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Comparing parasite communities in Atlantic Wolffish *Anarhichas lupus* around Iceland for stock discrimination

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Comparing parasite communities in Atlantic Wolffish *Anarhichas lupus* around Iceland for stock discrimination

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Thesis submitted in partial fulfillment of a
Baccalaureus Scientiarum in Biology

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Útdráttur

Stór hluti nytjastofna fiska er annað hvort ofveiddur eða veiddur upp að því hámarks veiðiálagi sem stofninn þolir. Án viðeigandi stjórnunar á veiðum geta fiskistofnar dregist saman eða jafnvel hrunið. Markmið þessarar rannsóknar var að afla grundvallar upplýsinga um snýkjudýra samfélög Steinbíts *Anarhichas lupus* á íslenskum fiskimiðum ásamt því að veita innsýn í hvaða snýkjudýr, ef einhver, er hægt að nota til líffræðilegra merkinga í þeim tilgangi að aðgreina Steinbíts stofna. Steinbít frá mismunandi svæðum við Ísland var safnað, hann krufinn og snýkjudýrum þeirra var, safnað og þau greind. Með greiningu snýkjudýra mátti sjá mismun á uppbyggingu snýkjudýra samfélaga steinbíts eftir hvar hann var veiddur. Einnig var rannsakað hvaða snýkjudýr hentuðu til að aðgreina stofna steinbíts. Niðurstöður rannsókanrinnar benda til þess að munur sé á milli þeirra snýkjudýra samfélaga sem er að finna í steinbít, eftir því á hvaða svæðum fiskarnir voru veiddir. Þar sem að fræðilegar heimildir um snýkjudýra fánu Steinbíts á Íslandsmiðum eru af skornum skammti og efnið lítið rannsakað leggur þessi rannsókn grunnin af innsýn í snýkjudýra samfélög sem finnast í steinbít við Ísland.

Abstract

A large proportion of marine fish stocks are either overfished or exploited to the level of maximum sustainable yield. Without proper management fishing stocks might decrease or collapse. The aims of this study are to provide a usable foundation of host-parasite associations for Atlantic wolffish *Anarhichas lupus* in Icelandic waters, and to provide insight into what parasites, if any, may be used for stock discrimination of *A. lupus*. The available academic literature on parasites of *A. lupus* in Icelandic water is very limited, therefore this study supplies a foundation of the parasite communities of *A. lupus* in Icelandic waters. Specimens of *A. lupus* were sampled from different location around Iceland and examined for parasites. With the parasites identified, a variation in parasite communities between sampling location could be observed. Candidates for potential biological tags could be observed within the parasite communities and are suggested herein.

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Acknowledgments

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1 Introduction

1.1 Stock discrimination

A sizeable proportion of marine fish stocks are at this time either overfished or exploited to the level of maximum sustainable yield. Without proper management these stocks might decrease or even collapse (Ward, 2006). Stock discrimination and structure are fundamental components of fisheries management and stock assessment. Due to the fact that a lot of marine fisheries exploit mixed stocks it is imperative to identify and qualify the multitude of stock structure components that comprise these mixed stocks (Begg et al., 1999). The ability to identify a fish stock is necessary for its management and so that the allocation of catch between competing fisheries and or sectors can be done fairly. It is also important for the recognition and subsequent protection of nurseries and spawning locations to ensure an optimal harvest (Begg & Cadrin, 2016).

The techniques and methods that can be used to produce the necessary information for stock structure estimates can be divided into three types: (1) Natural methods such as morphometric, meristic analysis, parasites as biological tags and genetic studies; (2) The use of artificial tags attached either internally or externally; and (3) Use of biological parameters with regard to life cycles (MacKenzie & Abaunza, 1998). Use of parasites as biological tags relies on the fact that fish can only become infected with parasites whilst within their endemic area. A parasites endemic area is the geographical region where conditions are favorable for parasite transmission (MacKenzie & Abaunza, 1998). The benefits of parasites as tags compared to artificial tags include that they can be better suited for the study of small and or delicate fish species where artificial tags often pose a difficulty, i.e., are less invasive. The use of parasites as tags is generally less expensive because the samples can be obtained from routine sampling programs as opposed to specialty-organized tagging expeditions. Biological tags also dismiss doubts concerning potentially altered behavior of artificially tagged fish (MacKenzie & Abaunza, 1998). There are some criteria a parasite must meet to be ideal as a biological tag. It is, however, rare that a single parasite fulfills all those criteria, so some trade-offs are often required. The listed criteria are as follows: (1) there should be a significant difference in the level of infection in the host depending on the subject's location in the study area; (2) the parasite should persist in the host long enough for the purpose of the study, for instance for stock assessment and recruitment studies the ideal life span of the parasite should exceed 1 year, compared to seasonal migration studies where parasites with a life span less than a year can be used; (3) if a parasite has a complex life cycle with two or more stages further information is required such as which biotic and abiotic factors affect transmission between hosts; (4) the level of infection should be comparatively constant from year to year; (5) the detection and identification of the parasite should be relatively easy; and (6) parasites who alter host behavior or cause selective mortality should be avoided (MacKenzie & Abaunza, 1998).

In 2004 parasites were used as biological tags for stock identification of three different stocks of Argentine hake *Merluccius hubbsi* in the south-west Atlantic. The study examined 344 hake specimens and found 26 species of parasites. For the sake of minimizing the effect of host size and or age on the parasite burden only mature fish of comparable lengths were included in the analysis. With the use of univariate analysis on parasite populations of infected hake and infra community descriptors in addition to discriminant analyses researchers were able to successfully separate the hake population into two distinct stocks, two of the aforementioned stocks shared most of their biological indicators and were therefore too similar to confirm separate stocks (Sardella & Timi, 2004). In a study like this one there are varying environmental factors and conditions that influence the distribution of zooplankton and other hosts involved in the parasites life cycle that can be determining factors when it comes to the differences observed among the hake stocks (Sardella & Timi, 2004).

1.2 Atlantic wolffish *Anarhichas lupus*

The Atlantic wolffish *Anarhichas lupus* is a demersal sedentary species. They are often found in pairs or alone (McCusker & Bentzen, 2010). The Atlantic wolffish is widely distributed around the North Atlantic. The species has considerable economic importance and has seen a decrease in population in recent years (Gunnarsson et al, 2013). *Anarhichas lupus* is currently listed as a species of special concern by the Canadian species at risk act (Gunnarsson et al., 2013). Their eggs are up to 6 mm in diameter, making them among the largest among teleosts (McCusker & Bentzen, 2010). Wolffish are believed to have internal fertilization. This belief is supported by sperm cell characteristics and the presence of genital papillae in males along with the wolffish reproductive habits. The female spawns fertilized eggs in an adhesive (Le François et al., 2007). The eggs are deposited in nests that the male then guards until hatching. Once the larvae emerge, they are about 20 mm in length and are negatively buoyant making them sink to the bottom once they stop swimming (McCusker & Bentzen, 2010). It is because of this large size, negative buoyancy, and their relative rarity in surface water that wolffish larvae are thought to be less subject to ocean currents compared to other marine species (McCusker & Bentzen, 2010). Once the juveniles reach about 100 mm length, they become nearly entirely bottom dwelling (McCusker & Bentzen, 2010). *Anarhichas lupus* individuals enter the fishing stock at the age of four years (Hafrannsóknastofnun, 2022). *Anarhichas lupus* females in the warmer waters of the Westfjords become fecund on average about 64 cm in length then being around 11 years old. Those of the Northern shore becoming fecund later or around 73 cm in length and 14 years of age (Gunnarsson et al., 2022). A stomach analysis done on *A. lupus* revealed that up to 85% of their food intake consists of invertebrates, with the largest contributors being crabs (22%), whelks (22%), brittle stars (16%), scallops (12%) and sea urchins (10%), with the remaining 15% consisting of fish, the most abundant being redfish (12%) (Templeman, 1985).

The Atlantic wolffish around Iceland undergoes two annual migrations, in autumn mature individuals move from shallow water out to deeper waters to spawn. Following the end of the spawning season they move back to shallow waters to feed (Gunnarsson et al., 2013). *A. lupus* prefers to spawn in depths of around 140 - 200 m. They can be found all around Iceland favoring depths of 40 - 180 m (Jónsson, 1982).

