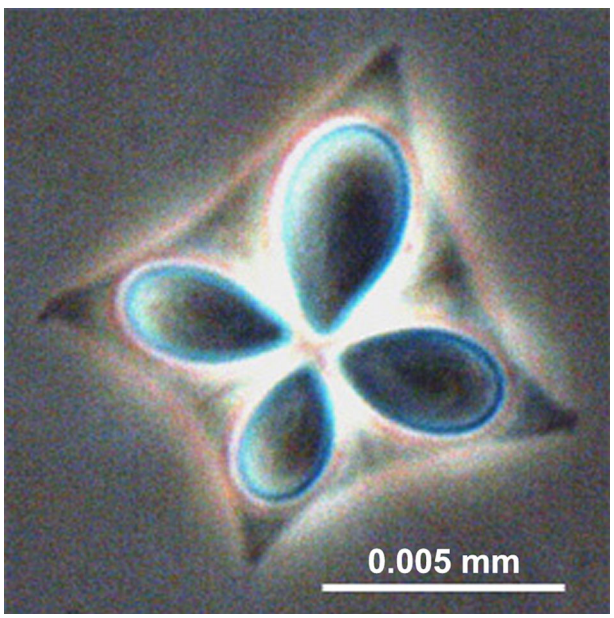

FIGURE 3 | A single spore of Kudoa thyrsites with four nematocysts (polar capsules) clearly visible. (phase contrast. Levsen, 2015).

machine preheated to 95°C for 30 min. and cooled at 4°C. Subsequently 50 µL of neutralization buffer (40 mM Tris-HCl, pH 5) was added to the tube, which was vortexed and incubated for 10-15 min. The tube was centrifuged to collect cellular debris and the supernatant removed and diluted 1:10 with nuclease-free water for subsequent PCR reactions.

Presence/absence tests were performed on the diluted lysates based on a previously published method validated for *K. thyrsites* (Funk et al., 2007). Each reaction well contained 4µL of QuantiTect, 0.25 µL of *Kudoa* Probe, 0.4 µL of *Kudoa* Primer-F, 0.4 µL of *Kudoa* Primer-R and 2.95 µL of nuclease-free water. 2 µL of DNA template was added to bring the reaction volume to 10 µL. Samples were 1:10 dilutions of the HotSHOT lysate. *Kudoa* Probe: VIC-TATCGCGAGAGCCGC-MGB. *Kudoa* F-primer: TGGCGGCCAAATCTAGGTT. *Kudoa* R-primer: GACCGCACACAAGAAGTTAATCC. Positive controls were included in each plate run that were obtained from a previous analysis of visibly infected tissue that yielded consistent amplification in PCR runs. The negative control replaced DNA templates with 2µL of nuclease-free water. Reactions were carried out in 96-well plates using an Applied Biosystems StepONE Plus realtime PCR platform. Samples were analysed in triplicate, corresponding to the three muscle sub-sample tissues extracted independently. The amplification profile was 50°C for 2 mins and 95°C for 15 mins then 45 cycles of 94°C for 15 s and 60°C for 1 min. The Ct value was defined as 20 times the standard deviation of the baseline fluorescent signal. Samples were classified as positive for *K. thyrsites*, if more than 1 of the 3 subsamples exhibited positive amplification.

Plankton Samples Collected From Faroe Plateau

Water Column Sampling

Water column samples were collected on the Faroese Shelf from January through to December (2018) with weekly to biweekly resolution (61.9703 °N. 6.8805 °W) as part of the Faroese Marine Ecosystem Observing (FAMEOS) program. Approximately 9 L of sample water were collected in acid-washed carboys following thorough rinsing (four times) with sample water. Seawater samples were collected using a pump (6m) from a land-based sampling facility connected to the fjord. The depth of the fjord is 18m and is well-mixed throughout the year due to tidal currents. A sample volume of 1.5 L replicate was collected onto 0.2 µm sterivex filters (Millipore; #SVGP01050) using a peristaltic pump and silicon tubing. Between each sampling event, the silicon tubing was stored in a solution of 10% v/v HCl. Prior to sampling, the tubing was flushed with 5 volumes of distilled water and 1 L of sample water prior to connecting sterivex tubing. Upon completion of the filtration the Sterivex filters were capped and stored at -80°C until extraction.

