MASTER'S THESIS

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Prevalence and load of *Anisakis spp.* in marine fish in the context of a rapidly changing marine environment

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Abstract

Climate change has caused substantial changes to marine ecosystems in recent decades, and a following consequence of this might be an increase in marine disease. Parasitic nematodes are an important mediator of disease in marine ecosystems. The *Anisakis simplex* species complex has apparently increased in abundance and prevalence in recent years. These parasites can have damaging effects on their hosts and can promote a higher incidence of zoonosis in humans. In this project, fish from the fjords near Bodø, Norway, were examined to determine the prevalence and abundance of *Anisakis* in the visceral organs and body cavity, and molecular genetic analyses conducted to investigate whether there has been any interbreeding of the parasite populations leading to eventual hybridization. The overall health of the fish hosts was calculated using the hepatosomatic Index (HSI) and Fulton's body condition factor (K), and these indices were used to assess whether parasite burden had any effect on the health. The second part of this study consisted of examining a collection of historic fish samples from the same area, where the visceral organs and body cavities were examined to assess parasite prevalence and load. The load and prevalence of parasites in the historic samples were then compared to those of contemporary samples. Statistical comparisons showed a significant increase in the abundance and prevalence of parasitic nematodes in the contemporary fish; *Anisakis* were found in 71.5% fish from the contemporary samples, and in 50.9% fish in historic samples. These differences were reviewed in the context of possible consequences of environmental change.

Keywords: Anisakis simplex, climate change, introgression, parasites, teleosts, Arctic, molecular analysis

Acknowledgements

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Elin-Marita B. Kristiansen

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1.0 – Introduction

1.1 – Changing climates and the changing marine environment

Climate fluctuates and changes over time, a consequence of natural cycles and contributing factors such as natural disasters and glacial periods (Khairullina *et al.*, 2019). These natural drivers include changes in the orbital cycle of the Earth, aerosol loading in the atmosphere due to large volcanic eruptions as well as changes in the Sun's energy output (Ghil, 2002). Post industrial revolution, these natural cycles have been disturbed by anthropogenic effects such as CO2-emissions due to industrialization, as well as physical changes such as landscape engineering for settlement and agriculture to sustain the sudden explosion in human population numbers globally (Godber and Wall, 2014). An increase of carbon dioxide as well as water vapor, methane and ozone in the atmosphere leads to an increased greenhouse effect that traps heat in the atmosphere (Kweku *et al.*, 2018). The increase of carbon dioxide emissions also affects the oceans, ultimately leading to changes in seawater carbonate chemistry and reduction of the ocean's pH-level (Kroeker *et al.*, 2010). These actions, among countless others are harming the environment and changing biomes and habitats. The area on the planet that has been most noticeably and rapidly affected by these changes is the Arctic, which in turn affects the rest of the planet (Thomas *et al.*, 2022). Many of the species inhabiting the ocean are sensitive to change, this is in particular during juvenile and larval stages of development, as well as invertebrates that undergo calcification during development (e.g. mollusks, corals, crustaceans) (Kroeker *et al.*, 2010). A warming ocean can lead to range shifts of species that have been native to certain areas, and a subsequent influx of anomalous non-native species, ultimately leading to disturbances in the food webs and destruction of habitats (AMAP, 2021). Disturbance of food webs can precipitate ecological cascades, where the effect of removal of a significant species in an ecosystem leads to overabundance and/or underabundance of other species or trophic levels in that ecosystem. A study by Myers *et al*., showed that reduction of large sharks (apex predators) in an ecosystem ultimately lead to an increase of the their prey (the mesopredators). The increase of mesopredators in the food web led to a reduction of bivalves, an important food source for them. The significance of apex

predators in an ecosystem is highlighted as an important component of top-down control of populations, and contributes to stability of ecosystems (Myers *et al.*, 2007).

This study was conducted in the sub-Arctic Ocean and will therefore focuses on the changing climate of the Arctic and sub-Arctic Oceans over the last 150 years; as well as considering the consequences climate change can have on host-parasite interactions, and the consequences of an increase in parasite load and prevalence on the environment. The Arctic and polar environments are particularly vulnerable to climate change, and it has been shown that with air temperature increase there are consequences such as intensification of the hydrological cycle, downward trends in sea ice thickness, and Spring snow cover and duration (Box *et al.*, 2019). The Arctic has warmed on average around 2°C over the last 50 years, which is more than double the global average. This is causing the sea- and land ice covering areas of the Arctic to melt rapidly. As a consequence of reduction in sea ice thickness and coverage, the albedo has changed, with land and ocean masses that previously covered by ice now exposed, leading to a net absorption of heat from the sun, rather than reflection of sunlight from snow and ice cover (Thomas, 2021).

The consequences of a warming Arctic will have implications over a much larger area due to feedback mechanisms that respond to change, eventually producing major consequences. In the Arctic and sub-Arctic areas, climate change happens rapidly, and major disturbances or perturbations can have devastating effects on the local communities.

The temperature of the Arctic Ocean and its surroundings (i.e. Barents sea and Norwegian sea) are dominated by two currents: the cold and fresh Transpolar Drift Stream (Thomas, 2021) and the warm and salty Atlantic Ocean Inflow (Timmermans and Marshall, 2020). These currents affect weather patterns and the high- and low-pressure systems located above them, inevitably affecting climate change in the Arctic. The Atlantic Ocean inflow provides warm ocean water from the Gulf of Mexico (the Gulf Stream), and largely regulates the average temperature in the Arctic. Increased melting of ice will create a layer of fresher, lighter layer of water at the surface on the Atlantic inflow. This meltwater will act as a lid over the inflow, stopping water that enters the area from cooling, which ultimately prevents it from sinking deeper, weakening the drift (Heiderich and Todd, 2020). A change in ocean circulation and

weather patterns could lead to devastating consequences for ecosystems worldwide (Timmermans and Marshall, 2020), as these ocean currents also provide an important influx of nutrients for the food webs (Wenegrat *et al.*, 2020).

In addition to climate change, there are several anthropogenic disturbances on the oceanic ecosystems in the Arctic and sub-Arctic as well. The list includes, but is not limited to, human pollution such as farming and aquaculture industries, as well as fisheries. All these anthropogenic effects have, and will continue to lead to pollution, whether it be run-off from fertilizing agents from land-based farming causing eutrophication in the coastal areas of the oceans (Howarth, 2008), so-called ghost nets from fishing industries (Deshpande *et al.*, 2020), and general disturbances caused by aquaculture taking place in the ocean, and especially in coastal areas (Olaussen, 2018). Overfishing is probably the most important contributor in the context of disturbing oceanic ecosystems, but management approaches based on single species as well as those based on ecosystems are continually being implemented (Schmidt *et al.*, 2019).

Climate change leads to abrupt changes in the ecosystems and ultimately impacts the abundance and diversity of pathogens; general predictions show that with a warmer and wetter climate, there will be increased indices of pathogen diversity and associated disease. The low biodiversity of the Arctic makes it is highly vulnerable to invasions of disease causing pathogens (Kutz *et al.*, 2009). With these changes, host-parasite relationships also appear to be impacted. Temperature increases seem to influence parasite metabolism, suggesting the spread of parasites might become more efficient. Increased temperatures could potentially also expand the window of time during which parasites can infect the host, e.g. during the winter months, where normally the parasites would be dormant, could lengthen allowing the parasites to infect hosts over a longer period of time throughout the year (Lõhmus and Björklund, 2015). With increased productivity and fitness of parasites, hosts must adapt accordingly to avoid severe infection. Some host adaptations include selection of mates that appear to be healthier (Aeschlimann *et al.*, 2003), avoidance behavior (Karvonen, Seppälä and Valtonen, 2004), and immune responses to infection (Karlsson *et al.*, 2020).

A meta-analysis published in 2020 suggests evidence of a significant increase in the number of *Anisakis* found in fish since 1962 until 2015, and this could potentially be linked to disturbances in the marine ecosystems and a changing climate. An increase in *Anisakis* implies

a higher risk of *Anisakis* in humans and cetaceans, and can compromise fishery industries globally. It is likely that the increase of *Anisakis* is partly attributable to the increased abundance of cetaceans (final hosts) due to legislative or regulatory protections of cetaceans/marine mammals being enforced in more recent years (mid-80's onward) (Fiorenza *et al.*, 2020).

As mentioned in this study by Fiorenza et al., there are a multitude of consequences that come from an increase of *Anisakis* in marine life, and these can have a variety of impacts on host-parasite interactions; such changes may include changing of the genotypes causing allele frequencies in a population of parasites to change over multiple generations. Generally for parasitic nematodes in the Arctic, empirical evidence and modelling approaches demonstrate that climate change is driving range shifts, seasonal changes in phenology and increases of abundance and presentation (Aleuy and Kutz, 2020).

A changing climate can also lead to immigration and emigration of species native to different areas, increasing the likelihood of transporting parasitic species from one location to another (Brooks and Hoberg, 2007). If a foreign parasite (with a direct life cycle) is introduced to a new area with potential hosts, it must be able to establish in the new environment. This includes the ability to survive in the new habitat with unknown abiotic parameters, such as temperature and salinity in marine habitats, as well as having the ability to switch between hosts. After these obstacles have been overcome in the initial introduction, the parasite will pose as a threat to the native population of potential hosts (Lymbery *et al.*, 2014). Parasites introduced to naïve hosts often have a higher virulence than in hosts they have co-evolved with. This is known as the "naïve host syndrome", and the co-evolution between host and parasite is often viewed as a contest between host resistance and/or tolerance, and parasite virulence. Introduced parasites will, according to the "naïve host syndrome", be more likely to induce more severe cases of infection and pathogenic consequences in naïve hosts, and naïve hosts are more likely to acquire infection than hosts that have co-evolved with the parasite (Mastitsky *et al.*, 2010).

Introgression, or hybridization, plays an important role in host-parasite relationships and can lead to increased infection numbers by increasing host range and the ability to withstand new environmental conditions, in contrast to their parental origin (Huyse *et al.*, 2009). The

instances mentioned here can lead to destabilization of well-established host-parasite relationships, and thus lead to outbreaks of the pathogen in a population.

Environmental change can lead to changes in the severity or prevalence of disease due to complex changes in host-parasite relationships, particularly in parasites that have environmental transmission stages where they persist outside the host. However, knowledge on how climate change will affect and drive pathogen-host evolution is limited (Altizer *et al.*, 2013), thus studying these relationships might offer valuable insight into how such interactions might change under the pressure of a changing environment. Another important implication in studying the change of parasite-host interactions temporally is to investigate what the baseline for prevalence and load of parasitic infections has been historically. Many studies regarding these long-term interactions of hosts and parasites have been studied and documented, but obtaining historic samples to establish the baseline of prevalence and parasite load in hosts is a major limitation, and often the timescale of these interactions is a limiting factor.

1.2 – General features of a parasitic lifestyle

Parasitism is generally defined as "A symbiotic interaction in which one organism, the parasite, derives its nourishment from another organism, its host, which is harmed in the process." (Campbell *et al.*, 2018). However, parasitism is by no means beneficial for the host, as might be implied in Campbell's definition, because parasites exploit the hosts and divert resources for the growth, fecundity, and survival (Sorci and Garnier, 2019). The effects of parasites in an ecosystem have previously been ruled as insignificant due to their low biomass compared to the rest of the trophic groups in an ecosystem, however, parasite-mediated effects may play a big role in population dynamics, energy flow, and alteration of interspecific competition, appearing as important drivers of biodiversity. Further, parasites can alter community structure through instances such as manipulation of host behavior and increasing the host susceptibility to predators. These effects, when combined with those effects parasites have on the abundance, survival and fecundity of hosts, leads to a modification in how energy flows through communities (Hudson, Dobson and Lafferty, 2006). Parasitic infections can also lead to secondary opportunistic infections from other pathogens, often through lesions in the skin being exposed to bacteria and other pathogens present in the water (Beck *et al.*, 2008).

Metazoan parasites are some of the most threatened and under-protected animals on Earth that are affected by global changes, and extinction events of parasites could lead to devastating effects for ecosystem function and stability (Carlson *et al.*, 2020). Conservation efforts for parasites are poorly understood and oftentimes conservation effects for wildlife in general tend to exclude or ignore parasites as an important factor for the preservation of a functioning ecosystem (Nichols and Gómez, 2011).

Parasites have been suggested as useful early indicators of pollution in marine ecosystems due to their complex life cycles that depend on host diversity, as well as the free-living stages of many parasite species being sensitive to change. Therefore, the abundance or increase of parasites in an ecosystem could be used as an indicator of instability (Mackenzie, 1999). However, the use of parasites as indicators of ecosystem instability as a stand-alone method is not a very strong approach. The use of other indicators of instability in ecosystems, such as sediment contamination and other laboratory analyses, combined with parasite abundance/increase monitoring, is suggested to increase the validity of this approach (Williams and Mackenzie, 2003).

One of the most important factors contributing to the survival, reproduction, and transmission of parasites in general seems to be directly linked to increased temperatures. Higher water temperatures can lead to induced stress in fish and ultimately increase host susceptibility to parasitic infection, culminating in an overall decline in fish health (Marcogliese, 2001).

1.2.1 – Nematodes

Nematoda, commonly known as roundworms, is likely the most abundant phylum in the biosphere, with about 23 000 described species. There is at least one described species of parasitic nematode in all terrestrial plants and larger animals, the true number of species might then be closer to one million, and possibly even more. Nematodes are of great importance when it comes to regulation of plants and animals, and by estimation of number of species of parasitic nematodes per host it is suggested there may be in the order of 25 000 nematode parasites in vertebrates alone (Blaxter and Koutsovoulos, 2015).

Distinguishing Nematoda from other worm phyla is simple, as nematodes have thin and long bodies, and do not possess segmentation. Within-phylum species determination is more

difficult, as nematodes often look extremely similar and require specialist nematologists with appropriate training and experience in different groups to distinguish species at a morphological level. A well-trained eye may be able to determine species, but without such expertise taxa cannot be differentiated (Seesao *et al.*, 2016). More efficiently, within-phylum species determination can be done using molecular methods, such as using species-specific genetic markers (Paoletti *et al.*, 2018).

Since parasitic nematodes are very common and easily recognizable, they are convenient target organisms when studying abundance of parasites in an ecosystem. These nematodes are also very resilient; they will stay intact even if the specimen nematodes are retrieved from has gone through freezing and fixing in e.g., ethanol. Nematodes have also been used in several studies for a long time, meaning background information on nematodes is very well documented compared to other parasitic species that could be used in such a study. Parasitic nematodes have important implications for medical and veterinary science, and are thus well documented in the fields of animal and public health (Jex, Gasser and Schwarz, 2019).

Consequently, the above-mentioned characteristics of parasitic nematodes are particularly helpful in studies where historic material is available to quantify prevalence and load of parasitic nematodes in hosts. This assessment can then be compared to the load and prevalence of parasitic nematodes in contemporary samples to gain insight into how load and prevalence has changed over time. These indices of load and prevalence in historic vs. contemporary samples can be examined in the context of data on environmental change and other ecosystem perturbations. This allows further exploration of the effects parasites have on the species infected, perhaps suggesting potential consequences of increased parasite prevalence and load in the future.

1.2.2 – Anisakis

Anisakis simplex is a parasitic nematode that infects fish and marine mammals and can accidentally infect humans who ingest raw or undercooked fish or cephalopods that are infected with the L3 larvae (third stage). The genus *Anisakis* consists of nine species that have been identified to date (Mattiucci *et al.*, 2018).

Pseudoterranova decipiens is another zoonotic nematode in the same family as *A. simplex*. *P. decipiens* infects fish and marine mammals, much like *A. simplex*, but the preferred final hosts are seals/pinnipeds. *P. decipiens* is seemingly less pathogenic in humans than *A. simplex*, and is less studied as regards for its zoonotic potential (Fiorenza *et al.*, 2020). *A. simplex* and *P. decipiens* are very similar in appearance but can be separated by observing the coloration of the larvae*, A. simplex* appear white or transparent, whereas *P. decipiens* appear more red or brown in coloration (Buchmann and Mehrdana, 2016) (Figure 1).

Figure 1. *Anisakid larvae retrieved from fish stomach. The smaller, white to transparent larvae are assumed to be Anisakis simplex, whereas the bigger, red larvae is assumed to be Pseudoterranova decipiens.*

Approximately 20,000 cases of anisakiasis have been reported worldwide to date, with 90% of those reported cases coming from Japan (Pravettoni, Primavesi and Piantanida, 2012). However, the true number of cases seem to be grossly underestimated due to difficulties in confirming a diagnosis of anisakiasis; mild cases (symptomless and asymptomatic cases), misdiagnosis and even unreported cases may offset the true number of infections worldwide

(Bao *et al.*, 2019). An increase of *Anisakis* could be problematic because it will affect public health as well as having an economic impact, but most important are its potential effects on marine ecosystems, possibly increasing the risk of disease.

Anisakis infections can lead to pathological effects in fish, such as red-vent syndrome in Atlantic salmon, and stomach crater syndrome in Atlantic cod, as well as severe inflammatory responses in infected individuals. Red-vent syndrome is caused by *A. simplex* infecting salmon and migrating to the vent of the fish, causing the vent to swell or hemorrhage. This leads to substantial damage to the vents in Atlantic salmon and can sometimes result in secondary opportunistic infection of pathogenic bacteria, which in some cases can lead to fatality (Beck *et al.*, 2008). *Anisakis* infections can cause substantial damage to the stomach wall and mucosa of fish (Fiorenza *et al.*, 2020). After ingestion of *Anisakis* larvae, they penetrate the stomach wall of their hosts, and settle in the body cavity and surrounding organs, such as the liver (Figure 2). Severe inflammatory responses have been associated with heavy infections. Atlantic cod has been associated with numerous and repeated migrations of *Anisakis* through the stomach wall leading to deformations in the tissue, and thus the term "stomach crater syndrome" has been applied (Buchmann and Mehrdana, 2016). In cetaceans, *Anisakis spp.* infections can cause ulcerative lesions that might compromise the health of the host. In severe cases of infection, peritonitis and death can be a consequence of infection, caused by profuse hemorrhaging and stomach perforation due to migration of *Anisakis* in the body cavity and internal organs (Pons-Bordas *et al.*, 2020). Considering the effects *Anisakis* infections have on the above-mentioned hosts, it is not unlikely that *Anisakis* infections can cause similar pathological effects in other hosts as well. However, the pathological effects of *Anisakis* in their selected hosts is not necessarily well documented but can to some degree be compared to the pathological effects parasitic nematodes have on other vertebrate hosts. For example, the parasitic nematode *Ascaris lumbricoides* is usually asymptomatic in humans, but 8-15% of infections are associated with symptoms such as lung inflammation, abdominal distension, and intestinal obstruction. *Ascaris* infection is also associated with malnutrition in the host, with weight loss and appetite loss. Helminth infections seem to interfere with cognitive abilities of the host, who may present with inflammatory reactions and lesions caused by migration of the parasite through tissues (Dold and Holland, 2011). The associated symptoms

and pathological effects *Ascaris* have on their host can be compared to some of the symptoms and pathological effects of *Anisakis* infections in fish.

