EXPLORING THE CAUSES OF RED VENT SYNDROME IN WILD ATLANTIC SALMON (*SALMO SALAR*) FROM COASTAL WATERS AROUND SCOTLAND

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ABSTRACT

In 2005, Atlantic salmon (*Salmo salar L.*) migrating to the United Kingdom exhibited swollen, haemorrhagic vents, symptoms not previously recorded. The condition was latterly termed Red Vent Syndrome (RVS), and subsequently observed across the North Atlantic. RVS has been pathognomonically associated with one of the most abundant parasites within the marine environment, the ascaridoid nematode *Anisakis simplex*, which also causes Anisakiasis in humans. Although *A. simplex* is commonly found in Atlantic salmon, heavy infestation of the vent region is novel, and the expression of RVS has not been prevalent in other fish species. Red Vent Syndrome has been well studied, however, the causes of the condition, and the reasons driving the novel site of infestation exhibited by *A. simplex*, have not been clarified. The aim of this PhD therefore, is to provide new information regarding the underlying factors of the infestation of the vent region by *A. simplex*, and the emergence of RVS. This study therefore: i) assessed the relationship between nematode burdens within the viscera and musculature, in comparison to the vent in 117 adult Atlantic salmon; ii) compared the genetic structure of *A. simplex* present in the vent region and the viscera using the entire nuclear internal transcribed spacer (ITS) region; iii) investigated migratory route and feeding ground of Scottish salmon populations using stable isotope analysis of dorsal muscle tissue and parasite component communities and, iv) assessed the expression of the cytokine TNF-α1 within vent muscle tissue using (q)RT-PCR, in relation to RVS severity. Phylogenetic analyses have shown that it is *A. simplex sensu stricto* infesting the vent region. The results show that there is a significant positive relationship between the nematodue burden in the body (viscera and musculature) and in the vent region. Isotopic signatures of salmon populations showed no significant differences, however, *A. simplex* intensities between populations on the East and North coasts of Scotland suggest geographical differences in

A. simplex transmission pathways. Finally, the expression of TNF-α1 is not significantly different between RVS severity, and nematode burden. Out of the four studied factors, increasing nematode intensities in Atlantic salmon populations, and the significant positive relationship of nematode intensities between the body (viscera and musculature) and the vent, are likely to explain the infestation of the vent by *A. simplex*. The underlying causes of RVS however remain uncertain and require further research. With incidences of RVS observed across a number of populations over a large spatial area, regional and global effectors such as warming sea surface temperatures, and the North Atlantic Oscillation are expected to play key roles in its aetiology.

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List of Abbreviations

β–ME – β- Mercaptoethanol

1SW- One sea-winter

AFA- Alchohol-formalin-acetic acid

AGD – Amoebic Gill Disease

AIC - Akaike information criterion

AMO- Atlantic Multidecadal Oscillation

ARA – Arachidonic acid

B2M - Beta-2 microglobulin

BBC – British Broadcasting Company

BLAST – Basic local alignment search tool

BKD- Bacterial Kidney Disease

BT – Boring tooth

cDNA – Complimentary deoxyribonucleic acid

CIPRES – Cyberinfrastructure for Phylogenetic Research

COX1- Cytochrome oxidase subunit 1

COX2- Cytochrome oxidase subunit 2

CT – Cycle threshold

CTL - Cytotoxic T-lymphocytes

DNA- Deoxyribonucleic acid

EEF1A2 - Eukaryotic elongation factor-1α-2

EiF3EA - Eukaryotic translation initiation factor 3 subunit E

F1 – First generation hybrids

GAPDH - Glyceraldhyde-3P-dehydrogenase

GI – Gastro-intestinal

HSI – Hepatosomatic index

HKY - Hasegawa-Kishino-Yano Model

IgM – Immunoglobulins

IHC - Immunohistochemical

IL- Interleukin

IPNV – Infectious Pancreatic Necrosis Virus

ITS – Internal transcribed spacer

L3 – Third larval stage

MEGA- Molecular evolutionary genetics analysis

MCMC- Monte Carlo Markov chain

MHC - Major Histocompatibility Complex

ML- Maximum Likelihood

MSW – Multi-sea-winter

MS222 - Tricaine mesylate

mtDNA- Mitochondrial DNA

NAO – North Atlantic Oscillation

NASCO – North Atlantic Salmon Conservation Organisation

NC1- Negative control group 1

NJ- Neighbour-Joining

NK – Natural killer cells

nMDS – Non-metric dimensional scaling

PKD – Proliferative Kidney Disease

PPT- Parts per thousand

PC1 – Positive control group 1

PCR-RFLP- PCR-restriction fragment length polymorphism

(q) RT-PCR - Quantitative reverse transcription PCR

rDNA – Nuclear ribosomal deoxyribonucleic acid

RFLP – Restriction fragment length polymorphism

RPM – Revolutions per minute

RNA – Ribonucleic acid

rRNA- Ribosomal ribonucleic acid

RVS – Red Vent Syndrome

SIA- Stable isotope analysis

S.L. – *Sensu lato*

S.S. – *Sensu stricto*

SST – Sea surface temperatures

TG1 – Treatment group 1

Th – Helper T-cells

T-reg – Regulatory T-cells

TNF-α- Tumour necrosis factor-alpha

UDN- Ulcerative Dermal Necrosis

VOT- Visceral organ topography

YWHAZ - Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta

Quantitative Parasitology Terms and Definitions

Abundance - The number of individuals of a particular parasite species per host examined.

Prevalence – The proportion of infested hosts among all the hosts examined.

Intensity – The number of individuals of a particular parasite species per infected organ/host in a sample

Chapter 1

General Introduction

1.1 The Atlantic salmon

The fish family Salmonidae forms a small part of the infraclass Teleostei, a term which is derived through the Greek: *teleios*, "reaching the end" and *osteon*, "bone”. First arising during the Triassic period (Santini *et al*., 2009), Teleostei are represented by nearly 30,000 (approximately 28,897) species and account for nearly half of all known species of vertebrates (Ravi & Venkatesh, 2018). The family Salmonidae contains some of the most commercially valuable and renowned fish species within it’s 11 genera including graylings (*Thymallus* spp.), whitefishes (*Coregonus* spp.), charr, (*Salvelinus* spp.) and within *Salmo*, the Atlantic salmon (*Salmo salar* L.). Atlantic salmon is an anadromous species whose native range spans the entire North Atlantic (Fig. 1.1), and has become emblematic of this region (Larrat *et al*., 2013).

Figure 1.1. Geographic distribution of Atlantic salmon in the North Atlantic (Adapted from ICES, 2007).

As an anadromous species, Atlantic salmon possess a complex lifecycle involving a number of maturation stages within both freshwater, and oceanic habitats, before the life cycle is completed (Fig. 1.2).

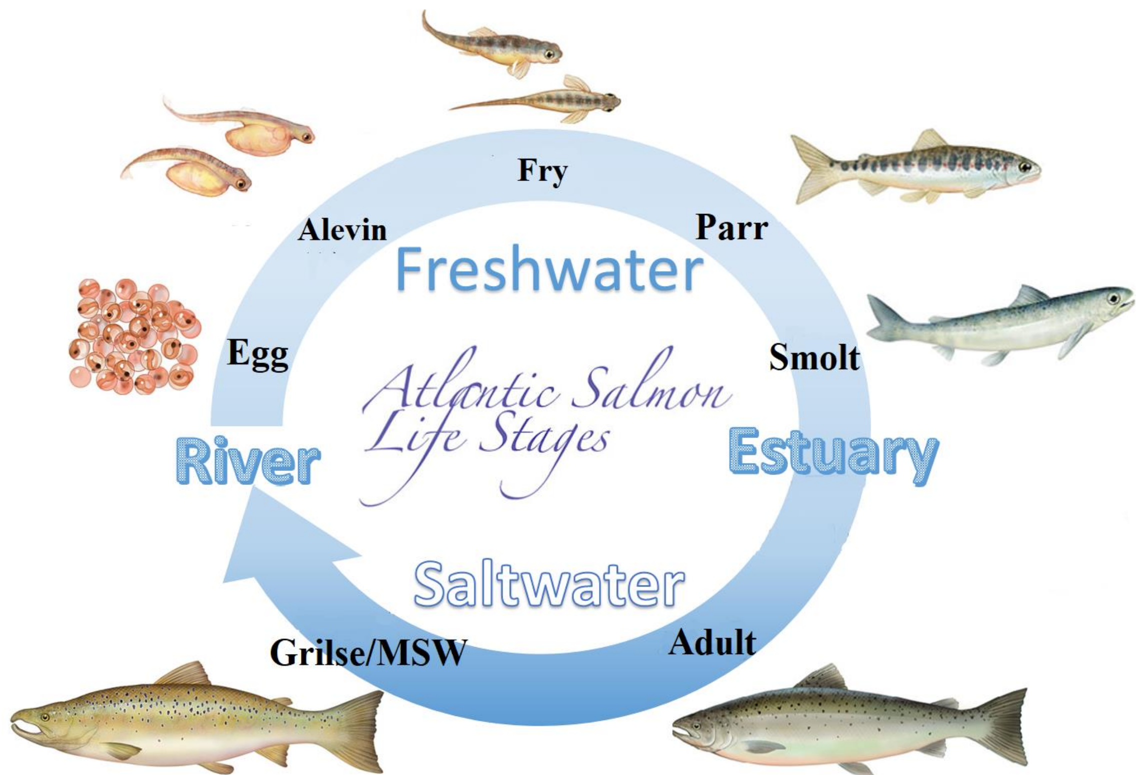

Figure 1.2. Life stages of the Atlantic salmon (Adapted from Miramichi Salmon Association, 2015).

1.1.1 Eggs, Alevins and Fry

Spawning commonly occurs in the autumn/winter, with Atlantic salmon eggs buried within gravel nests known as redds. Alevins emerge from redds the following spring and subsequently occupy high flow velocity regions (Marschall *et al*., 1998). The developmental rate of the embryo to hatching and maturation to lecithotrophic alevin however, is dependent on a number of physical e.g. habitat availability, and environmental factors e.g. temperature (Malcolm & Soulsby, 2002). Once alevins have absorbed their yolk sacs and emerge from the gravel to feed, they are known as fry (Miramichi Salmon Association, 2015).

1.1.2 Parr, Smoltification and Post-smolt migration

The rate at which fry become parr is highly variable and is dependent on feeding and growth opportunities (Marschall *et al*., 1998). Parr utilise covered habitats offering refuge from predators, and from the high energetic costs of remaining within high flow velocity (Stickler *et al*., 2008) in preparation for the post-smolt sea migration. The process of smoltification is triggered by a combination of individual size and photoperiod and is characterised by parr developing the osmoregulatory ability to respire in seawater (Bjornsson & Bradley, 2007). Although the smolt migration generally takes place during spring and early summer, environmental cues such as water discharge and water temperature affect both the rate and nature of the downstream migration (Thorstad *et al*., 2012).

1.1.3 Adults

Migratory routes of salmon populations differ, with the majority of salmon from the United Kingdom using northward flowing currents to aid migration to nursery feeding grounds which include the North Norwegian Sea (Hansen *et al*., 1993), the North Atlantic (Booker *et al*., 2008; Hansen & Jacobsen, 2003) and sometimes habitats off West Greenland (Hansen & Quinn, 1998). As an obligate pelagic species, they remain predominantly in the upper ten metres of the water column where they access the majority of their prey species such as zooplankton, crustaceans and smaller fish (Mills, 2003). Adults remain here for 1-5 years (Jutila *et al*, 2003) until they return to their natal spawning grounds as grilse (1 winter at sea (1SW)) or Multi-Sea-Winter (MSW) salmon. The ability to return to natal rivers stems from the process of olfactory imprinting during the smoltification, allowing this targeted migration to occur (Dukes *et al*., 2004). During the return migration, Atlantic salmon cease active feeding (Kjellman, 2015), and on re-entry to freshwater, begin a period of fasting (Doucett *et al*., 1999a). During the final stages of the spawning migration energy from somatic and visceral tissues is mobilised, with lipid

reserves decreasing from over 11% to 2% during this period (Shearer, 1992). In addition to the energetic resources required for this migration, individuals also undergo large-scale morphological transformations (Kacem *et al*., 2000), and changes in osmoregulation and calcium balance on transition from sea to freshwater (Persson *et al*., 1998). The costs of this migration are often significant, with a number of post-spawned salmon (kelts) suffering mortality post-spawning (Scottish Natural Heritage, 2015).

This complex life cycle involving temporal and spatial separation of the anadromous life stages of Atlantic salmon, exposes individuals to a wide variety of pathogens and parasites, which occur within freshwater and marine environments.

1.2 Protozoan and Metazoan Parasitic Organisms

Living in two disparate ecosystems, Atlantic salmon interact with distinct groups of pathogens, which reside in either freshwater or marine environments (Margolis, 1982a, b; Soleng & Bakke, 1997). According to Bakke & Harris (1998), the diverse pathogenic fauna known to infect wild and farmed populations of Atlantic salmon comprises at least 225 species from a diverse array of taxa including Crustacea, Mollusca, Protoctista and several helminths (Table 1.1). These parasites and pathogens of Atlantic salmon can be categorised as viral, bacterial, protozoan and metazoan parasitic groups, based on morphology, phylogeny and life history traits. Although there is a wide spectrum of host-parasite interactions, some of which being mutualistic (Berland, 2006), the central concept to the definition of parasitism, is that an infestation has deleterious effects on the host (Barber *et al*., 2000). Parasitism is a fundamental feature of life within ecosystems (Viney & Cable, 2011) and its success is demonstrated by some form of parasitism in at least half of all known existing organisms (Palm & Klimpel, 2007).

Viruses and bacteria are generally defined as small, rapidly *in situ* reproducing pathogens (Anderson & May, 1979). Although the innate immune response of teleosts plays a key role in the primary defence to infection, and driving adaptive immunity (Anderson & May, 1979; Whyte, 2007), bacterial, viral and fungal disease outbreaks have been historically reported in Atlantic salmon (Mackie *et al*., 1935; Poppe *et al*., 1989; Roberts, 1993; Rogers *et al*., 1998; Murray *et al*., 2012). Atlantic salmon exposure to pathogenic bacteria such as *Aeromonas salmonicida* (Lehmann & Neumann, 1896) and *Renibacterium salmoninarum* has led to outbreaks of furunculosis (Mackie *et al*., 1935) and bacterial kidney disease (BKD) (Murray *et al*., 2012) respectively. Furthermore, outbreaks of infectious salmon anaemia (ISA) (Rodgers *et al*., 1998) and infectious

pancreatic necrosis (IPN) (Poppe *et al*., 1989) caused by the infectious salmon anaemia virus (ISAV) and the togavirus respectively, can result in servere mortality rates. Although viruses exist in wild salmon populations, high mortality rates of infected salmon make viral infections hard to identify (Bakke & Harris, 1998). However, viral outbreaks have been frequently reported in farmed Atlantic salmon (Bakke & Harris, 1998; Rodgers *et al*., 1998). Exposure to fungi (*Saprolegnia* sp.) has also caused outbreaks of ulcerative dermal necrosis (UDN) throughout the 20th century (Roberts, 1993). These common, outbreak causing bacteria, viral and fungi oscillate between non-existent and dangerously large populations (Bakke & Harris, 1998).

Table 1.1. The total number of infectious species reported from wild and domesticated (ranched/hatchery) Atlantic salmon in marine and freshwater habitats (Adapted from Bakke & Harris, 1998; Wallace *et al*., 2017).

Group	Number of Species
Virus	9
Monera	21
Protoctista	27
Animalia	
Hirudinea	3
Helminths	
Monogenea	11
Digenea	41
Cestoda	35
Nematoda	29
Acanthocephala	20
Crustacea	13
Mollusca	3
Acarina	2
Fungi	11
Total Number	225

The cnidarian Myxozoa (Sterud *et al*., 2007), in addition to a wide range of protists including ciliophorans and flagellates also infect salmonids (Bakke & Harris, 1998). The highly pathogenic ciliate *Ichthyophthirius multifiliis* causes greater economic losses worldwide than any other freshwater fish parasite (Cross, 1994), and has been reported in freshwater salmon hatcheries (Wootten & Smith, 1980). Ciliophorans (e.g. C*apriniana, Chilodonella, Epistylis, Scyphidia, Trichodina*, and *Trichophry*) are ubiquitous in freshwater environments (Basson & Van As, 2006). Although the majority of these parasites are frequently found in reared freshwater salmon, only trichodinids have been associated with the death of kelts (Khan, 1991). In cases of intense infection, trichodinids induce excessive mucus secretion, epithelial sloughing and lesions leading to bacterial infection, and subsequently death (Khan, 1991).

Metazoan parasites (e.g. fish lice, tapeworms, and nematodes) generally have low reproductive rates, are larger in size and with long generation times (Bakke & Harris, 1998). Metazoan parasites can cause visible harm to heavily infected individuals, becoming pathogenic and predispose individuals to secondary bacterial and fungal infections resulting in mortality (Johnson & Paull, 2011). More commonly however, they are detrimental to a host's overall fitness (Barber *et al*., 2000), and can affect migratory behaviour, growth rate (Bakke & Harris, 1998), and gonad development (Ferrer-Maza *et al*., 2014). Metazoan parasites within a host/population however, do not normally oscillate to the same extremes as bacterial or viral infections in nature and therefore, do not have such severe consequences on wild host populations (Amundsen *et al*., 1997).

Although both protozoan and metazoan parasites can be generally referred to as parasites, not all are pathogenic i.e. cause disease (Holmes, 1996). Aquatic pathology identifies diseases that have the potential to influence population dynamics and growth in natural environments (Bakke & Harris, 1998). Parasitic infestation and diseases however, are typically multifactorial, complex and interactive in nature (McVicar, 1997), and

notoriously hard to detect within freshwater and marine environments in wild populations of fish (McVicar, 1997). Often the large spatial and temporal scales encountered (Bakke & Harris, 1998), coupled with potential host mortality due to mass infestation of metazoan parasites or microbial disease (Miller *et al*., 2014), make conclusive cause-effect relationships for both metazoan and protozoan parasites and their hosts difficult to determine (McVicar, 1997).

A paradigm of the complex aetiology of aquatic diseases in wild populations is demonstrated by the case of Ulcerative Dermal Necrosis (UDN). Ulcerative Dermal Necrosis was originally classified as a salmon disease and noted for its impact in the late 19th century (Roberts, 1993). Ulcerative Dermal Necrosis then disappeared in the 1960's and although both the temporal and spatial spread is strongly reminiscent of an infectious agent (Anderson, 1982), the causative agent remains unknown.

Despite difficulties in quantifying the presence and effects of pathogenic diseases, it is the interactions between the environment, the host, and the infectious agent(s) that result in disease (McVicar, 1997). Changes in these interactions, give the potential for some previously benign or innocuous infectious agents to become pathogenic (McVicar, 1997).

1.2.1 Host-Parasite-Environmental Interactions

Host-parasite relationships are in a dynamic equilibrium and are in constant co-evolution (Barrett, 1986). The life characteristics involved in parasitism, such as reproductive isolation through host switching, increases the likelihood of evolution in comparison to other life histories. Events of parasite speciation and diversification therefore, are thought to have occurred on multiple occasions (Barber & Poulin, 2002; Blaxter *et al*., 2004). These genetic based changes are the most common cause of changes in resistance and pathogenicity within a parasite-host relationship (McVicar, 1997).

Genetic heterogeneity in salmonid and salmon populations influences disease and parasitic resistance (Mackie, 1935; Bakke & Harris, 1998), and leads to differential aggregation of pathogens according to the relative susceptibility of the host (Chevassus & Dorson, 1990; Wakelin, 1994). A clear example of heterogeneity affecting resistance is the susceptibility of Norwegian salmon to the monogenean *Gyrodactylus salaris* (Malmberg, 1957), while the Baltic salmon population is less susceptible (Bakke *et al.*, 2004).