DNA was extracted directly from the Sterivex cartridges using a modified protocol of the Qiagen Dneasy Blood and Tissue Kit (Qiagen, #69504). Sterivex cartridges were removed from the freezer and allowed to defrost at room temperature for 20 min. A final check on residual volume was performed by expunging air through the Sterivex cartridge with a 50mL sterile syringe. The male nipple of the Sterivex cartridge was flame sealed and extraction reagents were added directly inside of the cartridge using sterile filter pipette tips. Extraction proceeded according to the manufacturer's instructions with the following modifications.

A volume of 720 µL of buffer ATL and 80 µL of proteinase K was added directly to the interior of the Sterivex cartridge. The female inlet was capped with a male luer-lock cap. The Sterivex cartridges were then placed in a rotary spinner and incubated at 56°C for 2 h. The cartridges were rotated 90° around their central axis every 30 min to ensure even coverage of the filter roll with extraction solution. The lysis solution was removed from the Sterivex cartridge using a sterile 3 mL luer-lock syringe and transferred to sterile DNase-free 2mL Eppendorf tubes. The Eppendorfs were pulse vortexed (10 s) and spun down in a mini-centrifuge. Subsequently, 600 µL of extraction solution was transferred to a clean 2 mL Eppendorf tube, followed by 600 µL of buffer AL solution. These Eppendorf tubes were pulse vortexed (10 s) to mix and centrifuged. Taking each Eppendorf in turn, 600 µL of ethanol was added followed by pulse-vortexing and centrifugation. This extraction solution was added to the Qiagen DNEasy Blood and Tissue kit spin columns in 3x ~ 600 µL aliquots. The spin column procedure followed the manufacturers instructions. DNA was eluted from the spin column in 120 µL of nuclease-free water following an incubation period of 5 min at 37°C. The flow-through from the first elution step was pipetted back onto the column for a second elution under identical conditions.

DNA concentrations in the extracts were measured with a Qubit Fluorometer and Qubit dsDNA HS assay (Q32854). Following the manufacturer's instructions, DNA extracts were frozen at −20°C until further processing (Salter et al., 2019). Presence-absence tests for *K. thyrsites* were carried out according to the qPCR protocol described in section 2.2.2. Single filter replicates were analysed in triplicate on non-diluted DNA extractions. Inhibition controls were carried out on seawater samples using a commercially available assay (Techne TKIT06035). An Applied Biosystems StepOnePLUS realtime PCR platform was used for amplification. DNA extract aliquots (40 µL) were spiked with 5 µL of control template. Each PCR reaction mixture of 20 µL contained 5 µL sample (including control template), 10 µL of 2X qPCR mastermix (TKITMM01), 2 µL of control primer/probe (TaqMan hydrolysis probe) and 3 µL of nuclease-free seawater. Template and primer/probe sequences are proprietary (Techne). PCR reactions were performed under thermocycler conditions of 2 mins at 95°C and 50 cycles of 10 s at 95°C and 60 s at 60°C. Fluorogenic data was collected through the VIC channel. Inhibition was tested for in four technical PCR replicates of each sample. A comparison of Ct values between samples including internal control template and nuclease-free seawater with internal control template was used to diagnose inhibition, where a Ct shift of >3 was considered as inhibitory (Salter et al., 2019). Inhibition was not detected in any of the seawater samples.

Statistical Analysis

Mean-Square Contingency Coefficient (Φ)

Statistical agreement between the visual and molecular detection methods was determined from binomial presence-absence data and calculated from mean square contingency coefficients (e.g. Salter et al., 2019), a metric commonly referred to as Φ (Cheetham and Hazel, 1969). Visual and molecular detection rates were treated as binary variables (1 = presence and 0 = absence). The two independent detection methods were compared in a 2x2 contingency table. The phi coefficient (Φ) was calculated according to Equation 1 and can be considered as analytically equivalent to the Pearson's product moment correlation taking values that range from -1 (negatively correlated) to 1 (positively correlated).

$$\phi = [(ad) - (bc)]\sqrt{(a+b)(c+d)(a+c)(b+d)} \quad (1)$$

where a = presence-presence, b=presence-absence, c=absence-presence and d= absence-absence.