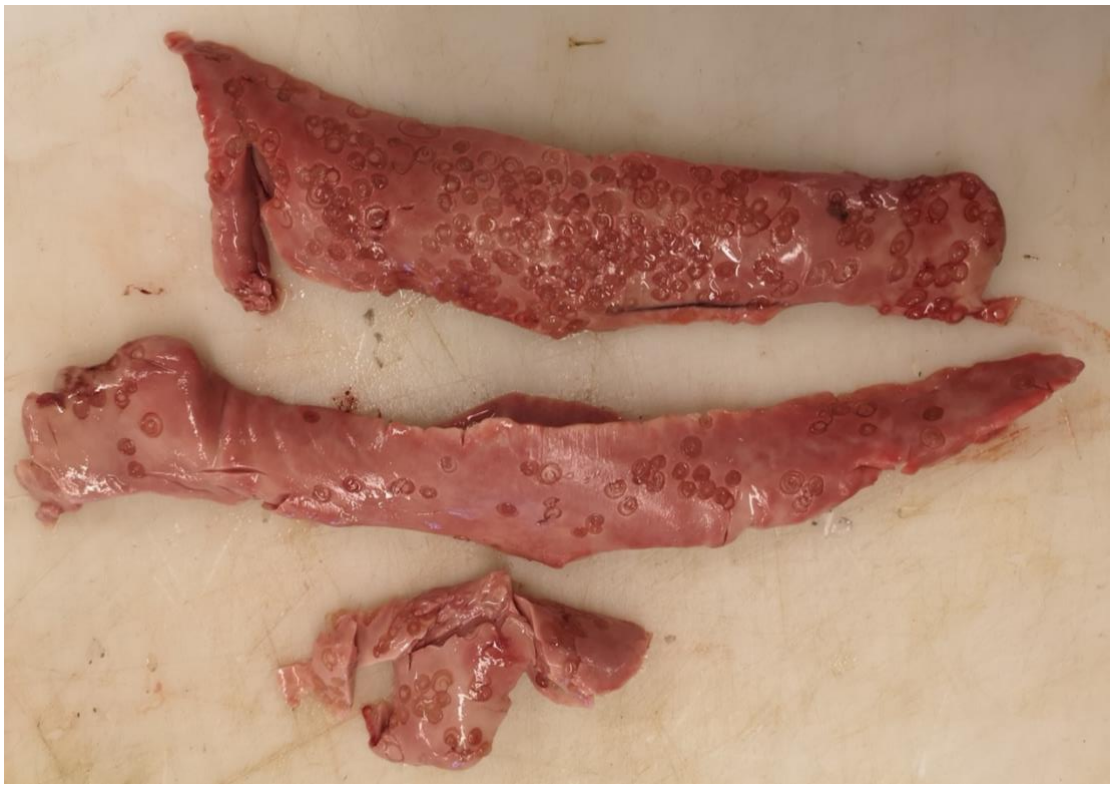

Figure 2. *Liver of Saithe (Pollachius virens) heavily infected with encapsulated Anisakis spp. larvae.*

Identifying some of the root causes regarding the apparent increase in *Anisakis* prevalence is important because of the impact infection has on human and fish health, and subsequent economic losses. Increase in *Anisakis* prevalence may be attributable to range shifts of both intermediate and definitive hosts affected by climate change, or even changes in resilience and fitness of *Anisakis* due to hybridization between sibling species of the *Anisakis simplex* species complex*,* or increased gene flow between formerly isolated populations. A study by Roca-Gerones *et al.* shows evidence of hybridization between *Anisakis simplex* and *Anisakis pegreffi,* but the hybrids only persisted in the larval (L3) stage, and none was found in the adult stage of the nematode (Roca-Geronès *et al.*, 2021). The hybrids found in the study by Roca-Geronès *et al.*, were not morphologically different from non-hybrids and were only detected using molecular markers. Thus, this study demonstrates the utility of molecular tools to detect genotypes that identify different species and indicate introgression between them.

Utilizing molecular methods to properly identify which species of *Anisakis* are present in the fish is extremely important. The difficulty of morphological identification of *Anisakis* and sibling species implies that estimates of the composition of anisakids in a host/population might be grossly distorted, and hybrids might be overlooked. Consequently, without molecular markers it is difficult to determine whether there is introgression between different species of *Anisakis* present in the fish sampled. Studies have shown that genetic markers derived from internal transcribed spacers (ITS) of nuclear ribosomal DNA can accurately identify a number of morphospecies or cryptic species of anisakids (Zhu *et al.*, 2002). The ITS region evolves faster than other regions in the genome, which makes this region efficient in detecting genetic variation within species (Umehara *et al.,* 2006). The mitochondrial genome (mtDNA; COX1 or COX2 markers) has also been proven useful in determining homologies or gene arrangements among individuals but might not be useful for detecting recent divergences among populations or species. The COX2 marker has been shown to be particularly useful in investigating the genetic variability of *Anisakis* nematodes, and resolving different haplotypes amongst them (Froeschke and von der Heyden, 2014). COX1 is a gene region in the mitochondria of Metazoa and has been widely accepted as a standard marker for a broad range of animal phyla. The COX1 gene bears characteristics that has made it very essential in evolutionary studies (Rach *et al.*, 2017). The choice of markers heavily depends on the research question but is also dependent on the data already available. For nematodes in the genus *Anisakis,* ITS-1 has shown to be highly effective in distinguishing species, and COX2 has been shown to be a highly effective marker to distinguish haplotypes and genetic variability within the genus *Anisakis* (Froeschke and von der Heyden, 2014). Therefore, combining ITS-1 and COX2 should be a highly effective method in detecting introgression or mixing between populations.

The apparent increase in *Anisakis* abundance and prevalence could also be attributed to increased marine functional connectivity (MFC). MFC is the movement of marine organisms that may result in interbreeding among isolated populations, increasing e.g., the exchange of beneficial genes in populations leading to higher fitness overall. Functional connectivity is especially important for isolated species and populations, as it contributes to stability and adaptive potential among those populations (Bradbury *et al.*, 2009). MFC and outcrossing between these previously isolated populations may allow interbreeding of formerly inbred

populations which have now low heterozygosity, reflected in an increased number of homozygous loci. Interbreeding between such populations, which by now may have purged the genetic load associated with many of their deleterious alleles through loss of individuals homozygous for them, can lead to higher fitness amongst their progeny, a phenomenon known as heterosis. This masks partially recessive deleterious mutations due to increased genome-wide heterozygosity in the (hybrid) offspring (Gagnaire, 2020).

The general life cycle of *Anisakis* includes four juvenile or larval (L1, L2, L3, L4) stages and one adult stage. Juveniles develop in an egg capsule, and after hatching, move on to a transport or intermediate/paratenic host. The nematode is then transmitted from the intermediate host and develops into its adult stage in the gut of the final/definitive hosts. Teleost fish are paratenic hosts, which are then usually eaten by Cetaceans (*Anisakis spp.*) or pinnipeds (*Pseudoterranova spp.*) which serve as definitive hosts, and eggs produced by the adult larvae are shed into the environment with the feces, and a new life cycle begins (Figure 3). *Anisakis* can be termed as permissive, in that they are accepted by a wide variety of paratenic hosts, allowing them to persist very successfully in the ecosystem (Klimpel *et al.*, 2004) (Figure 4). Climate change in the polar areas might contribute to the extension of the range in which *Anisakis* are able to exist. However, eggs and newly hatched larvae are dependent on the stability of the environment to be able to continue the life cycle (Rokicki, 2009). Abiotic factors such as temperature, salinity and light appear to influence the hatching time and survival of *Anisakis simplex* eggs and newly hatched larvae. An experimental approach demonstrated that hatching time varied inversely with temperature, and light exposure reduced the time needed for hatching. Survival of newly hatched larvae increased with salinity but decreased with temperature. The parameters for survival and hatching of larvae suggests that *Anisakis simplex* is adapted to off-shore pelagic marine environments (Højgaard, 1998).

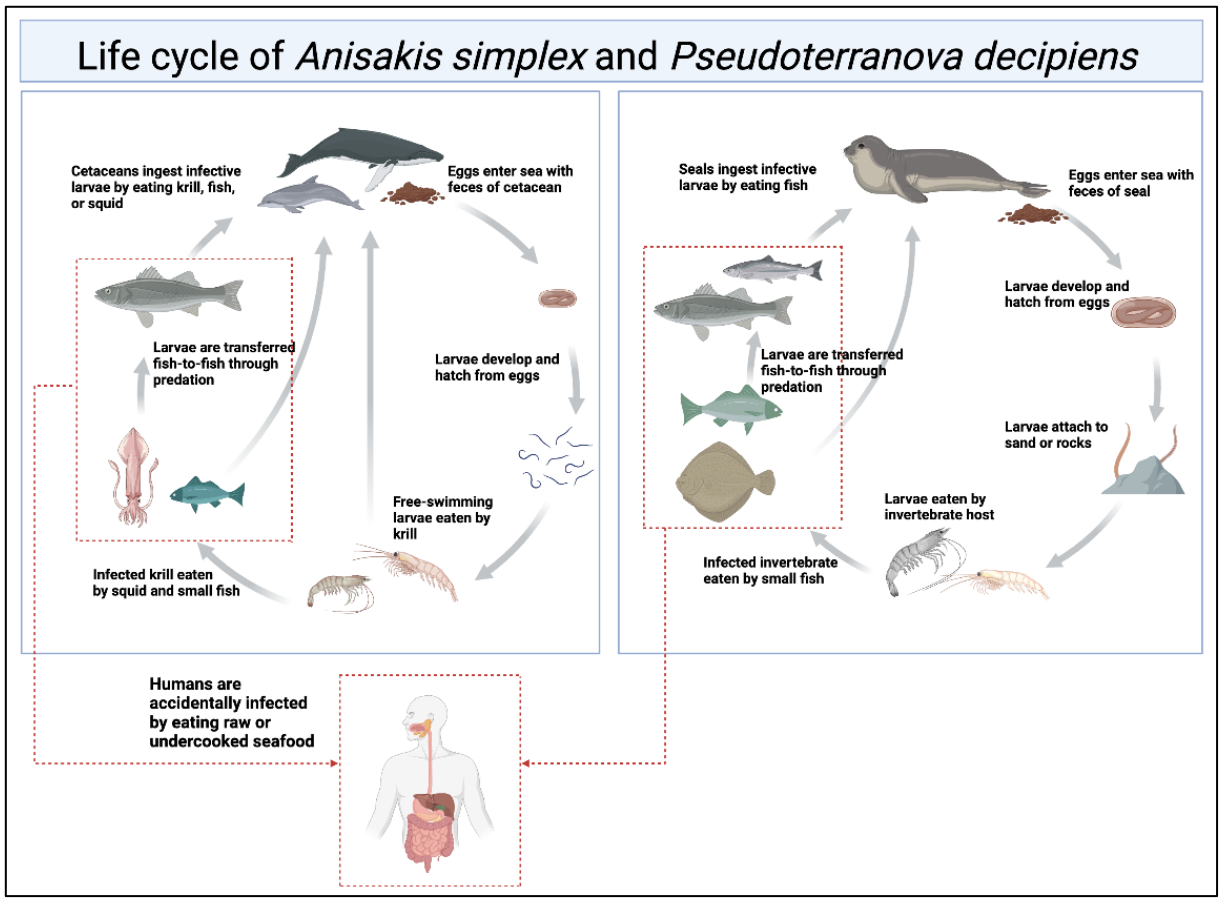

Figure 3. *Life cycles of Anisakis simplex and Pseudoterranova decipiens. Both parasites are shed into the environment, develops and hatch, and after this their life cycles vary slightly. A. simplex larvae are free-swimming in the water column, whereas P. decipiens attach to sand and rocks. This contributes to the differences between them, A. simplex is tied closely to the pelagic offshore environment, and P. decipiens is tied to the demersal in-shore environment. Both have copepods as their first host, and afterward small fish, and eventually predatory fish that ultimately lead to their final hosts; cetaceans and pinnipeds. Humans are accidentally infected by eating raw or undercooked seafood. (Figure created in biorender.com by the author, and figure is modified from Measures, L. N., 2014, "Anisakiasis and Pseudoterranovosis".)*

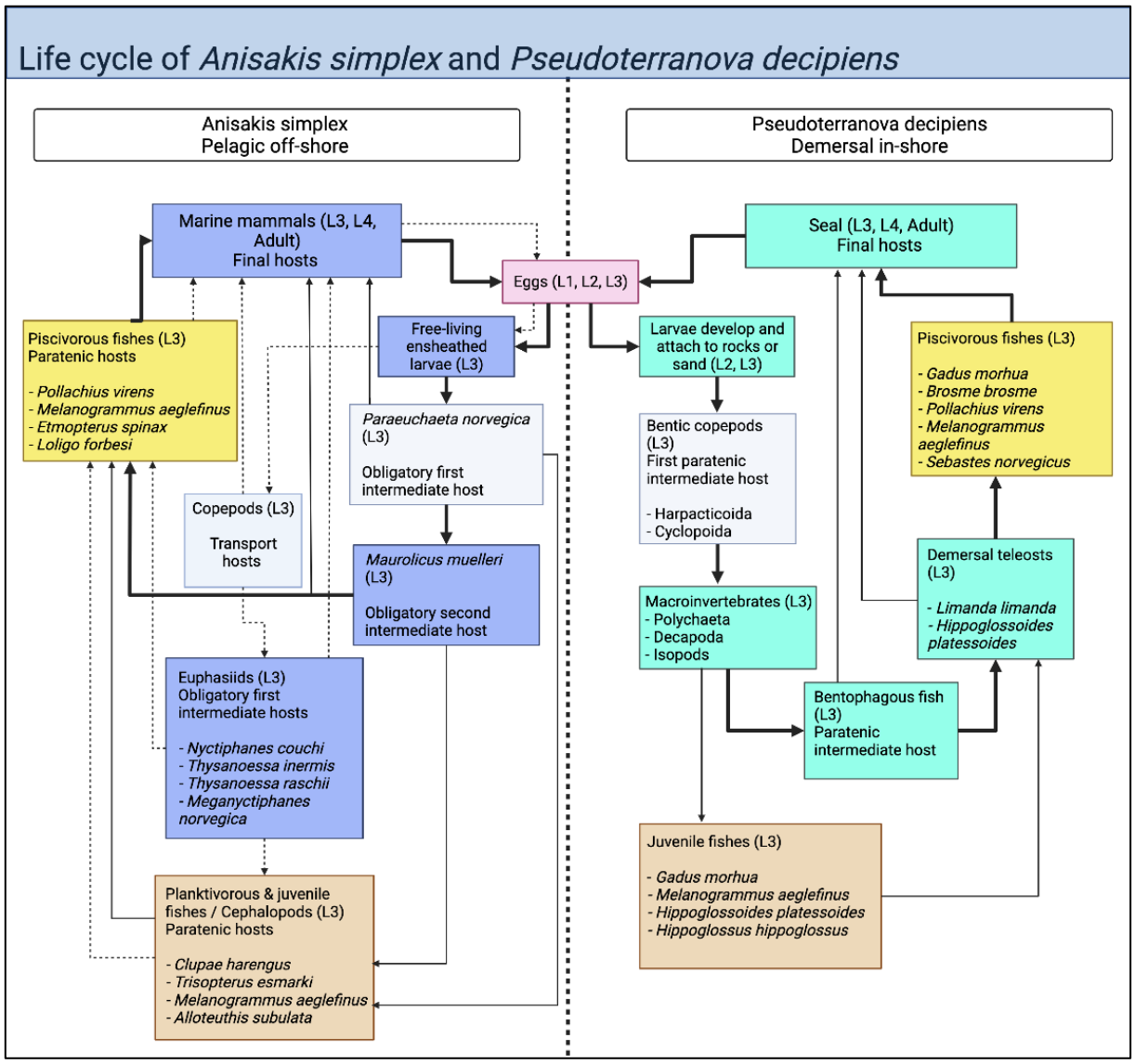

Figure 4. *Schematic life cycles for Anisakis simplex and Pseudoterranova decipiens. For both species, bold lines indicate the main life cycle, fine lines the secondary life cycle, and dashed lines the general life cycle in oceanic areas for A. simplex only. The boxes are color- coded; pink, yellow, light blue, and beige, are transmission stages where the two parasites might overlap and co-infect the same hosts, and where color represents what animal the parasites are infecting, e.g., beige represent juvenile fish, which can be infected by both A. simplex and P. decipiens. (Figure created in biorender.com by the author, and is modified from Klimpel, S., 2004, "The life cycle of Anisakis simplex in the Norwegian Deep (northern North Sea)".)*

1.3 – Host-parasite interactions and co-evolution

An important aspect of the survival of both host and parasites is co-evolution. The parasite utilizes the body of the host as a resource to survive and complete its life cycle and is therefore dependent of the survival of the host population. The hosts counteract parasitic infections through gained resistance and tolerance over time. Therefore, for both partners in a host-parasite relationship to be successful, survive and co-evolve, virulence – for the parasite - and resistance and tolerance - for the host - need to be balanced (Klemme, Hyvärinen and Karvonen, 2020).

A lack of co-evolution between host and parasite can lead to significant reduction, or even extinction of the host population because the hosts have not had the chance to develop resistance and tolerance to the parasites. This is demonstrated in a study on *Gyrodactylus salaris* in Swedish and Norwegian Atlantic salmon populations. The Swedish Atlantic salmon stocks have co-evolved with *G. salaris* and developed resistance and tolerance to the parasite, whereas the sibling population of Atlantic salmon in Norwegian rivers were naïve to this parasite, and thus unable to cope with infection. When *G. salaris* were accidentally introduced to the Norwegian salmon rivers, it resulted in extirpation of some Norwegian stocks. This is due to a combination of the virulence *G. salaris* evolved over many years of co-evolution with the Swedish salmon stocks, and the lack of tolerance and resistance to the pathogen in the Norwegian salmon stocks (Karlsson et al., 2020). This study demonstrates some of the issues that can occur when a parasite or pathogen is introduced to a new area where the hosts have not been exposed to it before.

A common misunderstanding in parasite-host interactions implies that parasites evolve to become avirulent or to an optimum-level of virulence to continue exploiting the host population and remain conspicuous. These implications can be supported in lab experiments but are rarely a reflection of virulence and transmission in natural populations, due to variables that might be ignored or remain unmeasured. It is also important to consider that during lab experiments or experimental infection approaches to measure virulence in a pathogen, the transmission occurs under controlled environments and during experimental approaches, and transfer of pathogens is often in much higher numbers, and of genetically less diverse infections, compared to those in natural environments. Virulence and transmission in parasites are closely linked to their genetic variation and conditions in host

populations, as well as environmental conditions (Ebert and Bull, 2003), which consequently can be difficult to replicate in an experimental setup. However, parasites depend on the survival of their host, and parasites that kill the host before transmission contribute less to the overall population, and are thus selected against (Karlsson et al., 2020).

Host resistance to parasites is an intricate system, depending on both biotic and abiotic factors. Hosts that evolve greater resistance to parasites may have a disadvantage to, for example, seasonal temperature fluctuations, which can lead to lower host survival (Ferris et al., 2020), or lower fecundity resulting in the production of fewer offspring. When hosts' fecundity is lowered and production of offspring is decreased, the parasite population also suffers negative consequences, meaning that the parasites will now have fewer hosts to infect. This sometimes results in an ecological feedback-effect, when lowered resistance in the host population is selected for, parasites can return to infect hosts that have lower resistance (Koskella, 2018).

In addition to resistance, hosts develop tolerance to parasitic infections, which does not necessarily stop, or eliminate infections, but rather is a coping mechanism to reduce the impact of infection. Tolerance is different from resistance because tolerance does not limit population growth of the parasite (Karlsson et al., 2020), and if tolerance is the predominant trait selected for in the host population, it will have a positive effect on the prevalence of parasites (Råberg, 2014).

Range shifts, whether they are induced by climate change or an accidental introduction of parasites (or hosts) to a new habitat can lead to modifications in host-parasite relationships (Lõhmus and Björklund, 2015), and/or hybridization in the pathogen (King et al., 2015).

1.4 – Hybridization in *Anisakis*

According to Arnold, "natural hybridization involves successful mating in nature between individuals from two populations, or groups of populations, which are distinguishable on the basis of one or more heritable characters" (Arnold, 1997).

Hybridization can be beneficial for parasites because it allows for genetic mixing which can act as a bigger "working surface" for selection in traits that can increase efficiency of host exploitation. In some extreme cases, hybridization can lead to phenotypes that are not

common to or intermediate between the parental populations, leading the hybrid offspring to diverge completely from their parents, eventually even becoming reproductively isolated from them (King et al., 2015). Hybrid offspring may also have great advantages when it comes to facing rapid ecological changes compared to their parental populations. This is because the phenotypes and associated alleles obtained through hybridization are available immediately, compared to those obtained through random mutations (Stelkens et al., 2014).

Hybridization can have major evolutionary consequences, either by promoting or preventing divergence depending on the viability of the hybrids. Adaptive traits could be acquired which in turn can lead to higher or lower fitness, and can also lead to phenotypic changes of the pathogen or parasite, such as invasion of new hosts, new geographical area and even site of infection (Mattiucci *et al.*, 2016).

However, it is not always the case that the hybrid offspring will be more successful than their parents. Previous studies have shown that even if there is hybridization, offspring may not even be viable and live through adulthood, and ones that do will gain or lose some morphological characteristics, which can have both positive and/or negative effects on the offspring. This case is however unclear in terms of hybridization within the anisakidae family, as *Anisakis* hybrids have not been researched well, and the viability of these offspring remains ambiguous (Roca-Geronès *et al.*, 2021).