Anarhichas lupus is an economically important species to Iceland with annual catches ranging from 7.500 - 9.500 tons in the last 10 years (Hafrannsóknastofnun, 2022). There is evidence to suggest that the *A. lupus* population is composed of two separate stocks. A Western stock and an Eastern stock. There is an observable difference in growth and maturity between the two stocks. The western stock shows faster growth and reaches maturity at a younger age and smaller size compared to the eastern one. It is suggested that there are abiotic factors such as temperature that contribute to this difference (Gunnarsson et al., 2013).

This study aims to see if identification is possible from which stock an individual *A. lupus* originates, with the use of parasite communities as biological tags. The Marine and Freshwater Research Institute of Iceland (MFRI) manages *A. lupus* as one stock despite the evidence that suggests there being two separate stocks. If stock discrimination with the use of parasite communities proves to be an easy and reliable method for identification that could affect and or change the outcome of the annual catch quota proposal, given out by the MFRI.

1.3 Research Question & Hypothesis

The focus of this research is to uncover what parasites make up the parasite fauna of Atlantic wolffish around Iceland and if stock discrimination of Atlantic wolffish around Iceland, based on the variation of their parasite communities between the two catch locations, is possible.

Firstly, I hypothesize that there is enough variation in parasite communities to allow us to allocate individual wolffish to different stocks.

Secondly, I hypothesize that we will see certain groups and or species of parasites that will be more useful as biological tags for stock discrimination than others.

2 Materials & Methods

2.1 Sample collection

Fish were collected by commercial fishermen and shipping from northern localities to Reykjavík was facilitated by the MFRI. One of the localities was off the coast of Grindavík in the south of Iceland (fish collected by car from Sandgerði), and the other out from the coast of Dalvík in the north of Iceland. The fish landed in Dalvík were then shipped to a fish market in Reykjavík where we could pick them up. A total of 19 specimens were collected, 6 from the southern location and 13 from the northern location see table 2-1. Each specimen was then given a serial number for future reference. The coordinates where each fish was caught were recorded along with the depth at which they were caught (Table 2-1).

Table 2-1 Individual catch data for each Atlantic wolffish sampled.

Sample	Date (collection)	Date (sampled)	TL (cm)	Sex	Location	Latitude	Longitude	Depth
WF 01	10.02.2023	15.02.2023	69	M	North	N66°06´33	W18°33´31	80m
WF 02	10.02.2023	16.02.2023	69	M	North	N66°06´33	W18°33´31	80m
WF 03	10.02.2023	16.02.2023	75	F	North	N66°06´33	W18°33´31	80m
WF 04	10.02.2023	17.02.2003	65	F	North	N66°06´33	W18°33´31	80m
WF 05	10.02.2023	17.02.2023	74	M	North	N66°06´33	W18°33´31	80m
WF 06	20.02.2023	21.02.2023	93	F	North	N66°03´33	W17°34´41	125m
WF 07	20.02.2023	22.02.2023	60	F	North	N66°03´33	W17°34´41	125m
WF 08	20.02.2023	22.02.2023	73	M	North	N66°03´33	W17°34´41	125m
WF 09	01.03.2023	02.03.2023	84	M	South	N63°57´057	W22°48´20	77m
WF 10	01.03.2023	03.03.2023	64	M	South	N63°57´057	W22°48´20	77m
WF 11	01.03.2023	05.03.2023	69	F	South	N63°57´057	W22°48´20	77m
WF 12	20.03.2023	21.03.2023	75	F	South	N63°55´37	W22°46´49	68m
WF 13	20.03.2023	22.03.2023	61	M	South	N63°55´37	W22°46´49	68m
WF 14	20.03.2023	22.03.2023	70	F	South	N63°55´37	W22°46´49	68m
WF 15	31.03.2023	01.04.2023	77	F	North	N65°42´44	W20°26´03	53m
WF 16	31.03.2023	02.04.2023	79	F	North	N65°42´44	W20°26´03	53m
WF 17	31.03.2023	03.04.2023	72	M	North	N65°42´44	W20°26´03	53m
WF 18	31.03.2023	03.04.2023	58	F	North	N65°42´44	W20°26´03	53m
WF 19	31.03.2023	04.04.2023	60	M	North	N65°42´44	W20°26´03	53m

2.2 Initial examination and necropsies

Once samples were brought to the laboratory, they were kept on ice until the initial examination and necropsies could be performed. When the examination was conducted, the fish were measured to the nearest cm in total length with the use of a measuring board, each fish was then examined for ectoparasites, mouth, gills, nostrils, skin, and fins. The nostrils were flushed out with a 8 ‰ saline solution, and the nasal flush examined for parasites.

After the external examination the eyes, gills, brain, and cranial fluid were removed and examined for parasites under a dissecting microscope. The sagittal otoliths were collected from each fish and placed in envelopes with their corresponding fish serial number along with the total length of the fish and stored for further research. The body cavity was examined for parasites before the internal organs were processed. Internal organs: (1) gonads; (2) heart; (3) intestine (4) liver; (5) spleen; (6) stomach and (7) mesenteries, were isolated, labeled and then frozen for later examination. While the gallbladders and urinary bladders were stored in 96% ethanol for later examination. Once the organs had been collected and processed the fish were filleted and deskinned, and the flesh and skin were then examined for parasites on a candling table. No mucus or blood smears were collected.

2.3 Collection of parasites

Once observed, parasites were counted, collected, and given a preliminary identification based on the morphological characteristics and their attachment site. Internal organs were defrosted, and the external and internal surfaces were examined in a saline solution under a microscope. All parasite samples were stored in 96% ethanol to allow for molecular confirmation of identification. Once a parasite was collected, it was cataloged with information on which fish and what organ it came from, whether they were internal or external in relation to the organ as well as the number of parasites of that functional group that were observed. Presence and abundance data for each parasite group for each organ of every fish were recorded to be used for statistical analysis and hierarchical clustering analysis on a distance matrix based on Euclidean distances between the parasite communities of different *A. lupus* individuals.

2.4 DNA extraction

Two samples of each parasite type from every site of infection, of two fish, from either location were chosen for molecular work along with all parasite samples with an identification of unknown. Copepods, isopods, and leeches were not sampled for DNA extraction and PCR. A total of six parasite groups were processed for molecular work: (1) Adult trematodes; (2) larval acanthocephalans; (3) larval cestodes; (4) monogeneans; (5) nematodes and (6) unknown. Each sample selected for molecular work got a new ID with the initials of the examiner and the number in which they were processed, for example AR 001 refers to Alex Rafn sample number one for DNA extraction. Before any work on the DNA extractions could be done, work surfaces and equipment were wiped down with distilled water and then again with 70% EtOH.

Parasite samples were decanted into a small petri dish and then isolated under a dissecting microscope. Adult trematodes, monogeneans and unknown (not exceeding 1 mm^3) were placed whole in a new Eppendorf with their corresponding ID. For nematodes, a 1 mm long, cross-section was cut away with a sterile scalpel and then placed in the new Eppendorf, the two ends were then placed back in 96% EtOH for storage as hologenophores (*sensu* Pleijel et al., 2008). Larval acanthocephalans and larval cestodes were cut open with a sterile scalpel and 1 mm^3 of internal tissue was collected, that tissue was then placed in a new Eppendorf with their newly assigned DNA label.

For the DNA extraction a master mix was made comprising of: (1)10 µl of fish buffer (Devlin et al., 2004); (2) 1 µl of 20% tween 20; (3) 2 µl of proteinase-K and (4) 2 µl of MQ H_2O, for each sample with an additional 2 doses of every component to cover errors in the distribution of the master mix into the samples. 15 µl of the master mix were added to every DNA sample.

Once the master mix had been added to the samples, they were then placed on a hot block at 65 °C for 2 hours to optimize the activity of proteinase K, each sample was flicked and spun down in a mini centrifuge every 20 min for the duration of the 2 hours. After 2 hours on the hot block at 65 °C the temperature of the block was increased to 95 °C in order to inactivate the proteinase K. When the hot block reached 95 °C the samples were left there for 10 minutes, before they were removed from the hot block and let cool down at room temperature for a minimum of 20 minutes. After the samples had cooled down, they were placed in a freezer until PCR could be conducted.