Chi-Squared and Fisher's Exact Tests

Following the recommendation of (Rósza et al., 2000) we define prevalence as the proportion of examined individuals that tested positive for *K. thyrsites*. Statistical comparisons of prevalence were based on the analysis of 2x(n) contingency tables, where rows represented the binomial categories of infected or not infected and (n) the number of columns representing categorical descriptor variables (e.g. year, age class). If all cells of the contingency table had expected frequencies >5 we used a Chi-squared test, if this assumption was not met, and or the contingency table was 2x2, we used a Fisher's exact test, significance level,$\alpha = 0.05$.

Logistic Regression (Generalised Linear Model)

Statistical analyses were run in the R environment (RStudio Team, 2020) using base R and the aod package (Lesnoff and Lancelot, 2019). We used a logistic regression to model the odds of infection as a binomial outcome from a linear combination of the following predictor variables: latitude (location), time (month), age (year class), size (weight) and gender (M/F). Predictor variables were continuous, except for gender, which was included as a binary variable. Model fit was measured by examining if the model including predictor variables fits significantly better than a null model (intercept only). The test statistic compares the residual deviance of the two model versions from distributed Chi-squared values with degrees of freedom equal to the number of predictor variables in the fitted model. The statistical significance of model coefficients was examined from Chi-squared tests, significance level,$\kappa\alpha = 0.05$.

Prevalence Confidence Intervals

Prevalence data are presented with 95% confidence intervals, calculated according to the following Equation 2:

$$p' - Z_\alpha \sqrt{\frac{p'q'}{n}} \leq p \geq p' + Z_\alpha \sqrt{\frac{p'q'}{n}} \quad (2)$$

where p' = x/n, x = samples positive for *K. thyrsites*, n = number of samples, q' = 1-p', $\alpha = 0.05$, $Z_\alpha = 1.96$

Welch Two Sample t-Test

The inter-annual differences between length and age parameters of the sampled mackerel were determined using a Welch two sample t-test to account for unequal sample size/variance in 2017 and 2018.

RESULTS

Detection of Infections in Soft-Flesh Specimens From Factory Processing

A total of 104 soft-flesh samples obtained from the factory processing plant in 2015 were examined both by microscope smears and qPCR (**Table 2**). The proportion of *K. thyrsites* spores (**Figure 2**) was 98% from microscope smears compared to 90% from qPCR. No samples scored positive by qPCR and negative by microscopy. In some cases it was possible to observe multiple spores in the soft-flesh mackerel specimens (**Figure 3**). The mean square contingency co-efficient calculated from the 2x2 contingency table was 0.43. A Fisher's exact test also demonstrated a significant association ($p<0.001$) of detection outcome from the two methodological approaches.

Prevalence in Faroese Commercial Fishery

The mackerel sampled in 2017-2018 ranged from 2-14 years (**Figure 4A**) corresponding to a length range of 28.1-41.1 cm (**Figure 4B**) and weights of 190-660g (data not shown). Fish 5 years and older dominated in both 2017 (median = 6 years) and 2018 (median = 8 years). The size at age was also comparable between 2017 and 2018 (**Figure 4C**). Welch's two-sample t-test showed that on average mackerel sampled in 2017 were significantly younger ($p<0.001$) and smaller ($p<0.01$) than in 2018.

The overall prevalence of *K. thyrsites* detected by qPCR was 4.1% (95% confidence interval (CI): 2.5-5.7%). Prevalence was higher in 2018 (4.8%, CI: 2.7-6.8%) compared to 2017 (2.7%, CI: 0.4-5.1%) but the overlapping confidence intervals as well as a Chi-squared test indicated there was no significant association between sampling year and overall prevalence ($p>0.05$). The range in the proportion of fish infected was 0-14%, with the maximum value measured on the Faroese shelf in October 2018 (**Figure 1** and **Table 1**). Positive PCR detection of *K. thyrsites* was detected in fish as young as 2 years (29.8 cm and 195g) and up to 14 years (40 cm and 595g). Two of the 24 positive PCR samples were negative for the presence of *K. thyrsites* spores when examined under the microscope.