The Iberian Peninsula has been suggested by Abattouy et al. as a bimodal hybrid zone for *Anisakis simplex* and *A. pegreffi* due to reported findings of hybrids and co-occurrence of these anisakid sibling species (Abattouy *et al.*, 2016). Evidence of sympatric co-existence of adult forms of *A. simplex* and *A. pegreffi* have been found in the stomach of a common dolphin, which could lead to hybridization (Abollo *et al.*, 2003). The findings by Abollo et al. support the suggestion of the Iberian Peninsula being a hybrid zone.

Additional studies have been conducted on the hybridization between *A. simplex* and *A. pegreffi*, based on gene expression patterns. One such study showed that hybridization between these two species occurred in geographical areas (FAO 27 area; North-eastern part of the Atlantic Ocean) where they are sympatric. The hybrids were distinguished using a diagnostic genetic marker, and hybrids that were detected differed from their parental origins in gene expression patterns in the L3 larval stage. The hybrids had strong parent-of-origin

effects and have been deemed particularly interesting in the study of speciation of nematodes (Llorens *et al.*, 2018).

Modelling approaches to understand the distribution of *Anisakis* has been done, focusing on biotic and abiotic factors to decide their distributions. Some species of *Anisakis* overlap in distribution, opening the possibility of hybridization and connectivity between species. In one such study, occurrence of *Anisakis* spp. and their definitive host species and their respective abiotic factors were combined, providing an in-depth model of where ranges overlap (Kuhn *et al.*, 2016).

1.4.1 – Genomic consequences of hybridization

Another important aspect in hybridization is the genomic consequences that occur following hybridization events. When parasites undergo hybridization, useful genes can be introduced through introgression and can lead to extended host ranges and increased disease potential for hybrid offspring. Higher virulence in hybrid parasites can also lead to alteration of the co-evolutionary relationships between hosts and parasites and thus increase evolutionary pressure for host resistance (King *et al.*, 2015). Hybridization is often assumed to lead to an increase in fitness of offspring, but it can also lead to a decrease in fitness or accumulation of maladaptive traits, also known as outbreeding depression, where the hybrid offspring are less successful than their parental origins. In some cases, hybrid offspring might not be able to infect hosts that are well-adapted to the (original) parasite, due to long-term coevolution between host and parasite. However, the hybrid offspring may still be able to infect other allopatric hosts that have not evolved alongside the original, co-evolved parasite (Dybdahl *et al.*, 2008).

Transgressive hybrids are offspring that express phenotypes outside what is considered the normal range of variation observed in either of the parental gene pools. This might be advantageous for the hybrid offspring because it can allow them to better adapt to novel conditions (for example, conditions caused by climate change or migrating to a new area), ultimately increasing the likelihood for divergence from their parental origin (Dittrich-Reed and Fitzpatrick, 2013).

Transposable elements (TE) have been shown to promote speciation by inducing rapid genomic changes, which might lead to adaptation and diversification. When the insertion of TEs in the genome is successful, it can lead to adaptive radiation by forming distinct species which are adapted to new ecological niches (Feiner, 2016). Divergence, or speciation, can also be initiated by cytonuclear incompatibilities that often occur as a consequence of hybridization (Sambatti *et al.*, 2008). Cytonuclear incompatibilities can occur in hybrids when there is a mix between the highly co-evolved cytoplasmic and nuclear loci from different origins; leading to a mismatch due to unbalanced inheritance (Barnard-Kubow, So and Galloway, 2016).

Parasites that are (transgressive) hybrids might lead to an increased incidence of disease, especially if they are introduced to new potential hosts that are susceptible to them.

1.5 – Fish variation in the study

In this thesis there are several different species of fish introduced, ranging from Atlantic Cod (*Gadus morhua*) to Blackmouth catshark (*Galeus melastomus*) (Table 1). Teleost fish are extremely diverse and makes up a great portion of all extant vertebrate species, making them very valuable in studies regarding evolution, ecology, behavior, and much more (Volff, 2005).

A wide range of teleost fish species provides a bigger picture in host variation of *Anisakis,* as there are several hosts involved in the life cycle of *Anisakis*, and it can be transferred from one intermediate host to another due to ingestion of infected individuals. This leads to accumulation of *Anisakis* in predatory fish *(Mattiucci et al., 2018)*.

Historic samples in this study consisted of two species, Cod (*Gadus morhua*) (Lofoten, 1880) and herring (*Clupea harengus*) (Bodø, 1850). These two species were chosen mostly due to the availability in museum collections, but also because these fish species were well represented around the Bodø/Lofoten area. Historic material was gathered after the initial sampling, so as to correspond to what has been found in the contemporary samples, although herring was not present in contemporary samples. Historic material was collected because it can provide particularly relevant insights into the historic baseline of *Anisakis* infections, thus fixing the context for comparisons of prevalence and load of *Anisakis* in contemporary samples. In previous studies, which indicate increasing prevalence and abundance of *Anisakis*

in contemporary material, this historic baseline has been absent. Consequently, it has been difficult for those earlier workers to estimate whether the contemporary prevalence and loads of *Anisakis* reflect a return to the once naturally higher levels, or have now escalated beyond what were naturally maintained lower levels than those seen in more recent investigations (See Fiorenza *et al.,* 2020).

Table 1. *Overview of the fish species that have been dissected, and are reported to have evidence of Anisakis spp. and Pseudoterranova spp.. + indicates that there are reported infections, whereas - indicates no proof or reported findings.*

Fish species	***Anisakis* spp.**	**Pseudoterranova spp.**	**Supporting papers**
Gadus morhua	+	+	(Mehrdana *et al.*, 2014)
Scomber scombrus	+	+	(Levsen *et al.*, 2018) (Paoletti *et al.*, 2018)
Pollachius virens	+	+	(Klimpel *et al.*, 2004) (Piccolo *et al.*, 1999)
Melanogrammus aeglefinus	+	+	(Pierce *et al.*, 2018)
Eutrigla gurnardus	+	-	(Levsen and Karl, 2014)
Galeus melastomus	-	-	
Limanda limanda	-	+	(Jensen, Andersen and Clers, 1994)
Trisopterus esmarkii	-	-	
Brosme brosme	+	+	(Ferrantelli *et al.*, 2015) (McClelland, 2002)
Chimaera monstrosa	-	-	
Etmopterus spinax	+		(Klimpel, Palm and Seehagen, 2003)
Sebastes norvegicus	+	+	(Bakay, 2017)
Lophius piscatorius	+	-	(Abollo, Gestal and Pascual, 2001)
Dipturus spp.	-	-	
Clupea harengus	+	-	(Levsen and Lunestad, 2010)

Hagfish (*Myxine glutinosa*), which is an important component of the food webs and nutrient transportation in the ecosystem (Glover and Weinrauch, 2019), can provide insight in the widespread transmission of *Anisakis* between species. Hagfish were explored as a possible (transport) host for *Anisakis* in this study. They serve as prey for marine mammals, larger cephalopods and sharks, which leads to the possibility that hagfish may provide another route

of transmission of *Anisakis* to larger hosts (Luo *et al.*, 2016). In addition to providing an alternative route of transmission of *Anisakis*, Hagfish may also play a role in hybridization of different *Anisakis* species. This is because of the scavenger lifestyle of Hagfish, and it can be assumed that throughout their lifetime of scavenging, they will ingest several different anisakids. When hagfish are eventually eaten by cetaceans and pinnipeds, they can thus bring those different species of *Anisakis* to their final hosts, potentially facilitating hybridization of compatible *Anisakis* sibling species.

1.6 – Fjord biology

Fjords represent a wide variety of habitats for a massive range of different species, all in a very defined and closed location. Each fjord possesses some unique characteristics such as differences in salinity, depth and temperatures, nevertheless it represents a microcosm of ocean habitats in an accessible location (Jordà Molina *et al.*, 2019). Due to the unique characteristics of fjords and their accessibility, the convenience in studying various topics ranging from behavior of different species to the intricacies of food webs is apparent. If the physio-chemical parameters of a fjord have been properly characterized, studies requiring observational data or gene-environment data are more easily provided than in offshore environments. Additionally, Fjords are of great interest in studying anthropogenic effects such as eutrophication in the ecosystems (Brattegard *et al.*, 2011).

The ecological characteristics and nature of fjord ecosystems in the Arctic and sub-Arctic are under pressure from climate change, and there have been reports of fish species expanding their range further north as a consequence of this (Perry *et al.*, 2005). An effect of climate change and species expanding their range can lead to increased species turnovers, which again can lead to disturbances that can disrupt ecosystem services (Cheung *et al.*, 2009).

1.7 – Aims of the study

Recent literature mentions an increase of *Anisakis* in marine fish during the last 50-100 years, both in numbers of human infections and prevalence in marine life. Therefore, the aim of this Master thesis is to investigate that claim by comparing historic samples with contemporary samples of fish to obtain an insight to confirm or deny these claims. This can be relevant to certain aspect of conservation and public health, such as how these infections are distributed in the marine and human food chain and affect fish and marine mammal populations that are not thriving, as well as from a medical standpoint, as *Anisakis* can cause reactions and disease in humans that have consumed infected fish. The biggest problem, however, is not for humans, as *Anisakis* cannot survive for long in the human body with infection lasting for only a few days and does not transmit between individuals. But in marine life they persist and reproduce, thus increasing the risk of disease with increased load and prevalence.

Here we examine parasite infections in several landings of fish from Sørfjorden in Gildeskål Kommune and compare the infection rates of Anisakids in these landings with infection rates of historic samples. Assessment of infection prevalence and load, and whether parasitic infections are influenced by various biological characteristics of the different fish species has been investigated, to see whether claims of an increase in marine disease and parasitic infections can be substantiated, with the focusing on species of *Anisakis spp*. Potential explanations for the increase of *Anisakis* in marine life were explored using statistical and molecular methods and sampling of *Anisakis* from contemporary and historic fish.

Questions of interest in this study are: whether fish health, age, sex and other morphometric traits are correlated with the parasite load of individuals, and if it is true that there has been an increase in *Anisakis* (infections) in fish over the past 100 years, and why. The historic baseline of *Anisakis* prevalence and load in fish before the time of modern commercial fisheries and whaling impacted Arctic ecosystems was discussed.

Hypothesis 1: The prevalence and load of *Anisakis* in teleost fish has increased over the past 100 years.

Null hypothesis: There has been no significant difference in the prevalence and load of *Anisakis* in teleost fish over the past 100 years.

Hypothesis 2: Worms of the *Anisakis simplex* species complex show evidence of introgression.

Null hypothesis 2: Worms of the *Anisakis simplex* species complex show no evidence of introgression.

2.0 – Materials and methods

2.1 – Intestinal system of fish presented in the study

All teleosts have more or less the same intestinal (guts) systems with some slight variations depending on the species, but sharks have a somewhat different intestinal system. The distal intestine of sharks is formed in a spiral, which allows a greater surface area for food to be digested in a smaller intestinal volume than in other fish (Figure 5). This space-efficient design of shark guts is due to other organs taking up the limited space in the body cavity, such as the large liver (Carrier, Musick and Heithaus, 2012).

The intestines and stomach of fish is where the main nutrient uptake takes place from food ingested but is also usually the site parasites such as *Anisakis* choose and are therefore the main focal point in the fish anatomy this thesis is based upon. *Anisakis* larvae can either be free floating in the stomach and intestines of the fish, or they can be embedded (encysted) into the lining of the stomach and other surrounding organs and musculature (Buchmann and Mehrdana, 2016). *Anisakis* will take up nutrients that the fish is trying to utilize, which may affect the overall health condition of the infected fish. The intensity of the parasite load may thus relate to and so assist in determination of the health condition of fish.

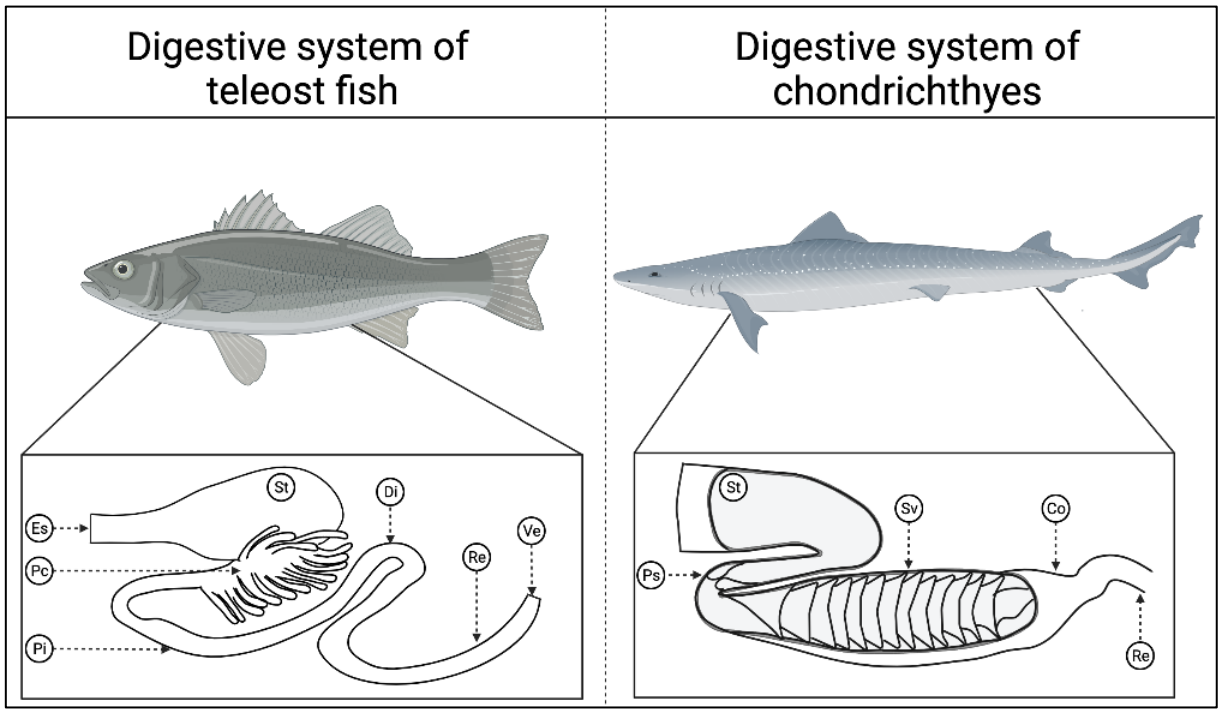

Figure 5. *Digestive system of teleost fish and Chondrichthyes. In both types of fish, the digestive system was removed and examined for parasites. Teleost anatomy; Es: Esophagus, St: Stomach, Py: Pyloric caeca, Pi: Proximal intestine, Di: Distal intestine, Re: Rectum, Ve: Vent. Chondrichthyes anatomy; St: Stomach, Ps: Pyloric sphincter, Sv: Spiral valve, Co: Colon, Re: Rectum. Figure created in biorender.com by the author.*

2.2 – Hepatosomatic index and Fulton's body condition factor K

The hepatosomatic index (HSI) is a calculation of the liver weight in proportion to the total body mass of the fish. The formula for calculating HSI is:

$$\frac{Liver\ weight}{Total\ body\ weight} \times 100$$

Both measures of weight is in grams (g) (Al-Ghais, 2013).

This index is used as an estimation of a high or low energy reserve in individuals, with a high HSI value indicating that the energy reserve is high, and a low value indicating the energy reserve is low. This varies from one fish species to another; some are naturally leaner than others. The HSI value of an individual can also be affected by the temperature, breeding season and pollution, but most importantly the food availability, which ultimately can be affected by the parasite load of the individual (Nunes *et al.*, 2011). HSI has also shown to differ between males and females, both during and outside the breeding season (Sharma and Ram, 2020).

Because the HSI tends to vary amongst species, and sexes within species, it can be difficult to determine what is considered normal; especially for a study like this, where the samples consist of several different species. Comparing HSI-values of the species in question with other scientific studies can therefore be helpful in determining what can be considered a normal range.

Fulton's body condition factor K is an alternative condition index that can be used to determine health by measuring the weight and length of fish. The formula used to calculate this index is:

$$K = \frac{100000 \times W}{L^3}$$

W is weight (g), and *L* is length (mm).

Here, it is assumed that fish with a K-factor close to 1 are in better condition than others of the same species and similar length, as they might have a higher energy reserve than fish with a lower K-factor (Robinson *et al.*, 2008).

2.3 – Sampling

Fish used in this study were provided by the trawler Oscar Sund, based in Inndyr, Nordland. They were caught using a trawling net, trawling over 1.2 nautical miles (NM) for 45 minutes at a depth between 223-254m in Sørfjorden, Gildeskål kommune (Figure 6). The first catch was caught 12.10.2021, second catch 03.01.2022, third catch 26.04.22. An additional catch was obtained through gill nets in Mørkvedbukta, Bodø (ca 25m depth), 25.03.2022. The landings consisted of several species of teleost fish, and a few elasmobranch species. The fish were dissected approximately 24h after they were caught, and another batch of fish were dissected approximately 48h after capture. The rest of the fish were kept in a freezer (-20°C) for some days and then dissected after approximately 24h of thawing. Additional data was provided from an unpublished master thesis sampling parasites in teleost fish in Saltenfjord. A total of 137 fish were sampled, which was approximately 265.35 kg combined.

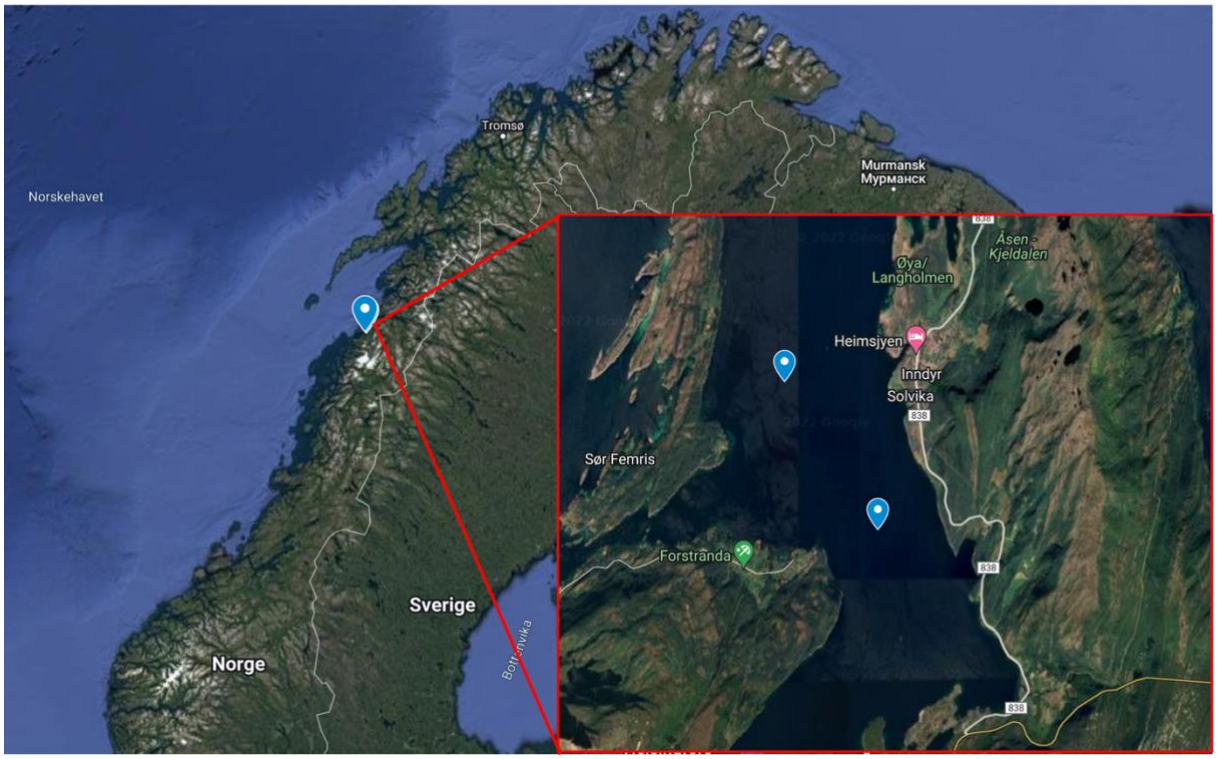

Figure 6. *Area for trawling.* Depth: 223-254m, Area: Sørfjorden, Gildeskål kommune, time: 45 min, distance: 1.2 nautical miles (NM).