In some cases however, non-genetic multiple stressors including both physiological and environmental influences can also affect this relationship (Dybdahl & Krist, 2004; Todd *et al.*, 2008). Ecosystem dynamics such as species interactions, marine fish prey resources, community composition, food availability and food web structure are driven for example, through temperature changes (Pörtner, 2002; Pörtner & Peck, 2010). Changes of which, can directly influence the occurrence and transmission of parasites, particularly in temperate zones (Karvonen *et al.*, 2010).

1.3 The Emergence of Red Vent Syndrome

For over 100 years, parasitisation by larvae of the nematode *Anisakis simplex sensu stricto* (Fig 1.3) (Family: Anisakidae) has been reported for commercial fish species such as Atlantic cod (*Gadus morhua L.* 1758) (Mouritsen *et al*., 2010; Nadolna & Podolska, 2014), Atlantic herring (*Clupea harengus L.* 1761) (Levsen & Lunestad, 2010), hake (*Merluccius L.* 1758) (Ferrer-Maza *et al*., 2014) and Atlantic salmon (Carmichael, 1863).

Figure 1.3. ***Anisakis simplex*** **third-satge larvae (30 mm total length) recovered from the body cavity of an Atlantic herring (Buchmann & Mehrdana, 2016).**

Third-stage larvae (L3) are most commonly found in an encapsulated stage within the body cavity tissues e.g. the external surfaces of visceral organs including the gut, pyloric

caeca and liver and surrounding mesenteries (Noguera *et al*., 2009; Noguera *et al*., 2015) but can also be found in the muscle and fat tissue of fishes (Noguera *et al*., 2009; Noguera *et al*., 2015). The encapsulation of larvae is a result of the cellular immune response of the host (Buchmann & Mehrdana, 2016). Larvae are encapsulated in a thick layer of host cells which places larvae in an inactive stage which can last for at least three years (Buchmann & Mehrdana, 2016). In 2005 however, returning Atlantic salmon were observed with bleeding, swollen, and haemorrhagic urogenital papilla regions, also known as the vent (Beck *et al*., 2008). These symptoms were subsequently pathognomonically associated with large numbers of un-encapsulated *A. simplex* larvae observed in gross legions around infested vent tissue (Levsen & Berland, 2012), also referred to as 'hyper-infestation' (Noguera *et al*., 2009). Prior to 2005, these symptoms, along with the presence of *A. simplex* in the 'novel' infestation site of the vent region as defined by Crompton (1973), had not been reported in any species of fish. These symptoms were later referred to as Red Vent Syndrome (RVS) (Beck *et al*., 2008; Noguera *et al*., 2009), and were recorded in Iceland (Helgason *et al*., 2008), Norway (Mo *et al*., 2010) and Canada (Larrat *et al*., 2013) amongst others. Prevalence of RVS in the United Kingdom is temporally and spatially variable (Pert *et al*., 2009). Although prevalence of RVS was typically lower between 2008-2012 (ICES, 2009a; 2010; 2011), RVS reached its highest prevalence in the time series for some European stocks in 2013 (ICES, 2014). Currently, only the rivers Tyne, Dee, and Lune are monitored for RVS in the UK and prevalences of 4%, 22% and 19% respectively were recorded in 2016 (ICES, 2017). Anisakid infestations of commercially important fish species, such as cod, herring, haddock (*Melanogrammus aeglefinus* L. 1758), mackerel (*Scomber scombrus* L. 1758), monkfish (*Lophius piscatorius* L. 1758) and whiting (*Merlangius merlangus* L. 1758) have been well studied (Noguera *et al*., 2009; Murphy *et al*., 2010). However, there is a distinct paucity of research involving wild Atlantic salmon. Since the emergence of RVS

in 2005, a number of studies such as Beck *et al*. (2008), Noguera *et al*. (2009 & 2015), Mo *et al*. (2010) Senos *et al*. (2013), and Larrat *et al*. (2013) have conducted research into anisakids and Atlantic salmon. These studies have prompted a number of main hypotheses for the underlying cause/s of RVS, and the infestation of the vent region by *A. simplex* (s.s.). Firstly, a different species of *Anisakis* may be the cause of the novel infestation site of the vent (Noguera *et al*., 2009). Alternatively, the 'hyper-infestation' of the vent by *A. simplex* (s.s.) (Noguera *et al*., 2009) may be a result of increasing nematode intensities within Atlantic salmon (Senos *et al*., 2013), subsequently referred to in this study as the 'hyper-infestation hypothesis'. High *Anisakis* sp. prevalence and intensities are commonly reported in fish species including cod (Wootten & Waddell, 1977), and herring populations in the Baltic Sea (Horbowy & Podolska, 2001) and North Sea (Levsen & Lunestad, 2010). Increasing larval nematode infestation of cod and whiting since the 1950's was observed by Wootten & Waddell (1977). Furthermore, between 1992 and 1997, 30-40% increases of larval *A. simplex* intensities in Baltic Sea herring was observed by Horbowy & Podolska (2001). To date however, there has been no observation of *Anisakis* sp. within the vent region of these species. Research into *A. simplex* distribution is commonly focussed on edible muscle tissue which potentially results in the vent region to be overlooked during analyses. The observation of RVS symptoms has been almost solely exclusive to populations of Atlantic salmon, with only one case reported in brown trout (*Salmo trutta* L.) in 2007 (Noguera *et al*., 2009). Therefore, there is also a suggestion that inter- and intra-specific differences in the immune response, may also play a key role during RVS.

Histological analyses of the vent tissue have revealed that cellular components of the innate immune system such as degranulating eosinophils and occasionally melano-macrophages play a major role during the expression of RVS symptoms (Dezfuli *et al*., 2007; Beck *et al*., 2008; Noguera *et al*., 2009). Since these studies significant progress in

our understanding of the immune response of teleosts has been achieved. The transcription of a number of pro-inflammatory cytokines including type I and II Tumour Necrosis Factor-α genes has been reported in Atlantic salmon, (Hong *et al*., 2013). TNF-α1 is a pro-inflammatory cytokine mediating the proliferation of epithelial cells in mammals (Ip *et al*., 1992). It is also involved in rapid recruitment of phagocytic granulocytes to regions of tissue damage similar to those seen in the vent region during RVS expression (Garcia-Castillo *et al*., 2004 Roca *et al*., 2008). TNF-α1 could therefore play a major role within RVS affected tissue.

There is currently no definitive answer to the cause/causes of RVS. The presence of *A. simplex* within the vent region seems to increase the likelihood of RVS, but it is not the sole determining factor of its expression (Larrat *et al*., 2013). Although it is thought that RVS and chronic anisakid infestations do not cause mortality (Kent & Fournie, 1993; Noguera *et al*., 2009), there is evidence to suggest that they can detrimentally affect gonadal energy reserves in hake (Ferrer-Maza *et al*., 2014), and can induce mortality in fish larvae (Adroher *et al*., 2004). Wild populations of Atlantic salmon have exhibited multi-decadal declines in recruitment, resulting in the lowest stock abundances since the 1970's (Fig. 1.4) (Friedland *et al*., 2009). There is concern that the emergence of RVS will have further detrimental impacts on the wild population. Furthermore, 85% of wild populations of Atlantic salmon have been categorised as being vulnerable, endangered, or critically endangered (WWF, 2001). Since the industrial revolution, there have been widespread declines and extirpations within their natural range (Parrish *et al*., 1998; Cowx & Van Zyll De Jong, 2004) however, within the last 50 years; these declines have become unprecedented with stocks falling to historical lows (ICES, 2005; Todd *et al*., 2008). Within Scottish waters commercial landings have declined from approximately 500,000 fish in the 1970's, to below 20,000 in 2015 (Wallace, 2010; Marine Scotland Official Report, 2016), which has led to the moratorium on the retention of salmon caught

in coastal waters of Scotland since 2014 as part of the Wild Fisheries Review (2014). Although causal factors for their decline include habitat degradation (McCormick *et al*., 1998; Parrish *et al*., 1998), climate change (Todd *et al*., 2008), and overfishing within marine environments (Pauly *et al*., 2002), viral infection and parasitic infestation are having an increasing influence on wild populations of Atlantic salmon (Forseth *et al*., 2017).

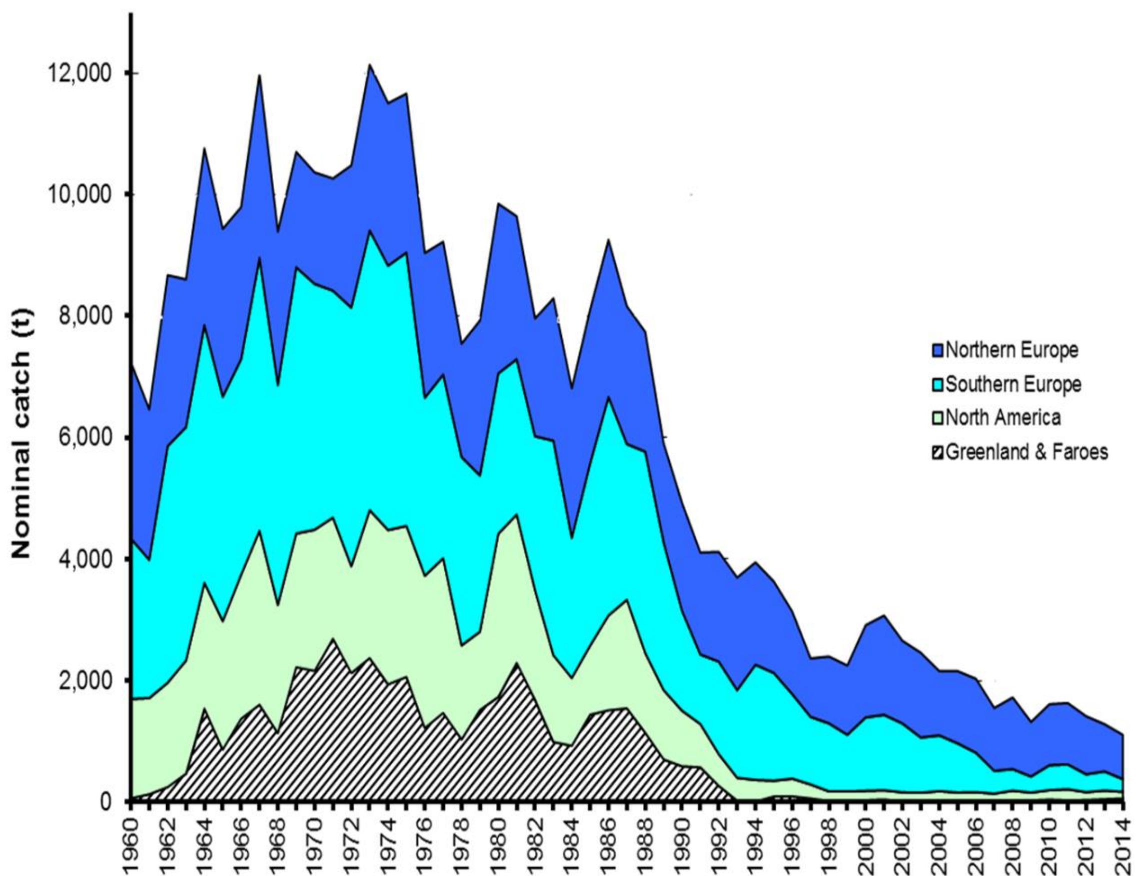

Figure 1.4. Nominal catches of wild Atlantic salmon (tonnes fresh weight) in four North Atlantic regions, 1960–2006 (Adapted from ICES, 2015).

1.4 *Anisakis simplex*- a cosmopolitan parasitic nematode

The nomenclature of anisakid nematode species belonging to the genus *Anisakis* (Dujadin, 1845) has been surrounded by confusion and controversy (Mattiucci *et al.*, 2014) due to no morphological differences between species. As a fundamental unit of biology comparable in importance to genes and cells, the ability to recognise and classify a species is imperative (De Quieroz, 2007) and has similarly been surrounded in controversy for a substantial length of time (De Quieroz, 2007). Controversy of species delimitation is a result of a number of subgroups of biologists advocating a number of biologist subdivisions 'species concepts' which are at the least, partially incompatible (De Quieroz, 2007). Mayden (1997) listed 24 different species concepts however, the most commonly used include the biological (Mayr, 1942), morphological (Cronquist, 1978), ecological (Van Valen, 1976), evolutionary (Wiley, 1981), cohesion (Templeton, 1994), phenetic (Ridley, 1993), phylogenetic (Arnold & Stace, 1989) and pluralistic species concepts (Campbell & Reece, 2002) (Table 1.2).

However, the recent use of genetic and molecular methodologies, and the adoption of a biological species concept (BSC), has resulted in a widely accepted taxonomy for anisakid nematode species (Mattiucci *et al.*, 1997; Mattiucci & Nascetti, 2008). One of the main outcomes has been the discovery of three sibling species within one of the species, *Anisakis simplex* (Rudolphi, 1809) (Nematoda, Anisakidae) (Mattiucci *et al.*, 2014). Although originally believed to be a cosmopolitan species that is parasitic in a wide variety of definitive hosts (Davey, 1971), *Anisakis pegreffii*, *A. simplex* (s.s.), and *Anisakis simplex* 'C' (Mattiucci *et al.*, 1997) have now been identified and together form the "*A. simplex* species complex" (Mattiucci *et al.*, 2014). As part of the "*A. simplex*

species complex", these three species are found in marine waters around the globe (Fig. 1.5).

Table 1.2 Alternative contemporary species concepts (i.e., major classes of contemporary species definitions) and their properties (Adapted from De Quieroz (2007).

Species Concept	Properties
Biological	Interbreeding (natural reproduction resulting in viable and fertile offspring)
Ecological	Same niche or adaptive zone (all components of the environment with which conspecific organisms interact)
Evolutionary	Unique evolutionary role, tendencies, and historical fate
Cohesion	Phenotypic cohesion (genetic or demographic exchangeability)
Morphological	Species are the smallest groups that are consistently and persistently distinct, and distinguishable by ordinary means
Phylogenetic	Heterogeneous
Phenetic	Form a phenetic cluster (quantitative difference)
Genotypic cluster	Form a genotypic cluster (deficits of genetic intermediates; e.g., heterozygotes)

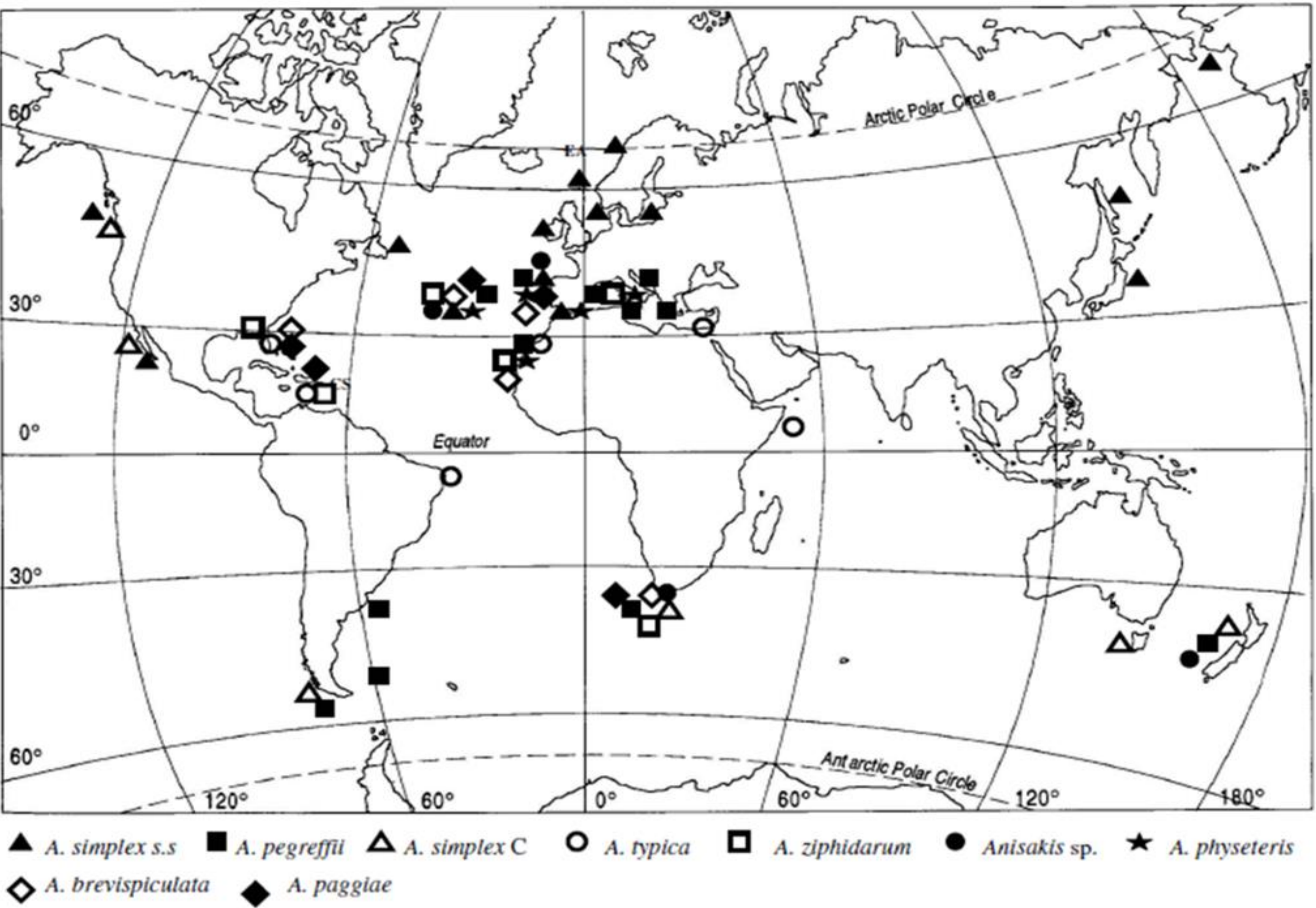

Figure 1.5. **Geographic distribution of *Anisakis* sp. (Adapted ftom Mattiucci & Nascetti., 2008).**

Anisakis simplex needs more than one obligatory host to complete its life cycle, also referred to as a heteroxenic life cycle (Klimpel *et al*., 2004; Berland, 2006). This strategy involves a number of intermediate and paratenic (not necessary for the development of the parasite) hosts (Klimpel *et al*., 2004; Berland, 2006; Audicana & Kennedy, 2008) (Fig. 1.6). Adult parasites live in the stomach of cetaceans and, following copulation, fertilised but un-embryonated eggs are expelled with the faeces (Køie *et al*., 1995; Køie, 2001).