A logistic regression model was used to test if the odds of prevalence could be explained by a linear combination of the following predictor variables: latitude, month, weight, age and gender. Model fit was tested by examining the residual deviance for the model including predictors with a null model (intercept only). Residual deviance was 180 on 578 degrees of freedom and null deviance was 200 on 583 degrees of freedom. The distributed Chi-squared value on 5 degrees of freedom (number of predictor variables) was 20.13, corresponding to significance level of $p<0.005$. The logistic regression model including predictor variables thus had a statistically significant better fit than a null model. The coefficients within the model and their statistical significance were as follows: latitude (-0.67, $p<0.01$), month (0.39, $p<0.001$), age (0.18, $p>0.05$), weight (-0.001, $p>0.05$) and gender (0.32, $p>0.05$). Consequently, only geographical location and month were significant predictor variables. The negative co-efficient for latitude (°N) indicates a decrease in the log odds of prevalence moving northward, whilst the positive coefficient for month indicates an increase in the log odds of prevalence later in the year.

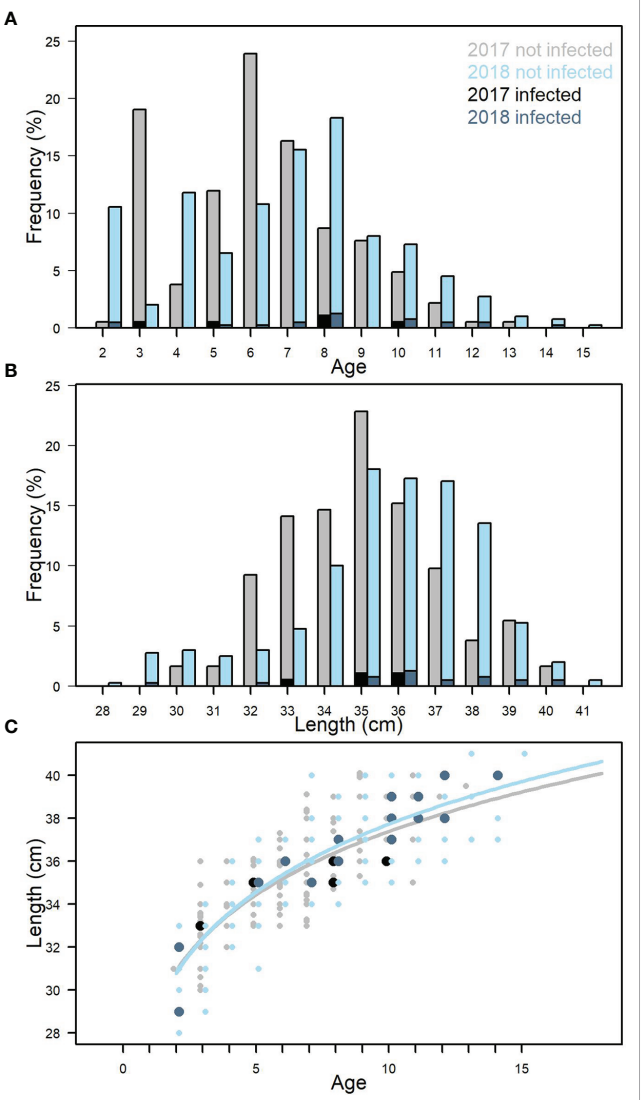

FIGURE 4 | Age distribution for all samples from 2017 and 2018 **(A)**. Length distribution for all samples from 2017 and 2018 **(B)**. Length-weight relationship for all samples. 2017 and 2018 **(C)**. Black and dark blue indicate fish infected with *Kudoa thyrsites*.

Sampling locations were classified into different regions within the study area (see **Tables 1**, **2**). The Faroese shelf had highest prevalence (8.2%, CI: 4.1-12.4%, n=170), followed by the British Area (2.7%, CI: 0-6.3%, n=75) and the Northern area (1.4%, CI: 1.4% 0-2.7%, n=289). A Chi-squared test of the 2x3 contingency table showed that prevalence was significantly associated with region ($p<0.05$). Since age was only just outside statistical significance in the logistic regression, we also examined differences categorically. The oldest fish (>10 years) had the highest prevalence (10.8%, CI: 4.2-17.5%, n= 83), followed by

medium (6-9 years) fish (3.1%, CI: 1.2-5.0%, n=322) and young (2-5 years) fish (2.8%, CI: 0.4-5.2%). A Chi-squared test on the contingency table showed a significant association between age category and prevalence ($p<0.01$).