Historic samples were provided by the Natural History Museum in Bergen, by Nicolas Straube. Fish were selected based on location and species. The length, weight, and liver weight of selected fish were measured. Dissection of historic collection fish was done by opening the

abdominal cavity, and any parasites present in samples were counted, then stored in ethanol. A total of 51 fish were sampled, which was approximately 26.3 kg combined.

2.3.1 – Morphometrics and species determination

Before dissection, fish were measured by standard length, from the snout to the base of the tail, and weighed using a scale. Age of fish was determined by counting the annuli on the scales of the fish using a stereo microscope, although was not used for later analysis. All morphometrics were recorded for later further analysis. Species identification of the fish was done based on morphology. If species determination was unclear, books, Google, and a trained eye (Morten Krogstad, pers. commun.) was used to assist.

2.3.2 – Dissection

Fish were dissected using a scalpel, tweezers, and scissors, depending on the morphology/species; but in all fish the abdominal cavity was completely opened to ensure thorough examination of the organs as well as the abdominal cavity. The liver of each individual was retrieved after dissection and weighed. The liver was also checked for attached parasites. The stomach, intestines and pyloric caeca were removed from the abdominal cavity and stored temporarily in a plastic bag. The stomach and attached organs were either kept frozen (in -20°C) or brought to the stereo microscope for further examination and retrieval of parasites.

During dissection the sex of the fish was decided by inspection of the gonads. In elasmobranchs this was done by examining whether claspers were present or not in males and developed egg(s) (capsules) in females. Age of elasmobranchs was noted down as immature or mature depending on the presence or absence of claspers or egg(s) (capsules). Age of the elasmobranchs was done slightly different than in teleosts, since sharks do not have scales where annuli can be counted, they were recorded as mature if they had visible claspers or egg capsules, and those that did not were recorded as “immature juvenile”. All parasites that were retrieved during this stage of dissection were stored in tubes with 96% ethanol.

2.3.3 – Stomach dissection and parasite retrieval

The stomach, pyloric caeca and intestines were carefully examined for parasites before dissection. The stomach was dissected lengthwise using a scalpel, scissors and tweezers and examined under a stereo microscope. The contents of the stomach were removed and thoroughly examined, and parasites extracted. After stomach contents were removed, the tissue of the stomach was examined both on the inside and the outside and any remaining parasites present were extracted. After parasites were extracted from the stomach, the same method was followed to extract parasites from the intestine.

The pyloric caeca were also examined in the same manner as the stomach and intestines, but due to the extremely high number and small size of pyloric caeca in some of the samples it was not done as thoroughly as the stomach and intestines, but consistency was kept throughout the sampling. All parasites extracted were counted and then stored in tubes with 96% ethanol.

2.4 – Genetic identification

A selection of parasites was taxonomically identified by Scott P. Lawton based on genetic identification. The genetic identification of *Anisakis* was done using PCR with COX2-markers and ITS region. To ensure accurate genetic identification and possible differentiation of the species, the ITS region was also analyzed in the samples. COX2 and ITS were chosen as markers to identify the species of nematodes, because they have been proven effective in distinguishing species within the genus, as well as being recognized as good markers to identify different haplotypes of the genus *Anisakis.* All genetic analysis was conducted by Scott P. Lawton, Scotland's Rural College: Inverness, GB.

2.5 – Data analysis

Data analysis was conducted in JMP (SAS Institute Inc. 2016 JMP® 13 Deployment Guide for Annually Licensed Macintosh Versions. Cary, NC: SAS institute Inc.). Linear models were conducted to check for any correlation between nematode numbers and length, nematode numbers and HSI and K-factor, and to compare contemporary and historic fish in general.

ANOVA was also conducted, to further investigate the differences between the groups, and finally, principal component and discriminant analyses were conducted to visually show how the two groups, historic and contemporary fish, differed.

Linear models and principal component analysis (PCA) are helpful tools to make multivariate data easy to read. Linear models are used to explore relationships between variables and see whether there is any correlation between selected variables that have been chosen. PCA is used to predict relatedness between variables, and any eventual clustering in the biplot will assist in deciding whether there are any differences or similarities within groups in the dataset.

Discriminant analysis is used to examine whether there are any significant differences between groups based on chosen predictor variables. Discriminant analysis is similar to PCA, because this analysis also produces a biplot. However, the discriminant analysis shows how canonical variables summarize variation between categories or groups.

ANOVA is a statistical formula used to compare variances across means in different groups. Therefore, ANOVA has been used in this study to compare HSI and liver weight between historic and contemporary fish.

Maximum likelihood (ML) phylogenetic reconstruction, haplotype mismatch analysis and a haplotype network were made based on the genetic analyses. ML were visualized in FigTree, and haplotype network was made in PopArt; all by Scott P. Lawton. Maximum likelihood phylogenetic reconstructions provide insight in how closely related samples are, shown as branches on a tree-like phylogeny. Proximity of individuals on this tree reflect their relatedness based on the molecular marker used. An outgroup is used as a reference to provide comparison within the ingroup and allows for the phylogeny to be rooted.

Haplotype networks show relations identified in the ML and provides insight into where the populations overlap and what geographic origin they have. Mutational events separating populations or individuals are also shown in the network, providing some relative information of how much time has passed since separation. Haplotype mismatch analysis is used to further demonstrate relatedness within populations, and whether there are many or few divergent haplotypes present in the populations.

3.0 – Results

3.1 – Biological data

In contemporary samples, a total of 137 fish were sampled (Figure 7). Out of all of these, nematodes were found in 98 fish, ranging from a minimum of one nematode to a maximum of 458. The mean distribution of nematodes from contemporary samples is 38.32 per fish. The calculated HSI values ranged from 0.0495 to 24.1, with HSI values increasing with higher load of *Anisakis*. The K-factor ranged from 0.33 to 2.5 (Table 2).

Table 2. *Host size and infection parameters of sampled contemporary fish. Values (size, HSI, K-factor and total no. of Anisakis) are given as mean ± SD (range) and prevalence of infection given in percentage.*

Fish species	Weight (g)	Length (cm)	HSI	K-factor	Total no. of *Anisakis*	% Prevalence
Cod (n=73)	2327.92 ± 137.55 (610-6731)	58.1 ± 1.21 (37-86)	3.1 ± 0.17 (1.16 - 8.43)	1.09 ± 0.03 (0.76 - 2.11)	41.64 ± 3.86 (0-120)	95.9
Anglerfish (n=3)	4935 ± 672.74 (4070-6260)	59.33 ± 2.33 (55-63)	2.42 ± 1.25 (0.05-4.3)	2.34 ± 0.14 (2.07-2.5)	1.33 ± 1.33 (0-4)	33.3
Flounder (n=17)	385 ± 13.05 (295-495)	31.5 ± 0.4 (29.5-35)	1.06 ± 0.07 (0.5-1.65)	1.23 ± 0.03 (0.95-1.54)	0.18 ± 0.13 (0-2)	0.12
Saithe (n=16)	2343.94 ± 211.89 (960-3643)	53.81 ± 1.97 (42.5-69)	4.48 ± 0.72 (1.04-11.72)	1.49 ± 0.1 (0.9-2.45)	111.81 ± 30.96 (0-458)	0.94
Skate (n=2)	5090 ± 490 (4600-5580)	101.5 ± 2.5 (99-104)	9.21 ± 0.79 (8.42-10)	0.49 ± 0.01 (0.47-0.5)	0	0
B.M. catshark (n=7)	763.86 ± 68.99 (564 – 1025)	51.86 ± 1.94 (45-59)	5.61 ± 0.43 (4.32-7.88)	0.55 ± 0.04 (0.34-0.68)	0	0
Cusk (n=2)	1866 ± 16 (1850-1882)	49 ± 0 (49)	6.54 ± 1.63 (4.91-8.18)	1.59 ± 0.01 (1.57-1.6)	60.5 ± 59.5 (1-120)	100

Grey gurnard (n=3)	531 ± 116.48 (320-722)	34.33 ± 3.48 (28-40)	3.3 ± 0.23 (3.04-3.75)	1.29 ± 0.1 (1.13-1.46)	37.67 ± 22.93 (3-81)	100
Mackerel (n=1)	677 ± 0 (677)	36 ± 0 (36)	2.66 ± 0 (2.66)	1.45 ± 0 (1.45)	9 ± 0 (9)	100
Chimaera (n=4)	1259.25 ± 229.36 (730-1850)	50.75 ± 1.89 (47-56)	21.58 ± 0.99 (19.27-24.1)	0.93 ± 0.08 (0.7-1.05)	0	0
Haddock (n=4)	1799 ± 2.99 (41-55)	46.5 ± 2.99 (41-55)	7.24 ± 3.23 (2.9-16.8)	1.72 ± 0.16 (1.51-2.18)	30 ± 17.8 (0-70)	50
Redfish (n=1)	1843 ± 0 (1843)	46 ± 0 (46)	1.62 ± 0 (1.62)	1.89 ± 0 (1.89)	32 ± 0 (32)	100
N. pout (n=1)	243 ± 0 (243)	25.5 ± 0 (25.5)	3.3 ± 0 (3.3)	1.47 ± 0 (1.47)	19 ± 0 (19)	100
V.B. lanternshark (n=3)	237 ± 59.66 (170-356)	34.33 ± 0.67 (33-35)	17.2 ± 1.05 (15.29-18.9)	0.58 ± 0.13 (0.4-0.83)	0	0

A total of 51 fish were sampled from historic collections. Out of all the historic samples nematodes were found in 26 fish, ranging from a minimum of one nematode, to a maximum of 26. The mean distribution of nematodes from contemporary samples is 1.75 per fish. The calculated HSI values ranged from 3.63 to 8.4, with HSI values increasing with higher load of *Anisakis*. The K-factor ranged from 0.3426 to 5.4375 (Table 3).

Table 3. *Host size and infection parameters of sampled historic fish. Values (size, HSI, K-factor and total no. of Anisakis) are given as mean ± SD (range) and prevalence of infection given in percentage.*

Species	Weight (g)	Length (cm)	HSI	K-factor	Total no. of *Anisakis*	% Prevalence
Cod (n=30)	517.1 ± 15.12 (333-645)	38.8 ± 1.62 (25-55)	5.04 ± 0.17 (3.63-8.4)	1.08 ± 0.1 (0.34-2.59)	0.8 ± 0.16 (0-2)	50
Herring (n=21)	513 ± 6.48 (435-570)	26.19 ± 0.73 (20-35)	4.8 ± 0.1 (3.9-5.6)	3.07 ± 0.22 (1.33-5.44)	3.10 ± 1.58 (0-26)	52

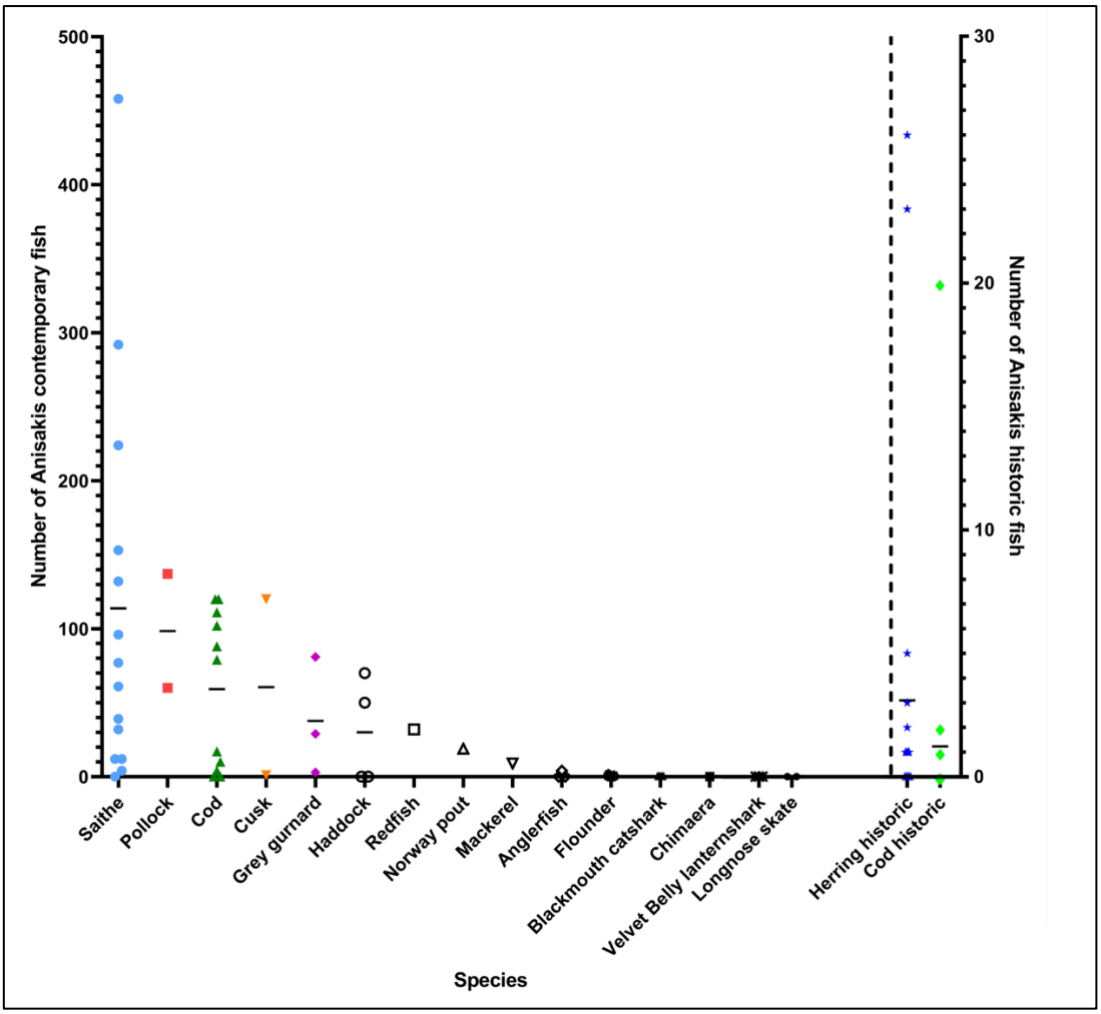

Figure 7. *Summary figure for the number of Anisakis in contemporary and historic fish. Contemporary fish are to the left of the plot, and historic fish are to the right of the plot. Contemporary and historic fish are separated by a dotted line, and the scales for number of Anisakis are different on the two y-axes; left y-axis ranging from 0-500, right y-axis ranging from 0-30, to reflect maximum number of nematodes in the sampled fish. A vertical line is shown for all counts of Anisakis in the species, which represents the mean number of Anisakis. For more details, see Tables 2 and 3.*

3.2 – Models

3.2.1 – Linear models and ANOVA

Linear models and ANOVA analyses were made based on size differences between historic and contemporary samples. Here, the data was trimmed to Cod (*Gadus morhua*) only, in the size class 25-55 cm.

One way ANOVA show that historic fish (H, blue) have significantly higher hepatosomatic index (HSI) than contemporary fish (C, red), r^2=0.3 (Figure 8).

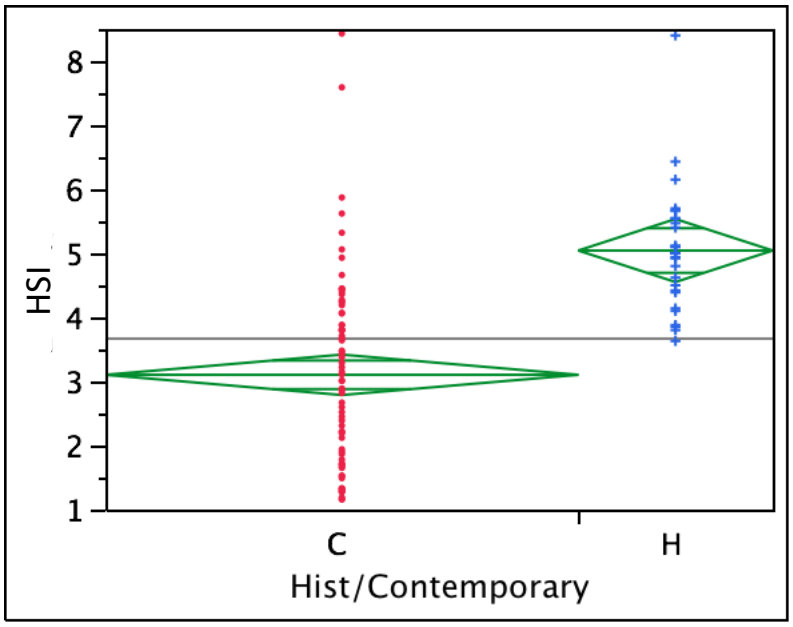

Figure 8. *One way ANOVA comparing hepatosomatic index (HSI) between contemporary (C, red) and historic (H, blue) fish. HSI is higher in historic fish compared to contemporary fish, r^2=0.3, p value = <.0001, F ratio = 43.3013.*

One way ANOVA shows that historic fish (H, blue) have smaller livers than contemporary fish (C, red), r^2=0.1242 (Figure 9).

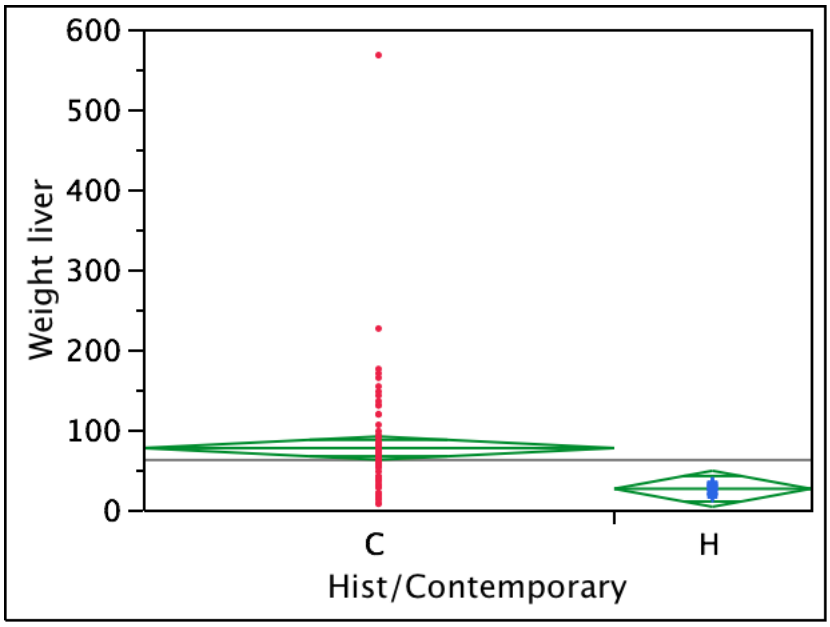

Figure 9. *One way ANOVA comparing liver weight between contemporary (C, red) and historic (H, blue) fish. Livers of historic fish are smaller than the livers of contemporary fish, r^2=0.1242, p value = 0.0003, F ratio = 14.1813.*

The distribution of nematodes across samples limited to cod only, size class 25-55 cm, is not normally distributed, and also does not follow the Poisson curve (Figure 10).

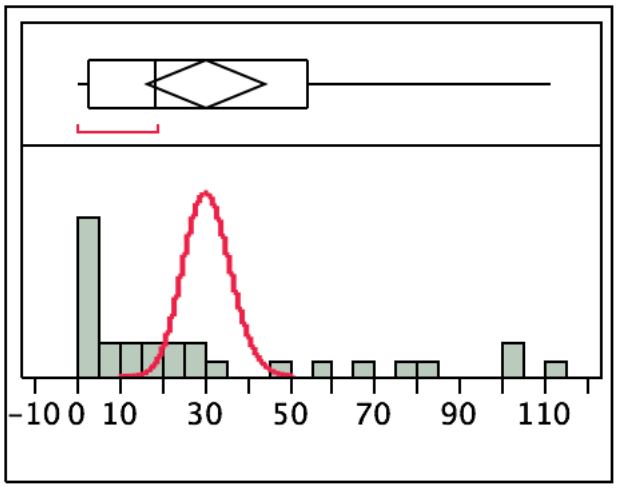

Figure 10. *Distribution of all nematodes across historic and contemporary samples limited to cod only in the size class 25-55 cm. The distribution is not normal, and does not fit a Poisson distribution. Nematode numbers: 30.35 ± 35.42 (0-111).*

The linear regression model based on number of nematodes by length in contemporary (red dots on the graph) and historic samples (blue dots on the graph) show a positive correlation, r^2=0.167319 (Figure 11).