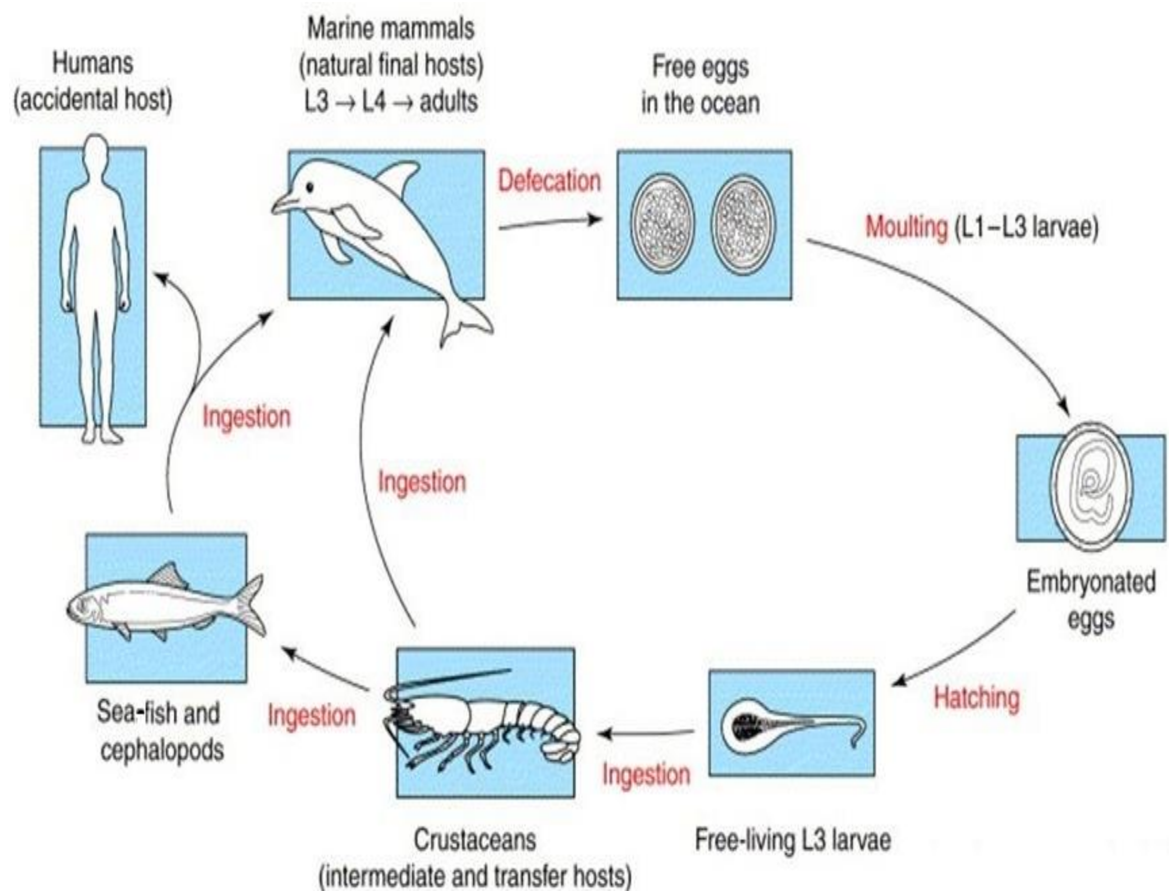

Figure 1.6. Life cycle of *Anisakis* sp. (Adapted from Audicana *et al*., 2002)

These eggs develop and then hatch, releasing a free-living *Anisakis simplex* third stage larvae (L3), which when ingested, infects intermediate hosts such as oceanic krill and copepods through penetration of the gut wall (Køie *et al*., 1995). Fish and cephalopods ingest planktonic crustaceans or other fish and cephalopods infested with L3 larvae,

which are subsequently ingested by marine mammals to close the life cycle (Audicana & Kennedy, 2008).

Both cetaceans as definitive hosts, and teleosts as intermediate hosts play central roles in the life cycle of *Anisakis* sp. Regional changes in *Anisakis* sp. abundance has previously been linked to the presence of definitive hosts (Platt, 1975; Strømnes & Andersen, 2000). Increases in Atlantic cod infestation by *Anisakis* sp. have been related to periodical concentrations of the pilot whale (*Globicephala melaena* Traill, 1809), in the fjords of the Faroe Islands (Harrison & King, 1965). Northward migrations of cetaceans have also been linked to 'Spring Rise' peaks of infestation seen in saithe (*Pollachius virens* L.), cod and redfish (*Sebastes marinus* L.) in central Norway (Strømnes & Andersen, 2000). Furthermore, in marine mammal communities of Scotland, there have been marked changes since 1980 (MacLeod *et al*., 2005). There have been observed declines of cold water species such as the killer whale (*Orcinus orca* L.) and long-finned pilot whale (*Globicephala melas* Traill, 1809), and the addition and increasing occurrence of new warm water species including Fraser's dolphin (*Lagenodelphis hosei* Fraser, 1956) and pygmy sperm whale (*Kogia breviceps* Blainville, 1838). These changes in marine mammal communities have the potential to result in changes in *Anisakis* sp. abundance in Scottish waters.

The ecological importance of some teleosts are characterised through their ability to cover large geographical distances as migratory species, possess large geographical boundaries, and as consumers of crustaceans and smaller fish (Barber *et al*., 2000). Species such as Atlantic cod (Nadolna & Podolska, 2014; Mouritsen *et al*., 2010), herring (Levsen & Lunestad, 2010), hake (Ferrer-Maza *et al*., 2014) and Atlantic salmon (Noguera *et al*., 2009) are commonly documented as hosting large numbers of parasitic nematodes in the wild. The heteroxenic strategy of *A. simplex* relies on larval transmittance through predation by trophically higher intermediate and paratenic predators (Berland, 2006).

Infestation by *A. simplex* has led to a classification as 'one of the most important problems for the fishing industry' (Abollo *et al*., 2001a; Noguera *et al*., 2009; Lunneryd *et al*., 2015). In addition to reducing the quality of the flesh in commercial fish species (Noguera *et al*., 2009), they also cause Anisakiasis in humans when raw or undercooked fish is consumed (Nagasawa, 1990; Chai *et al*., 2005; Umehara *et al*., 2007).

Human infestation by *Anisakis simplex* was first described in the 1960's (Van Thiel, 1962; 1976). While the symptoms of Anisakiasis such as severe abdominal pain, malnutrition, and vomiting are unpleasant, it was recently discovered that severe hypersensitivity reactions can be caused through the ingestion of dead worms in fish and may be more prevalent and dangerous than the infestation itself (Audicana *et al*., 2002; Audicana & Kennedy, 2008). In addition to allergic symptoms caused by the *Anisakis* infestation "gastroallergic anisakiasis", true anaphylactic reactions can occur following exposure to the allergens present within dead worms through food-borne, airborne, or skin contact routes (Audicana *et al*., 2002; Heffler *et al*., 2016). Improved molecular diagnosis tools and increased awareness of this parasitic disease have led to an increase in the recorded number of cases during the past 20 years in many parts of the world (Deardorff *et al*., 1991; Couture *et al*., 2003; Bourée *et al*., 1995).

Thesis Aims and Objectives

There remains a number of knowledge gaps underlying the cause(s) leading to the infestation of the vent region by *A. simplex* (s.s.), and the exhibition of RVS symptoms. There has been no study assessing speciation of *A. simplex* (s.s.) in Atlantic salmon in Scotland since 2009, and to the best of our knowledge, there has been no study investigating its presence in Atlantic salmon caught off the West coast. Furthermore, research will build on previous studies investigating the 'hyper-infestation' hypothesis by expanding on the methodologies e.g a more representative sample. To date, there has been no investigation of the presence of inter-population differences in migratory route and

feeding grounds used by Atlantic salmon in Scotland using stable isotope analysis and parasitic component communities, which could directly influence parasitic burden, and exhibition of RVS. Finally, there has been no assessments of levels of expression of the pro-inflammatory cytokine TNF-α1 in different RVS severities. The aim of this study therefore, is to elucidate *A. simplex* infestations of wild Atlantic salmon populations in Scotland, with a specific focus on the aforementioned knowledge gaps.

To achieve this aim, as well as fill the knowledge gaps, this study has the following objectives:

1. Identify possible genetic differences between *A. simplex* specimens found in the vent, and the viscera of Atlantic salmon.
2. Assess the intensity of *A. simpl*ex infestation in the whole fish, the muscle tissue and the viscera, in comparison to the vent region thereby testing the 'hyper-infestation' hypothesis.
3. Investigate any location preference of *A. simplex* within Atlantic salmon through an experimental infestation challenge.
4. Assess the relationship between migratory route and diet between Atlantic salmon populations of different natal rivers.
5. Test the levels of genetic expression of cytokine TNF-α1 in different Red Vent Syndrome severities.

Thesis Layout

This thesis entitled "Exploring the causes of Red Vent Syndrome in wild Atlantic salmon (*Salmo salar*) from coastal waters around Scotland" consists of seven chapters, four of which will present data addressing the objectives outlined above:

Chapter 2 – Molecular phylogeny of *Anisakis simplex* larvae infesting the vent region of Atlantic salmon.

This chapter elucidates any genetic differences between *Anisakis* specimens found in the vent region, and throughout the viscera of RVS salmon. The conserved ribosomal ITS region was used for phylogenetic analyses.

Chapter 3 – The 'hyper-infestation' hypothesis: A case study using Atlantic salmon from Scotland.

This chapter enhances the knowledge of anisakid infestation intensities within populations of Atlantic salmon around Scotland. Anisakid infestations within portions of muscle, organs within the viscera, and the isolated vent region was enumerated to investigate the relationship between infestation intensity within the body, and within the vent. This relationship is referred to as the 'hyper-infestation' hypothesis. This chapter also includes the description of a controlled experiment investigating location preferences of *Anisakis simplex* when infesting Atlantic salmon with *Anisakis*.

Chapter 4 – Identification of potentially different migratory routes and feeding grounds of Atlantic salmon populations of Scotland using stable isotopes and parasite component communities.

This chapter will report on the results of the first stable isotope analyses of muscle and scale tissue of Atlantic salmon populations sampled from the North, East and West coasts of Scotland. Furthermore, parasite communities found in/on Atlantic salmon will be analysed. Assessments of both $\delta^{13}C$, $\delta^{15}N$ and parasite component communities will aid

identification of any differences in migratory route and feeding ground between these populations.

Chapter 5 – Assessing the levels of expression of the cytokine TNF-α1 in response to increasing RVS severity, and nematode intensity.

This chapter describes for the first time the levels of expression of the pro-inflammatory cytokine TNF-α1 in the surrounding muscle tissue of the vent regions exhibiting different severities of Red Vent Syndrome symptoms, and nematode intensities. *Anisakis. simplex* are also present within the vent region of Atlantic salmon not exhibiting RVS, thus the assessment of the expression of TNF-α1 reveals whether increasing nematode burdens stimulate an increase in immune response intensity.

Chapter 6 – General Discussion.

This chapter synthesises the main findings of the study in a broader context and relevance, critically reviews the methods used, and identifies areas for future research.

Chapter 7 – General Conclusions.

Chapter 2

Molecular phylogeny of *Anisakis simplex* larvae infesting the vent region of Atlantic salmon

2.1 Introduction

Species of vertebrate parasites occupy nearly every available niche or habitat within a specific host (Secombes & Chappell, 1996). Although some of these parasitic species are commonly found in restricted or specific sites (or microhabitats) (Holmes, 1973), other species exhibit low specificity and can be observed in multiple locations within a host (Holmes, 1973). In some parasitic species, observed specificity to a restricted microhabitat within a host is so invariant that it is frequently used as a critical character in taxonomic classification e.g. heartworms or liver flukes (Sukhdeo & Bansemir, 1996). Other parasitic species such as nematodes in the *A. simplex* species complex however, exhibit low site specificity and possess the ability to migrate to new microhabitats, or undergo complex migrations, resulting in its presence at multiple sites (Holmes, 1973).

Within susceptible hosts, *A. simplex* is commonly found in the alimentary canal and musculature (Secombes & Chappell, 1996; Strømnes & Andersen, 1998). Its prevalence in the alimentary canal is believed to be driven by i) the ease of access to the host, ii) their transmission method, iii) a ready provision of attachment sites and nutrients, as well as iv) a relatively non-aggressive immune response (Secombes & Chappell, 1996). Due to their ability to migrate through tissues (Haarder *et al*., 2013), *A. simplex* can be found encapsulated i) in the body cavity tissues including the external surfaces of the gut, pyloric caeca, liver and fat tissue and surrounding mesentries, and ii) the musculature (Strømnes & Andersen, 1998; Noguera *et al*., 2009).

In 2005, the emergence of Red Vent Syndrome (RVS) in wild Atlantic salmon was observed along with large numbers of non-encapsulated L3 *A. simplex sensu stricto* larvae in the vent region (Beck *et al*., 2008). Although the presence of *A. simplex* in Atlantic salmon has been reported for over 100 years (Carmichael, 1863), it has never previously

been reported within this microhabitat. Moreover, the vent region is considered a 'novel' infestation site in this parasite-host relationship (Noguera *et al*., 2009). The observation of *A. simplex* larvae in this novel microhabitat, and their association with the cause of RVS therefore (Larrat *et al.*, 2013), has called into question whether *A. simplex* found in the vent are genetically different to those found in more common microhabitats (Noguera *et al*., 2009).

2.1.1 Nematoda & Their Potential for Evolution

Evolution is a fundamental feature of parasitism (Kochin *et al*., 2010). Parasitism and other less extreme versions of exploitative symbioses have evolved at least 223 times in 15 phyla (Blaxter *et al*., 2004; Weinstein & Kruis, 2016). Nematoda have acquired parasitism in up to 18 separate occasions (Blaxter & Koutsovoulos, 2015; Viney, 2017). Therefore, this phylum exhibits quicker rates of evolution than any other (Blaxter *et al*., 2004). It is the features of the nematodes themselves, and that of their life-cycles, that is believed to be especially conducive to the ability to evolve parasitism (Viney & Cable, 2011). Firstly, nematodes are moulting animals with typically four larval stages preceding the adult stage (Viney & Cable, 2011). Moulting usually occurs during transmission between host species, and with each moult, the complex extracellular collagenous cuticle is re-modelled and is accompanied by physiological and other changes (Viney & Cable, 2011). Finally, the free-living nematode species have a facultative arrested larval form known as the dauer larva (Crook, 2014). This life-history stage offers the most probable driver of the original evolutionary transition from being free-living to being associated with a host prior to parasitising it (Viney & Cable, 2011; Crook, 2014). Dauer larvae possess a specialised morphology and metabolism, allowing them to persist in an environment until conditions improve, when they resume development (Crook, 2014), and bear striking similarities to the infective larval stage of parasitic nematodes (Viney & Cable, 2011). Furthermore, sexual reproduction is a key feature of nematode parasitism

(Tinsley, 2004), and adaptation is driven by both geographical (Nuismer, 2006) and host (Blouin *et al*., 1995) isolation. Thus, the life history characteristics of parasitic helminths can lead to high rates of inter- and intra-specific gene flow (Blouin *et al*., 1995). This ability to evolve at a rapid rate, is crucial in the co-evolutionary arms race for ecological advantage within the dynamic equilibrium of every host-parasite relationship (Blaxter *et al*., 2004).

The species within the "*A. simplex* species complex" (Mattiucci *et al*., 2014) are one example of parasitic nematodes exhibiting high rates of gene exchange (Abollo *et al*., 2003). Originally it was believed that *Anisakis simplex* is a cosmopolitan species parasitic in a wide range of definitive hosts (Davey, 1971). Through the introduction of molecular approaches, such as multi-locus allozyme electrophoresis (Nascetti *et al*., 1986) and DNA based methods such as PCR restriction fragment length polymorphism (PCR-RFLP) (D'Amelio *et al*., 2000) and direct sequencing of nuclear ribosomal and mitochondrial DNA (Abollo *et al*., 2003; Noguera *et al*., 2009), our understanding of their phylogeny and nomenclature has greatly improved (Mattiucci *et al*., 2014). With three species now identified within *Anisakis simplex sensu lato* (*Anisakis pegreffii*, *A. simplex* (s.s.), and *A. simplex 'C'*) (Mattiucci *et al*., 1997), forming the "*A. simplex* species complex" a number of these sibling species have been observed to sympatrically occur within the same geographical areas (Abollo *et al*., 2003). The Pacific coasts of Canada harbour both *A. simplex* (s.s.) and *A. simplex 'C'* (Abollo *et al*., 2003), whilst *A. simplex 'C'* and *A. pegreffii* are both found in the South Atlantic Ocean (Abollo *et al*., 2003). In European waters, *A. simplex* (s.s.) and *A. pegreffii* occur sympatrically in the central Mediteranean Sea (Farjallah *et al*., 2008), and in coastal regions surrounding the Iberian Peninsula (Abollo *et al*., 2003; Martin-Sanchez *et al*., 2005).

With sympatrically occurring species, the promotion of local genetic diversity of a parasitic population increases in likelihood (Dybdahl *et al*., 2008). Based on the idea that

different parasite genotypes are limited to infecting a different subset of host genotypes, parasites are expected to impose time-lagged selection against common host genotypes, leading to the maintenance of local genetic diversity in host populations (Dybdahl *et al*., 2008). Through the promotion of local genetic diversity, parasitic populations are able to constantly evolve within the co-evolutionary arms race against their hosts (Peters & Lively, 2007; Dybahl *et al*., 2008). Larvae of *A. simplex* have been observed within the same intermediate hosts (Abollo *et al*., 2001a), as well as adult forms within the same definitive hosts in these sympatric areas (Abollo *et al*., 2003). The potential for gene exchange and hybrid development can be seen in recombinant genotypes of *A. simplex* species observed in both Gallician waters, and the Alboran Sea (Abollo *et al*., 2003; Mattiucci *et al*., 2016) and observed hybrids of the closely related species complex of *Pseudoterranova decipiens* (Krabbe, 1878) (Paggi *et al*., 1991). However, first generation hybrids (F1) of both *Pseudoterranova* and *Anisakis* species are believed to be sterile (Paggi *et al*., 1991; Mattiucci *et al*., 2016).

The occurrence of interspecific hybridisation within these 'hybrid zones' (Abollo *et al*., 2003) could have major evolutionary consequences for both anisakids and their hosts. The presence of recombinant genotypes has the potential to promote or prevent divergence (Mattiucci *et al*., 2016), and raises questions about epidemiological and ecological aspects of these parasites. As a consequence of the new allelic combinations produced by hybridisation, enhanced phenotypic characteristics have the potential to result in better host exploitation (Detwiler & Criscione, 2010; King *et al*., 2015). Furthermore, hybrids have the potential to infect a greater range of host species (Criscione *et al*., 2007; King *et al*., 2015). Interspecific hybrids of *Schistosoma haematobium* (Bilharz, 1852) and *Schistosoma bovis* (Bilharz, 1852) for example, have been observed in both *Bulinus globosus* (Morelet, 1866) and *Bulinus truncatus* (Audouin, 1827), the intermediate snail hosts of *S. haematobium* and *S. bovis* respectively (Huyse *et al*., 2009;

Webster *et al*., 2013). The potential for infestation of new host species is also highly likely, due to greater adaptation to both biotic and abiotic factors leading to a potentially wider geographical range than 'parental' taxa (Miura *et al*., 2006; Dlugosch *et al*., 2015; Mattiucci *et al*., 2016). Hybridisation has led to *Phytophthora* spp. to become pathogenic to alder trees (Brasier *et al*., 1999) and *Zymoseptoria pseudotritici* (Schröt, 1894) infecting new host species of grasses (Stukenbrock *et al*., 2012). A hybridisation event in the case of the monogenean species *Gyrodactylus* sp. led to a permanent host switch from grayling (*Thymallus thymallus L.*) to Baltic salmon (Kuusela *et al*., 2007), with additional secondary and tertiary recombination events observed on farmed rainbow trout and salmon in the Russian lake Kuito (Kuusela *et al*., 2007). Most importantly however, hybridisation can also lead to improved avoidance of recognition and resistance from a hosts' adaptive immune system leading to higher infectivity and pathologies within parasite-host relationships (Gandon *et al*., 2002; Ebert & Bull, 2003). Hybridisation of *Phytophthora* led to a range of new species of aggressive pathogenicity (Brasier *et al*., 1999). Similarly, hybrid lineages of the amphibian zoosporic fungus *Batrachochytrium* are hyper virulent to many hosts (Farrer *et al*., 2011), and caused dramatic outbreaks of chytridiomycosis, which have led to mass mortalities and extinctions of some frog populations (Skerratt *et al*., 2007; King *et al*., 2015). Whether changes in host microhabitat can be driven by hybridisation remains unclear.