2.5 PCR protocol

Before PCR protocols were carried out, work surfaces and equipment were wiped down with distilled water and then again with 70% EtOH to prevent contamination of the samples. DNA samples from the extraction were thawed and then flicked and spun in a mini centrifuge. They were then placed on ice. A specific master mix was prepared for the different parasite groups. For each reaction including a positive and negative control for every PCR that was performed, with the exception of acanthocephalans for which no positive control was available. Each DNA sample was assigned a new tag for their PCR reaction. Each tube of the tube strips used for the PRC reactions was then labeled with its specific number. A list was made of the PRC labels and their corresponding DNA sample, that would be added for the reaction.

Table 2-2 Components of the PCR master mix

Chemical ingredients	**Volume**
2x MyTaq	12.50 µl
Primer 1	0.35 µl
Primer 2	0.35 µl
DD H_2O	11.30 µl
Total amount pr. sample	24.5 µl

When making the master mix 2 additional doses were included to account for any error in the distribution of the master mix for PCR reactions. The master mix was allocated to 200 µl tube strips and then 0.5 µl of the appropriate DNA sample was pipetted into each tube.

Table 2-3 Primer targets and target sizes for the different parasite functional groups.

Parasites	Primers	Target	Target size
Monogeneans, Cestodes and Unknowns	T01N and T13N (Harper & Saunders 2001)	5' end of 28s rDNA	1400-1900 base pairs
Trematodes	BD3 and 536 (García-Varela & Nadler 2005)	5' end of 28s rDNA	1200 – 1700 base pairs
Nematodes	93 and 94 (Nadler et al. 2005)	Internal transcribed spacer	1000 base pairs
Acanthocephalans	LCO1490a and HCO2198 (Folmer et al 1994)	Cytochrome oxidase subunit 1	750 base pairs

The strips were then placed in a thermo-cycler and the appropriate protocol for each parasite functional group ran (see table 2-4). Once the run time of the thermo-cyclers protocol had finished samples were removed from the thermos-cycler, and placed in a labeled rack and moved to a refrigerator to be stored at 4 °C.

Table 2-4 Thermo-cycler protocols for the different parasite functional groups.

Parasites	Thermo-cycler protocol
Monogeneans, Cestodes and Unknowns	94°C for 4 min 38 cycles: • 94°C for 30sec • 50°C for 30 sec • 72°C for 2 min 72°C for 7 min
Trematodes	94°C for 1 min 35 cycles: • 94°C for 60 sec • 50°C for 60 sec • 72°C for 90 sec 72°C for 10 min
Nematodes	94°C for 3 min 35 cycles: • 94°C for 30 sec • 52°C for 30 sec • 72°C for 60 sec 72°C for 7 min
Acanthocephalans	94°C for 3 min 35 cycles: • 94°C for 60 sec • 40°C for 60 sec • 72°C for 60 sec 72°C for 7 min

Once every protocol had finished the thermo-cycler would cool the samples down to 4°C, and would maintain that temperature indefinitely, unless the protocol was turned off manually. This is to prevent the deterioration of the samples once the protocol ends.

2.5.1 PCR gel electrophoresis

To ensure the viability of the PCR product samples were tested using gel electrophoresis in order to visualize the outcome. Gel electrophoresis is a technique where the DNA fragments in the PCR product are pulled through a gel matrix by an electric current and separated according to size. This process works because DNA in itself is negatively charged and can therefore be moved along the gel toward the positively charged electrode, with an electric current. For this a 1.5% agarose gel was made, by mixing 100ml of 1x TAE (Tris base, acetic acid, ethylenediaminetetraacetic acid) with 1.50 g of agarose powder. The solution was swirled in a 250 ml Erlenmeyer flask and then microwaved until the TAE reached a boiling point and the powder fully dissolved. To this mixture 5.0 µl of ethidium bromide was added, and the addition of the EtBr was performed in a fume hood. The mixture was then poured into a casting tray with two 20 pronged rakes, making 40 wells in the gel once it had solidified. After letting the mixture cool down in the casting tray, the rakes were removed, and the gel was placed in an electrophoresis chamber. More 1x TAE was then poured into the chamber until the gel was submerged. For gel electrophoresis to work every sample must have a loading dye added to it to weigh down the molecules of the sample and prevent them from floating up and out of their wells. Roughly 1 µl of the loading dye was pipetted for each sample on a strip of parafilm, the droplets of dye where then diluted with 3.0 µl of distilled H_2O, before 2.0 µl of the PCR product was added every droplet with exception of two droplets for each gel, where 2.0 µl of a molecular ladder solution replaced the PCR product. The molecular ladder was loaded into the first wells of each row of the gel, and the droplets containing the PCR products were placed in the other wells. Once every droplet had been pipetted into separate wells of the gel, the electrophoresis chamber was closed, and the voltmeter of the power supply was set to 80 volts to initiate the run. The gels were run for 40 min in the chamber or until the smallest band had made it past the halfway point of the gel. Once the electrophoresis run was finished the gels were removed from the chambers and imaged. The gels were placed under a closed cone with a digital camera at the top and photographed whilst being backlit by ultraviolet light. The ultraviolet light luminates the DNA fragments in the gel showing a distinct band where the fragments had been dragged through the gel. The visualization of the bands confirmed viable DNA fragments in the PCR product. If a sample showed no bands after the electrophoresis, the PCR product had failed, and no viable DNA fragments were in the PCR product.

2.5.2 PCR purification

Once successful PCR products had been confirmed with gel electrophoresis, all successful samples were purified. ExoSap was used for enzymatic cleaning and amplification of the PCR products.The ExoSap hydrolyses the surplus primers and nucleotides. 10.0 µl of PCR products from each successful sample was pipetted in a new tube strip and each well labeled with the PCR ID of the PCR product. The samples were placed on ice to keep them cool since the ExoSap is very temperature sensitive. Once all the tube strips were ready the ExoSap was taken from a freezer and placed on ice to protect it from temperature fluctuations. 2.0 µl of the ExoSap were then added to every sample intended for purification. The samples were then placed in the thermo-cycler and run on a specific ExoSap protocol where samples were held at 37°C for 15 min and then heated up to 80°C for 15 min. The samples were then moved to a freezer to be stored at -20°C. Purified samples were then sent Microsynth in Germany for sequencing.

2.6 Data analysis

For data analysis R studio (version 4.2.1) was used, and an algorithm was trained to make a predictive model separating the parasite data by location. To train the algorithm, it was made to process a dataset compiled of morphological parasite identifications and the site of attachment of each parasite. After the algorithm had processed the dataset, which was compiled of all parasites cataloged it could correctly assign each fish to their general sampling site (north or south) based on their parasite fauna.

3 Results

A total of 19 specimens of *A. lupus* were sampled in this study, 13 specimens came from the three sampling sites north of Iceland, and six specimens from the two sampling locations out from the southern part of Iceland. From the north there were a total six male specimens ranging in size from 60 to 74 cm in total length, and seven female specimens ranging in size from 58 to 93 cm in total length. The specimens from the south totaled three males ranging in size from 61to 84 cm in total length, and three females ranging in size from 69 to 75 cm in total length. The overall sex ratio for all specimens was nine males and ten females. On average the specimens from the northern sampling sites were infected with more parasites 157,46 individual parasites, that number drops to 80.54 if one single outlier is omitted, over 1000 trematodes were counted in the intestine of WF 19. The specimen from the southern sampling sites harbored on average 102.50 individual parasites.

Figure 3-1 Map of catch sites and depths for each group of specimens sampled.

Table 3-1 Parasite abundance data for every examined site of infection by location.