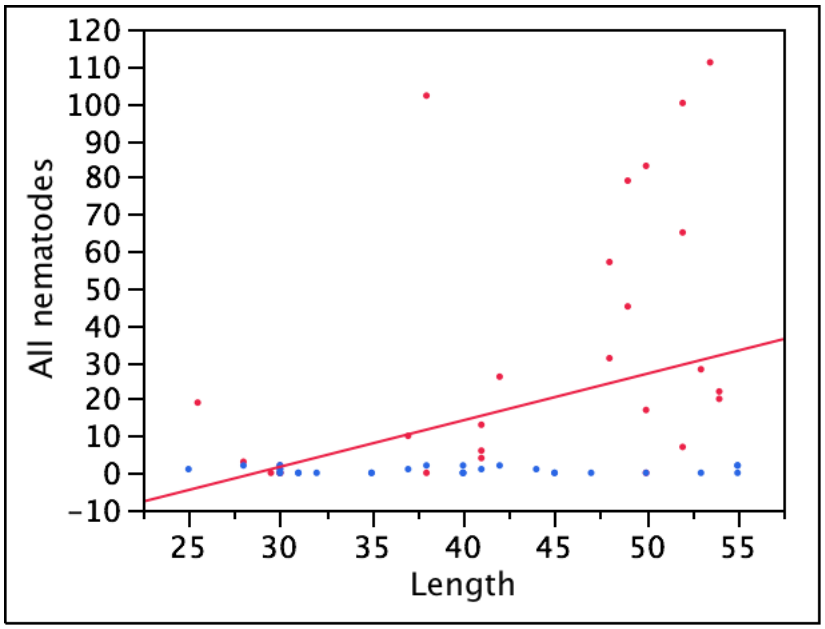

Figure 11. *Bivariate fit of all nematodes by length in cod only, size class 25-55 cm. The linear regression line shows a positive correlation, r^2=0.167319, p value = 0.0014, F ratio = 11.2527. Red dots are contemporary samples, and blue dots are historic samples.*

Linear regression model based on all nematodes by liver index in contemporary samples only shows a positive correlation, r^2=0.124191 (Figure 12).

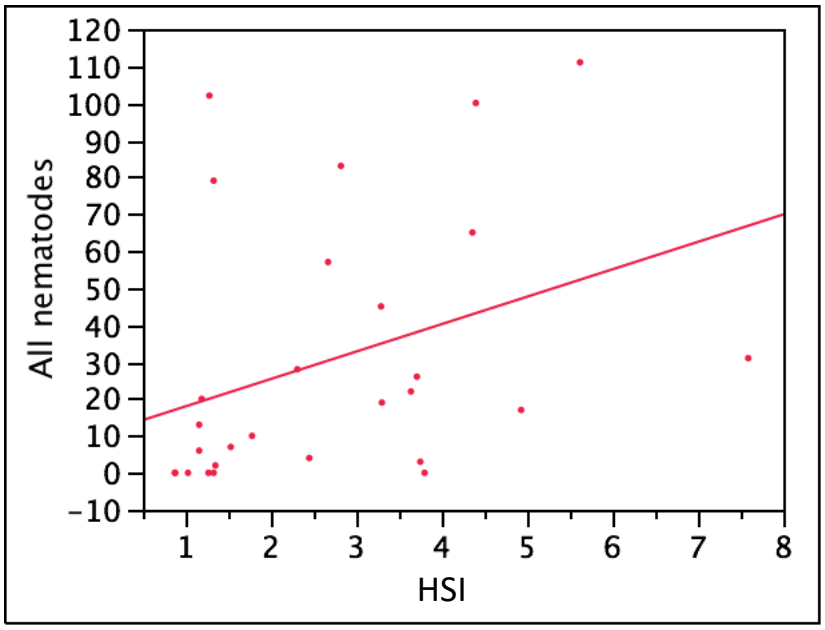

Figure 12. *Bivariate fit of all nematodes by liver index (contemporary samples only). The linear regression line shows a positive correlation, r^2=0.124191, p value = 0.0659, F ratio = 3.6868.*

Linear regression model based on number of nematodes by liver index (HSI) in contemporary (red dots on the graph) and historic samples (blue dots on the graph) show negative correlation, r^2=0.026127 (Figure 13).

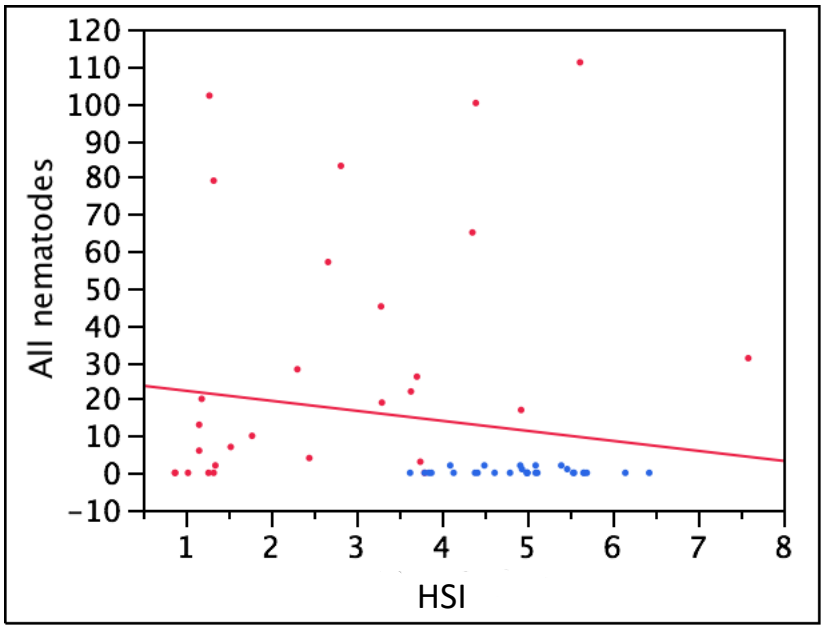

Figure 13. *Bivariate fit of all nematodes by liver index (Contemporary samples = red dots, historic samples = blue dots). The linear regression line show negative correlation, r^2=0.026127, p value = 0.2094, F ratio = 1.6097.*

Linear regression models based on the K-factor by all nematodes and length in contemporary (red dots on the graph) and historic (blue dots on the graph) fish. No significant correlation between K-factor and nematode burden in either of the groups of fish r^2=0.032742 for contemporary and historic fish together, r^2=0.031083 for only contemporary fish (Figure 14).

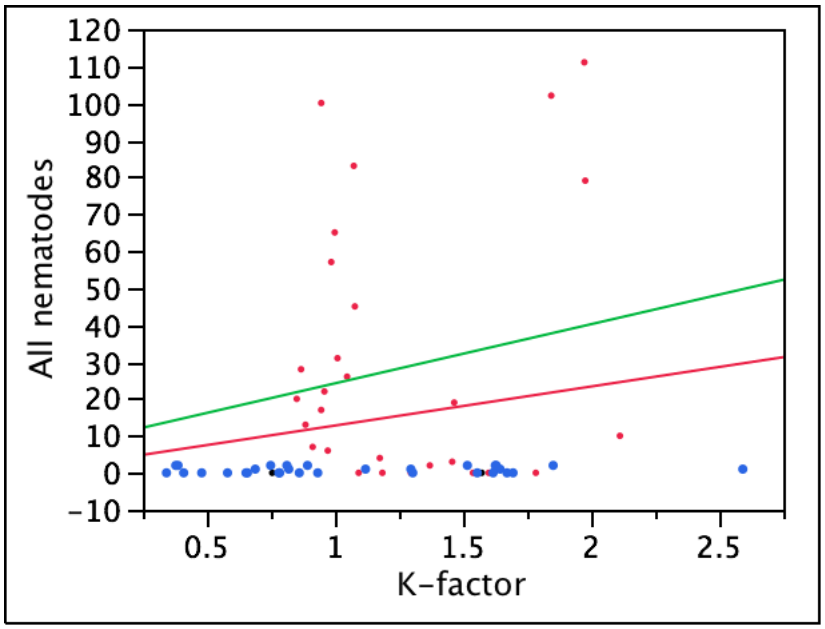

Figure 14. *Bivariate fit of K-factor by nematodes in historic (blue dots) and contemporary (red dots) fish. Red correlation line is contemporary and historic fish combined, and green correlation line is only contemporary fish. No significant correlation between K-factor and nematode burden in combined regression, r^2=0.032742, p value = 0.1665, F ratio =1.9633. No significant correlation between K-factor and nematode burden in contemporary fish only regression, r^2= 0.031083, p value = 0.3514, F ratio =0.8982.*

3.2.2 – Principal component and discriminant analysis

Principal component (Figure 15) and discriminant analysis (Figure 16) both show that the two groups, contemporary (red dots) and historic (blue dots) fish, are different, in terms of parasite burden, as well as other morphometric differences. Scree plot (top right quadrant of Figure 15) for principal component analysis shows how much of the variation is accounted for by each principal component, which can also be seen in Table 4. For example, the first two principal components account for 80.03% of the total variation in the data, and with the addition of the third principal component, 93.6% of the total variation in the data is explained. Thus, the three first principal components have been chosen to represent the data in the score plot, and these numbers are displayed on the graph axes of the score plot.

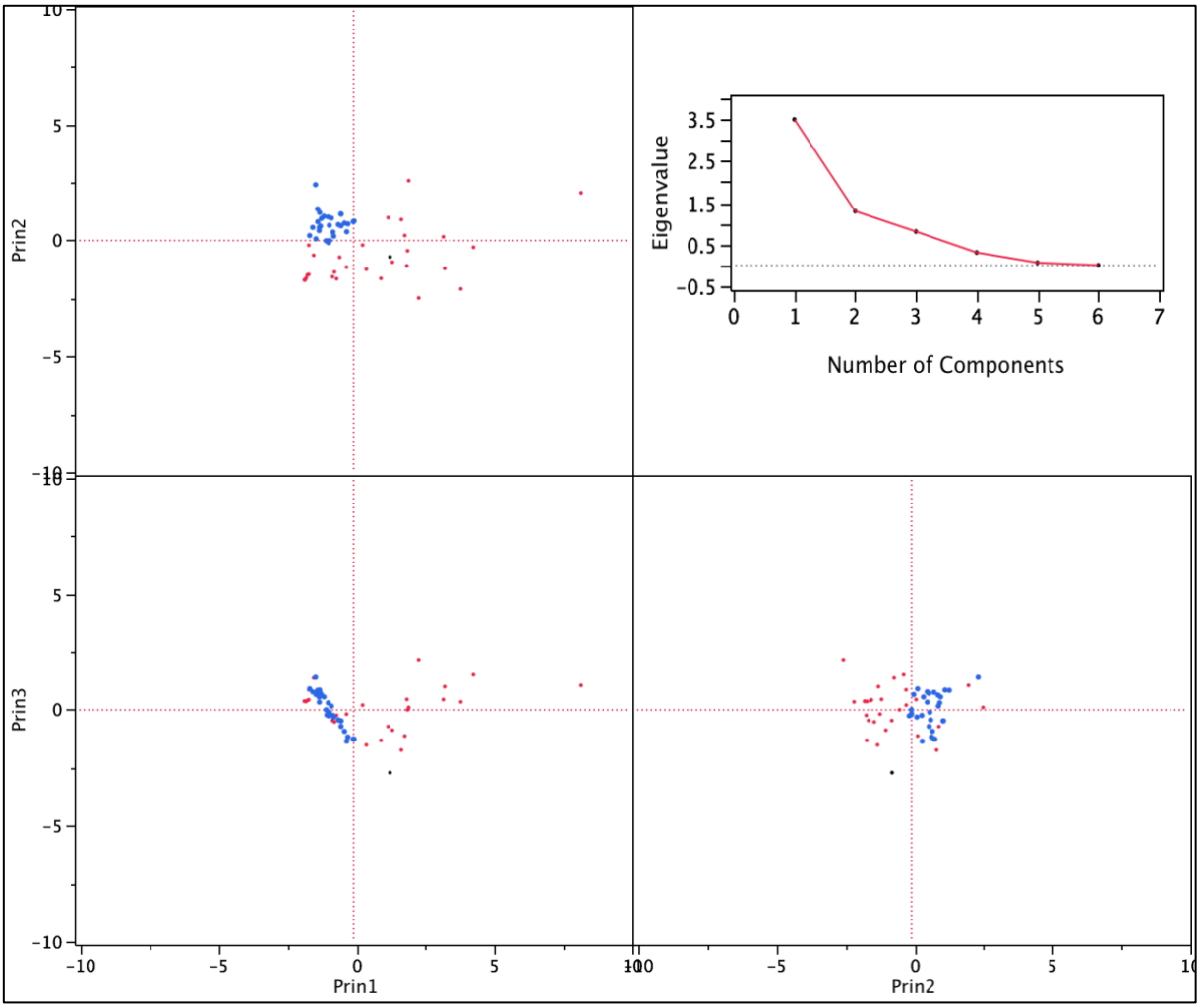

***Figure 15**. Score plot for all values in contemporary (red dots) and historic (blue dots) fish. Shows clear differences between those two groups. The difference is caused mainly by the fact that historic fish have a lower nematode burden and smaller livers than contemporary fish. Scree plot (top right corner) is showing the eigenvalues and number of components of the PC biplot is shown in the top right corner.*

***Table 4.** Principal components / Factor analysis. Shows how much of the variation in the data is accounted for. The three first principal components account for 93.6% of the variation in the data, and thus have been selected for the score plot to represent the data.*

Number	Eigenvalue	Percent	Cum Percent
1	3.5010	58.351	58.351
2	1.3007	21.678	80.029
3	0.8133	13.554	93.583
4	0.3104	5.174	98.757
5	0.0687	1.145	99.902
6	0.0059	0.098	100.000

Discriminant analysis separating the nematode burdens to high (H, 5 nematodes and above) and low (L, less than 5 nematodes) shows that the historic fish are in the low class (Figure 16). This model is based on Cod only in the size range 25-55 cm from historic and contemporary samples to compare like-with-like. Table 5 shows that the model has 69% accuracy, and the first eigenvalue accounts for 100% of the variation in the data. Wilks' Lambda value for the first principal component is 0.5194952.

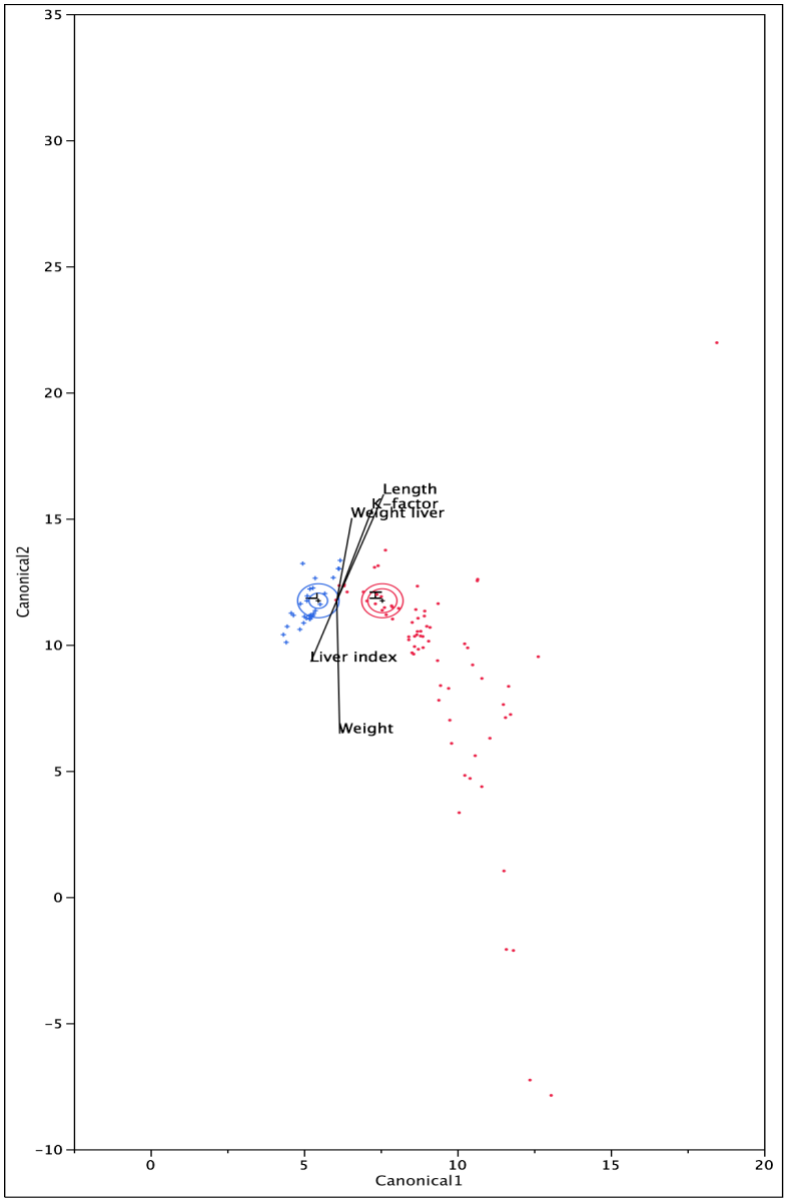

Figure 16. *Discriminant analysis with K-factor with low (L) and high (H) nematode burdens. Here, it implies that the historic fish (blue dots on the graph) are those with the lowest numbers of nematodes, whereas the contemporary fish (red dots on the graph) are those with the highest numbers of nematodes. This model has 69% accuracy.*

Table 5. *Eigenvalues for the discriminant analysis. The first eigenvalue accounts for 100% of the variation in the data, and canonical correction shows that the model has 69% accuracy.*

Number	Eigenvalue	Percent	Cum Percent	Canonical Corr
1	0.92494554	100.0000	100.0000	0.69318452
2	1.9599e-15	0.0000	100.0000	0
3	4.7682e-17	0.0000	100.0000	0
4	3.41e-17	0.0000	100.0000	0
5	-2.941e-17	0.0000	100.0000	0

Discriminant analysis to separate historic (blue) and contemporary (red) fish show that there is a difference within those groups, and HSI and K-factor are the best parameters to separate groups (Figure 17). Historic fish have lower K-factor and higher HSI than contemporary samples. Table 6 shows that the model has 85% accuracy, and the first eigenvalue accounts for 100% of the variation in the data. Wilks' Lambda value for the first principal component is 0.2831844.