The oceanic distribution of sibling species within the *A. simplex* complex is primarily dependent on the migration of their final cetacean hosts such as *Delphinidae*, *Monodontidae, Phocoenidae*, and *Balaenopteridae*, and the availability of appropriate copepod species as first intermediate hosts (Murphy *et al*., 2010). *Anisakis simplex* (s.s.) is commonly observed in regions such as the mid-Atlantic ridge (Klimpel *et al*., 2008) and wider North Atlantic (Murphy *et al*., 2010), making areas of sympatry with *Anisakis*

pegreffii in the North East Atlantic (Kuhn *et al*., 2016), like those off the South West of England (Mattiucci *et al*., 2014) highly likely.

Parasites with high host specificity are predicted to limit gene flow between host species, as conspecific parasites are infecting different hosts (Archie & Ezenwa, 2011). Generalists such as *A. pegreffii* and *A. simplex* (s.s.) however, might induce high rates of gene flow between parasites found in a number of host species, and foster increased genetic exchange between parasites infecting different hosts (Archie & Ezenwa, 2011). The potential broad capacity for rapid evolutionary change in these species (Mladineo & Poljak, 2014) means the potential formation of another 'hybrid zone' within these waters are a major concern.

2.1.2 *Anisakis* sp. *in the Vent Region of Atlantic salmon*

After the emergence of RVS in 2005, several molecular studies on the genetic identity of *Anisakis* sp. infesting the vent region have been carried out. Mo *et al*. (2010) analysed the partial mitochondrial cytochromeoxidase 2 (COX2) sequence of one nematode each from the vent region of 16 fish from six different rivers. All specimens were identified as *A. simplex* (s.s.) and showed the highest similarity to COX2 sequences (GenBank accession numbers GQ338428 – GQ338436) from *A. simplex* (s.s.) recovered from Atlantic salmon in the northeast Atlantic Ocean (Murphy *et al*., 2010). However, the use of the COX2 gene has shown considerable degrees of intra-specific variability due to its higher evolutionary rate in both *A. simplex* (s.s.) and *A. pegreffii* (Abattouy *et al*., 2016).

Another commonly used set of genes in these studies are ribosomal DNA (rDNA) genes (Mattiucci *et al*., 2014), an important multigene family. One unit of rDNA consists of three rRNA encoding genes separated by the nuclear ribosomal Internal Transcribed Spacer region (ITS) (Umehara *et al*., 2006). The ITS region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions (Umehara *et al*.,

2006). The level of variation in this region makes it suitable for the detection of genetic variation within species (Mattiucci & Nascetti, 2008; Jahantab *et al.*, 2014; Umehara *et al.*, 2007).

Noguera *et al.* (2009) used the ITS region in conjunction with the partial cytochrome oxidase I (COX1) mitochondrial DNA gene (mtDNA). These genes were analysed for 41 nematodes from the body cavity, and 43 from the vent of RVS affected Atlantic salmon. Based on restriction fragment length polymorphism patterns (RFLP) using the restriction endonucleases HinfI and HhaI (D'Amelio *et al.*, 2000; Umehara *et al.*, 2007), all samples were identified as *A. simplex* (s.s.) (Noguera *et al.*, 2015). COX1 sequences were also identified as *A. simplex* (s.s.) and showed distinct differences to *A. pegreffii* sequences (Noguera *et al.*, 2009). Within the ITS sequence analysis however, three specimens (vent) displayed a C/T heterogeneity at position 305 (GQ143709) and a single specimen (body), which displayed a C/T heterogeneity at position 305 and also at position 173 (GQ143711) in relation to the sequence of *A. simplex* (s.s.) (AY826723). Analysis of the ITS region of *Anisakis* sp. isolates from Atlantic salmon in Scotland therefore, is likely to show potential speciation events, and will provide an update on the presence of speciation in this region.

This chapter provides further clarification on the potential presence of genetic speciation or hybridisation events of anisakids within Atlantic salmon from the coastal waters of Scotland. DNA isolated from anisakids from Atlantic salmon returning to the West coast of Scotland, and from the Sandback hatchery of the River Spey were used. The Atlantic salmon population returning to the West coast of Scotland has previously not been analysed in associated molecular studies by Noguera *et al.* (2009) and Mo *et al.* (2010). This chapter hypothesised that the ITS region of anisakids found within the vent, of RVS affected Atlantic salmon within the western population, will exhibit genetic differences to those from the Sandbank hatchery, and to anisakids found within the body cavity, and

potentially provide a link to the novel infestation site of the vent, and exhibition of RVS symptoms.

2.2 Materials and Methods

Samples of adult Atlantic salmon aged as 1SW (see Chapter 4 for methodology of aging) were obtained from Douglas Hall wild capture fisheries (Sandyhills, West Coast of Scotland, UK (54°52'45.7"N 3°43'46.9"W)). One specimen exhibiting severe RVS (see Chapter 3.2.1 for assessment criteria) was chosen for this study. Additionally, one Atlantic salmon aged as 1SW without RVS was sampled from wild captured kelts at the Sandbank hatchery on the River Spey (East Coast of Scotland, UK) (57°21'0.001"N 3°18'21.188"W)). Both fishes were euthanised by a concussion blow to the head and subsequently frozen at -20 °C within a maximum of six hours. After being thawed overnight at 4 °C, fish were examined for the presence of *Anisakis* sp. larvae within the vent region and the body cavity (see Chapter 3.2.2 for dissection protocol)

2.2.1 *Isolation of Anisakid Larvae*

During the dissection, approximately ten *Anisakis* sp. L3 (stage 3 larvae) were removed from the body cavity, and the vent region of each Atlantic salmon using sterile forceps. Isolated nematodes were washed with physiological saline (Sigma-Aldrich, Irvine, UK) to remove any potential contaminants from Atlantic salmon tissue and then stored in 100% molecular grade ethanol (Thermo Fisher Scientific, Loughborough, UK) for subsequent molecular analysis.

2.2.2 DNA Extraction

One larva each, isolated from both regions of the two Atlantic salmon was selected for molecular analysis. Whole nematodes (~2 mg) were removed from ethanol, cut into smaller pieces using a sterile scalpel, and placed in 1.5 ml micro centrifuge tubes with 180 µl of buffer ATL, and homogenised using the TissueLyser LT (Qiagen, Manchester, UK) for 3 minutes at 50 Hz. The solution was subsequently mixed with 20 µl of Proteinase

K solution (20 mg/ml) (Qiagen, Manchester, UK) and incubated at 56 °C for 2 hours, vortexing the mixture for 15 seconds every 30 minutes until completely lysed. Genomic DNA was extracted from the resulting homogenate using the DNeasy® extraction kit (Qiagen, Manchester, UK) according to the manufacturer's instructions and eluted in 100 µl elution buffer.

2.2.3 PCR Product Amplification

The entire nuclear internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA gene and ITS2) of the nematodes' nuclear ribosomal DNA (rDNA) was amplified using the primers NC5 (5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'TTAGTTTCTTTTCCTCCGCT-3') at a concentration of 10 pM/µl following Noguera *et al*. (2009). Each reaction mixture contained 2.5 µl genomic DNA, 1 µl of each primer, 1x puReTaq Ready-to-go PCR beads (GE Healthcare, Quebec, Canada) and 20.5 µl of PCR grade water (Sigma Aldrich, Irvine, UK) with a final volume of 25 µl. PCR was carried out using the 2720 Thermal Cycler (Applied Biosystems, Warrington, UK) following the cycling conditions outlined by Zhu *et al*. (1998) and Noguera *et al*. (2009): 94 °C for 5 minutes (initial denaturation); 30 cycles of 94 °C for 30 seconds (denaturation), 60 °C for 30 seconds (annealing), and 72 °C for 30 seconds (extension) followed by a final extension at 72 °C for 5 minutes. A negative control with no DNA template was included to detect any contamination that may have occurred. The ITS PCR products were screened on a 1% agarose gel using the stain GelRed™ along with a 1 kb plus DNA Ladder GeneRuler™ (Thermo Fisher Scientific, Loughborough, UK) and documented using the ChemiDoc™ XRS+ with Image Lab™ Software (Bio-Rad, Watford, UK) (Fig 2.1)

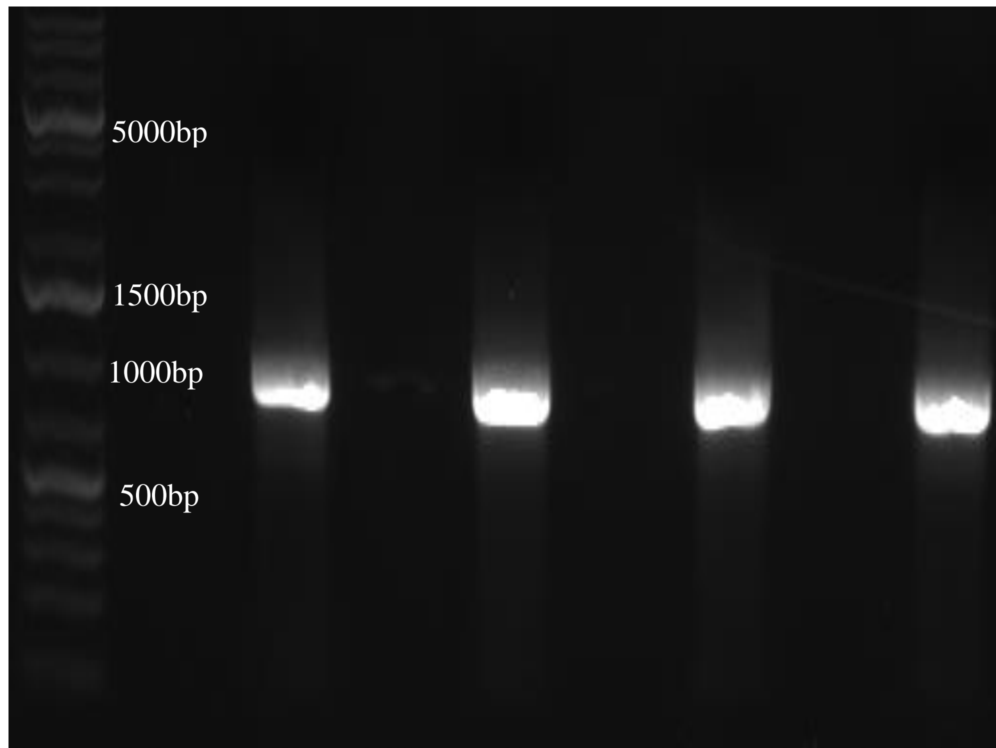

Figure 2.1. PCR products of the ITS rDNA region (~950bp) (ITS-1, 5.8S, and ITS-2) of (from left to right) body and vent from West coast Atlantic salmon, and body and vent from Sandbank Hatchery Atlantic salmon.

2.2.4 PCR Product Purification

PCR products corresponding to the expected size (~950 bp) were cut out of the gel using a sterile razor blade and underwent purification using UltraClean™ 15 DNA Purification kit (MO Bio, Carlsbad, California). This was conducted through the addition of 500 µl of Ultra Salt to the gel, and incubation at 55 °C. The solution was mixed occasionally by hand until the gel had completely melted (~5 minutes). A total of 6 µl of Ultra Bind was added to each sample and mixed by inversion at room temperature for 5 minutes. The mixture was then centrifuged for 1 minute at 13,000 g. After the supernatant was discarded, the Ultra Bind pellet was re-suspended in 900 µl of Ultra Wash and centrifuged for 1 minute at 13,000 g. The supernatant was removed, and the pellet dried at 55 °C for 10 minutes. Finally, the pellet was re-suspended in 13 µl of DNA free water (Sigma Aldrich, Irvine, UK). DNA quality and concentration was then assessed using the NanoDrop2000 spectrophotometer (Nanodrop Technologies, Wilmington, USA).

Purified PCR products were premixed with 1 µl of NC5 or NC2 primers at a working concentration of 10 pM/µl and diluted to a concentration of 5 ng/µl in accordance with submission guidelines of the sequencing company (Eurofins Genomics, Wolverhampton, England).

2.2.5 Sequencing and Phylogenetic Analysis

The new sequences were initially BLAST identified and then aligned with verified sequences of species within the *A. simplex* species complex and wider sibling species within the genus *Anisakis* available in GenBank (for specimen codes and GenBank accession numbers see Table 2.1). In total 15 sequences (incl. four isolates from this study) were included in the analysis.

A 15-sequence dataset was aligned using ClustalW (Thompson *et al*., 1994) and manually fine-tuned using Molecular Evolutionary Genetics Analysis (MEGA) 6 software resulting in 855 unambiguously aligned sites (Tamura *et al*., 2013). Phylogenetic trees were inferred using two inference methods: maximum likelihood (ML) using MEGA6, and Bayesian posterior probability using MrBayes 3.2.6.p (Ronquist & Huelsenbeck, 2003) with the latter being executed on the CIPRES portal (Miller *et al*., 2010). MEGA6 and PhyML both selected a Hasegawa-Kishino-Yano (HKY) model of nucleotide substitution, with a discrete gamma distribution (5 categories) following the Akaike Information Criterion (AIC) (Posada & Crandall, 1998). ML inference was conducted with complete deletion of gaps and missing data and used a Nearest Neighbour-Interchange ML heuristic method and bootstrap searches using 1,000 pseudo replicates. The Initial tree was constructed using a neighbour-joining (NJ) starting tree.

Bayesian analysis was performed on the same alignment dataset, with the program MrBayes set to include four Monte Carlo Markov chains (MCMC; default temperature = 0.2). A total of 7,000,000 generations were calculated, with trees sampled every 100

generations. When the average split rate fell below 0.1, the program would terminate. Posterior probability values shown in consensus trees were determined after discarding trees from the burn-in-period. For each dataset the burn-in was estimated to include the first 3,500,000 generations.

Table 2.1 *Anisakis* species and GenBank Accession numbers for the ITS regions included in alignments and subsequent analysis.

Species	GenBank Accession Number (ITS)
Anisakis simplex sensu stricto	AY826723
Anisakis sp. SAN-2004 isolate N136	AY821740
Anisakis sp. SAN-2004 isolate N243	AY821749
Anisakis simplex 'C'	JX535519
Anisakis pegreffii	AB196671
Anisakis typica	JQ912690
Anisakis brevispiculata	JQ912694
Anisakis paggiae	EU624345
Anisakis physeteris	JQ912693
Anisakis nascetti	JQ912692
Anisakis ziphidarum	AY826725
Outgroup	
Ascarus suum	AB110023 & FJ418786
Toxicara canis	AJ002435 & FJ418788

Both phylogenetic trees were rooted using two outgroup species: the nematode parasite of pigs *Ascaris suum* (Goeze, 1782) (GenBank accession nos. AB110023 and FJ418786), and the nematode parasite of canids *Toxocara canis* (Werner, 1782) (GenBank accession

nos. AJ002435 and FJ418788) where sequences were combined to span the entire ITS region as used in previous studies (Cavallero *et al*., 2011).

A pairwise distance calculation based on Kimura's two-parameter model (Kimura, 1980) was performed on a dataset of 635 nucleotides (excluding gaps and missing data), and 921 nucleotides (including gaps and missing data) using MEGA6 with bootstrap searches performed using 1,000 pseudoreplicates.

2.3 Results

The phylogenetic analyses were carried out on 15 aligned nuclear ITS sequences comprising the four sequences of *Anisakis* sp. from Atlantic salmon within this study, 11 verified sequences of species within the genus *Anisakis* and two outgroup species (*T. canis* and *A. suum*) that were used in previous studies (Paggi *et al*., 1998; Noguera *et al*., 2009; Cavallero *et al*., 2011).

2.3.1. Molecular Phylogeny Based on Maximum Likelihood (ML) Analysis

The phylogenetic tree inferred through ML analysis (Fig 2.2) presented a tree topology with two strongly supported clades (bootstrap value: 100) within the well supported clade of sequences from species belonging to the genus *Anisakis*. The first clade consisted of the *A. simplex* species complex comprising *A. simplex* (s.s.), *A. pegreffii, A. simplex 'C'*, two *Anisakis* species from two different northern Pacific marine mammals, the northern right whale dolphin (*Lissodelphis borealis* Peale, 1848) and the harbour porpoise (*Phocoena phocoena L.*) and the four *Anisakis* sp. isolates obtained in this study. The two species *A. nascetti* and *A. ziphidarum* clustered together with weak support (43%) and formed the sister group to the *A. simplex* species complex with moderate support (76%). The second strongly supported clade, also known as the '*A. physeteris*-complex' (Mattiucci *et al*., 2014) was represented by *A. paggiae*, *A. physeteris* and *A. brevispiculata*. *Anisakis typica* formed the sister group to all other sequences representing a separate lineage at the base of the tree, which was also strongly supported (100%). All four *Anisakis* sp. isolates from this study branched together with very weak support (3%). The sister group containing *A. simplex* (s.s.) and the *Anisakis sp.* SAN-2004 isolate was also weakly supported (12%).

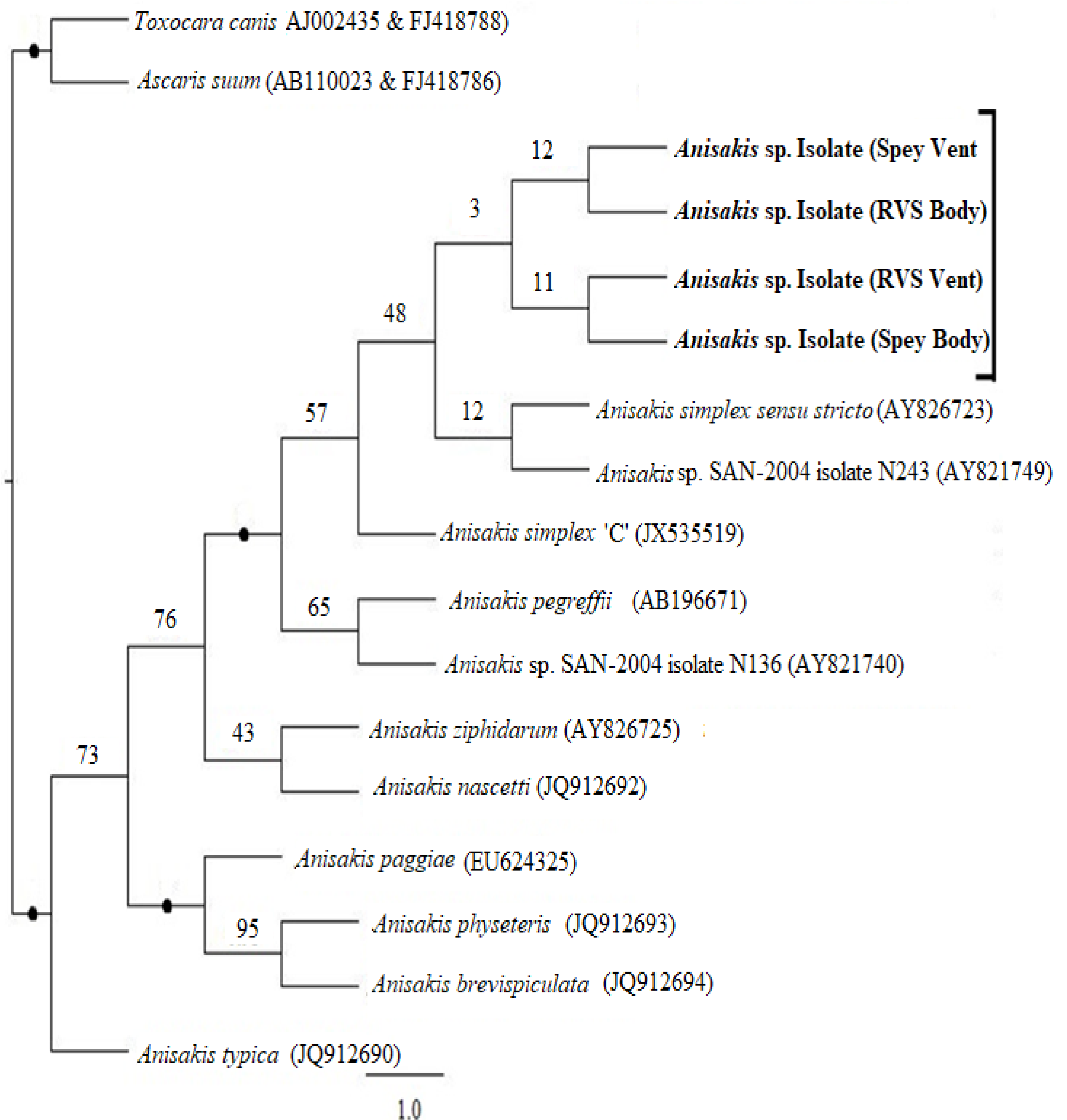

Figure 2.2. Maximum likelihood tree of 15 ITS rDNA sequences and 855 unambiguously aligned sites. ML tree inferred using HKY+G substitution model. Numbers at branches denote ML bootstrap percentages. Black dots on branches denote bootstrap percentages of 100%.