Site of attachment	Parasite identification	North n = 13	South n = 6
External exam	Sea Leech	0.15	2.50
	Monogeneans	0.62	3.83
	Nematodes	0.00	0.17
	Unknown	0.00	0.17
Nasal flush	Monogeneans	0.00	0.50
	Nematodes	0.00	0.17
Brain	Unknown	0.08	0.17
Gills	Copepods	0.92	0.17
	Monogeneans	0.00	3.00
	Isopods	3.23	0.67
	Sea Leech	0.15	0.17
	Nematodes	0.00	0.33
	Acanthocephalan	0.62	0.83
	Unknown	0.38	0.50
Flesh	Nematodes	2.54	4.50
Body cavity	Nematodes	4.08	1.50
Spleen	Nematodes	0.08	0.00
	Trematodes	0.08	0.00
	Unknown	0.08	0.00
Heart	Nematodes	0.00	0.17
Liver (exterior)	Nematodes	0.54	2.83
	Cestode (larvae)	0.00	0.50
	Trematodes	0.23	0.00
Liver (interior)	Nematodes	0.08	0.00
Liver	Unknown	0.08	0.00
Stomach (exterior)	Nematodes	0.15	0.00
	Trematodes	2.00	0.00
Stomach (interior)	Nematodes	0.08	4.33
	Trematodes	1.92	0.17
	Cestode (larvae)	0.08	0.00
Intestine (interior)	Nematodes	0.31	19.50
	Nematodes (adult)	0.00	0.17
	Trematodes	89.69	16.50
Intestine (exterior)	Nematodes	0.31	2.50
	Trematodes	0.69	1.50
Intestine	Unknown	0.08	0.00
Gonads	Cestode (larvae)	2.15	0.17
	Nematode	0.08	0.00
	Unknown	0.00	0.17
Mesenteries	Trematodes	0.46	7.50
	Nematodes	1.00	1.17
	Cestode (larvae)	1.69	0.83
	Unknown	0.31	0.00
Gallbladder	Cestode (larvae)	42.62	28.00
	Nematodes	0.08	0.17
	Unknown	0.00	0.17

All parasite identifications in table 3-1 are based on morphological identification. Parasite abundance is a parameter of parasite infection and is considered a multifactorial variable of both biotic and abiotic factors. Abundance may be considered highly variable by species, both host and parasite (Amarante et al., 2016).

$$\text{Parasite Abundance} = \frac{Total\ number\ of\ parasites}{Number\ of\ infected\ hosts + Number\ of\ uninfected\ hosts}$$

Table 3-2 Parasite Intensity data for every examined site of infection by location.

Site of attachment	Parasite identification	North n=13	South n=6
External exam	Sea Leech	2.00	5.00
	Monogeneans	2.67	4.60
	Nematodes	0.00	1.00
	Unknown	0.00	1.00
Nasal flush	Monogeneans	0.00	3.00
	Nematodes	0.00	1.00
Brain	Unknown	1.00	1.00
Gills	Copepods	2.00	1.00
	Monogeneans	0.00	9.00
	Isopods	42.00	4.00
	Sea Leech	2.00	1.00
	Nematode	0.00	1.00
	Acanthocephalan	2.67	5.00
	Unknown	1.67	1.00
Flesh	Nematodes	4.71	6.75
Body cavity	Nematodes	10.60	9.00
Spleen	Nematodes	1.00	0.00
	Trematodes	1.00	0.00
	Unknown	1.00	0.00
Heart	Nematodes	0.00	1.00
Liver (exterior)	Nematodes	2.33	4.25
	Cestode (larvae)	0.00	3.00
	Trematodes	3.00	0.00
Liver (interior)	Nematodes	1.00	0.00
Liver	Unknown	1.00	0.00
Stomach (exterior)	Nematodes	1.00	0.00
	Trematodes	26.00	0.00
Stomach (interior)	Nematodes	1.00	8.67
	Trematodes	8.33	1.00
	Cestode (larvae)	1.00	0.00
Intestine (interior)	Nematodes	2.00	39.00

	Nematodes (adult)	0.00	1.00
	Trematodes	194.33	33.00
Intestine (exterior)	Nematodes	4.00	15.00
	Trematodes	4.50	4.50
Intestine	Unknown	1.00	0.00
Gonads	Cestode (larvae)	9.33	1.00
	Nematodes	1.00	0.00
	Unknown	0.00	1.00
Mesenteries	Trematodes	1.50	9.00
	Nematodes	4.33	2.33
	Cestode (larvae)	2.75	2.50
	Unknown	4.00	0.00
Gallbladder	Cestode (larvae)	46.17	42.00
	Nematodes	1.00	1.00
	Unknown	0.00	1.00

All parasite identifications in table 3-2 are based on morphological identification. Parasite intensity is a variable that affects the ability of stock discrimination with the use of parasites as biological tags. The intensity of infection should remain relatively stable on a sessional as well as annual basis for parasites to be eligible as biological tag (MacKenzie & Abaunza, 1998).

$$\text{Parasite Intensity} = \frac{Total\ number\ of\ parasites}{Number\ of\ infected\ hosts}$$

Table 3-3 Parasite Prevalence data for every examined site of infection by location.

Site of attachment	Parasite identification	North n=13	South n=6
External exam	Sea Leech	7.69%	50.00%
	Monogeneans	23.08%	83.33%
	Nematodes	0.00%	16.67%
	Unknown	0.00%	16.67%
Nasal flush	Monogeneans	0.00%	16.67%
	Nematodes	0.00%	16.67%
Brain	Unknown	7.69%	16.67%
Gills	Copepods	46.15%	16.67%
	Monogeneans	0.00%	33.33%
	Isopods	7.69%	16.67%
	Sea Leech	7.69%	16.67%
	Nematodes	0.00%	33.33%
	Acanthocephalan	23.08%	16.67%
	Unknown	23.08%	50.00%
Flesh	Nematodes	53.85%	66.67%
Body cavity	Nematodes	38.46%	16.67%
Spleen	Nematodes	7.69%	0.00%
	Trematodes	7.69%	0.00%
	Unknown	7.69%	0.00%
Heart	Nematodes	0.00%	16.67%
Liver (exterior)	Nematodes	23.08%	66.67%
	Cestode (larvae)	0.00%	16.67%
	Trematodes	7.69%	0.00%
Liver (interior)	Nematodes	7.69%	0.00%
Liver	Unknown	7.69%	0.00%
Stomach (exterior)	Nematodes	15.38%	0.00%
	Trematodes	7.69%	0.00%
Stomach (interior)	Nematodes	7.69%	50.00%
	Trematodes	23.08%	16.67%
	Cestode (larvae)	7.69%	0.00%
Intestine (interior)	Nematodes	15.38%	50.00%
	Nematodes (adult)	0.00%	16.67%
	Trematodes	46.15%	50.00%
Intestine (exterior)	Nematodes	7.69%	16.67%
	Trematodes	15.38%	33.33%
Intestine	Unknown	7.69%	0.00%
Gonads	Cestode (larvae)	23.08%	16.67%
	Nematode	7.69%	0.00%
	Unknown	0.00%	16.67%
Mesenteries	Trematodes	30.77%	83.33%
	Nematodes	23.08%	50.00%
	Cestode (larvae)	61.54%	33.33%
	Unknown	7.69%	0.00%
Gallbladder	Cestode (larvae)	92.31%	66.67%
	Nematodes	7.69%	16.67%
	Unknown	0.00%	16.67%

All parasite identifications in table 3-3 are based on morphological identification. Parasite prevalence is a variable that represents the proportion of the population that is infected with the parasite of interest.

$$\text{Parasite Prevalence} = \frac{Number\ of\ infected\ hosts}{Number\ of\ infected\ hosts + Number\ of\ uninfected\ hosts}$$

3.1 Sequences from successful PCRs

Table 3-4 Molecular confirmation of identification for sequenced parasites from the North.

PRC ID	Extraction ID	Parasite	Host ID	Organ	BLASTn ID
36	AR 013	Nematode	WF 04	Body cavity	***Anisakis simplex***
59	AR 036	Nematode	WF 01	Body cavity	***Anisakis simplex***
71	AR 048	Nematode	WF 04	Body cavity	***Anisakis simplex***
82	AR 059	Nematode	WF 01	Body cavity	***Anisakis simplex***
68	AR 045	Nematode	WF 04	Flesh	***Anisakis simplex***
88	AR 065	Nematode	WF 01	Gallbladder	***Anisakis simplex***
47	AR 024	Nematode	WF 03	Gonads	***Anisakis simplex***
78	AR 055	Nematode	WF 01	Intestine EXT	***Anisakis simplex***
42	AR 019	Nematode	WF 01	Intestine INT	***Anisakis simplex***
48	AR 025	Nematode	WF 03	Intestine INT	***Anisakis simplex***
64	AR 041	Nematode	WF 04	Liver EXT	***Anisakis simplex***
74	AR 051	Nematode	WF 04	Liver EXT	***Anisakis simplex***
46	AR 023	Nematode	WF 01	Liver INT	***Anisakis simplex***
58	AR 035	Nematode	WF 03	Mesenteries	***Anisakis simplex***
84	AR 061	Nematode	WF 01	Mesenteries	***Anisakis simplex***
85	AR 062	Nematode	WF 03	Mesenteries	***Anisakis simplex***
52	AR 029	Nematode	WF 03	Spleen	***Anisakis simplex***
75	AR 052	Nematode	WF 04	Stomach EXT	***Anisakis simplex***
57	AR 034	Nematode	WF 01	Stomach INT	***Anisakis simplex***
62	AR 039	Nematode	WF 01	Stomach INT	***Anisakis simplex***
45	AR 022	Nematode	WF 01	Flesh	***Pseudoterranova decipiens***
67	AR 044	Nematode	WF 01	Flesh	***Pseudoterranova decipiens***

The diversity of sampled nematodes from *A. lupus* caught in the north of Iceland (table 3-5) is minimal with *Anisakis simplex* being the predominant species making up 20 out of 22 samples sequenced and being found in 12 different infections sites. The other two samples being *Pseudoterranova decipiens.*

Table 3-5Molecular confirmation of identification for sequenced parasites from the South.