Seawater Analyses

Water column samples were analysed from the Faroese shelf spanning a full seasonal cycle from February-November in 2018 (**Table 3**). During the year, temperature ranged from 6.2-10.5°C, peaking in September. Pre-bloom chlorophyll (chl < 1 μg L^{-1}) nitrate concentrations were 11.6 ± 0.4 μmol L^{-1} and drawn down to 3.6 μmol L^{-1} on 5th June corresponding to a first phytoplankton bloom peaking at 3.7 μg chl L^{-1}. A second larger phytoplankton bloom (6.6 μg chl L^{-1}) occurred on 21st August, followed by a rapid decline. DNA was successfully extracted from every sample and extract concentrations ranged from 1.2-8.4 ng μL^{-1} (average = 5.4 ng μL^{-1}). All samples successfully amplified for a PCR inhibition control with Ct scores ranging from 23.16 ± 0.14 to 24.12 ± 0.17, which in all cases deviated <3 Ct from a negative control (nuclease-free water) spiked with a control template. None of the water samples were positive for *K. thyrsites*.

DISCUSSION

Prevalence of *K. thyrsites* in Faroese Fishery

K. thyrsites was confirmed as the causative agent of myoliquefaction of Atlantic Mackerel within the Faroese wild fishery. Investigations of myoliquefaction in various fish species in Icelandic waters have identified *K. islandica* as the causative agent (Kristmundsson and Freeman, 2014), although it has not yet been identified in Atlantic mackerel. The morphology of *K. thyrsites* and *K. islandica* is quite distinct, where the former has one enlarged polar capsule, clearly visible in our samples (**Figure 3**). The vast majority of specimens obtained from a factory processing plant tested positive for *K. thyrsites* using both microscope smears and PCR. There was good statistical agreement between the two techniques, enabling us to apply qPCR to a large number of (>500) natural samples. It is unclear why we could not observe spores in a small fraction of the samples. There is some anecdotal evidence of spores disappearing in soft-flesh mackerel specimens (Levsen, personal communication), which could be related to spatial heterogeneity within the muscle sample itself. The focus of the present study was not to describe the intensity of infection. However, it is possible that quantifying variation of intensity across regions or age categories, along with manual muscle texture testing (Levsen et al., 2008) could explain some of the small discrepancies in detection. Future work integrating prevalence, intensity and muscle texture of *K. thyrsites* infected mackerel, and other *Kuoda* genera, is generally required (Giulietti et al. 2022).

There was spatial and temporal variability evident in the prevalence of *K. thyrsites* in mackerel comprising the Faroese wild fishery. Logistic regression modelling showed that both more northerly latitudes and later sampling months increased the log odds of detecting prevalence. Different regions were also statistically associated with *K. thyrsites*, with the shallow Faroese shelf environment displaying highest prevalence. Previous studies in the Northern North Sea have also documented temporal variability of *K. thyrsites* in mackerel (Levsen et al., 2008), including inter-annual differences (Levsen, 2015). Although our prevalence estimates are similar to those reported in the North Sea, we did not detect statistically significant inter-annual variability.

Separating spatial and temporal variability in migratory fish such as Atlantic mackerel remains challenging. For example in

TABLE 3 | Biogeochemical characteristics of Faroese shelf waters during eDNA survey of *K. thyrsites*.