2.3.2 Molecular Phylogeny Based on Bayesian Analysis

Bayesian phylogenetic analysis revealed a tree topology that differed from that of the ML tree. The main clade comprising all different *Anisakis* species was strongly supported. The clade representing the '*A. physeteris*-complex' represented by *A. paggiae*, *A. physeteris* and *A. brevispiculata* was moderately supported (Bayesian Posterior Probability: 0.96) and formed the sister clade to all other *Anisakis* species that formed a strongly supported clade as well. The biggest differences within Bayesian analysis compared to ML analysis was the *A. simplex* species complex, which contained *A. simplex* (s.s.), *A. pegreffii, A. simplex 'C',* and the four *Anisakis* sp. isolates obtained in this study which had weak support (0.89). With Bayesian inference, *A. simplex 'C'* was the most basal species in this group. The *Anisakis* sp. isolate from the body cavity of the RVS affected Atlantic salmon formed a sister group to both the strongly supported clade of *A. pegreffii* and *Anisakis* sp. SAN-2004 isolate N136 (1.00), and the moderately supported clade (0.97) containing *A. simplex* (s.s.), *Anisakis* sp. SAN-2004 isolate N243, and the other three *Anisakis* sp. isolated in this study, the latter showing a polytomic branching pattern. Furthermore, *A. nascetti*, *A. ziphidarum* and *A. typica* branched as a separate lineage each at the base of the main *Anisakis* clade.

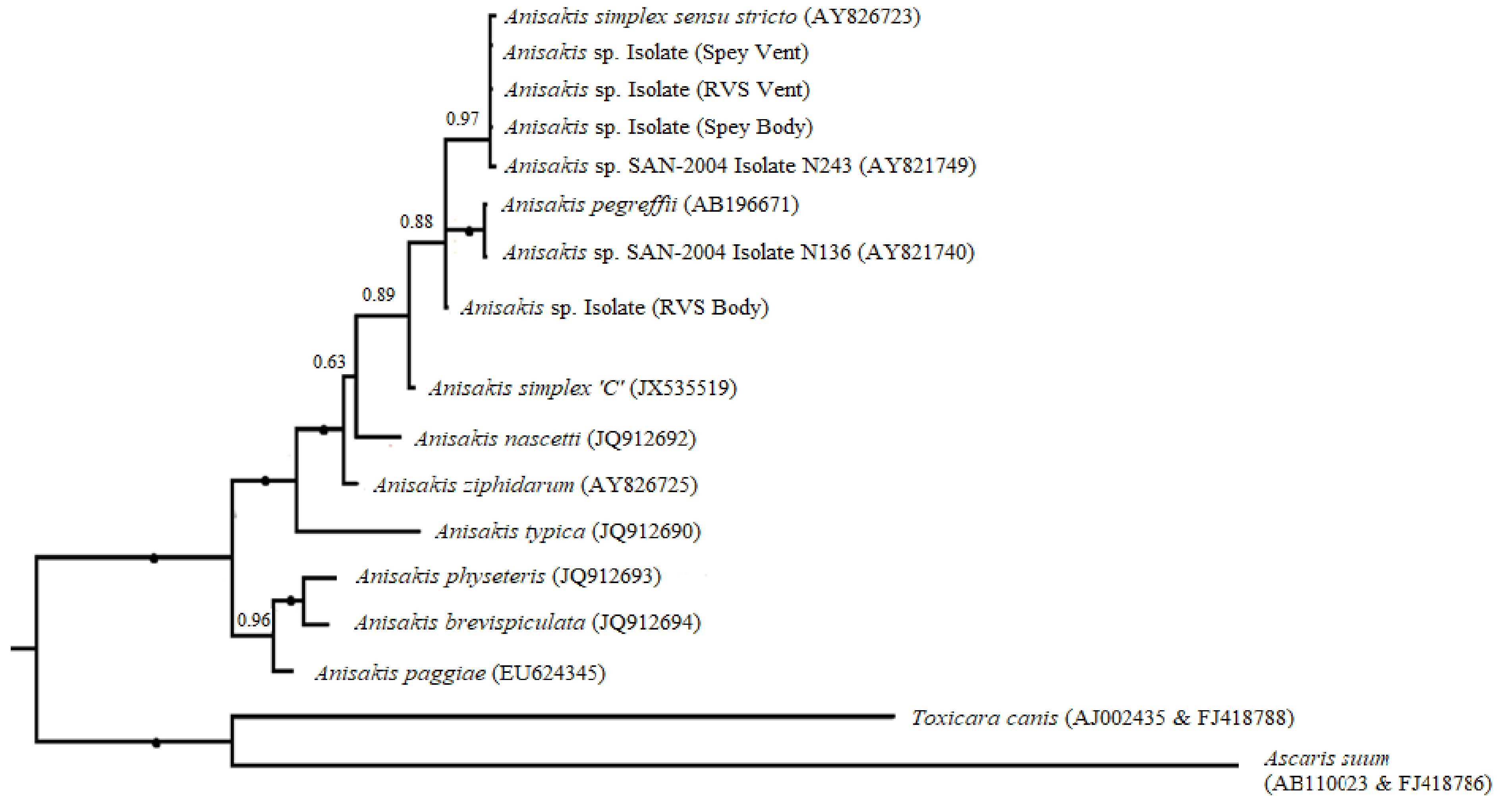

Figure 2.3. Bayesian tree inferred from alignment sequences of the ITS region of *Anisakis* sp. Numbers at branches denote Bayesian posterior probabilities (0.0-1). Black dots on branches denote posterior probabilities of 1.

In addition to the 15 aligned nuclear ITS sequences analysed in Bayesian and ML phylogenetic trees, two ITS sequences of *Anisakis sp*. isolated by Noguera *et al*. (2009), which had shown C/T heterogeneity were subsequently included (GQ143709 & GQ143711). Following alignment within MEGA6 using ClustalW (Thompson *et al*., 1994), ITS sequences were checked for any potentially clade-specific differences and positions of heterogeneity between *Anisakis* sp. included in the phylogenetic analysis. A number of base-pair positions (5, 60, 509, and 629) were observed to differentiate between species within the *A. simplex* species complex, and those within the *A. physeteris*-complex (Table 2.2) At one base-pair position (217), the *Anisakis* sp. sequence isolated from the body cavity of RVS Atlantic salmon displayed a C along with *A. simplex* sp., *A. simplex 'C'* (JX535519) and *A. pegreffii* (AB196671). This differentiated to the T displayed by *A. simplex* (s.s.) (AY826723) and the other *Anisakis* sp. isolates (Table 2.2), while *Anisakis* sp. sequences obtained from Noguera *et al*. (2009) exhibited a Y. Species within the *A. physeteris*-complex displayed a gap at this position.

Table 2.2. Alignment of ITS sequences (855bp) from *Anisakis* spp., Highlighted base at position 217 displays heterogeneity in *Anisakis* sp. recovered from the body of Atlantic salmon exhibiting Red Vent Syndrome. Dots (.) indicate the same base pair as *A. simplex* (s.s.) and hyphen (-) denotes missing base.

Sequence		…	5	…	60	61	…	153	…	156	…	217	…	233	…	494	…	497	498	…	503	…	509	…	558	…	629	…	832	…	834	835
Species	Accession Number																															
A. simplex (s.s.)	AY826723		A		A	G		A		T		T		T		C		T	G		G		A		C		G		A		C	T
Anisakis sp. Hatchery Vent	-		.		.	.		.		.		.		.		.		.	.		.		.		.		.		.		.	.
Anisakis sp. RVS Vent	-		.		.	.		.		.		.		.		.		.	.		.		.		.		.		.		.	.
Anisakis sp. RVS Body	-		.		.	.		.		.		C		.		.		.	.		.		.		.		.		.		.	.
Anisakis sp. Hatchery Body	-		.		.	.		.		.		.		.		.		.	.		.		.		.		.		.		.	.
A. simplex genotype a	GQ143709		.		.	.		.		.		Y		.		.		.	.		.		.		.		.		.		.	.
A. simplex genotype c	GQ143711		.		.	.		.		.		Y		.		.		.	.		.		.		.		.		.		.	.
A. simplex sp.	AY821740		.		.	.		.		.		C		C		.		.	.		.		.		.		.		.		.	.
A. simplex sp.	AY821749		.		.	.		.		.		.		.		.		.	.		.		.		.		.		.		.	.
A. simplex 'C'	JX535519		.		.	.		.		.		C		.		.		.	.				.		.		.		.		.	.
A. pegreffii	AB196671		.		.	.		.		.		C		C		.		.	.		.		.		.		.		.		.	.
A. paggiae	EU624345		G		G	T		G		A		-		C		T		C	A		.A		G		T		T		T		T	G
A. ziphidarum	AY826725		G		G	T		G		.		-		C		.		.	.		.		G		T		T		T		T	A
A. typica	JQ912690		G		G	.		G		A		-		.		T		.	A		A		G		T		T		.		.	A
A. nascetti	JQ912692		G		G	T		G		A		-		C		.		.	A		A		G		T		T		T		T	A
A. physteris	JQ912693		G		G	T		.		A		-		C		T		C	A		A		G		T		T		T		T	G
A. brevispiculata	JQ912694		G		G	T		G		A		-		.		T		C	A		A		G		.		T		T		T	G

2.3.3 Kimura Sequence Divergence

The results of the pairwise distance calculation based on Kimura's two-parameter model (Kimura, 1980) on the dataset excluding gaps and missing data showed that there was no divergence between all four *Anisakis* isolates obtained in this study (0.0%) in comparison to *A. simplex* (s.s.) sequence (AY826723) (Table 2.3). Low divergence between the four anisakis isolates within this study was also seen for *A. pegreffii* (0.1%), *A. simplex* 'C' (0.1%) and the *Anisakis* sp. isolated from the Northern right whale dolphin (0.1%). Sequence divergence between all four *Anisakis* isolates from this study and *A. paggiae*, *A. physeteris* and *A. brevispiculata* were 12.5%, 13% and 13.4% respectively. Divergence between *A. physeteris* and *A. paggiae* exhibited the largest divergence between these three latter species (5.4%), with the lowest of 3.7% being observed between *A. physeteris* and *A. brevispiculata*. The largest divergence however, was between the four *Anisakis* isolates and *A. typica* (14.3%).

The paired deletion scenario showed very low levels of divergence (0.1%) between the *Anisakis* sp. isolate from the body cavity of RVS Atlantic salmon and *A. simplex* (s.s.) as well as *Anisakis* sp. isolates from the vent of RVS Atlantic salmon (0.1%), and from the body (0.0%) and vent (0.0%) of the hatchery salmon. The *Anisakis* sp. isolate from the body cavity of RVS Atlantic salmon showed lower levels of divergence to both *A. pegreffii* (0.1%), and *Anisakis* sp. isolated from the Northern right whale dolphin (0.1%) in comparison to the other three isolates within this study (0.3%). Once again, the largest sequence divergence was between *A. simplex* (s.s.) and *A. typica* (17.8%). Similarly, high divergence of *A. simplex* (s.s.) was also shown in comparison to *A. paggiae*, *A. physeteris* and *A. brevispiculata* sequences with values of 14.9%, 15.2% and 15.7% respectively. Within the *A. physeteris* species complex, sequence divergences between *A. physeteris* and *A. paggiae* was the largest (7.4%), with A. physeteris and *A. brevispiculata* being the lowest (4.7%).

Table 2.3 Sequence divergence of ITS rDNA sequences of *Anisakis* sp. Values observed represent percentage sequence divergence within the complete deletion of gaps and missing data (*a*), and partial deletion (*b*). Highlighted results show sequence divergence of *Anisakis* sp. recovered from the vent and body of Atlantic salmon and *A. simplex* (s.s.)

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *A. ziphidarum*	-													**a**
2. *A. nascetti*	5.9	-												
3. *A. typica*	13.2	14.8	-											
4. *A. physeteris*	12.9	15.9	18.6	-										
5. *A. brevispiculata*	14.1	16.5	18.4	3.7	-									
6. *A. paggiae*	11.6	14.1	16.3	5.4	5.1	-								
7. *A. pegreffii*	4.3	6.8	14.4	12.8	13.6	12.3	-							
8. *A. simplex* 'C'	4.4	7.2	14.3	12.8	13.2	12.3	0.3	-						
9. *Anisakis* sp.	4.4	7.2	14.4	13.2	13.6	12.7	0.3	0.3	-					
10. *A. simplex* sp.	4.3	6.8	14.4	12.8	13.6	12.3	0.0	0.3	0.3	-				
11. *A. simplex* - Spey Body	4.4	7.0	14.3	13.0	13.4	12.5	0.1	0.1	0.1	0.1	-			
12. *A. simplex* - Fishery Body	4.4	7.0	14.3	13.0	13.4	12.5	0.1	0.1	0.1	0.1	**0.0**	-		
13. *A. simplex* - Fisheries Vent	4.4	7.0	14.3	13.0	13.4	12.5	0.1	0.1	0.1	0.1	**0.0**	**0.0**	-	
14. *A. simplex* - Spey Vent	4.4	7.0	14.3	13.0	13.4	12.5	0.1	0.1	0.1	0.1	**0.0**	**0.0**	**0.0**	-
15. *A. simplex* (s.s.)	4.4	7.0	14.3	13.0	13.4	12.5	0.1	0.1	0.1	0.1	**0.0**	**0.0**	**0.0**	**0.0**

Sequence	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1. *A. ziphidarum*	-													
2. *A. nascetti*	7.2	-												**b**
3. *A. typica*	16.8	17.9	-											
4. *A. physeteris*	14.8	18.3	20.8	-										
5. *A. brevispiculata*	15.8	18.8	20.3	4.7	-									
6. *A. paggiae*	13.0	16.6	19.4	7.4	6.0	-								
7. *A. pegreffii*	5.6	8.1	18.1	15.0	15.8	14.9	-							
8. *A. simplex* 'C'	5.6	8.6	17.9	15.0	15.5	15.0	0.6	-						
9. *Anisakis* sp.	5.7	8.4	18.1	15.3	15.8	15.2	0.4	0.8	-					
10. *A. simplex* sp.	5.6	8.1	18.1	15.0	15.8	14.9	0.0	0.6	0.4	-				
11. *A. simplex* - Spey Body	5.7	8.3	17.9	15.2	15.7	15.1	0.3	0.6	0.1	0.3	-			
12. *A. simplex* - Fishery Body	5.7	8.3	17.9	15.2	15.6	15.1	0.1	0.5	0.3	0.1	**0.1**	-		
13. *A. simplex* - Fisheries Vent	5.7	8.3	17.9	15.2	15.6	15.1	0.3	0.6	0.1	0.3	**0.0**	**0.1**	-	
14. *A. simplex* - Spey Vent	5.7	8.3	17.9	15.2	15.6	15.1	0.3	0.6	0.1	0.3	**0.0**	**0.1**	**0.0**	-
15. *A. simplex* (s.s.)	5.7	8.3	17.8	15.2	15.7	14.9	0.3	0.6	0.1	0.3	**0.0**	**0.1**	**0.0**	**0.0**

2.4 Discussion

The aim of this study was to provide further clarification on the potential presence of genetic speciation or hybridisation events of anisakids within Atlantic salmon from the coastal waters of Scotland. Using the entire nuclear internal transcribed spacer (ITS) region (ITS1, 5.8S rDNA gene and ITS2), DNA of anisakids was analysed from an Atlantic salmon population returning to the West coast of Scotland exhibiting RVS symptoms, and from the Sandback hatchery of the River Spey not exhibiting RVS using both Bayesian, and maximum likelihood approaches.

2.4.1 Molecular Phylogeny of Anisakis sp.

Results of maximum likelihood phylogenetic analysis revealed that all *Anisakis* sp. isolates from both Atlantic salmon were positioned together, and closely related to both *A. simplex* (s.s.) (AY826723) and *Anisakis* sp. SAN-isolate N243 (AY821749). The observed low support levels of 0.12 and 0.03 for this tree topology suggests that these branches are interchangeable, revealing a lack of genetic distance between *Anisakis* sp. isolates, and *A. simplex* (s.s.). Therefore, the new isolates are most likely *A. simplex* (s.s.).

The tree topology also revealed a poorly supported sister clade containing *A. nascettii* and *A. ziphidarum* (0.43), and a third clade of the '*A. physeteris* species complex' containing *A. paggiae*, *A. physeteris* and *A. brevispiculata*, and *A. typica* as a separate lineage. The separation of the '*A. physeteris* species complex' and '*A. simplex* species complex' into two distinct clades conform to those previously identified through similar molecular analyses (Cavallero *et al*., 2011) and are consistent with the morphological observations (shape and length of ventriculus, length of spicules, and position of vulva), which have historically guided the subdivision of the genus into the two subgenera of *Skrjabinisakis* and *Anisakis* respectively (Mozgovoi, 1953). *Skrjabinisakis* was later synonymized with

Anisakis (Davey, 1971). The validity of this taxon was subsequently confirmed by both genetic and morphological data (Mattiucci *et al*., 1986).

Bayesian analyses however, revealed some differences between the two tree topologies, the biggest of which revolved around the *A. simplex* species complex, which was weakly supported (0.89). The basal species within this complex was *A. simplex* 'C', and although three of *Anisakis* sp. isolates were positioned alongside *A. simplex* (s.s.) (AY826723) with strong support (100), the *Anisakis* sp. isolate recovered from the body cavity of RVS affected Atlantic salmon however, formed a separate branch. Following further analysis of this isolate, a base-pair difference was observed at position 217 in comparison to other isolates and *A. simplex* (s.s.). At this position, the *Anisakis* sp. isolate from the body cavity within RVS affected Atlantic salmon exhibited a C in concurrence with *A. simplex* sp., *A. simplex 'C'* (JX535519) and *A. pegreffii* (AB196671), in contrast to the T exhibited by *A. simplex* (s.s.) (AY826723) and other isolates within this study. In comparison to the sequences obtained from Noguera *et al*. (2009) (GQ143709 & GQ143711), the Y exhibited at this position by both sequences correspond to the C/T heterogeneity observed at position 305 described by Noguera *et al.* (2009) within these sequences. Furthermore, with no observed differences in base pair or missing data between *Anisakis* isolates and *A. simplex* (s.s.) (AY826723) within this study, the heterogeneity at this position is driving the 0.1% Kimura sequence divergence for this sample within the pairwise deletion scenario.