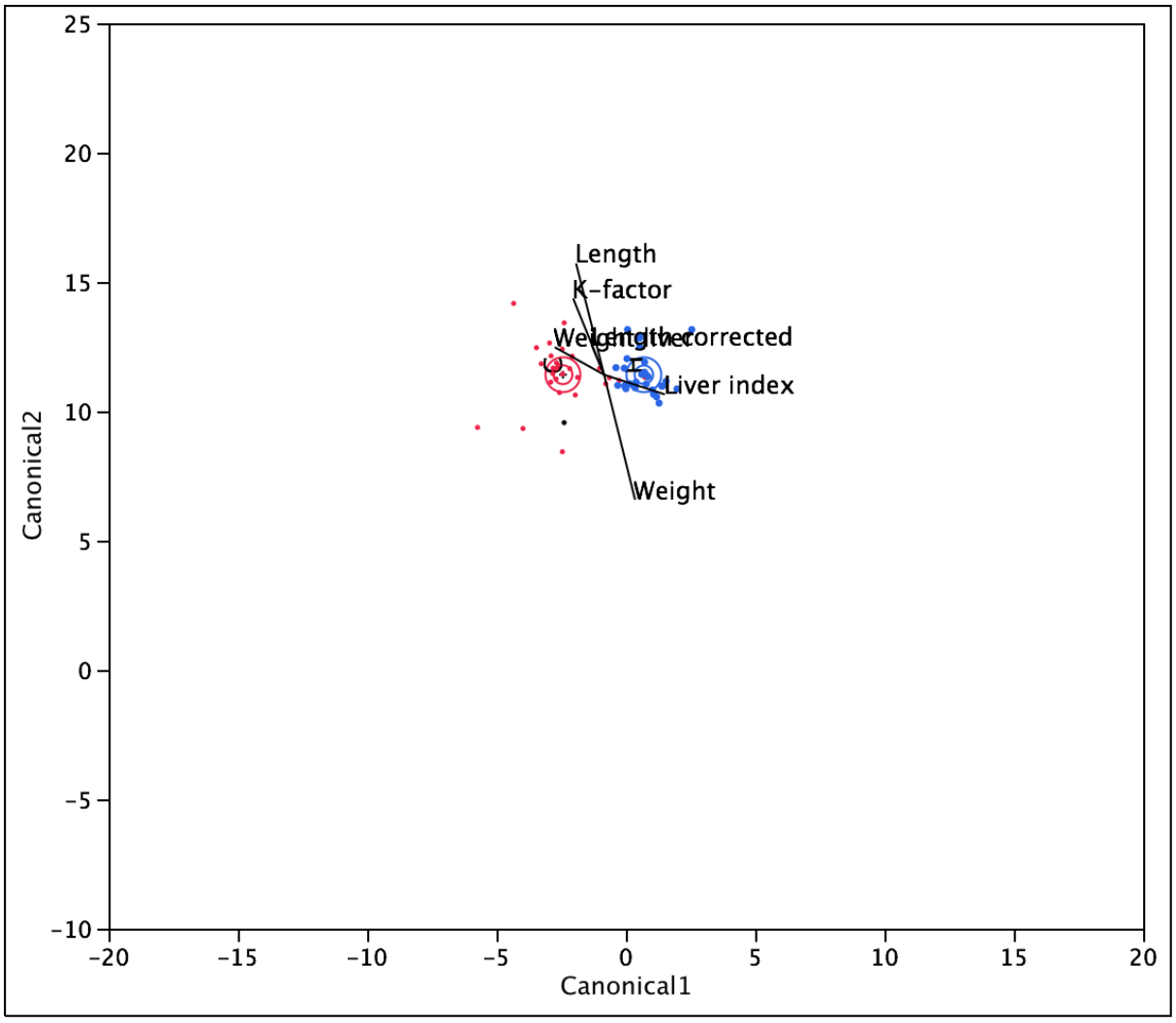

Figure 17. *Discriminant analysis built to separate historic (blue), and contemporary (red) fish based on morphometrics. K-factor and liver index (HSI) are the best components that show variance between groups.*

Table 6*. Eigenvalues for the discriminant analysis. The first eigenvalue accounts for 100% of the variation in the data, and canonical correction shows that this model is 85% accurate.*

Number	Eigenvalue	Percent	Cum Percent	Canonical Corr
1	2.5312681	100.0000	100.0000	0.84664964
2	5.3785e-16	0.0000	100.0000	0
3	5.5196e-17	0.0000	100.0000	0
4	7.6645e-18	0.0000	100.0000	0
5	-1.41e-16	0.0000	100.0000	0
6	-6.193e-16	0.0000	100.0000	0

3.2.3 – Chi-Square analysis

Chi-square analysis was conducted to demonstrate difference in the incidence of infection in historic and contemporary fish (Table 7). This analysis was conducted on Cod (*G. morhua*) only from historic and contemporary samples, to compare like-with-like. The number of infected historic fish are much fewer, and the infected contemporary fish are much greater, than might be expected in the Chi-square contingency table.

***Table 7**. Chi square analysis to test difference of the incidence of infection between historic and contemporary fish. P-value is calculated with 1 degree of freedom, and Yates correction has been used.*

Individual fish +ve/ -ve for Anisakis	Historic		Contemporary		Total
+ve	15 (24.9)	3.94*	68 (58.1)	1.69NS	**83**
-ve	15 (5.1)	19.22***	2 (11.9)	8.24**	**17**
	30		**70**		**100**

Chi^2 =	3.94	+	1.69	+	19.22	+	8.24	= 33.09 p>0.001***
	Historic fewer +ve p =0.05*		Contemporary close expected NS		Historic more -ve p>0.001***		Contemporary fewer -ve p> 0.005**	

Being more conservative and limiting the Chi-square analysis to Cod only in the size class 25-55 cm, slightly different numbers are obtained (Table 8). The significant result for historic fish expected infected in the previous table is now not significant (NS). Historic fish observed not infected is much higher than expected values in the Chi-square test.

***Table 8.** Chi square analysis to test the difference of the incidence of infection between historic and contemporary fish. P-value is calculated with 1 degree of freedom, and Yates correction has been used.*

Individual fish +ve/ -ve for Anisakis	Historic		Contemporary		Total
+ve	15 (21.7)	2.38NS	30 (23.3)	1.7NS	**45**
-ve	15 (8.3)	4.63*	2 (8.8)	6.1**	**17**
	30		**32**		**62**

Chi^2 =	2.38NS	+	1.7NS	+	4.63*	+	6.1**	= 14.81 p>0.001***
	Historic close expected NS		Contemporary close expected NS		Historic more -ve p>0.001*		Contemporary fewer -ve p> 0.005**	

3.3 – COX2 and ITS sequencing

40 nematodes from the contemporary samples were sequenced, and 38 of these returned with reliable sequences. Most nematodes were classified as *Anisakis simplex sensu stricto*, but some individuals had a very divergent COX2-haplotype. Of those individuals that had a divergent haplotype, a full phylogeny was done to further investigate where that haplotype might have derived from.

3.3.1 – Haplotype network analysis

Haplotype network analysis (Figure 18) shows relatedness between the sequenced nematodes. There are some major haplotypes present, size of circles shows that there are more haplotypes present, and the colors in each of the circles show where those haplotypes originate from. The vertical lines on the network represent mutations that separate the haplotypes from each other.

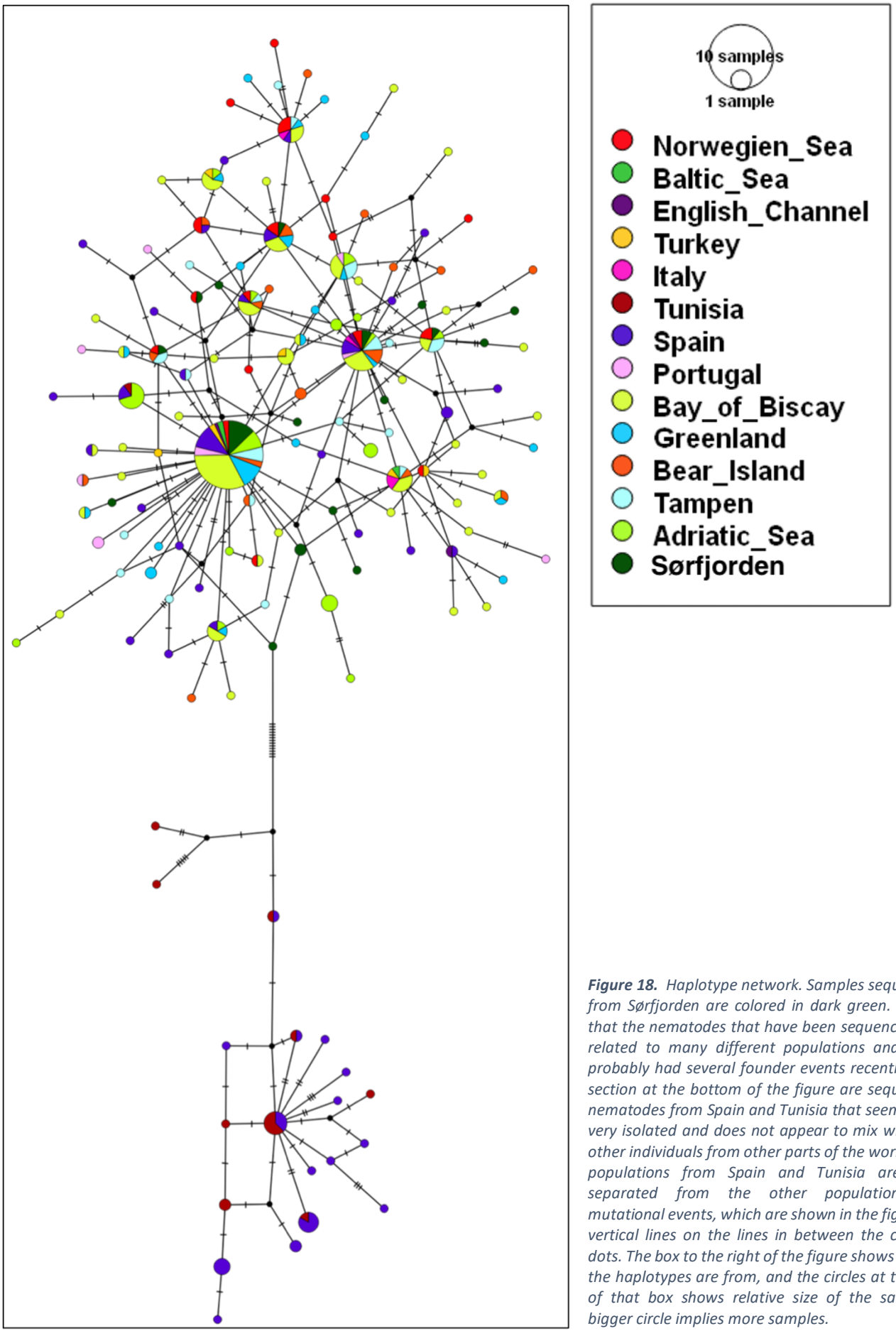

Figure 18. *Haplotype network. Samples sequenced from Sørfjorden are colored in dark green. Shows that the nematodes that have been sequenced are related to many different populations and have probably had several founder events recently. The section at the bottom of the figure are sequenced nematodes from Spain and Tunisia that seem to be very isolated and does not appear to mix with the other individuals from other parts of the world. The populations from Spain and Tunisia are also separated from the other populations by mutational events, which are shown in the figure as vertical lines on the lines in between the colored dots. The box to the right of the figure shows where the haplotypes are from, and the circles at the top of that box shows relative size of the samples; bigger circle implies more samples.*

3.3.2 – Pairwise mismatch analysis

Pairwise mismatch analysis for all sequences (Figure 19) show high differences between the different populations, the smaller peak that is separated is likely a reflection of what is shown in the haplotype network; separation between the bigger North-East Atlantic populations and the smaller Mediterranean populations.

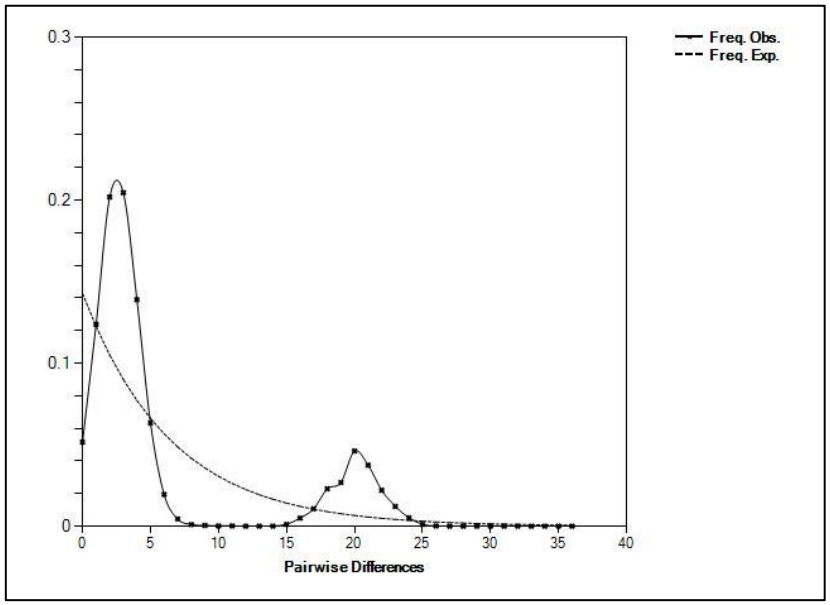

Figure 19. *Pairwise mismatch analysis for all sequences. Indicates that there are signatures of a potential global expansion event with a high frequency of low divergent haplotypes. The second small peak may be reflective of the small sub population from Spain and Tunisia as reflected in the haplotype network.*

Pairwise mismatch analysis for the sequenced samples from Sørfjorden (Figure 20) has a bimodal appearance, showing a stable population. A smaller peak is observed toward the end of the curve. All those peaks might suggest different clades present in the samples, but nonetheless a stable population since those peaks are discrete.

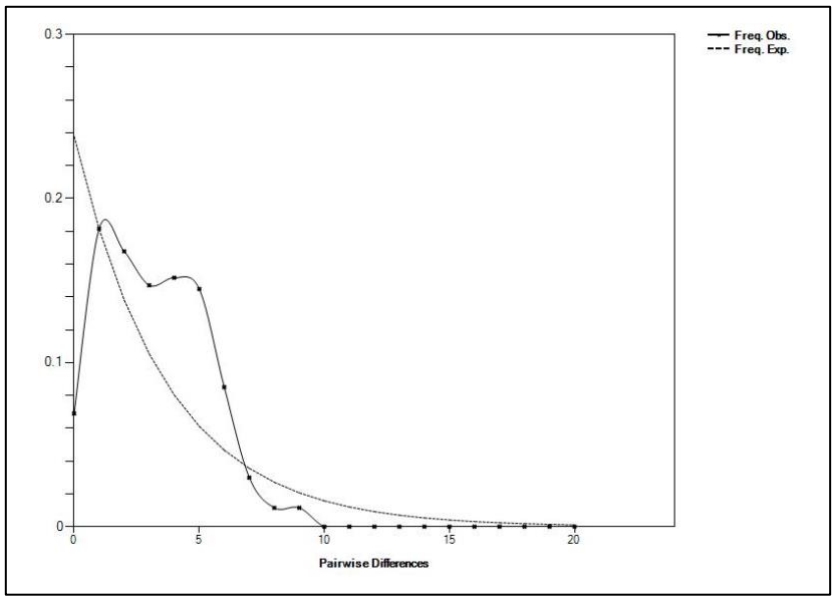

Figure 20. *Pairwise mismatch analysis for the sequenced samples from Sørfjorden. Bimodal appearance could suggest the population from Sørfjorden is stable but may also be reflective of a divergent "cryptic" species within the complex.*

3.3.3 – Maximum likelihood trees

Maximum likelihood phylogenetic reconstruction for COX2 (Figure 21) indicates a very clear separation or pattern in the different clades of *Anisakis* present, and only a few of the sequenced samples from Sørfjorden fall out of the *A. simplex* species complex and are grouped together with *Hysterothylacium aduncum*. All samples from Sørfjorden are represented in the figure with red boxes around groups, and four with red lines underneath.

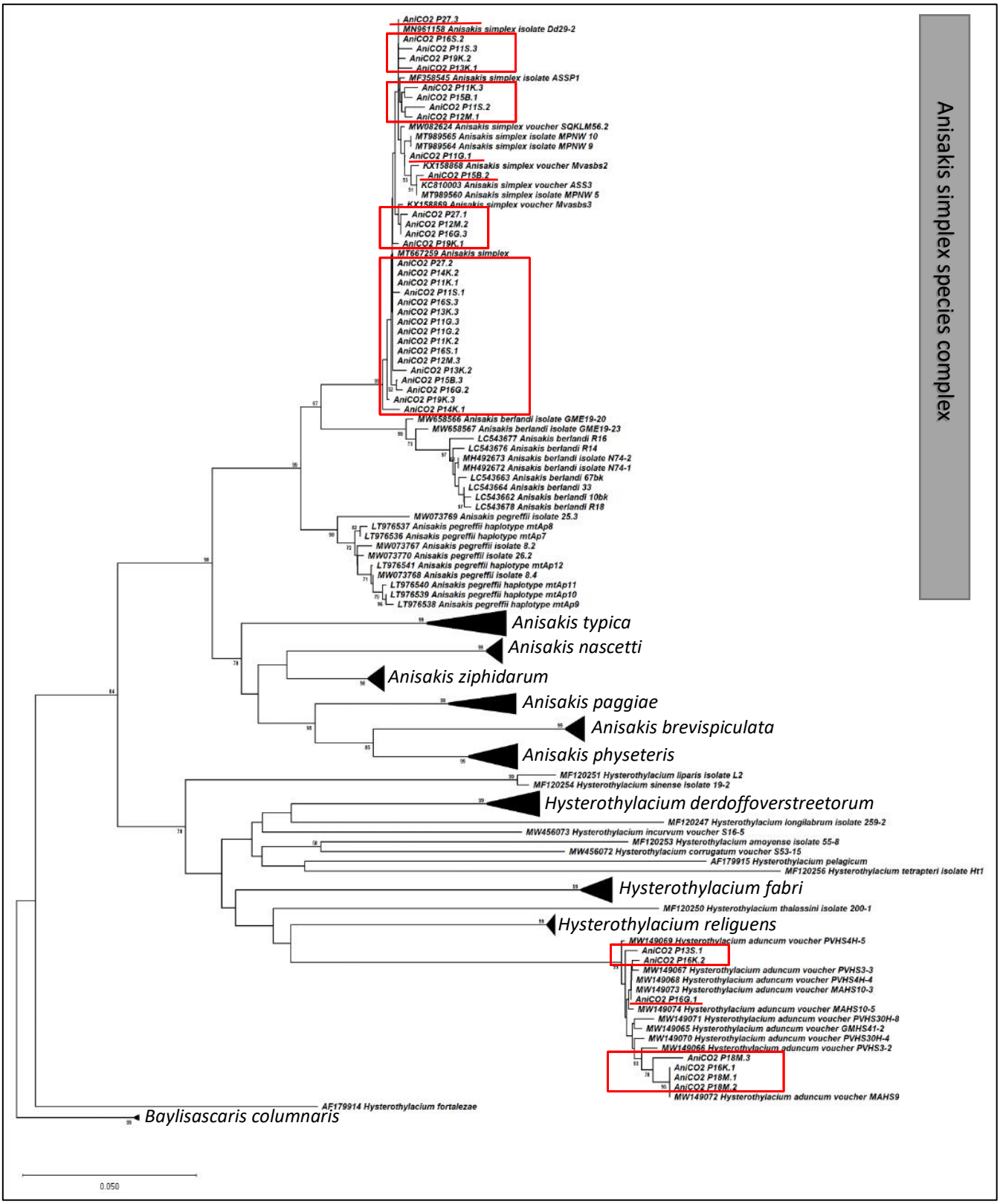

Figure 21. *Maximum likelihood Phylogenetic reconstruction of CO2 using the HKY+I+G model and 1000 bootstrap replicates for nodal support with only supports of 50> shown. Shows that almost all samples from Sørfjorden falls into the Anisakis simplex species complex, and only a few fall out of the complex, grouped together with Hysterothylacium aduncum. Baylisascaris columnaris was used as the outgroup. All samples from Sørfjorden are represented here with red boxes around groups, and four with red lines under them.*

Due to separation in the Maximum likelihood trees, X4 ratio calculations were made to distinguish if those clades have been formed through geographical variance or through speciation events. The X4 ratio compares the ratio of mean pairwise differences between two clades, and it is considered that if K/Θ>4, the compared groups represent distinct species. In the calculations, all compared groups exceeded the threshold (Table 9).

Table 9. *X4 ratio calculations on the clades is a conservative estimation based on divergence of none recombining markers that enables you to distinguish if clades are formed through geographical vicariance or potentially through speciation events - the general rule is to always go for the higher value output, and it is considered that if K/Θ>4, the compared groups represent distinct species. This shows that the A. simplex complex indeed fall out as three distinct species, however closely related. See yellow highlights.*

	A. simplex	*A.berlandi*				
	Clade 1	Clade 2	Clade 1 vs Clade 2			
Number of sequences	41	10				
K=D= total pairwise differences between clades			0.0608			
d = total pairwise differences within clades	0.00483	0.01065				
π =(d*(n/n-1)	0.00495075	0.011833333				
Θ =(π/(1-(4π/3)	0.004983647	0.01202303				
K/Θ	12.19990086	5.056961502				
	A. simplex	*A. pegreffi*	Clade 1 vs Clade 3	*A. pegreffi*	*A. berlandi*	Clade 2 VS Clade 3
Number of sequences	41	10		10	10	
K=D= total pairwise differences between clades			0.0504			0.0657
d = total pairwise differences within clades	0.00483	0.01111		0.01111	0.01065	
π =(d*(n/n-1)	0.00495075	0.012344444		0.012344444	0.011833333	
Θ =(π/(1-(4π/3)	0.004983647	0.012551025		0.012551025	0.01202303	
K/Θ	10.11307572	4.015608281		5.234632223	5.464512676	

Maximum likelihood phylogenetic reconstruction for ITS (Figure 22) indicates that the clear separation between clades as seen in the COX2 reconstruction is less visible, and most samples fall into a single, bigger clade, along with *Anisakis pegreffi* and *Anisakis berlandi*. ITS sequences of the nematodes from Sørfjorden still fall into the *Anisakis simplex* species complex, but the separation between the different species is less clear. Some of the nematodes from Sørfjorden fall out of the *A. simplex* species complex and are grouped together with *Hysterothylacium aduncum.* All samples from Sørfjorden are represented in the figure with red boxes around groups, and three with red lines underneath.