Date *dd/mm/yyyy*	Temperature *°C*	Nitrate *μmol L^{-1}*	Chlorophyll *μg L^{-1}*	Inhibition Control (Ct)	*Kudoa Thyrsites*
20/02/18	6.23	11.76	0.25	23.42 ± 0.08	A
20/03/18	6.19	11.58	0.3	23.39 ± 0.14	A
03/04/18	6.32	11.03	1.07	23.43 ± 0.18	A
10/04/18	6.19	9.47	2.01	23.32 ± 0.04	A
17/04/18	6.51	9.46	1.95	23.27 ± 0.24	A
01/05/18	6.95	8.07	1.46	23.16 ± 0.15	A
22/05/18	7.6	4.88	2.2	23.23 ± 0.16	A
05/06/18	8.38	3.6	3.71	23.21 ± 0.08	A
21/06/18	8.77	6.55	0.84	23.26 ± 0.15	A
10/07/18	9.4	7.22	0.95	23.41 ± 0.05	A
24/07/18	9.79	*n.d.*	0.73	23.24 ± 0.18	A
31/07/18	10.09	6.93	*n.d.*	23.79 ± 0.77	A
21/08/18	10.39	*n.d.*	6.64	23.37 ± 0.18	A
04/09/18	10.45	7.54	0.76	23.72 ± 0.10	A
18/09/18	10.37	7.75	0.71	23.75 ± 0.19	A
16/10/18	9.6	8.73	0.48	24.12 ± 0.17	A
13/11/18	8.85	10.11	0.21	23.92 ± 0.14	A
27/11/18	8.52	10.68	0.14	23.94 ± 0.16	A

Presence or absence (P/A) of K. thyrsites was determined by qPCR. Inhibition Control is an independent assay of DNA extracts to confirm absence of K. thyrsites is not caused by qPCR inhibition from environmental samples (see Materials and Methods). Four technical PCR replicates were analysed for each sample and data are reported as the mean Ct scores ± 1 standard deviation. n.d. denoted missing data.

our dataset, the observed prevalence in the British Area is associated with sampling in January. However, the Faroese shelf and the area north of the Faroe islands both had sample coverage during September and October, with notably higher prevalence observed on the Faroese shelf. Specific factors that influence regional patterns of infection could be related to water mass properties characterising different oceanographic regimes. For example there are notable differences in temperature and salinity properties that comprise different water masses found to the north of the Faroe Islands, as well as there is variability in the relative composition of water masses among years (Hátún and Chafik, 2021). All of the samples collected north of Faroes are from an environment influenced by relatively cold Norwegian North Atlantic Water, while those on The Faroe Shelf are from an environment characterised by warmer and more saline waters.

Prevalence of *K. thyrsites* in mackerel was evident across the entire range of age classes sampled in the Faroese wild fishery. Prevalence was highest in larger and older fish (>10 years) and is consistent with studies from the North sea (Levsen, 2015). However, notably we also detected *K. thyrsites* in two and three year old fish, clearly demonstrating the potential for infection in younger adults. Presumably the likelihood of parasite-host encounters scale as a function of age, although migratory patterns into potentially infective areas may also contribute. Traditional stock migration patterns have shown that spawning occurs in the southern areas (close to Spain, France and Ireland) from February-July. Adult mackerel (>2-3 years) distribute to the north during the feeding migration phase in summer and can be found in an area extending from Gibraltar (36°N) up to Svalbard (78°N) to the areas east of Greenland (Jansen et al., 2016; Nøttestad et al., 2016). In late autumn the mackerel return towards the wintering areas around Shetland Isles and stay there during winter. In January the mackerel start their southward migration to the spawning grounds (Jansen et al., 2012) and we detected infection in January in the British area. Interestingly, no *K. thyrsites* was detected in mackerel from the Faroese Shelf late spring/early summer (May and July). The spatial and temporal patterns observed in our data appear to suggest that mackerel migrating into Faroese fishing grounds become infected in other areas. Our observations need to be interpreted cautiously within the context of the sampling regime. Further research is required to fully elucidate spatial and temporal patterns in the occurrence of infection.