PRC ID	**Extraction ID**	**Parasite**	**Host ID**	**Organ**	**BLASTn ID**
86	AR 063	Nematode	WF 11	Mesenteries	***Anisakis pegreffi***
72	AR 049	Nematode	WF 14	Body cavity	***Anisakis simplex***
54	AR 031	Nematode	WF 13	External	***Anisakis simplex***
50	AR 027	Nematode	WF 12	Flesh	***Anisakis simplex***
55	AR 032	Nematode	WF 10	Flesh	***Anisakis simplex***
69	AR 046	Nematode	WF 10	Flesh	***Anisakis simplex***
89	AR 066	Nematode	WF 14	Gallbladder	***Anisakis simplex***
39	AR 016	Nematode	WF 14	Intestine EXT	***Anisakis simplex***
80	AR 057	Nematode	WF 01	Intestine INT	***Anisakis simplex***
38	AR 015	Nematode	WF 12	Liver EXT	***Anisakis simplex***
60	AR 037	Nematode	WF 11	Liver EXT	***Anisakis simplex***
77	AR 054	Nematode	WF 12	Liver EXT	***Anisakis simplex***
90	AR 067	Nematode	WF 11	Liver EXT	***Anisakis simplex***
53	AR 030	Nematode	WF 14	Mesenteries	***Anisakis simplex***
66	AR 043	Nematode	WF 11	Mesenteries	***Anisakis simplex***
87	AR 064	Nematode	WF 14	Mesenteries	***Anisakis simplex***
76	AR 053	Nematode	WF 13	Stomach INT	***Anisakis simplex***
35	AR 012	Nematode	WF 14	Gills	***Contraceacum osculatum***
79	AR 056	Nematode	WF 14	Intestine EXT	***Hysterothylacium aduncum***
83	AR 060	Nematode	WF 14	Intestine INT	***Hysterothylacium aduncum***
37	AR 014	Nematode	WF 13	Stomach INT	***Hysterothylacium aduncum***
81	AR 058	Nematode	WF 13	Intestine INT	Sequence Unsuccessful
49	AR 026	Nematode	WF 13	Nasal flush	Sequence Unsuccessful
44	AR 021	Nematode	WF 09	Stomach INT	Sequence Unsuccessful
91	AR 108	Nematode	WF 09	Intestine INT	Sequence Unsuccessful
113	AR 080	Trematode	WF 14	Stomach INT	***Fellodistomum fellis***

The diversity of the parasites sequenced from *A. lupus* caught in the southern region of Iceland (table 3-5) is higher than that of the northern region. With four species being represented in the sampling pool. *Anisakis simplex* is still the predominant species making up 16 of the 25 nematode samples sequenced. *A simplex* had nine different sites of infection. Three samples proved to be of *Hysterothylacium aduncum,* one sample was identified as *Contraceacum osculatum* and one sample of *Anisakis pegreffi*. The remaining four samples sent out for sequencing did not come back with a successful identification. The only trematode to provide a successful sequence came from a *A. lupus* sampled in the south. The sequence identified the trematode as *Fellodistomum fellis.*

Tables 3-5 and 3-6 represent all successful PCRs from the study. Apart from one trematode (PCR sample 113), which was identified as *Fellodistomum fellis*, the only PCRs to provide DNA fragments suitable for sequencing were the nematode samples. The majority of the nematode samples were successful in providing enough DNA fragments for sequencing.

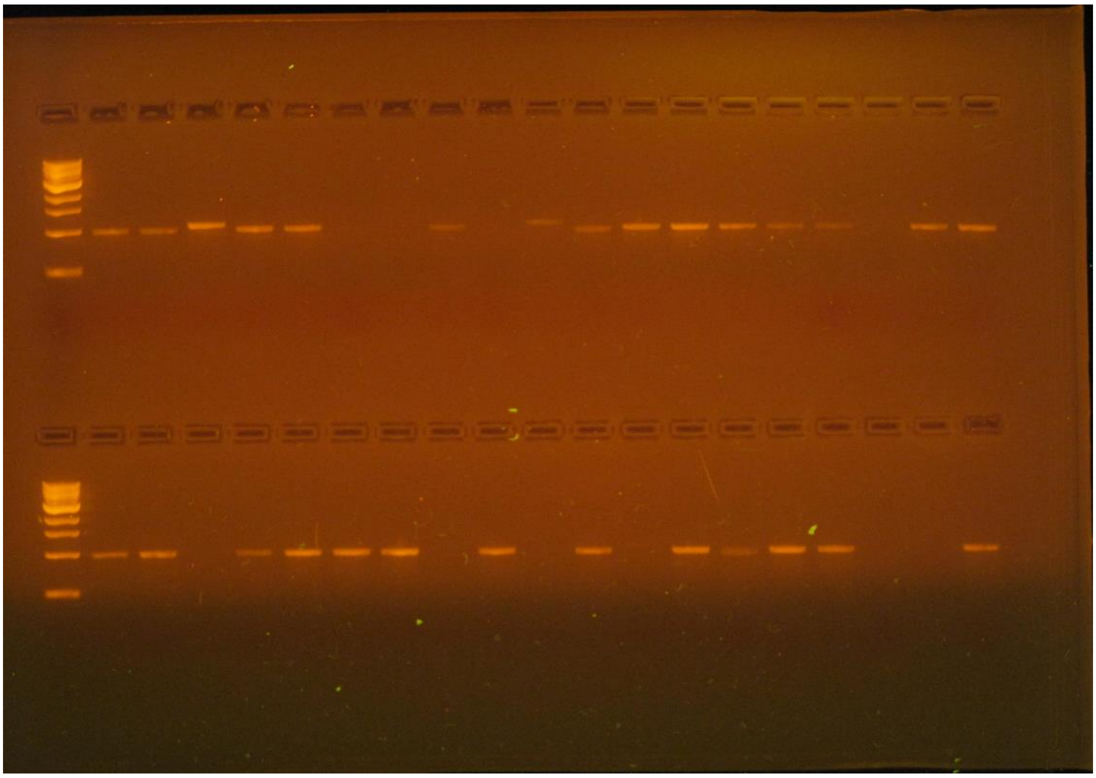

Figure 3-2 PCR gel electrophoresis of nematode samples 35-68.

The viable bands on the electrophoresis gel in Figure 3-2 show how far the DNA strands made it through the gel. The distance each band travels from the wells is a measure of how large the DNA fragments are. The wells on the far right are loaded with a ladder, providing multiple bands of a known size. From the top left the samples were loaded in order of processing, the first sample next to the ladder on the top is sample 35, the next one 36 and so on. The bands show that the DNA sequences of the samples are all a similar size. Sample 35 is from *Contraceacum osculatum* retrieved from WF 14, which had a Sequence length of 927 bases.