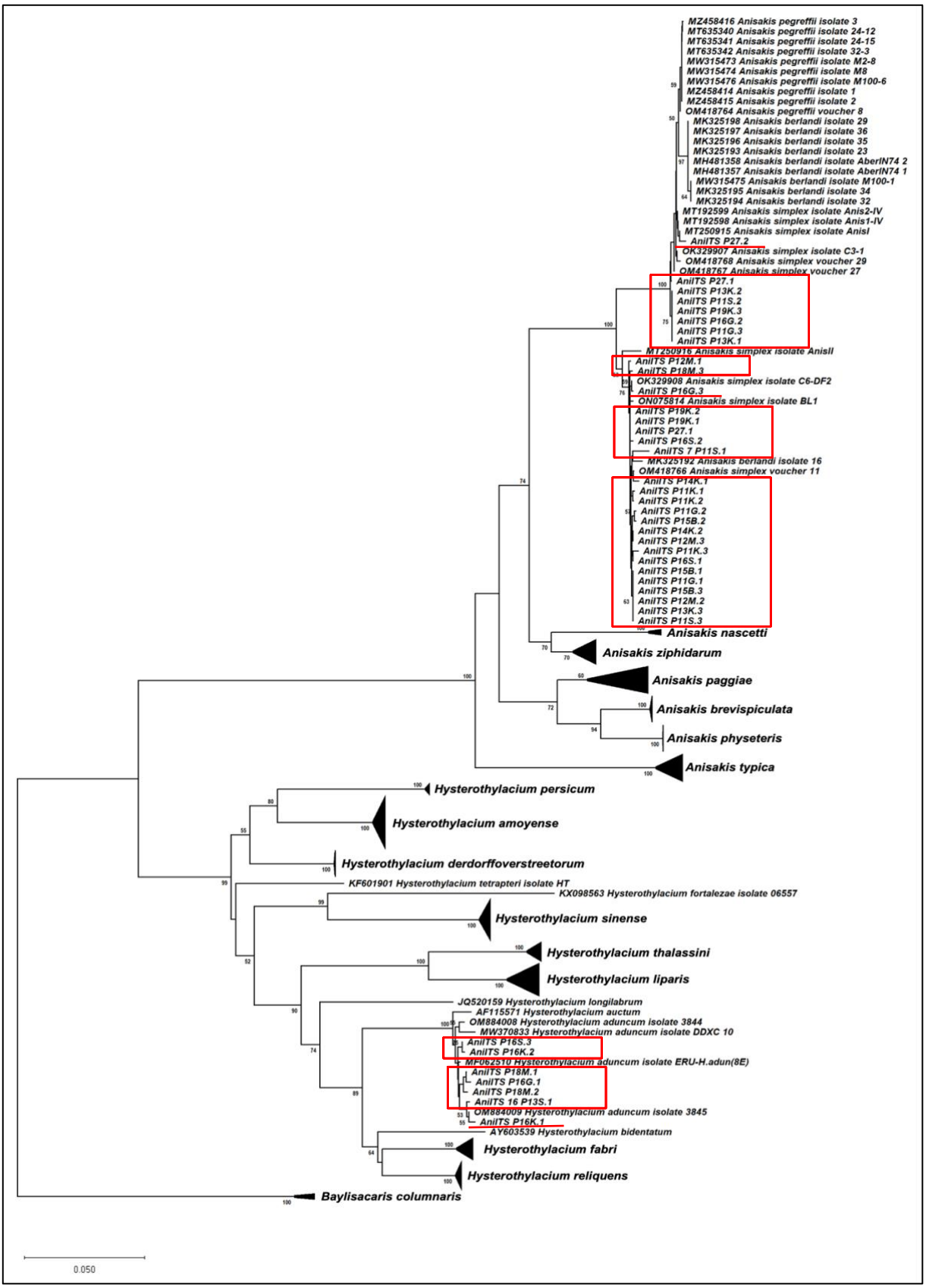

Figure 22. *Maximum likelihood tree based of ITS, produced using the HKY+G model with 1000 bootstrap replicates for nodal support. Only nodal support values of 50> are shown. Almost all nematodes from Sørfjorden are in the Anisakis simplex species complex, grouped together with Anisakis pegreffi and Anisakis berlandi, and a few fall out of the complex and are grouped together with Hysterothylacium aduncum. Baylisascaris columnaris was used as the outgroup. All samples from Sørfjorden are represented here with red boxes around groups, and three with red lines.*

4.0 – Discussion

The intention of this study was to investigate and compare the load and prevalence of *Anisakis* in contemporary and historic samples, reviewing this in the context of potential consequences of environmental change. Instances such as temperature change leading to range shifts in hosts and hybridization between *Anisakis* species, increased connectivity between populations (of hosts and parasites) leading to altered host-parasite interactions, and increased pressure from fisheries, amongst others are discussed as factors contributing to the apparent increase in these parameters.

4.1 – Potential factors contributing to higher prevalence and load of Anisakis

Based on the findings in this study, there is indeed a higher prevalence and load of *Anisakis* in contemporary compared to historic samples, and the baseline of infection historically appears to have been considerably lower than it is today.

4.1.1 – Eutrophication and stratification

Eutrophication in marine environments might have an influence on host-parasite relationships. With increased input of nutrients, primary production in the water column may increase. Consequently, zooplankton and copepods, that act as intermediate hosts for *Anisakis*, will increase. Following on from this cascading effects might then include the attraction of animals from higher trophic levels that feed on these species, which then attract predators, increasing the likelihood of higher trophic transmission of parasites. Greater food availability can lead to an increased opportunity for parasites to exploit host resources. However, greater abundance of food resources for hosts may also negatively affect the parasite community if the hosts are able to invest more resources into resistance (Budria, 2017). With very high nutrient input, toxic effects may also occur which in turn can lead to a decrease in parasitism (Lafferty, 2008). Eutrophication in the form of sewage effluents has been shown to affect the reproduction, growth, and sexual development of the European mollusk (*Planorbarius corneus*) due to high exposure to chemicals such as synthetic steroids and natural hormones, among others (Clarke *et al.*, 2009). It is not unlikely that eutrophication and exposure to these chemicals will also affect teleost fish and marine mammals in a similar way, and a possible outcome could be alterations of the host-parasite interactions. High levels of polychlorinated biphenyls (PCB), an endocrine disruptor, in

harbor porpoises have been associated with high parasitic worm burdens in individuals. Although these associations have been confounded with age, sex and cause of death in those individuals, and those individuals with highest parasite burdens were not the ones that had the highest PCB levels, PCBs remained an important contributory factor (Bull *et al.*, 2006).

Increased stratification in the marine water column might also be a contributing factor to higher incidence of parasitic nematodes in the marine food web. This is likely because highly stratified waters will limit the vertical migration of zooplankton and of larval nematodes, both of which become restricted to a smaller depth range, increasing the likelihood of those zooplankton becoming infected. Highly stratified waters might also contribute to overlap between zooplankton and their predators, which means the predators will also be more likely to ingest those infected zooplankton (Marcogliese, 2001). It has been shown that in stratified areas, haddock and whiting are more likely to be heavily infected with parasitic nematodes of *H. aduncum* due to feeding mainly on pelagic hyperiids, that serve as the intermediate host of this parasite (Klimpel and Rückert, 2005).

4.1.2 – Environmental change - impacts on final and intermediate hosts

Modern whaling began around the 1860's in northern Norway and has continued to the present day, expanding to several other countries partaking in the whaling industry, resulting in severe reduction of the targeted whale stocks globally (Tønnessen and Johnsen, 1982). Overexploitation of whales has led to a dramatic reduction of population numbers, and might have reduced overall genetic variation among populations, however such a claim is controversial. Low genetic diversity in small populations results in inbreeding depression, which can reduce immunity to disease and other environmental threats (Clapham, Young and Brownell Jr, 1999). Whaling industries were long thought to negatively impact the genetic diversity among targeted species, and research effort conducted on this implies that modern whaling has not been significant enough to reduce genetic diversity (Cypriano-Souza et al., 2018). However, in many instances these claims are based on genotypes derived from non-focal microsatellite loci. These tend to be highly polymorphic, and might not accurately estimate allele frequencies in the selected populations (Putman and Carbone, 2014). Microsatellites used as a measure of genetic diversity or relatedness between populations is

often unreliable. In one instance a researcher using focal microsatellites found cod (*G. morhua*) from two different sides of the Atlantic to be full siblings. This unlikely finding was attributed to problems with homology of microsatellite loci, the phenomena of homoplasy, where different mutations in the microsatellite of the genome produce similar but historically unrelated alleles that happen to correspond in these apparently related individuals. A better approach to tend to variation in whole genomes would be the use of next-generation sequencing (Yue, Kovacs and Orban, 2010).

The conservation of marine mammals may contribute to an increase of *Anisakis*. This is because marine mammals serve as the final host, and when marine mammals are not targeted and exploited, their population numbers increase for both marine mammals and *Anisakis.* Thus, the potential for *Anisakis* completing their life cycle is higher, which will ultimately increase the number of parasites (Shamsi, 2021). The study by Fiorenza et al. hypothesized that the increased number of *Anisakis* might not even be unnatural, and that these high numbers are representative of what the natural prevalence of this parasite would have been without decimation of cetaceans. The marine mammal populations bouncing back to what they were thought to be before overexploitation of these species consequently supporting high numbers of *Anisakis* (Fiorenza et al., 2020). Thus, it is important to interpret the increase of *Anisakis* after the years the legislative acts based on marine mammal protection were enforced with great caution.

Marine fishes along with marine mammals have also been under pressure from overexploitation for a long time, and it is apparent that many targeted stocks have suffered the consequences of loss in genetic diversity. This loss of genetic diversity leads to reduced adaptive capacity and make stocks vulnerable to environmental change (Pinsky and Palumbi, 2014). Other studies regarding limited genetic variance in overexploited fish populations have been conducted, and it is apparent that limited genetic variance in populations will increase the susceptibility of disease and pathogenic and parasitic infections in hosts, and increase the transmission of pathogens (Campbell et al., 2010). Overexploitation of fish can also lead to decreased transmission of parasites directly caused by the removal of hosts; especially for those parasites that have host dependent life cycles (specialists). This could seem beneficial for fisheries because number of parasites is reduced, but it also proves that parasites can be used as indicators of the status of the fish stocks (density/populations) (Lafferty, 2008).

Increased temperatures in marine habitats have been linked to species migrating away from the equator towards the poles in search of cooler temperatures they are better adapted to. This leads to the formation of new predator-prey interactions (Richardson *et al.*, 2021). Environmental change such as increased water temperatures can contribute to the modification of ranges in which hosts of *Anisakis* are able to exist. The modification of host ranges impacts the infective potential of *Anisakis* because infected hosts can transport *Anisakis* from one locality to another. If the new hosts that the parasites are transferred to are susceptible to infection, it increases the chance of *Anisakis* being transferred to its final host and completing its life cycle in new areas (Klimpel and Palm, 2011).

When parasites are transferred to a new area, they are likely to encounter closely related cryptic species, which previously have been separated due to barriers such as distance. If those cryptic species are not fully speciated from their origin, as reflected in the genetic analyses of this study, they may interbreed with each other. Such interbreeding can promote the activation of transposable elements in the genomes of hybrid offspring, and/or heterosis. These genomic changes may significantly increase the fitness of some hybrids, giving rise to a much higher numbers of parasitic nematodes. The parasites that have been genetically identified in this study show a high likelihood of being cryptic clades that are now mixing again. The reason for this remains unclear. It could be due to transportation of hosts shedding eggs into the environment during migration and shifting parasite ranges, or it could be linked to environmental change. Genetic analyses of historic specimens may aid in resolving some of these issues, providing insight to whether there are similar or more well-defined patterns of relatedness of the three recognized clades between specimens of nematodes recovered from contemporary fish.

Human activities can also be directly linked to a direct “shortcut” in the life cycle of Anisakids. Due to increased attention of anisakiasis and anisakidosis in recent years, it seems that the practice of gutting fish at sea and discarding the organs at sea has become more normalized. Thus, the parasites present in the discarded fish organs are now back at sea, and the likelihood of predatory fish being infected might now be slightly increased (Mattiucci et al., 2018). However, it is difficult to measure how significant this is in respect of higher parasite prevalence but is something that should be considered. A way to account for this and research the significance of this gutting practice possibly contributing to an increase could be done by

sampling fish from areas where this is normal practice and compare this with an area close by where this is not normal practice.

4.1.3 – Increased virulence and transmission in Anisakis

The increase of *Anisakis* can also be due to higher resilience and adaptive virulence and transmission in the parasite. One important aspect in this study has been potential hybridization and backcrossing of different species of *Anisakis*, possibly leading to phenotypes with better evolutionary or adaptive potential being expressed in the parasite populations. Those phenotypes of the parasite can increase its ability to adapt to new environmental conditions, as well as the ability to infect novel hosts. Due to *Anisakis* being a permissive parasite, meaning *Anisakis* is accepted by a wide range of hosts, it seems to not be greatly affected by the decrease or decline of some intermediate hosts, and possess the ability to infect new hosts successfully. Increased virulence and subsequent high transmission rates of *Anisakis* might have significant implications for disease in the marine environment. These implications might manifest as more severe infections in already well-established hosts, or these nematodes might be able to infect new paratenic hosts. The elasmobranchs in contemporary samples all appeared to be free of infections, however, Spiny dogfish (*Squalus acanthias*) from the Celtic Sea have been shown to be infected with *Anisakis* (Bachelor's thesis, 2019, Elin-Marita B. Kristiansen, unpublished). Comparing these shark species might not be ideal, but it can be an indicator that *Anisakis* are infecting new paratenic hosts.

Increased virulence in *Anisakis* might be a reflection of increased marine functional connectivity brought by hosts transporting *Anisakis* around. Based on the haplotype network, it can be seen that there is a lot of mixing between populations of *Anisakis,* and this might manifest as higher virulence in the parasite.

Comparisons of contemporary and historic fish and their HSI indices with load of *Anisakis*, HSI increase with nematode burden. However, in the historic fish, livers were unusually small compared to contemporary fish (Figure 9). This might suggest that historic fish has suffered a more severe immunological response to infections than contemporary fish have. This comparison might be an indicator that virulence of *Anisakis* has decreased over time, but whether that is accurate or not is difficult to determine based on these comparisons alone.

4.2 – Differences in load and prevalence of *Anisakis* in contemporary and historic samples

There was an apparent difference in load and prevalence in temporal collections. In historic samples, fish had a generally low prevalence and load of parasites, with a maximum parasite number of 26. The average number of parasites found in fish from historic samples was 1.75, and the prevalence of infection was 50.9%.

In contemporary samples on the other hand, load and prevalence were much higher compared to that of historic samples, with a maximum parasites number of 458. The average number of parasites between fish from contemporary samples was 38.32, the prevalence of infection was 71.5%. The incidence of parasites in the fish; 39 fish were apparently uninfected, and 98 fish had *Anisakis*, and thus were infected. However, the majority of uninfected individuals were Chondrichthyes (n=16) and flounders (n=15). Only 8 individuals of the uninfected portion were other teleost species. All the flounders dissected were apparently free of *Anisakis*, except two, so it can be argued that in the sampling area, flounders appear to have a smaller chance than other teleosts of being infected by *Anisakis*. However, genetic analysis of the three anisakids retrieved from those flounders were shown to be *Hysterothylacium aduncum. H. aduncum* is a parasitic nematode that infects marine fish, especially teleosts. The life cycle of this parasite is not fully understood, but generally it seems not to involve marine mammals, and the transmission is dependent on highly stratified waters (Klimpel and Rückert, 2005).

Anisakis are unlikely to infect Chondrichthyes, so if they were excluded from the samples, only 23 individuals would be uninfected. As a corollary, Spurdog (*Squalus acanthias*) caught near the Isles of Scilly in the Celtic Sea, have been found to have *Anisakis* present in their spiral valve (Elin-Marita B. Kristiansen, 2019, Bachelors' thesis, unpublished data; see Appendix A for details). It is difficult to determine why the Chondrichthyans of this study showed no infections of anisakids, but Spurdog in the Celtic Sea did. Spurdog have also been reported to be infected by *Anisakis* in other studies (Henderson, Flannery and Dunne, 2002). One assumption that can be made here is that since the Spurdog were caught from an area where they have been very heavily exploited (Dell'Apa, Bangley and Rulifson, 2015), the population might have suffered reduced genetic variance, which potentially could increase the risk of contracting *Anisakis* infections. In addition to this, the Spurdog (from the bachelors' thesis)

that were infected with anisakids were all above 55 cm in length, and as the sharks grow older, their diet diversifies, and thus the risk of contracting anisakids might be higher. The sharks sampled in this study rarely exceeded 55 cm in length, and additionally none of them was a Spurdog. Spurdog feed on a wide range of prey, including squid, mackerel, and herring, which all are hosts of *Anisakis* (Murdy and Musick, 2013). Velvet belly lanternsharks and Blackmouth catsharks both feed on bottom invertebrates such as shrimps and cephalopods, as well as small teleosts (Compagno, 1984). From the study area, it might be a combination of smaller specimens sampled, as well as different species, which might not feed on the same prey as the Spurdog, accounting for the elasmobranchs in this study to have no anisakid infections. The sharks sampled in this study are also closely associated to the demersal habitat, which is similar to the preferred habitat of Spurdog, but Spurdog have been known to migrate to the pelagic environment as well as the surface areas. *Anisakis simplex* is adapted to the pelagic offshore environment, which implies demersal species are less likely to be infected. However, these conclusions cannot be confirmed without further in-depth research. To account for this, sampling of Spurdog that reside in Norwegian waters could be done and compare *Anisakis* abundance and load of those Spurdog of the Celtic Sea.

Observing a higher abundance and prevalence of *Anisakis* in contemporary samples was expected, based on reported findings of increased incidences of infections in recent years (Fiorenza *et al.*, 2020). The study by Fiorenza *et al.,* only investigated the abundance of *Anisakis* from the 1960's until 2020, so the low numbers of *Anisakis* found in the present study only relates back to the 1960's. A study conducted in 1995 in the Barents Sea reported relatively low mean intensities of anisakids in coastal and oceanic cod (6.1 and 4.3, respectively) but the percentage of infected fish was high, 96% (Aspholm, 1995). Further back in time there is little information available on the abundance of *Anisakis,* thus the historic material obtained in this study gives valuable insight in establishing a historic baseline of abundance of the parasite and shows there is a steady increase of *Anisakis* in the marine environment.

In Northern Norway around the mid 1980's, there was a dramatic increase of anisakids in commercially important fish, which was likely a consequence of the Harp seal migrating to this area and spreading anisakids. The infections in the fish were sometimes so severe that fishermen were unable to sell the fish or cut fillets due to the high number of nematodes in

the fish. The anisakids are especially abundant in fish from shallow waters, in the springtime (K. Nilssen, April 2022, pers. comm.).

4.3 – Hepatosomatic index and Fulton's body condition factor K

The hepatosomatic index (HSI) has been calculated, and there seems to be a general trend of increased HSI with an increase of *Anisakis* load in contemporary fish (Figure 12). There are certain problems that occur when comparing HSI across several different species of fish due to the natural variation in HSI among species. Thus, comparing health indices like this is not necessarily representative; however, comparison of HSI between individuals of the same species of fish is more representative and has been applied here (Figure 12). It is apparent that HSI fluctuates among individuals of the same species, and individuals with great loads of *Anisakis* seem to have a higher HSI. It is likely that infected individuals have suffered liver damage due to *Anisakis* infections, which in turn can lead to a higher-than-average HSI compared to that of uninfected individuals.