Examples of heterogeneity within *A. simplex* specimens however are common e.g. with specimens infecting herring (*Clupea harengus L.*) in the Baltic Sea (AJ937670/AJ937671) displaying a T at position 173, and a specimen from Baltic Sea cod (*Gadus morhua L.)* displaying C (AJ225065) at this position (Noguera *et al*., 2009). Heterogeneities at these positions are unlikely to represent potential speciation events between anisakid species (Noguera *et al*., 2009). With similar heterogeneities between

sequences at position 278 showing C in *A. pegreffii* and T in *A. simplex* (s.s.), and 294 showing C in *A. pegreffii* and T in *A. simplex* (s.s.) (Mattiucci *et al*., 2016), none of these observed positions could be used as unambiguous diagnostic markers (D'Amelio *et al*., 2000). Therefore, the heterogeneity observed at position 217 in this sample, cannot be used as an unambiguous diagnostic marker either.

In the paired deletion scenario, only the *Anisakis* sp. isolate from the body cavity of RVS Atlantic salmon showed any divergence against *A. simplex* (s.s.) (AY826723) (0.1%). All other *Anisakis* sp. isolates in this study in either total deletion or paired deletion scenarios, showed no divergence (0.0%). Such low divergences suggest *Anisakis* isolates are highly likely to be *A. simplex* (s.s.).

Eight years have passed since the most recent genetic analyses of *A. simplex* (s.s.) in Atlantic salmon of Scotland by Noguera *et al*. (2009). With results strongly suggesting that all *Anisakis* sp. isolates can be identified as *A. simplex* (s.s.), it is highly unlikely that speciation of *A. simplex* (s.s.) has occurred to date in this region. The analysis of only two anisakid specimens isolated from one Atlantic salmon from the West coast in this study however, does not provide a full representative assessment of potential speciation within this population. A substantial increase in the number of *Anisakis* isolates from multiple West coast Atlantic salmon hosts are required, before the potential *Anisaki*s speciation within this population can be completely ruled out.

2.4.2 *Hybridisation of Anisakis Sibling Species*

One of the main ecological concerns within the *A. simplex* species complex is the future potential of hybridisation events. Presently, the observation of recombinant genotypes has been restricted to areas of sympatric coexistence between *A. simplex* (s.s.) and *A. pegreffii* for example in Gallician waters (Abollo *et al*., 2003), the Atlantic coast of Morrocco (Farjallah *et al*., 2006), and other areas within the Mediterranean (Mattiucci *et al*., 2016). Although these first generation hybrids were sterile and therefore not

representing true speciation (Mattiucci *et al*., 2016), if reproductive capability in hybrids was to occur, there is potential of increased pathogenicity or hyper-virulence in the infested host. This was seen in the amphibian zoosporic fungus *Batrachochytrium* (Farrer *et al*., 2011) that caused outbreaks of chytridiomycosis, which can result in mass mortalities and extinctions of whole frog populations (Skerratt *et al*., 2007; King *et al*., 2015).

Although in coastal waters surrounding the United Kingdom there has been no evidence of sympatric coexistence of *Anisakis* sibling species, there is the potential for this to change. The identification of a larvae possessing the mtDNA of *A. pegreffii*, and rDNA of *A. simplex* (s.s.) at Little Sole Bank off the South West of England, is the first case of mitochondrial introgression within the *Anisakis* genus (Abattouy *et al*., 2016), in an area much closer to the UK than the Mediterranean Sea. *Anisakis pegreffii* is the dominant species of *Anisakis* in the Mediterranean Sea, due to the abundance of various dolphin species, such as the common bottlenose dolphin (*Tursiops truncatus,* Montagu, 1821), which are their main definitive hosts (Mattiucci & Nascetti, 2006). However, *T. truncatus* are highly mobile and are already present within Scottish waters (Cheney *et al*., 2013). Furthermore, with ocean scale warming in the temperate North Atlantic, there has been noticeable encroachment of species such as warm water copepods (Beaugrand *et al*., 2002), and a number of cetaceans such as the striped dolphin (*Stenella coeruleoalba* Meyen, 1833), Fraser's dolphin (*Lagenodelphis hosei* Fraser*,* 1956) and pygmy sperm whale (*Kogia breviceps* Blainville, 1838) (MacLeod *et al*., 2005). Increases in the geographical range of *Anisakis* sp. is not uncommon e.g. recent observations of *A. typica* within the Adriatic (Smrzlić *et al*., 2012). The existence of common warm water intermediate and definitive hosts for *A. pegreffii*, are likely to accelerate sympatric co-existence of *A. simplex* (s.s.) and *A. pegreffii* and their potential recombinant genotypes within this region (Dlugosch *et al*., 2015).

Although the ITS region had previously been outlined as a robust method to identify sibling species within the *A. simplex* species complex (Mattiucci *et al*., 2014), recent evidence suggests that the use of a single marker lacks the power to decipher whether shared polymorphism between two taxa is caused by incomplete lineage sorting, historical introgression or current hybridization (Mattiucci *et al*., 2016). This study was originally designed to use both COX1 and COX2 mitochondrial markers in addition to analysis of the ITS region, but unfortunately fell short due to time constraints. Therefore, the results of this study cannot completely rule out any hybridisation events within coastal waters of Scotland.

The combined results of the phylogenetic trees and Kimura's two-parameter sequence divergence analyses suggest that *A. simplex* sp. found in the vent and body of Atlantic salmon exhibiting RVS symptoms and Atlantic salmon without RVS symptoms in this study are in fact *A. simplex* (s.s.). The drivers behind the presence of *A. simplex* (s.s.) within the vent region remain uncertain, but there is a growing body of evidence that suggests that it is unlikely to be due to a speciation event (Noguera *et al*., 2009; Mo *et al*., 2010). One possible explanation is that *A. simplex* has been present within the vent region previously, but has not been reported, because historically the protocol of commercial fisheries did not include parasitic surveys of the vent region (Noguera *et al*., 2015). In addition, large numbers of *A. simplex* within the belly flaps of the muscles (Karl *et al*., 2011) in regions close to the vent (Haarder *et al*., 2013) suggest that their presence within the vent could have occurred previously. The presence of *A. pegreffii* and *A. simplex* (s.s.) hybrids that are likely to show higher pathogenicity is a growing concern not only in the Mediterranean Sea (Mattiucci *et al*., 2016), and Gallician waters (Abollo *et al*., 2003), but also in waters closer to the United Kingdom e.g. Little Sole Bank (Abattouy *et al*., 2016). Use of a multi-marker approach as advocated within Mattiucci *et*

al. (2016) should be incorporated into future studies to monitor this situation within coastal waters surrounding the United Kingdom.

Chapter 3

The *'Hyper-Infestation'* hypothesis: A case study using Atlantic salmon from Scotland

3.1 Introduction

Metazoan parasites exhibit a wide range of specificity, both to a host, and to microhabitats within a host (Holmes, 1973). *Anisakis simplex* has a low host specificity utilising a large number of intermediate hosts in its heteroxenic life cycle before the definitive host (cetaceans) is reached (Audicana & Kennedy, 2008). Common intermediate hosts of *A. simplex* include small crustacean invertebrates (euphausiids and amphipods), crayfish, crabs, lobsters, and shrimp (Decapoda), squid and cuttlefish (Cephalapoda), and a range of commercially valuable fish species (Klimpel *et al*., 2004; Noguera *et al*., 2009; Levsen & Lunestad, 2010; Mouritsen *et al*., 2010; Ferrer-Maza *et al*., 2014; Nadolna & Podolska, 2014). The presence of *A. simplex* over large geographical scales (Mattiucci *&* Nascetti, 2006), coupled with their low host specificity, has resulted in approximately 200 fish species and 25 cephalopod species to be used as hosts worldwide (Klimpel *et al*., 2004).

Anisakis simplex infestations of Atlantic salmon are well documented and have been reported for over 100 years (Carmichael, 1863). *Anisakis simplex* larvae are most commonly found within the viscera and musculature of Atlantic salmon (*Salmo salar* L.) and other fish species (Strømnes & Andersen, 1998). The emergence of Red Vent Syndrome (RVS) in wild populations of Atlantic salmon (Fig. 3.1) however, has been linked to the observation of large numbers of *A. simplex* found in the vent region (Beck *et al*., 2008).

Red Vent Syndrome in wild Atlantic salmon, has been pathognomonically associated with large numbers of *A. simplex* larvae observed within the vent region (Beck *et al*., 2008; Noguera *et al*., 2009). The 'hyper-infestation' of the vent region by *A. simplex* larvae (Noguera *et al*., 2009), has prompted research into the causal factors behind the 'novel' infestation site (Mo *et al*., 2010; Senos *et al*., 2013; Larrat *et al*., 2013). Associated factors involved during *in vivo* migrations of anisakids including hepatotropic behaviour

(Horbowy *et al*., 2016), the number of intermediate hosts e.g 'personal history'of larvae (Wootton & Smith, 1975; Smith & Hemmingson, 2003), and infestation intensity (Mo *et al*., 2010), can drive *A. simplex* distribution within hosts and thus are likely significant factors affecting the choice of infestation site.

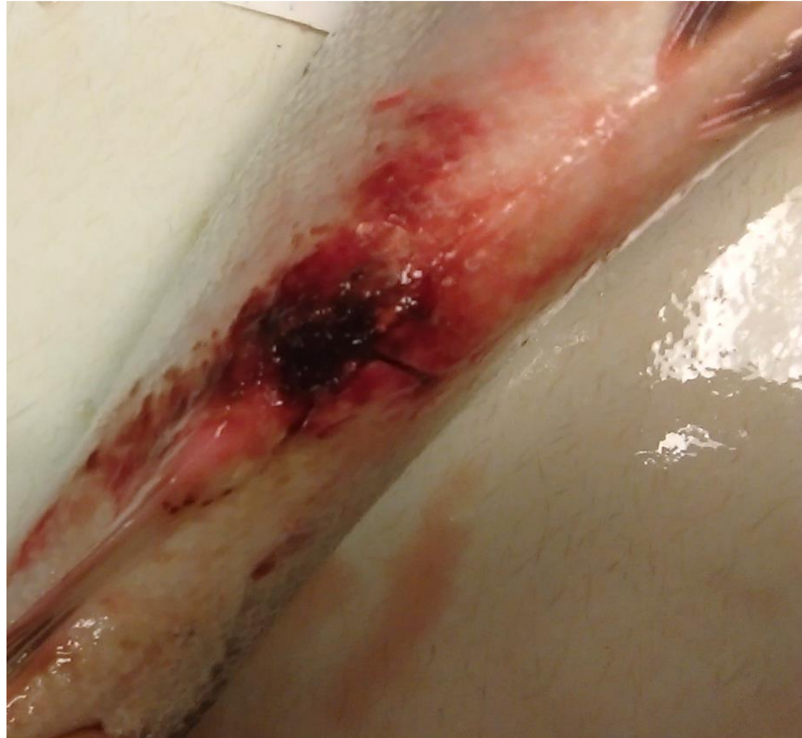

Figure 3.1. Haemorrhagic vent region – symptom associated with Red Vent Syndrome

3.1.1 The Hyper-Infestation Hypothesis

A parasite survey of 17 Atlantic salmon exhibiting RVS from south-east Norway revealed significant positive correlations between the total number of *A. simplex* larvae per fish and the number of larvae in the viscera, the musculature (Including and excluding the vent) and the vent ($p < 0.05$) (Senos *et al*., 2013). There was also a significant positive correlation between the number of larvae in the musculature and vent ($p < 0.05$) (Senos *et al*., 2013). Furthermore, comparisons of nematode intensities with historical data from similar studies carried out in the late 1960's, 1970's (Pippy, 1969; Beverley-Burton & Pippy, 1978), and more recently in 2010 (Wootten *et al*., 2010) (Table. 3.1) suggested

that a ten-fold increase in *A. simplex* burdens of Atlantic salmon has occurred in the North Atlantic and North Sea during the intervening period (Senos *et al.,* 2013).

Table 3.1. Historical comparisons of nematode abundances averages and ranges found in 1-sea-winter salmon (Senos *et al*., 2013)

Sampling Region	Number of Fish	Region of Infestation		Source
		Viscera	Musculature	
North-west Atlantic Ocean	140	2 – 13	2 - 9	**Pippy (1969)**
West Greenland	70	5.3	3.6	**Beverley-Burton & Pippy (1978)**
Montrose, River Spey, Armadale, North and North-East Scotland	5	N/A	39.4	**Wootten *et al.* (2010)**
Drammenselva river, South East Norway	17	54.5	22.8	**Senos *et al.* (2013)**

These results prompted Senos *et al*. (2013) to suggest that increasing nematode burdens triggered the infestation of the vent region of Atlantic salmon with larval *A. simplex*. The hypothesis states that increased inflammation of gastro-intestinal tissues (Ferguson, 2006) caused by increasing *Anisakis* intensities within more common sites of infestation (e.g. stomach, pyloric caeca, intestine), results in an inhospitable environment for other larvae (Murphy *et al*., 2010). Encapsulated nematode larvae found in the wall of the intestine can cause displacement, vacuolation, and necrosis of the circular muscle fibers and disrupt the stratum compactum (Murphy *et al*., 2010). Furthermore, their presence induces extensive cellular infiltration comprising eosinophilic granular cells, macrophages, lymphocytes, and fibrocytes in this region (Murphy *et al*., 2010). The induction of an increasingly severe immune response from the host and subsequent

inhospitable environment therefore, may cause larvae to pass further down the gastro-intestinal tract before migrating into the abdominal cavity (Murphy *et al.*, 2010)

To explore this potential explanation further for Atlantic salmon from coastal waters around Scotland, the factors controlling *A. simplex* abundances in coastal waters and within a host, as well as the theories behind their *in vivo* migratory routes need to be investigated.

3.1.2 Drivers of Anisakis simplex Abundance within a Host

The parasitic nematode *Anisakis simplex* possesses a heteroxenous life cycle and is transmitted through direct ingestion or the ingestion of infested prey (Køie *et al.*, 1995; Audicana & Kennedy, 2008). Due to the nature of heteroxenous transmission, significant positive correlations between a host's size and parasite intensity are common within many host-parasite relationships including *A. simplex* (Abaunza *et al.*, 1995; Wootten & Waddell, 1977). These relationships commonly result in large parasitic intensities accumulated over a hosts' lifespan (Petrie *et al.*, 2007; Wooten & Waddell, 1977). *Anisakis simplex* intensity within a host and its geographical distribution are dependent on a number of biotic and abiotic factors at different geographical scales (Kuhn *et al.*, 2016).

The development and dispersal of excreted propagules (eggs) is primarily influenced by physical parameters including salinity (Lei & Poulin, 2011), ocean currents (Baldwin *et al.*, 2011) and temperature (Højgaard, 1998). The abundance of eggs is also affected by the location and abundance of definitive hosts (Wootten & Waddell, 1977). Distribution of intermediate and adult stages however, is generally shaped through biotic factors involved in transmission pathways such as trophic interrelations between definitive, intermediate and transport hosts and their respective migrating behaviour (Marcogliese, 2002; Mattiucci & Nascetti, 2008; Kuhn *et al.*, 2011). Abiotic factors including land distance, mean sea surface temperature and range, depth, salinity, and primary production

are also important (Kuhn *et al*., 2016). Both abiotic and biotic factors can therefore drive geographical and seasonal differences (Abaunza *et al*., 1995; Konishi & Sakurai, 2002).

Greater infestations of Atlantic cod by *Anisakis* sp. reported within the Faroe plateau (Platt, 1975) have been related to periodical concentrations of the long-finned pilot whale, in the fjords of the Faroe Islands (Harrison & King, 1965). These periodic concentrations of cetaceans during their northward migrations and subsequent increased abundance of anisakid eggs are suggested to be the cause of the 'spring rise' peaks of *Anisakis* infestation seen in saithe, cod and redfish in central Norway (Strømnes & Andersen, 2000). There has also been substantial evidence that increasing grey seal (*Halichoerus grypus, Nilsson, 1820)* populations in coastal waters of Denmark are causing increased *Contracaecum osculatum* (Rudolphi, 1802), another anisakid nematode, infestation in Atlantic cod (Haarder *et al*., 2014).

The geographically driven differences in food availability and food web structure in particular, can have direct influences on levels of parasite abundance (Mouritsen *et al*., 2010; Larrat *et al*., 2013). Decreases in less heavily infested intermediate hosts such as krill, and increases in more densely infected paratenic hosts, such as Capelin (*Mallotus villosus* Müller, 1776) and Atlantic herring in the Gulf of St. Lawrence (Dufour *et al*., 2010), have been attributed to higher anisakid infestations in Atlantic salmon (Larrat *et al*., 2013), and Greenland cod (*Gadus ogac* Richardson, 1836) (Mouritsen *et al*., 2010) in Canada.

All Atlantic salmon used in comparisons of current and historic parasite abundances made by Senos *et al*. (2013), were one year at sea (grilse) with no distinct disparity in size and length. Size-based differences in Atlantic salmon can affect nematode intensities; therefore, the use of similar sized and aged fish only strengthens the case for observed increases in anisakid abundances over the last 40 years. The observed increase in nematode intensity in Atlantic salmon is most likely a consequence of a number of

geographical and environmental factors including changes in abundance and location of definitive host populations, dietary resources and seasonality.

3.1.3 Anisakis simplex: Transmission and Distribution within a Host

Although initially present within the digestive tract for the first hours after transmission (Køie *et al*., 1995; Køie, 2001), the possession of a boring tooth at the anterior end (Fig. 3.2) (Berland, 2006) enables *A. simplex* to migrate through tissues before they are encapsulated by the immune response of the host. Once 'encapsulated', inactive larvae wait for their next transmission to the second intermediate or the definitive host (Køie *et al*., 1995; Audicanca & Kennedy, 2008). This ability to migrate can lead to differences in distribution within a host. In marine fish species, more than 80% of *A. simplex* larvae are most commonly found in the viscera, with the remaining 20% found in the musculature (Strømnes & Andersen, 1998).

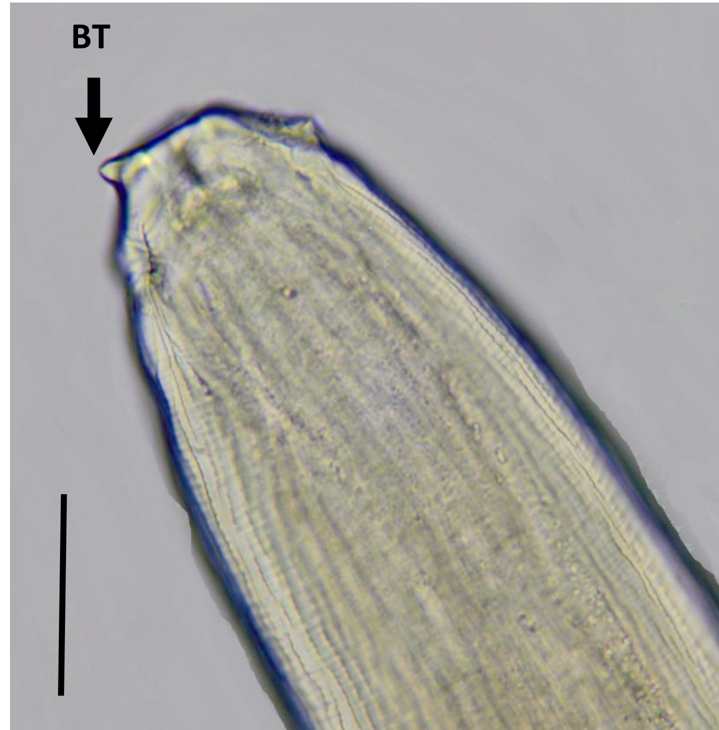

Figure 3.2. Boring tooth (BT) on cephalic end of *Anisakis simplex* L3 larvae. Scale bar = 50µm

Although *A. simplex* has previously been recorded in the hypaxial muscle close to the vent in Atlantic herring and grey gurnard (*Eutrigla gurnardus* Fraser-Brunner, 1938) (Levsen & Lunestad, 2010; Levsen & Karl, 2014), large numbers of *A. simplex* in the vent have never been reported in Atlantic salmon or in any other fish species prior to 2005. The majority of research into *Anisakis* site selection within a host has focussed on edible muscle tissue due to the associated public health concerns regarding Anisakiasis (Van Thiel, 1976; Audicana *et al*., 2002; Audicana & Kennedy, 2008). The relationship between *Anisakis* burdens within the viscera and muscle tissue has also been studied for other fish species (Smith & Wootten, 1975; Wootten & Waddell, 1977; Smith, 1984). Significant positive correlations were found between numbers of *Anisakis* in the viscera and musculature of Atlantic herring (*Clupea harengus* L. 1761) (Smith & Wootten, 1975) and whiting (*Merlangius merlangus* L. 1758) (Wootten & Waddell, 1977), but not in Atlantic cod (*Gadus morhua L.* 1758) (Wootten & Waddell, 1977).