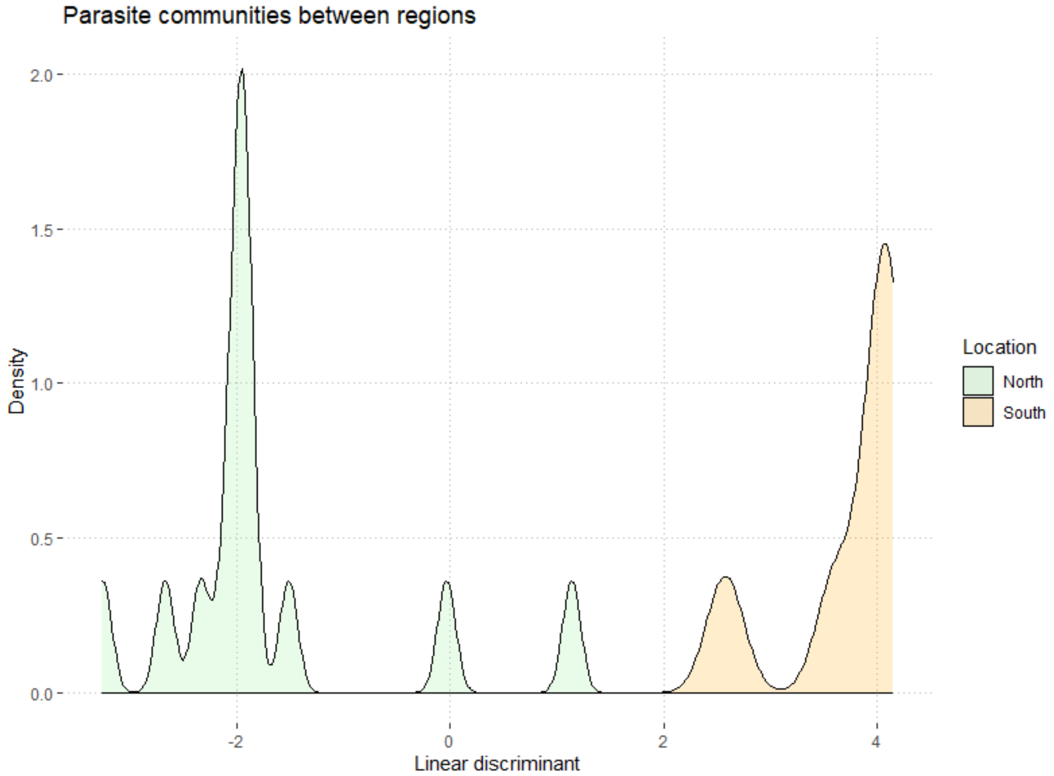

Figure 3-3 Liner discrimination of parasite fauna by location.

he linear discriminant plot shows that there is complete separation of the parasite communities. This is because certain groups of parasite infections have no significant overlap between the two locations.

Table 3-6 Assignment of specimens to locations based on parasite fauna.

	Actual location of origin	
Predicted location of origin	North	South
North	13	0
South	0	6

The algorithm had been trained to process the parasite data. It could correctly assign each *A. lupus* specimen to its location of origin. The outcome of the analysis and assignment of location of origin can be seen in table 3-6.

4 Discussion

The aim of the study is to catalogue the parasite communities of *A. lupus* in Icelandic waters and to see if stock discrimination is possible based on the variation of parasite communities between the two locations. To answer the question of what parasites, make up the fauna of *A. lupus* parasite communities, the 19 specimens collected were examined for ecto- and endoparasites. The findings catalogued and analysed. The results showed that there is a separation of parasite communities between *A. lupus* individuals originating from the North and the South.

4.1 Parasites of Atlantic wolffish

Recorded parasite infections in *A. lupus* are not as readily available as one might hope. There is some documentation although most do not center on parasites but rather mention them in passing. From those documents we can assume that a substantial proportion of the parasites we will encounter in the necropsies might be new to us due to a lack of prior research of Atlantic wolffish parasites in Icelandic waters. There are a few parasites that have been documented and they are *Platybdella anarhichae*, a leech most commonly found behind the pectoral fin (Jónsson, 1982). Although molecular identification of the leeches collected in this study, were not preformed, the description of *Platybdella anarhichae* match the leeches collected from *A.lupus* specimens. There is a distinct lack of recorded monogenean parasite infection of Atlantic wolffish in the North Atlantic. There only available documented case was a single specimen of *Gyrodactylus* errabundus, that was found on an Atlantic wolffish from a laboratory tank. The tank also held the monogeneans true host, a European eelpout *Zoarces viviparus* (Mo & Lile, 1998). Nematodes are not commonly found in the flesh of *A. lupus* but rather in the alimentary system with reports of *Contracaecum auduncum* infections in both the stomach and small intestine (Jónsson, 1982). *Anisakis simplex*, is a nematode species common in most teleost fish. *Anisakis simplex* eggs are released into the environment with feces of marine mammals. The eggs then develop in the water and reach a larval stage that can be ingested by crustaceans. Once *A. simplex* has infected the intermediate host the larvae migrates from the crustaceans intestine to its peritoneal cavity. Predatory fish then eat the infected crustacean. Once the intermediate host has been eaten, the larvae migrate to the liver and or muscle tissue of the fish. There the larvae can develop into a sexually mature worm, the females then start producing eggs that are excreted with the hosts fecal material and the lifecycle is complete (McConnaughey, 2014). A larvae of *Hepatoxylon trichiuri* a species of tapeworm which infects pelagic sharks as definitive hosts (Palm & Caira, 2008), has also been recorded in the small intestine of *A. lupus* (Jónsson, 1982). The tapeworms found during the necropsies and examination of organs, were all larval forms. The most prevalent infections were in the gallbladders. With a 93.3% prevalence in specimens from the north and 66.7% prevalence in specimens from the south.

There are also recordings of a sporozoan infection that was prevalent in about 10% of the total Icelandic catch of *A. lupus* in 1952. The infection is found in the flesh of the fish and gives the appearance of discontinuous veins (Jónsson, 1982). Instances of identification on a species level of *A. lupus* parasites are not very common. The species that have available data most are digenean trematodes. *Derogenes varicus* is a digenean trematode with a free living cercariae stage that swims with a furcate appendage. The cercariae are eaten by copepods, which are then preyed upon by small planktivorous fish. If planktivorous fish harbour immature *D. varicus*, piscivorous fish who feed on them have been found to be a suitable definitive host for *D. varicus* (Køie, 1979). It has been hypothesised that hermit crabs, who are a frequent prey item of *A. lupus*, could come into contact with *D. varicus* in its planktonic larval phase, and carry that infection to the wolffish (Bray, 1987). *Plagioporus indoneus* another digenean trematode, the life cycle is not known but studies of related species suggest that small crustaceans and amphipods could be the usual second intermediate host. Bray (1987) suggests that larger decapods could be the source of *P. indoneus* in Atlantic wolffish (Bray, 1987). *Brachyenteron pycnorganum*, is a digenean trematode that has been found in the intestine of *A. lupus*. little information is available on its lifecycle (Bray, 1987). Another instance of a recorded parasitic digenean in *A. lupus* is *Caudotestis nicolli* which was found in the intestine (Bray, 1987). The lifecycle is not fully known but we do know that it uses sculpins as an intermediate host (Gibson et al., 1996). *Fellodistomum agnotum* has been recorded in *A. lupus* in the White Sea. The identification of *F. agnotum* was based on morphological characteristics where the specimens were stained to emphasize their characteristics, the identification was then confirmed with the use of molecular data from PCR testing. Three gastropod species have been confirmed as the second stage intermediate host for *F. agnotum*, those species are: (1) *B. undatum*, (2) *B. scalariforme*, and (3) *N. despecta* (Krupenko et al., 2020). Both *B. undatum* and *F. agnotum* have been found in the stomach contents of wolffish during marine surveys (Delongueville, et al., 2021). *Fellodistomum fellis* is a digenean trematode that is known to infect *A. lupus*, it can be found in its adult stage in the gallbladder of *A. lupus*. The metacercaria have been found in (1) *B. undatum*, (2) *B. scalariforme*, and (3) *N. despecta* much like *F. agnotum (*Krupenko et al., 2020). *Neophasis anarrhichae*, is a digenean trematode with a two-host life cycle it infects the common whelk *B. udatum* as its intermediate host and through the predation on the whelk it reaches its definitive host wolffishes belonging the genus *Anarhichas* (Kremnev et al., 2021).