HSI was also calculated for historic samples, but here it might be problematic due to those samples being stored in ethanol or formalin, which might have desiccated the samples obscuring the relationship between body and liver masses. When the number of parasites was compared to the HSI in an analysis which combined both historic and contemporary samples, it showed a negative but non-significant trend of those two variables (Figure 13). However, it seems that the historic fish generally have a high HSI, but also fewer nematodes. This contrasts with the contemporary fish, which also seem to have a high HSI, but also a significantly higher number of nematodes across samples (Figure 12, Table 2). Due to the historic fish showing elevated HSI but low number of nematodes, it can be thought that these fish are suffering more severe consequences than the contemporary fish do, but this method is flawed, and this cannot be confirmed just by comparing these indices against each other. A more thorough approach would be to determine fat percentage in fish livers and the use of histology. It is suggested that fish with abnormally high percentages of fat cells in their livers will suffer negative consequences such as aberrations in metabolism and other physical properties. Liver histology can also provide a more in-depth description of the nutritional status of fish (Lu et al., 2013). The use of histological methods to examine and compare the livers of fish from both

contemporary and historic samples would thus give valuable insight in how those fish might have reacted to parasitic infections, making for an appropriate and feasible comparison.

In addition to this, Fulton's body condition factor (K) was calculated for the fish. K-factor was not significantly different between historic and contemporary fish, and there was no significant correlation between nematode burden and K-factor (Figure 14). Since K-factor is a general measure of health in fish by only calculating the relation of body mass and length of the fish to reveal how fat they are, it is not a good measure for health in wild fish, as there are too many unmeasured variables present that might obscure the value. Therefore, the K-factor does not necessarily provide much information on how the fish might react to parasitic infections.

It is important to take into consideration that the apparent difference in HSI between contemporary and historic fish could be due to the historic fish being preserved in ethanol or formalin for decades. The preservation could be a limiting factor when comparing HSI, and it might not reflect of what the true relationship between HSI and nematode burden is in those historic fish. Therefore, using histological methods to determine fat percentage of livers in contemporary and historic fish might potentially reveal associations it has not been possible to address here.

4.4 – Genetic analyses

Analysis of molecular markers revealed that the sampled nematodes mainly consisted of *Anisakis simplex s.s. (sensu stricto)*, however, some are of the species *Hysterothylacium aduncum,* which were found in flounders, cod, and saithe. The maximum likelihood (ML) trees of both the ITS and COX2 sequences shows the sampled nematodes could be split into two different groups: *Anisakis simplex* species complex, and *Hysterothylacium aduncum*.

However, the ITS and COX2 ML trees differ markedly. The COX2 ML tree shows the *A. simplex* complex falls out into three distinct clades. This was not reflected in the ITS ML tree, which suggests that recently there might have been mixing between the three different clades or incipient species identified by the COX2 tree. The fact that the ML tree based on the ITS sequencing does not show much separation between the different clades indicates that the barriers that should have separated these newly arisen clades may have been disrupted,

perhaps by anthropogenic effects. This claim is supported by the COX2 ML tree, and by the X4 ratio statistics (Table 9), which show there is a definitive separation between the three clades, but their lack of separation in the ITS ML tree suggests that populations began interacting again. Hence, the X4 ratio calculations on the clades suggests that the *Anisakis simplex* species complex consists of three distinct but closely related species/incipient species. The haplotype mismatch analysis shows that the nematodes genetically identified in this study are related to several different populations and have probably had experienced several founder events recently.

The COX2 haplotype network shows that the sampled *Anisakis* are related to the other North Atlantic populations. The many haplotypes separated by a single mutational step corresponds with the mismatch analysis, indicating that there have been several founder events recently. The haplotype network also shows that the Mediterranean (Tunisia and Spain) populations are isolated from others in the Northeast Atlantic. Isolation between the Mediterranean and Northeast Atlantic is confirmed by the many mutational steps between their distinct COX2 haplotypes. The pairwise mismatch analysis shows evidence of a recent population expansion, which might indicate high marine functional connectivity during colonization of North Atlantic environments during which clades might have rapidly diverged.

The findings of hybrids and sibling species of the *A. simplex* complex in the stomachs of common dolphins in Iberian Peninsula waters (Abbatouy *et. al*., 2016 and Abollo *et. al*., 2003) suggests that area may be an area of introgression for *Anisakis.* Similarly, areas in Gildeskål where the *Anisakis* specimens in this study came from might suggest another area for continuing introgression.

These analyses show that it is very likely that the anisakids have gone through species mixing, heterosis or incomplete transposable elements repression, which might have led to increased fitness in the parasites.

4.5 – Limitations of the study and future work

4.5.1 – Fish composition of the samples and health indices

In this study, contemporary fish were caught using a trawl, which resulted in several different species of fish, but unfortunately the total number of fish provided was low. The combination of having a small sample size and several different species has made it difficult to provide a comprehensive insight of the load and prevalence of *Anisakis* in the fish. As stated earlier, HSI tends to vary between species and even among individuals in the same species. This can be problematic, because comparing means of HSI between the different species does not seem representative here. Ideally, the sample size of fish would be larger and limited to for example only gadoids. Alternatively, larger sample sizes of the different species of fish would also have been ideal, as this could provide more accurate representations of HSI and *Anisakis* load among the different species. This would also allow a more in-depth analysis of the *Anisakis* load and prevalence in the different species and a more rigorous comparison of correlations between HSI and parasite load; as well as how *Anisakis* prevalence and load varies throughout the food web.

Coincidentally, the same applies for the K-factor used as an indicator of health in the sampled fish. Inaccuracy in the relative comparisons of HSI in fish are even more pronounced when K-factor is an optional measurement of health. This is because K-factor is a very limited measure of health if one wants to compare those indices between fish; K-factor should only be compared in between fish of the same species, but also the compared fish should be similar in size. Even then, given the numbers that represent a subsample of the fish, the K-factor does not take into account factors such as breeding season or pollutants. Thus, it is important to remember that the K-factor can be helpful in providing an indicator of health but is not something that is definitive and it must be interpreted with caution. K-factor is a good measure of health when fish are regularly monitored, and it is known what the fish have been feeding on; thus, it should be a good measure of relative health of fish in aquaculture industries, but not necessarily in wild fish.

The health conditions/indices of the fish of contemporary samples could also be further explored using other health indices, such as gonadosomatic index, which is an indicator of reproduction activity, and splenosomatic index, which is an indicator of immune activation. Head kidneys could also be sampled in the fish, to determine immune system activation.

Combining these indices with the already measured HSI would essentially eliminate any other possible causes that might influence the condition and weight of the fish; mature gonads increase weight in fish, especially female individuals, and splenosomatic index adds accuracy to assessments of the immune activity of the fish in response to infection. These additional health indices could be associated with the presence or absence of *Anisakis* and used to determine the effect of infection on fish health. These indices could also be used to test whether the hybrid parasites have a more severe pathological effect on their hosts.

4.5.2 – Parasite extraction methods

During writing of this thesis, I realized that the extraction of *Anisakis* larvae from livers and organs of infected fish could be done more thoroughly. Dissolving organs in acid digests (Pepsin-HCL) seems to be an efficient way of extracting parasites that might have migrated further into the tissue of the liver and various organs (Buchmann and Mehrdana, 2016). During dissection, only visible *Anisakis* were extracted from the livers, meaning *Anisakis* that have migrated further into the tissue may have been overlooked. However, this approach allowed for an appropriate comparison between contemporary and historic material, as the latter could not be subjected to more invasive examinations.

During dissection and examination of the abdominal cavity of the fish, only visible parasites were extracted. There is a possibility that the parasite load of fish was higher than that recorded, and this could be further investigated by thorough examination of the muscle (fillets) of the fish. This could be done by cutting thin strips of the fillet and using light sources to illuminate the muscle and possibly extracting more parasites. Additionally, it is also possible to detect Anisakids by using the so-called “UV-press method”, where fillets are pressed flat and flash frozen, and then after thawing, the fillets are illuminated by a UV light to detect any larvae, as frozen and thawed *Anisakis* glow under UV light (Karl and Levsen, 2011).

4.5.3 – Genetic identification

Ideally, the genetic identification of the parasites would have been done in tandem with the dissections and collection of quantitative data. This is an important limitation, because if the genetic identification was done earlier, a more thorough examination of the parasite genomes might have been possible. Additionally, more time would have allowed for the possibility of

sequencing the whole genome of some of the parasites, as well as sequencing the genomes of more nematodes. This would have indicated the level of introgression between the three clades and those genes affected. Another possibility here would be that, given more time, nematodes from historic samples could also have been sequenced. This would give a better insight into genetic variation among those nematodes from over 100 years ago and could be compared to that of contemporary nematodes.

4.5.4 – Hagfish

Another important limitation in this study is the fact that we were unable to do further experiments regarding potential transport hosts of *Anisakis*, such as hagfish. This is due to COVID related issues affecting sampling efforts. Here, an additional catch of fish would have been matched for size, species, and sex, half the catch dissected, and the load and prevalence of *Anisakis* noted. The other half of this catch would then be used as bait in hagfish-traps. Collected hagfish could then be dissected to determine whether the local species of hagfish could be potential paratenic hosts for *Anisakis*. If this additional experiment was included, it could potentially uncover an additional transport-route for *Anisakis*. Preliminary dissection of hagfish was done, before any of the other lab work took place. Approximately 30 hagfish were dissected, and *Anisakis* larvae found, in two hagfish. This idea of investigating *Anisakis* infections in hagfish came from an earlier study conducted in Taiwanese waters, as opposed to the sub-Arctic, and the species of hagfish that were dissected in that study (*Eptatretus spp.*) were not the same as the species that was dissected here (*Myxine spp.*). However, in this study, the authors found *Anisakis* in the different species of hagfish, and suggested they may serve as an important paratenic host of anisakids due to their scavenging life style (Luo et al., 2016).

4.5.5 – Data analysis

The data analysis used in this study has proven sufficient to explain the relationships between nematode burden and other indices in the two groups of fish, and the conclusions drawn. However, with a bigger dataset, it would be very beneficial to use other modelling approaches to demonstrate variability in the groups, such as Generalized Additive Models (GAMs). GAMs

could be used to investigate the influence nematode burden has on the species' lengths and liver indices, amongst others. A more detailed dataset could also be of great use in this thesis, incorporating hydrographic conditions from the areas where the fish were sampled. This could allow investigation of how environmental conditions in the water, such as salinity, dissolved oxygen, etc., affects parasite burden in the fish. Additionally, the comparisons of the existing data have been extremely limited, due to the small sample sizes. To ensure as accurate as possible comparison between historic and contemporary fish, the data was trimmed significantly, and thus the linear models and ANOVAs suffer from a dearth of data. Nonetheless, the data analyses that were done show some significant differences between the groups.

Additionally, a morphometric analysis of the fish jaws could have been an informative addition to this thesis. This might have indicated if there was an association with nematode load. Similarly, it would have been useful to examine mixed versus stratified waters to determine whether mixed or stratified waters had a higher abundance of amphipods; and if there would be a higher abundance of *Anisakis* in either of those. Molecular tools, especially COX2 would be used to determine infection in crustacea.

4.5.6 – Drawbacks associated with using parasitic nematodes

The use of parasitic nematodes in a study like this comes with certain drawbacks. The most apparent is the fact that retrieval or recovery of these parasites is done by dissecting fish (or other animals depending on the study). This provides ethical questions raised in the context of "The Three Rs" principle in animal experimentation. The use of historic material such as older scientific literature comes with another important drawback which is inaccuracy of species determination of parasitic nematodes. Recorded historic data and published papers sometimes refer to parasitic nematodes/*Anisakis* as "herring worm", "cod worm", or even "seal worm" which contributes to confusion for the reader, and lack of molecular tools to accurately describe species. In other instances, the identification of anisakid nematodes has relied purely on morphological differences between species. This is an unreliable approach, as this method of identification cannot be used on juvenile larvae due to undeveloped characteristics that would separate species (Anshary *et al.*, 2014). This excludes a descriptive picture of what the actual parasite composition present in the samples could be. In later years,

within-species determination of anisakid nematodes is significantly easier due to improved and more efficient molecular methods, but in some instances, for example in medical science, these tools are often ignored and infections in humans are still assumed to be caused by *Anisakis simplex* and other species tend to be ignored or overlooked (Rahmati *et al.*, 2020).

4.5.7 – Future studies

Despite numerous limitations in this study, the results have given informative and solid evidence that there has been an increase of *Anisakis* in contemporary fish, and the baseline for infection is much higher today than it appeared to be over 100 years ago. Possible future work on investigating historic and contemporary infection numbers of *Anisakis* in fish is promising. If a study like this would be repeated it could be beneficial to increase the sample size of both contemporary and historic samples. Obtaining historic material has proven to be tremendously difficult, museums and institutions that carry large collections of historic material are entitled to the preservation of their samples and collections, and thus it is often difficult to obtain samples for dissection. This is due to the invasive nature of collecting *Anisakis* worms from the fish, which involves dissection of the fish and therefore the specimen might not be useful for further study. It is also important to be able to collect contemporary samples from a wide range of locations to obtain a representative estimate on prevalence and load looks in a variety of different locations differing in physical and anthropogenic factors deemed to affect *Anisakis* uptake.

5.0 – Conclusion

The study produced two significant findings, the first being that hypothesis 1 can be accepted, because the abundance of *Anisakis* is suggested to have increased in teleost fish in recent years. The second finding of this study confirms that hypothesis 2 can be accepted, since the parasites that were genetically identified using ITS and COX2 markers showed evidence of introgression. The increased abundance of *Anisakis* in contemporary fish could be caused by a variety of factors, and the points made in this study merely scratch the surface of what those factors might be. It should be emphasized that many of those factors that may contribute to the increase of parasites are not necessarily well understood and should be elaborated on in future studies.

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Appendix A

Data from the unpublished bachelor's thesis titled "Parasites in the spiral valve of spiny dogfish (*Squalus acanthias*)".

Linear regression model for number of nematodes by fork length in Spiny dogfish (*Squalus acanthias*) aggregation (**Figure A1**). Positive correlation, r^2=0.03.

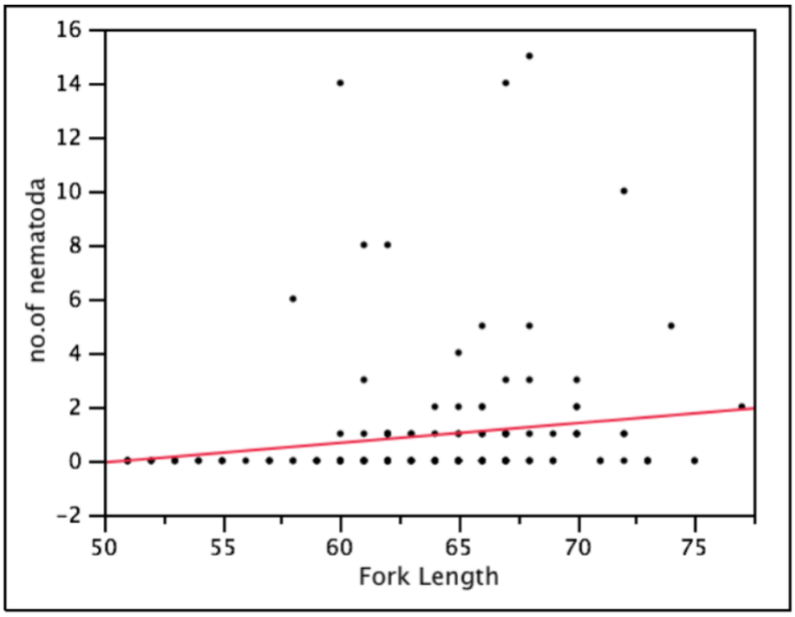

Figure A1. *Linear regression model for number of nematodes by fork length in Spiny dogfish. Positive correlation, r^2=0.03.*

Linear regression model for number of nematodes by HSI (hepatosomatic index) in Spiny dogfish. Red regression line shows individuals with nematodes present in the spiral valve, showing a positive correlation, r^2=0.11 (**Figure A2**).

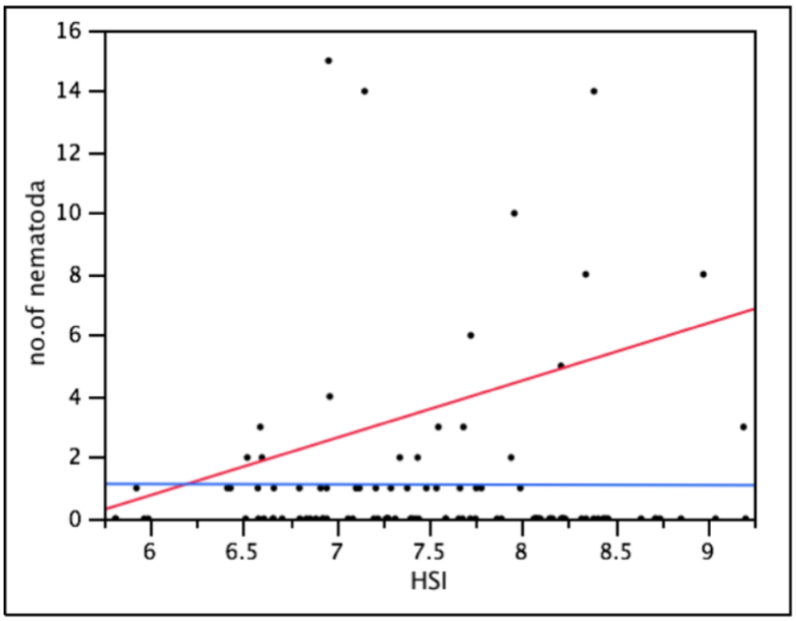

Figure A2. *Linear regression model for number of nematodes by HSI (hepatosomatic index) in Spiny dogfish. Red line is correlation line for infected individuals, positive correlation, r^2=0.11.*

Linear regression model for fork length by HSI. This was done to check whether the high liver index of the sampled individuals was due to the sharks being bigger (i.e., longer, therefore assumed older and bigger), but that did not seem to be the case. Negative correlation was demonstrated in this analysis, r^2=0.09 (**Figure A3**). Thus, it can be assumed that the individuals that had parasitic nematodes present in their spiral valves had some immune response to the infection.

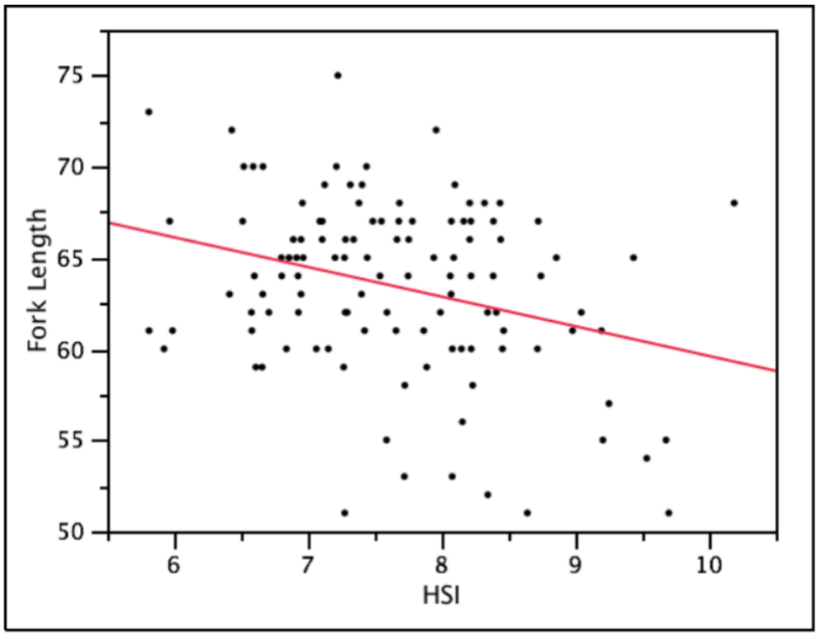

Figure A3. *Linear regression model for fork length by HSI. HSI decreases with length, longer (thus, older) individuals are leaner than shorter individuals. Negative correlation, r^2=0.09.*