In some parasite species, sites of infestation are highly specific, to the point where it can be used as a taxonomic characteristic. *Dirofilaria immitis* (Leidy, 1856) also referred to as the 'heartworm' for example, is found in the heart of canids. (Sukhdeo & Bansemir, 1996). The distribution of *A. simplex* within a host is dependent on its migratory potential and behaviour, which is influenced by a number of factors including 'personal history' (Wootton & Smith, 1975), host size (Young, 1972) and species (Wootten & Waddell, 1977; Noguera *et al*, 2009), and visceral organ topography (VOT) (Smith & Hemmingsen, 2003).

3.1.4 The 'Personal History' and Other Fitness Costs of Migration

With finite energetic resources, the number of intermediate and paratenic hosts used by an individual *A. simplex* can greatly reduce its ability to migrate through tissues (Smith & Hemmingsen, 2003). Although a maximum of three intermediate hosts can be used during its lifetime (Wootton & Smith, 1975), individuals that have a reduced 'personal

history' including fewer intermediate hosts, will have the greatest potential for migration (Smith & Hemmingson, 2003). Furthermore, Young (1972) proposed the presence of an optimal pre-encapsulation migratory distance within host tissues where trade-offs between migratory distances and associated fitness costs leads to an optimal migration range of anisakid larvae. This hypothesis states that high *A. simplex* abundances within muscle tissue are inversely related to the size of the infested fish. However, a number of studies yielded results contradicting this hypothesis (Noguera *et al*., 2009; Larrat *et al*., 2013). The distribution of *A. simplex* is therefore not only governed by a hosts' size (Strømnes & Anderson, 1998), but is more complex potentially involving visceral organ topography (VOT) and hepatotropic behaviour.

Higher *A. simplex* abundances in the left musculature of Atlantic cod in comparison to the right (Rae, 1963) has been supported by McClelland *et al*. (1985) and Brattey & Bishop (1992), resulting in the use of VOT as an explanatory tool of *A. simplex in vivo* migratory route. Before reaching muscle tissue, *Anisakis* sp. larvae must pass through a number of visceral organs and membranes (Fig. 3.3) the spatial arrangement and contiguity of which could have variable fitness costs to an individual depending on the route employed (Smith & Hemmingsen, 2003).

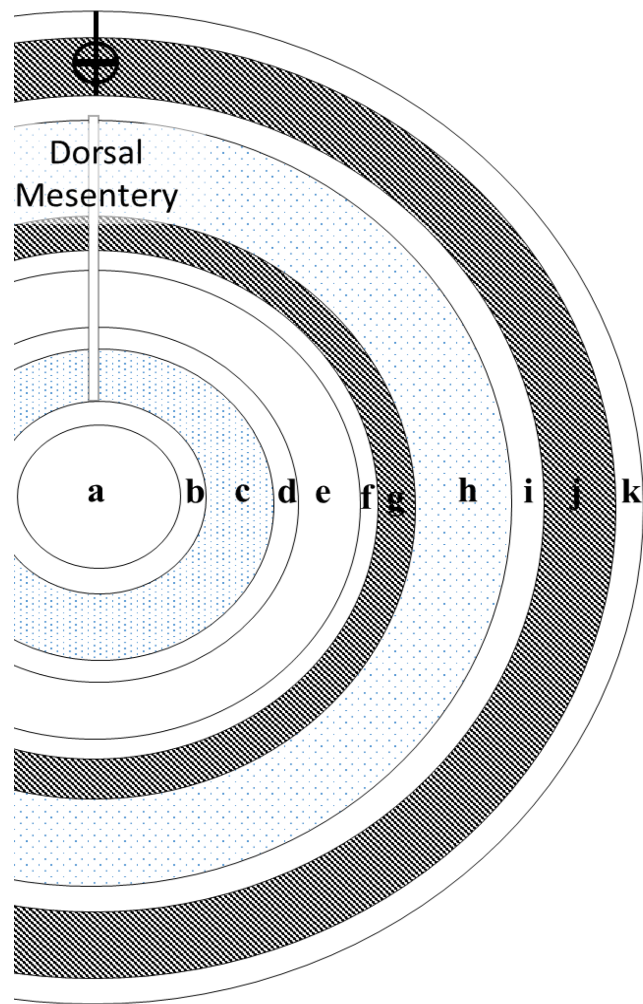

Figure 3.3. Diagrammatic transverse section through the body cavity of a gadoid fish illustrating the various barriers that a "successful" larval ascaridoid (or other macroparasite) has to breach in order to reach the host's body cavity and/or musculature; (*a*) gastro- intestinal tract lumen; (*b*) mucosa; (*c*) gastro-intestinal tract wall; (*d*) serosa; (*e*) body cavity; (*f*) liver capsule; (*g*) liver parenchyma; (*h*) mesenteries and connective tissues; (*i*) parietal peritoneum; (*j*) body musculature; (*k*) skin. (Adapted from Smith & Hemmingsen, 2003)

Although the liver (which commonly lies dorsally and to the right of the gut), might interfere with migrations of larval nematodes from the gut to the hypaxial (or epaxial muscle) on this side of the body (McCllelland, 2002; Smith & Hemmingsen, 2003), the liver is a common site of infestation (Horbowy *et al*., 2016) and is one of the earliest sites

of nematode infestation in post-smolt salmon (Murphy *et al*., 2010). Whether this hepatotropic behaviour is due to the 'soft' tissue being easier to penetrate (Berland, 2006), driven by nutrient availability, or a direct response to chemical cues however, remains uncertain (Bahlool *et al*., 2012).

In addition to being hepatotropic, the high burdens of nematodes found in belly flaps or epaxial muscle (Højgaard 1980; Karasev 1990; Berland, 2006), suggests they may also be lipophilic. Although initial migration is random, L3 *Anisakis* have the ability to detect and reside in high lipid areas short distances away (Strømnes, 2014). Burdens of *Anisakis* and *P. decipiens* larvae in the flesh of cod have been higher in winter time when liver lobes are much smaller (Berland & Hemmingsen, 1991). Whether these migrations are due to the reduction in distance between the liver and muscle, or reduction of lipid content in the liver remain unclear.

There is an increase in use of experimental challenges to explore microhabitat selection and *in vivo* migrations of L3 *A. simplex* within different fish species. For example, differences in *A. simplex* migration times have been demonstrated between juvenile and adult cobia (*Rachycentron canadum* Kaup, 1826) (Shih *et al*., 2010). Microhabitat preferences have also been elucidated between salmonid species (Bahlool *et al*., 2012; Haarder *et al*., 2013). In brown trout, *A. simplex* larvae were found mainly in the body cavity on the external surface around the stomach, but also between the pyloric caeca and intestine, while the majority of larvae found in rainbow trout (*Oncorhynchus mykiss* Walbaum, 1792) were partially or totally inserted between the pyloric caeca (Haarder *et al*., 2013). In Baltic salmon nematodes were dispersed in and on the spleen, head, kidney, liver, swim bladder and musculature (Haarder *et al*., 2013). Reasons for the inter-specific differences in microhabitat selection remain unclear, however, differing immune responses between tissues and species have been proposed as a potential factor (Abollo *et al*., 2001b; Bahlool *et al*., 2012).

This chapter investigated the relationship between the abundance and the distribution of *A. simplex* and other nematode species in Atlantic salmon and assessed its relationship with the severity of Red Vent Syndrome symptoms. This study tested the 'hyper-infestation hypothesis' on a sample size that is more representative than the study by Senos *et al*. (2013), to better understand this relationship. The results provide data on nematode intensities to clarify the current status of infestation in Atlantic salmon in coastal waters of Scotland. Analysis of the location and abundance of nematodes found within the right or left side of muscle tissue, and within the viscera, coupled with a novel infestation challenge, will further clarify *in vivo* migratory behaviour of *A. simplex* in Atlantic salmon. Finally, geographical differences in nematode abundance and intensity between the three Atlantic salmon populations was also assessed.

3.2 Materials and Methods

3.2.1 *Sample Sites*

Between June and September of 2015, a total of 117 Atlantic salmon was sampled from commercial inshore net fisheries from the East (Usan, Angus) (56°40'55.7"N 2°27'02.5"W; n = 57), North (Armadale, Sutherland) (58° 32′ 59.64″ N, 4° 5′ 22.2″ W; n = 26) and West (Sandyhills, Dumfries and Galloway) (54°52'45.7"N 3°43'46.9"W; n = 34) coast of Scotland (Fig. 3.4 & Fig. 3.5). Individuals were sampled haphazardly, however, a number of Atlantic salmon not exhibiting RVS symptoms were selectively chosen for comparison from East (n = 18), North (n = 3), and West (n = 4) sample sites. Samples ranged from 0.75 – 5.25 kg (mean = 1.85 ± 0.68 kg) in ungutted weight and 46.4 – 81 cm (mean = 57.6 ± 5.5 cm) in length. Based on scale analysis (see Chapter 4.2.1), all salmon were 1 sea-winter fish, so called grilse. All fish were euthanised by a concussion blow to the head, labelled, packed in ice at the sampling site and subsequently frozen at -20 °C within a maximum of 6 hours at Edinburgh Napier University.

Figure 3.4. Collection of Atlantic salmon at Douglas Hall Fisheries, Sandyhills, Scotland.

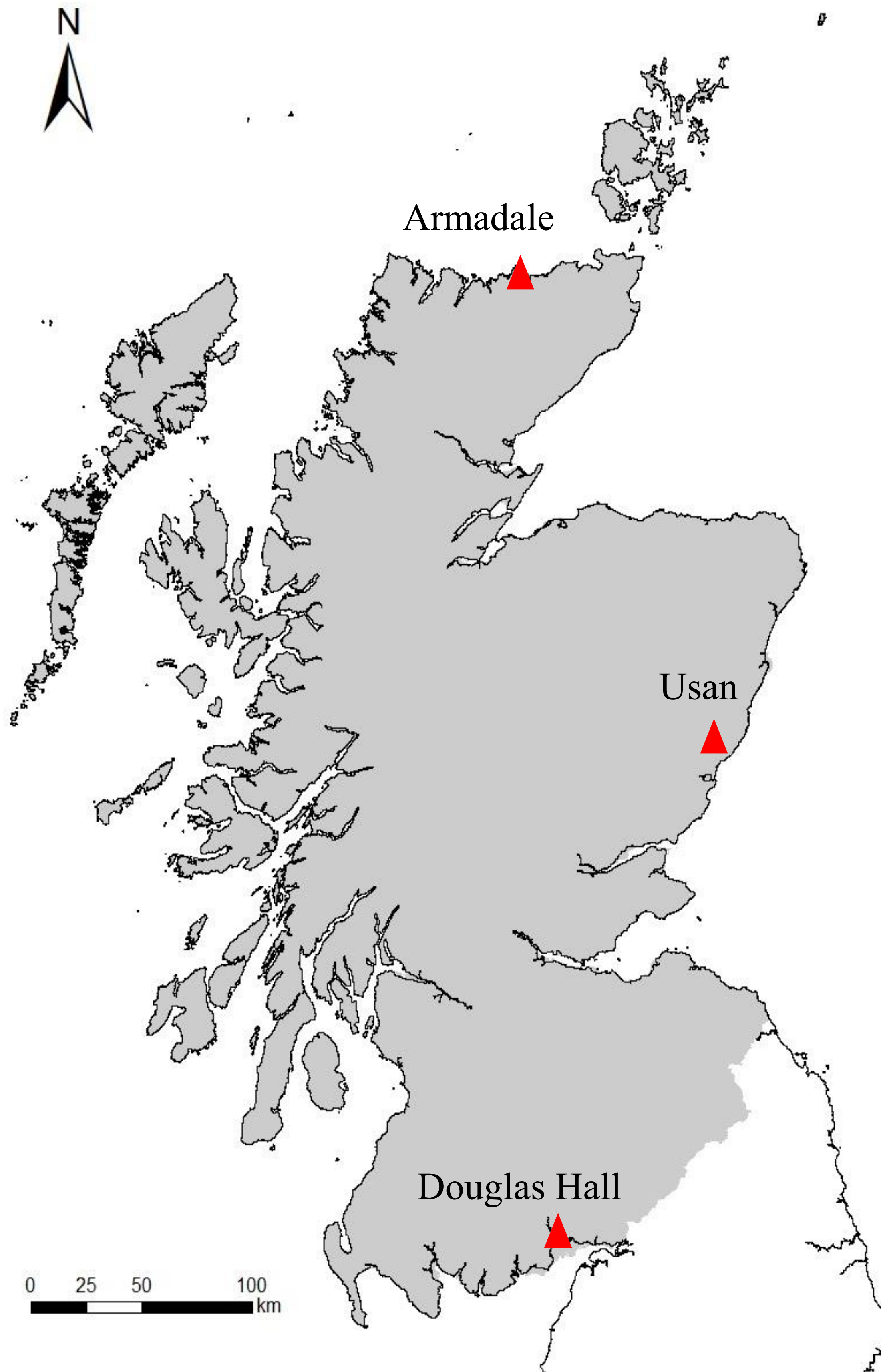

Figure 3.5. Locations of commercial Atlantic salmon fisheries, where samples were obtained in Scotland.

Photographs of the vent region were taken, and RVS severity (Fig. 3.6) was determined following the external observation guidelines provided by the Fisheries Research Services (now Marine Scotland) (FRS, 2008) (Table 3.2).

Table 3.2. Summary of the Fisheries Research Services (now Marine Scotland) external observation guidelines for defining Red Vent Syndrome severity (Adapted from FRS, 2008)

Red Vent Syndrome Severity	Physical Observations
I) Normal	No symptoms
II) Mild	Small red spots (petechial haemorrhage) and reddening around the vent.
III) Moderate	Obvious widespread redness surrounding the vent and initial swelling; haemorrhage, skin erosion and scale loss might be observed.
IV) Severe	Pronounced swelling and obvious erosion; tissue may protrude from the vent and bleeding may occur.

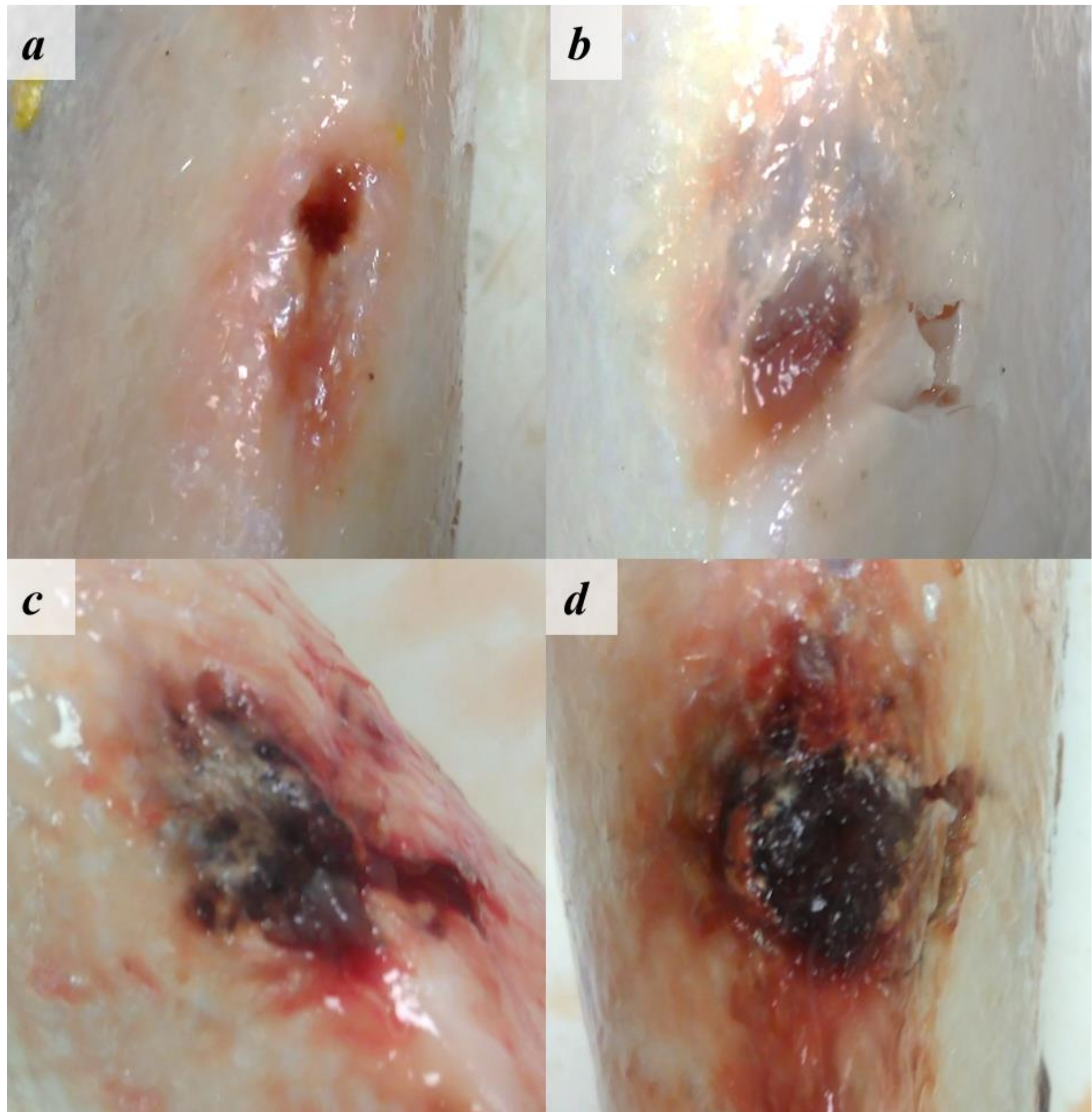

Figure 3.6. Examples of Atlantic salmon exhibiting (*a*) normal; (*b*) mild (*c*) moderate, and (*d*) severe Red Vent Syndrome

3.2.2 Dissection Protocol

Atlantic salmon were thawed overnight at 4 °C. Fish total weight and fork length (anterior tip of the fish to the fork of the caudal fin) were recorded to the nearest 0.5 cm. External examination for ectoparasites was then conducted including the skin, mouth, nasal and gill cavities. Muscle tissue was marked for separation using small incisions using a scalpel into the following portions described in Noguera *et al*. (2015) (Fig. 3.7): Front portions (l and r) were defined from the pectoral fins to the anterior end of the dorsal fin (corresponding to the body portion around the stomach, liver and pyloric caeca). The middle portions (l and r) covered the entire length of the dorsal fin base and the rear portions (l and r) reached from the posterior end of the dorsal fin to the anterior end of the anal area (minus the excised vent region). The peduncle region extended from the anal fin to the base of the caudal fin. The vent region includes the perianal region (the genital cavity and pore, the last portion of the urinary canal and the surrounding tissues of the posterior abdominal wall).