Non-Detection of *K. thyrsites* in Seawater Samples

In the current study we did not detect the presence of *K. thyrsites* in Faroese shelf seawater samples. Although it is challenging to conclude absence from non-detection in PCR-assays, it seems unlikely that extraction or inhibition of seawater samples could adequately explain the ubiquitous non-detection observed in our study. In all samples tested, DNA concentrations in extracts were reasonably high and an independent extraction control confirmed there was no PCR-inhibition of seawater samples used in the present study. Furthermore, specific multiplex inhibition controls for quantitative PCR of Atlantic cod (*Gadus morhua*) using the same extraction method has not revealed any specific inhibition of seawater samples collected on the Faroese shelf (Salter et al., 2019). Failure of the extraction methodology to specifically extract DNA from the *K. thyrsites* target also seems unlikely. The Qiagen extraction kit used in this study includes a vigorous bead-beating step that has been applied previously to successfully extract *K. thyrsites* rDNA from seawater filters (Jones et al., 2016). The seawater samples used in this study were obtained from an archive of a coastal environmental DNA monitoring program (FAMEOS) and so spiking parallel water samples with the possible range of un-envisaged targets was not feasible. However, future studies targeting *K. thyrsites* in natural seawater samples may consider the addition of spores as an additional control measure.

The non-detection of *K. thyrsites* in annual seawater samples from the Faroese shelf support a hypothesis that Atlantic mackerel are not locally exposed to the parasite (**Table 3**). This is further supported by the circumstantial observation that *K. thyrsites* has not currently been observed in farmed Atlantic Salmon on the Faroese shelf, unlike other areas (St-Hilaire et al., 1997a; St-Hilaire et al., 1997b; St-Hilaire et al., 1997c; St-Hilaire et al., 1998; Moran et al., 1999b). The Faroese shelf is the shallowest environment in our survey area and corresponds to the highest prevalence of *K. thyrsites* (**Table 1**). Considering that practically all known life-cycles of myxosporeans involve annelid worms as an alternate host (Yokoyama, 2003), tight pelagic-benthic coupling on a tidally-mixed shelf might be considered to promote encounter rates between actinospores and migratory fish hosts. It is clear from seawater screening close to aquaculture facilities suffering from *Kudoa* spp. infections that myxospores can be detected in seawater samples and UV irradiation of raw seawater reduces infection (Jones et al., 2016). A seawater study of *K. yasunagai* in infected aquaculture areas appears to suggest some seasonality of myxospore abundances that were linked to water temperature and ecology of the alternate invertebrate host (Ishimaru et al., 2014). Our seawater survey covered the typical annual temperature range of the Faroese shelf and growth and decline of phytoplankton blooms that might influence invertebrate ecology. To the best of our knowledge our seawater survey is the first attempt to detect *K. thyrsite* myxospores in seawater samples in association with wild fish infections. One unexplored infection pathway is through feeding. In particular, sampling regimes that target intensive feeding in Southern spawning areas (Jansen et al., 2021) may help identify infection pathways occurring in Atlantic mackerel prior to their northward migration.

CONCLUSIONS

In the present study, we have investigated the prevalence of *K. thyrsites* in Atlantic mackerel in the Faroese fishery. Examination of soft-flesh (myoliquefaction) specimens obtained from a Faroese processing factory demonstrated *K. thyrsites* as the causative agent. We found excellent agreement between

microscope smears and PCR detection of *K. thyrsites*, allowing us to apply more rapid molecular detection methods on a large number (>500) of mackerel specimens caught from the wild Faroese fishery. We observed infection across the entire age range (2-14 years) of mackerel and statistical analyses indicated spatial and temporal, but not inter-annual, variability in prevalence. Highest prevalence was observed on the relatively shallow, tidally mixed Faroese shelf. It was not possible to detect *K. thyrsites* spores in seawater samples from the Faroese shelf, despite sampling across the entire range of biogeochemical conditions occurring throughout the year. Taken together, with the absence of *K. thyrsites* infection in farmed salmon on the Faroese shelf, our data support the hypothesis that infection likely occurs in southern spawning areas and infected fish migrate north into the Faroese fishery. Future work investigating fish and water column samples in these southern areas could help resolve the spatial and temporal dynamics of *K. thyrsites* prevalence in Atlantic mackerel.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The samples were taken on sea onboard fishery or research vessels. The fish was cooled in RSW seawater onboard and later frozen on land. Thus all sampling occurred from already dead fish.

AUTHOR CONTRIBUTIONS

DH and IS developed the main ideas. EH constructed the maps and histograms. IS and DH wrote the manuscript with feed-back from EH. All authors approved the submitted version.

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