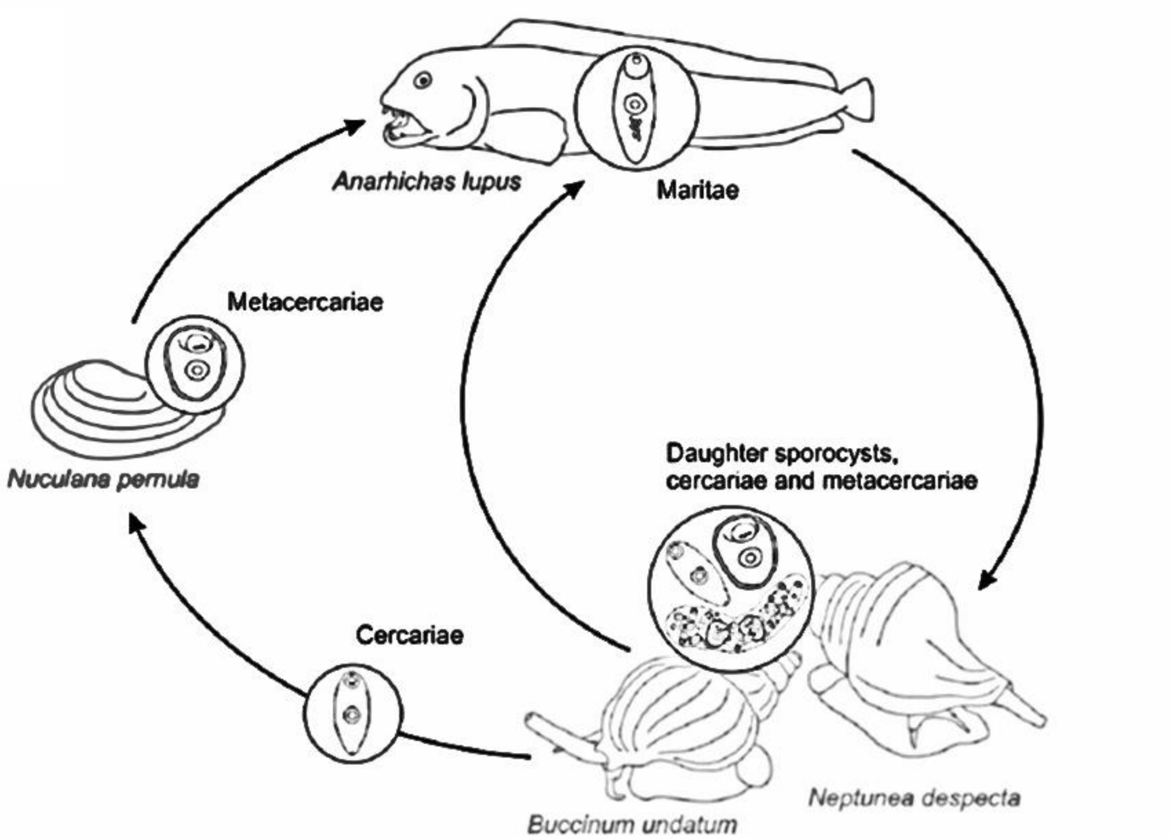

Figure 4-1 Lifecycle of Pseudozoogonoides subaequiporus (Kremnev et al., 2023).

Pseudozoogonoides subaequiporus is a digenean trematode that uses buccinid gastropods as their first intermediate and bivalves as second intermediate hosts, before infecting fish as their definitive host. For *A. lupus*, it is thought that the dominant way of transmission of *P. subaequiporus* is the direct consumption of the first intermediate hosts (Kremnev et al., 2023).

Clavellodes rugosus, is a copepod species that has been recorded in the gills of both *Anarchichas lupus* the Atlantic wolffish and *A. minor* the spotted wolffish. (Krøyer, 1837 as cited in Kabata et al., 1988).

Ascarophis morrhuae is a nematode that has been recorded as a parasite of *A. lupus* (Arai & Smith, 2016). *A. morrhuae* has a two-host life cycle where it uses hermit crabs as an intermediate host. In the hermit crab the larval worm encapsulates itself until the intermediate host is consumed by the definitive host. There it can reach maturity in the intestine. Once the worm has reached maturity it can release eggs that are excreted with the hosts faecal matter. The eggs are then eaten by the hermit crabs and the cycle is complete (McDermott et al., 2010).

4.2 Implications

Because the findings show such a strong separation of parasite communities between the two sampling location, parasite communities of *A. lupus* could be useful as biological tags for stock discrimination. A larger sample size would most likely provide a usable basis for stock discrimination. The use of parasites as biological tags has proven to be a more affordable, and less invasive method of stock studies compared to artificial tags. The use of parasites as biological tags is also often more opportunistic, since the samples can be collected on various research surveys and do not require a special tagging survey (MacKenzie & Abaunza, 1998).

4.3 Limitations

Even though the linear discriminant analysis shows 100% separation, the quality of the data is not ideal, the sample size of *A. lupus* specimens between the locations are not equal and vary by more than a factor of three. On top of that the overall sample size may not be sufficient to portray a realistic representation of the *A. lupus* population. For a more accurate representation a larger sample size would be needed. Estimations of the scale biodiversity of parasites indicate that they make up as much as half of all species on Earth (Poulin et al., 2016). A large proportion of parasites are aquatic during different stages of their life cycle. It should therefore be safe to assume that their representation in academic literature regarding marine ecology would be at least equal to the general ecological literature, if corrected for taxonomic and other biases. That is however not the case, a survey of publication trends covering the last decade estimated that on average 10% or more of published articles about general ecology tackled the subject of parasites and diseases. When it comes to literature of marine ecology that number is less than 5%. Showing that parasitological studies are vastly underrepresented in scientific literature (Poulin et al., 2016).

A catalog of parasite fauna provides a list of parasites to be expected during a parasitological survey. Host-parasite checklists are compiled of an extensive list including all known host parasite associations. The problem is that most of the available lists are based on morphological identifications and lack molecular conformations. These lists, though often very useful, are not an exhaustive list of all host and parasite associations that exist in a given region. The lists may encompass larger spatial and temporal scales, that often include host parasite associations not found within reginal study areas of parasite surveys (Poulin et al., 2015). Therefor the list of parasites of *A. lupus* that was assembled from other host-parasite check lists for this study does not necessarily provide a conclusive list of the parasite fauna of *A. lupus* in Icelandic waters. Given the scope and cost of molecularly confirming all parasites identification, from all specimens of *A. lupus* was not possible. There for two specimens of every parasite type from every site of infection of two fish from either location were selected for molecular conformation of identification. This means that the majority of parasites collected were not identified at a species level. Because not all parasite functional groups were included in the DNA extraction and PCR, and the fact that most of the PCRs were unsuccessful it seems likely that the species diversity of parasites is underrepresented. In fact, the only PCRs that were successful were the nematode samples and one trematode sample (table 3-5 and 3-6). The result show us that there are at least five different species of nematodes that infect *A. lupus* in Icelandic waters. But the sample size is not of a size large enough to accurately represent the nematode diversity of *A. lupus,* we can assume that the species that are present in this study could be the most prevalent once, especially *Anisakis simplex* which made up the majority of the sequence data, for both the northern and southern sampling pools.

4.4 recommendations

The data that was collected and processed did show that there is separation between the parasite communities, that result suggests that there are grounds for further research with a larger sample size. The sampling took place over a period two months, as stated by MacKenzie and Abaunza (1998), for parasites to be ideal as biological tags, their level of infection should remain relatively constant from year to year, that is data we do not have for the parasites of *A. lupus.* Since most of the parasites observed use intermediate hosts it would be safe to assume that seasonal, as well as annual changes in the abundance and density of the intermediate hosts populations could affect the level of infection seen in *A. lupus.* A multi-year study could answer this question and provide a sufficient data set to provide a reliable representation of the parasite fauna of *A. lupus,* with a more accurate conclusion of if, and what parasites can reliably be used for stock discrimination of *A. lupus* in Icelandic waters.

5 Conclusion

The available data on *A. lupus* host-parasite associations is less than optimal, and due to time constraints, a sample size large enough to accurately describe the parasite fauna of *A. lupus* in Icelandic waters was not obtainable. The data collected did however show a strong indication of separation of *A. lupus* individuals based on parasite communities variation between the two sampling locations. Since such a low number of the PCRs were successful the species diversity of *A. lupus* parasites is most likely underrepresented in the data. Therefore, the next recommended steps in compiling an accurate list of *A. lupus* parasites would be further samplings of *A. lupus* over a longer time frame and larger sample size. A larger temporal scale is needed to accurately account for sessional and or annual variation in parasite prevalence and intensity.

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Figure reference

Figure 0-1 Lifecycle of Pseudozoogonoides subaequiporus. Kremnev, G., Gonchar, A., Uryadova, A., Krapivin, V., Skobkina, O., Gubler, A., & Krupenko, D. (2023). No tail no fail: Life cycles of the Zoogonidae (Digenea). *Diversity*, *15*(1), 121. https://doi.org/10.3390/d15010121

Appendix

R-code for figure 2.

```
# Install and load required packages
install.packages("leaflet")
library(leaflet)

# Create a dataframe with location data
locations <- data.frame(
  Name = c("WF01-05 80m Depth", "WF06-08 125m Depth", "WF09-11 77m Depth",
"WF12-14 68m Depth", "WF15-19 53m Depth"),
  Lat = c(66 + 6/60 + 33/3600, 66 + 3/60 + 33/3600, 63 + 57/60 + 57/3600, 63 + 55/60 +
        37/3600, 65 + 42/60 + 44/3600),
  Lon = c(-18 - 33/60 - 31/3600, -17 - 34/60 - 41/3600, -22 - 48/60 - 20/3600, -22 - 46/60 -
        49/3600, -20 - 26/60 - 3/3600))

# Create a leaflet map object with grayscale tiles
map <- leaflet() %>%
  addTiles(urlTemplate = "http://{s}.tile.stamen.com/toner-lite/{z}/{x}/{y}.png")

# Add dot markers for each location
for (i in 1:nrow(locations)) {
  map <- map %>%
    addCircleMarkers(
      lat = locations$Lat[i],
      lng = locations$Lon[i],
      radius = 4,

# Adjust the radius to change the size of the markers
      fill = TRUE,
      fillOpacity = 1,
      fillColor = "red",
      color = "red",
      stroke = FALSE,
      popup = locations$Name[i] )
}

# Add label-only markers for each location with adjusted offsets
offsets <- list(list(x = +10, y = -15), list(x = +125, y = -15), list(x = 0, y = -15), list(x = 0, y
= 15), list(x = 0, y = -15))
for (i in 1:nrow(locations)) {
  map <- map %>%
    addLabelOnlyMarkers( lat = locations$Lat[i],
```

```
   lng = locations$Lon[i],
   label = locations$Name[i],
   labelOptions = labelOptions(
     noHide = TRUE,
     direction = "under",
     textOnly = TRUE,
     offset = offsets[[i]],
     style = list("font-size" = "14px") ) )
}

# Print the map
map
```

R-code for Linear discrimination

```
wolffish<- read.csv("ParaLocations for R (ARE).csv", sep=",", header=T)

install.packages(c("usethis", "cli", "remotes"))
install.packages("devtools")
library(MASS)
library(ggplot2)
library(readxl)
library(klaR)
library(psych)

options(repos = c(
  fawda123 = 'https://fawda123.r-universe.dev',
  CRAN = 'https://cloud.r-project.org'))

install.packages('ggord')
library(ggord)


###########

#Read file

###########

wolffish<- read.csv("Parasites for R (ARE).csv", sep=",", header=T)
str(wolffish)
summary(wolffish)
wolffish     <-na.omit(wolffish)
summary(wolffish)
```

```
##Remove column

wolffish=subset(wolffish, select=-c(Fish))
summary(wolffish)

#LDA
#pairs.panels(wolffish,gap=0, pch=21)
set.seed(123)

sample<-sample(c(TRUE, FALSE),nrow(wolffish), replace=TRUE, prob=c(1, 0))
#sample<-sample(c(TRUE, FALSE),nrow(wolffish), replace=TRUE, prob=c(0.6, 0.4))
train<-wolffish[sample, ]
test<-wolffish[!sample, ]

linear<-lda(Location~., data=train)
linear
attributes(linear)
par("mar")
par(mar=c(1,1,1,1))
p<-predict(linear,train)
ldahist(data=p$x[,1],g=train$Location)
dim(p$x)
str(p)

#ggord(linear,train$Species, xlim=c(-6,6), ylim=c(-6,8),vectyp="blank", txt=NULL)

#ggord(linear,train$Species, xlim=c(-6,6), ylim=c(-6,8))

#Training dataset

p1<-predict(linear,train)$class
tab<-table(Predicted=p1,Actual=train$Location)
tab
sum(diag(tab))/sum(tab)

#Testing dataset
#p2<-predict(linear,test)$class
#tab1<-table(Predicted=p2,Actual=test$Species)
#tab1
#sum(diag(tab1))/sum(tab1)


###########
```

```
#Groupings

wolffish_lda<-
lda(Location~EE_Le+EE_Mono+EE_Nema+EE_Uk+NF_Mono+NF_Nema+BR_Uk+GI_Cope+GI_Mono+GI_Iso+GI_Le+GI_Nema+GI_Acan+GI_Uk+FL_Nema+BC_Nema+SP_Nema+SP_Trema+SP_Uk+HE_Nema+LI_ext_Nema+LI_int_Nema+LI_ext_CesL+LI_ext_Trema+LI_Uk+ST_ext_Nema+ST_int_Nema+ST_ext_Trema+ST_int_Trema+ST_int_CesL+IN_ext_Nema+IN_int_Nema+IN_int_Nema_adult+IN_int_Trema+IN_ext_Trema+IN_Uk+GO_CesL+GO_Nema+GO_Uk+ME_Trema+ME_Nema+ME_CesL+ME_Uk+GB_CesL+GB_Nema+GB_Uk, data=wolffish)

wolffish_lda
print(wolffish_lda)
p<- predict(wolffish_lda, train)
p.df <- data.frame(LD1 = p$x, class = train$Location) #--- converting the prediction to data.frame
print(p.df)


#--- plotting the density plot --- #

library(ggplot2)
custom_colors <- c("lightgreen", "orange")
ggplot(p.df) + geom_density(aes(LD1, fill = class), alpha = 0.2) +
  scale_fill_manual(values = custom_colors) +
  labs(fill = "Density", x = "Linear discriminant", y = "Density") +
  ggtitle("Parasite communities between regions") +
  theme(panel.background = element_blank(),
panel.grid.major = element_line(color = "gray", linetype = "dotted")) +
labs(fill = "Location")

wolffish_ldapred<-predict(wolffish_lda,wolffish[,-1])
table(wolffish$Location,wolffish_ldapred$class)

wolffish_ldapred

p<- predict(wolffish_lda, train)
p.df <- data.frame(LD1 = p$x, class = train$Location) #--- converting the prediction to data.frame
print(p.df)

#--- plotting the density plot --- #

library(ggplot2)
ggplot(p.df) + geom_density(aes(LD1, fill = class), alpha = 0.2)
```

```
#wolffish_ldacv<-
lda(Location~EE_Le+EE_Mono+EE_Nema+EE_Uk+NF_Mono+NF_Nema+BR_Uk+GI_Cope+GI_Mono+GI_Iso+GI_Le+GI_Nema+GI_Acan+GI_Uk+FL_Nema+BC_Nema+SP_Nema+SP_Trema+SP_Uk+HE_Nema+LI_ext_Nema+LI_int_Nema+LI_ext_CesL+LI_ext_Trema+LI_Uk+ST_ext_Nema+ST_int_Nema+ST_ext_Trema+ST_int_Trema+ST_int_CesL+IN_ext_Nema+IN_int_Nema+IN_int_Nema_adult+IN_int_Trema+IN_ext_Trema+IN_Uk+GO_CesL+GO_Nema+GO_Uk+ME_Trema+ME_Nema+ME_CesL+ME_Uk+GB_CesL+GB_Nema+GB_Uk, data=wolffish, CV=T)

#ggord(linear, train$Location,xlim=c(-10,10), ylim=c(-10,5),axes=c("1", "2"),xlab='1st LD',ylab='2nd LD', ext=3)
#ggord(linear, train$Location,xlim=c(-5,5), ylim=c(-5,5),axes=c("1", "2"),xlab='1st LD',ylab='2nd LD', vectyp="blank", txt=NULL)
#ggord(linear, train$Location,xlim=c(-5,5), ylim=c(-5,5),axes=c("1", "2"),xlab='1st LD',ylab='2nd LD')
#Change Axes values if warning message "geom_segment"
```