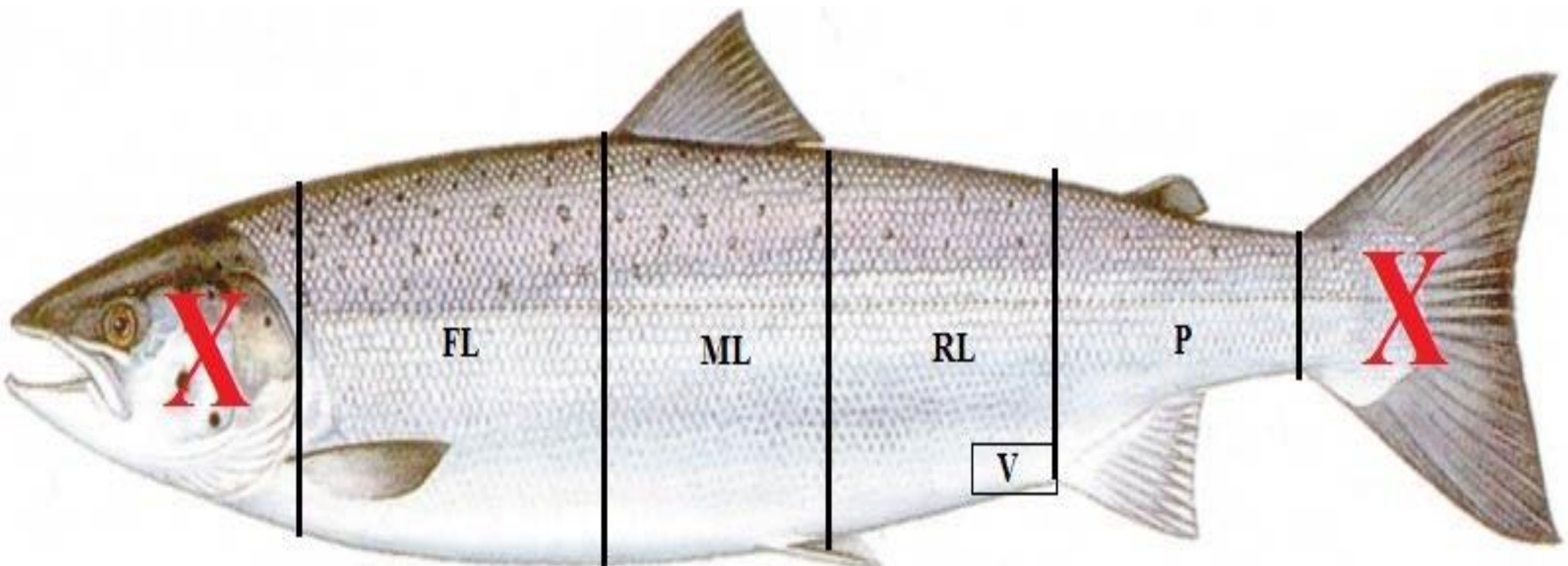

Figure 3.7. Muscle tissue was separated into front (F); middle (M); and rear (R) (left and right) portions, peduncle (P) and vent (V). Head and cadal fin sections (X) were not included in analysis. (Adapted from Noguera *et al*., 2015)

An incision was then made from the vent region up to the heart cavity using a scalpel. Organs were removed from the body cavity, and along with the vent region, the liver weight and gutted weight of the sample were recorded. Viscera weight was calculated using the difference between ungutted, and gutted weights. Mesenteries of organs, and the empty body cavity were examined for parasites. Finally, the eyes, and gill filaments were removed and placed in separate petri dishes containing 0.9% saline solution, as for all other removed internal organs. Muscle portions were separated, and the weight of the skeleton was recorded to enable the calculation of muscle weight. Muscle tissue was processed on a light box. All internal organs were flattened using inverted petri dishes and analysed under a dissecting microscope. Stomach and intestine however, were separated into the content and wall. The intestine was separated into anterior, middle and posterior portions of similar lengths. Every tenth salmon was kept for enzymatic digestion to ascertain levels of accuracy in nematode isolation.

3.2.3 Enzymatic Digestion

The enzymatic digestion method was adapted from Jackson *et al*. (1981) and Noguera *et al*. (2015) and involved the enzyme pepsin recovered from porcine submucosa. Secreted within gastric juices, this enzyme breaks proteins e.g. muscle tissue, down into smaller peptides, but due to their robust nature, individuals of *A. simplex* remain undigested. Carcasses were weighed, and the volume of required digestion fluid was calculated based on 1 L of fluid per 200 g of tissue. The digestion solution comprised of 25 g pepsin powder (Sigma-Aldrich, Irvine, UK) per litre of 0.85% NaCl. The pH of the solution was reduced to 2.0 using 3 N hydrochloric acid. Muscle tissue and digestive solution were placed in three litre buckets, which were incubated at 40 °C and agitated at 66 revolutions per minute (RPM) overnight. The content of each bucket was sieved through 1 mm and 0.5 mm mesh. Nematode larvae were collected from the remains in the mesh and enumerated.

3.2.4 Parasite Isolation and Identification

Nematodes were carefully removed from tissue samples with forceps and placed in a petri dish containing 0.9% saline solution. Samples were fixed in Davidsons' AFA fixative solution (acetic acid (1 part), formaldehyde (2 parts), and alcohol (3 parts)) for 24 hours and finally stored in glycerol-alcohol (glycerol (1 part), 70% EtOH (9 parts)).

Nematodes were cleared in a solution of equal parts 70% EtOH and glycerol until complete evaporation of EtOH had occurred and examined under a microscope. Morphological features including the visceral arrangement of the ventriculus, intestinal caecum and oesophagus (Fig 4.10) spicule features and the presence of lips (Fig 4.11) were used to identify the nematodes following the identification keys provided by Arai & Smith (2016).

3.2.5 Oral Infestation Challenge - Pilot Trial

A pilot infestation trial was run at the Ellis Aquarium at Marine Scotland – Science (Aberdeen, UK) during July-August, 2015 (Fig 3.8a). The aim of this pilot trial was to successfully infest Atlantic salmon, with *A. simplex*. A successfully infested fish is defined as an individual from which one or more live *Anisakis* are recovered free from the body cavity, its tissues (visceral organs) or muscle. If infestation was considered successful, the trial's objective was to investigate microhabitat selection of *A. simplex* within Atlantic salmon.

A total of 66 aquaculture-bred post-smolt Atlantic salmon was purchased from a Marine Harvest freshwater farm at Loch Lochy. Atlantic salmon ranged from 0.36 – 1.04 kg (mean = 0.63 ± 0.13 kg) in ungutted weight and 33 – 47 cm (mean = 39.5 ± 2.8 cm) in length and were subsequently acclimated in sea water (34 ppt) at 10 °C prior to commencement of the experiment. Atlantic salmon were fed daily to 1% body weight using the Skretting Atlantic smolt diet. As Atlantic salmon were fed pasteurised commercial feed pellets and would therefore not be infested with *A. simplex*, no pre-

screening test was carried out. Two weeks prior to the experiment, salmon were randomly allocated into two treatment groups (TG1 and TG2), and one negative control group (NC1) each comprising of 22 salmon and transferred to three separate 1 m diameter flow-through tanks (100 L h^{-1}) at 34 ppt and 10 °C. Salmon were starved 1 week prior to the experimental procedure. *Anisakis simplex* L3 larvae were obtained from the viscera of Atlantic herring from commercial landings at Denholms fisheries Ltd. (Peterhead, Scotland). Larvae were refrigerated in physiological saline until administration (24-48 hrs). Prior to administration, viability of nematodes was assessed under a stereomicroscope. Individuals that were motile and thus had the greater tissue migration potential were allocated to the decapsulated (TG2) treatment group. Encapsulated larvae were allocated to the encapsulated (TG1) treatment group. The procedures involving fish handling were conducted by a trained member of staff at the Ellis Aquarium in accordance with Home Office regulations. Atlantic salmon was immersed into a solution of MS-222 (Sigma-Aldrich, Irvine, UK) at a concentration of 50 - 75 mg/l until sufficient anaesthetic depth had been reached. At this point an oral gavage (Fig. 3.8*b*) (metal rod, flexible polyurethane feeding tube, and rubber stopper removed from a syringe) which was loaded with 20 encapsulated, 20 decapsulated *A. simplex* larvae, or feed pellets, was administered to into the stomach of TG1, TG2 and NC1 fish, respectively. Three fish from each group were exposed to lethal concentrations of MS-222 solution at a concentration 200 mg/l at 2, 7, 14, 21 and 28 days post infestation. All remaining fish were destructively sampled at the terminal sampling time of no more than 35 days post infestation. Following mortality, samples were labelled, bagged and frozen at -20 °C at Marine Scotland-Science. They were transported to Edinburgh Napier University where they underwent dissection following the dissection protocol (Chapter 3.2.2). All aspects of the experiment were carried out in accordance with Home Office Regulations (Project Licence: 70/7897; Personal Licence: IECA2B578).

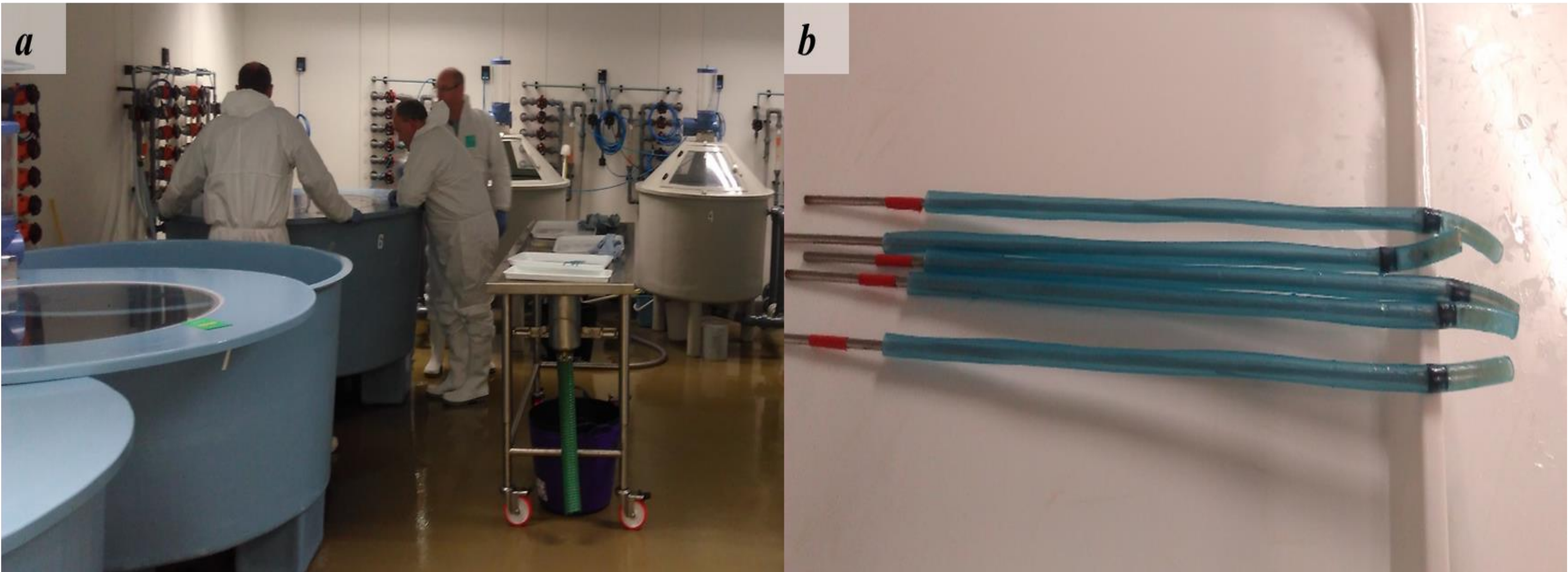

Figure 3.8. (*a*) The Ellis aquarium at Marine Scotland – Science, (*b*) the oral gavage equipment filled with *Anisakis*

3.2.6 Oral Infestation Challenge – Pilot 2

A second pilot challenge was run in recirculation tanks at Marine Scotland-Science (Fig 3.9*a*) in February (2016). A total of 10 aquaculture-bred post-smolt Atlantic salmon were purchased from Marine Harvest at Loch Lochy. Additionally, 5 rainbow trout were purchased from College Mill Trout Farm in Almondbank, Perthshire. Atlantic salmon ranged from 0.42– 0.78 kg (mean ± SD = 0.6 ± 0. 1 kg) in ungutted weight and 35 – 44 cm (mean ± SD = 40.9 ± 2.6 cm) in length, and rainbow trout 0.63 – 0.12 kg (mean ± SD = 0.63 ± 0.12 kg) in ungutted weight and 37.5 – 47.5 cm (mean ± SD = 42.1 ± 3.7 cm) in length. All fish were acclimated in sea water (34 ppt) at 10 °C prior to the commencement of the experiment. Atlantic salmon and rainbow trout were fed daily to 1% body weight using the Skretting Atlantic smolt diet. As both Atlantic salmon and rainbow trout were fed pasteurised commercial feed pellets and would therefore not be infested with *A. simplex*, no pre-screening test was carried out. The design of the experiment included one treatment group (TG1) comprising of five Atlantic salmon (T1), one negative-control group (NC1) comprising five Atlantic salmon (T2) and one positive control (PC1) comprising five rainbow trout (T3). As rainbow trout were successfully infested by *Anisakis* ≥21 days post-challenge in another study (Quiazon *et al*., 2011), their inclusion as a positive control in this experiment was to assess the validity of the experiment and associated procedures. Two weeks prior to the experiment, fish were transferred to 1 m diameter flow-through tanks (100 L h^{-1}) at 34 ppt and 10 °C and were not fed 1 week prior to the experimental procedure. TG1 and PC1 were each challenged by oral gavage with 20 unencapsulated *Anisakis* L3, with NC1 challenged by gavage with feed pellets only. In all treatments oral gavage was administered into the stomach.

Anisakis simplex larvae (L3) were obtained from the viscera of Atlantic mackerel from commercial landings at Denholms Fisheries Ltd. (Peterhead, Scotland). Larvae were transported on ice in physiological saline until administration < 12 hrs after isolation. A

net (mesh size 0.5 mm) was fixed over outflows of all tanks (Fig 3.9*b*) to ensure that regurgitation or egestion of nematodes could be captured and accounted for (Fig 3.9c). All fish from all groups were destructively sampled 21 days post infestation. Unless stated, all procedures followed the protocol within the pilot trial.

3.2.7 Statistical Analysis

Statistical analysis was carried out using Minitab 17 Statistical Software (2010) (Minitab Ltd, Coventry, UK). All data were checked for normality and homogeneity of variances. When assumptions were not met, the data were log10 or log10 (x+1 transformed). General Linear Models were used to test i) differences in prevalence and intensities of nematodes between regions, ii) distribution of nematodes within muscle portions, and iii) nematode intensities between different organs within the viscera. In the cases where the models were significant, Tukey's HSD Post-hoc test was used to determine significant differences and grouping using pairwise comparisons. One-way analysis of variance (ANOVA) was used to test i) the differences between nematode intensities found in the left and right side of the muscle tissue, and ii) differences in nematode intensities per fish, and in the vent, between male and female fish. A Chi-squared test was used to investigate differences in RVS severity between sexes. Regression analysis was used to explore the relationships between i) weight and nematode intensities in the viscera, vent and body (viscera and musculature), ii) nematode intensities in the vent and body (viscera and musculature), vent and viscera, and vent and muscle, and iii) nematode larvae per gram in the vent and body (viscera and musculature), vent and viscera, and vent and muscle. A full exploration of best line fits to the data using linear, quadratic, and cubic terms was performed on a) the whole population and b) each separate coastal population for each regression. Standard error of the regression S and R^2 values were used to assess the curve fitting effectiveness of the different model.

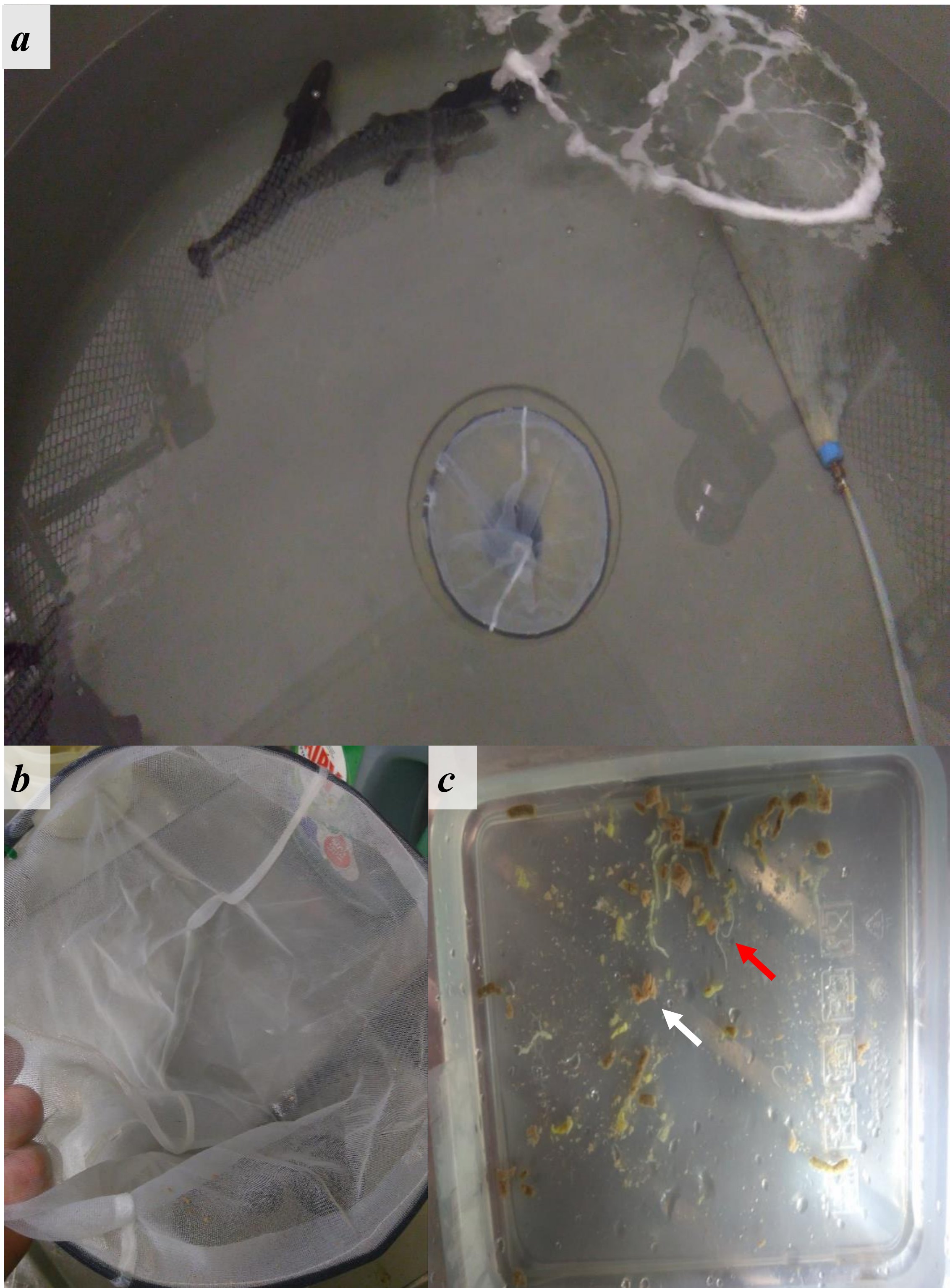

Figure 3.9. (*a*) **A circular tank at Marine Scotland – Science,** (*b*) **the netting system placed on outflow** (*c*) **faecal matter (white arrow) and nematodes (red arrow) collected from outflow nets.**

3.3 Results

A total of 30,925 nematodes were isolated from 117 dissected Atlantic salmon, belonging to three nematode species of the family Anisakidae. Using morphological features detailed in Chapter 4, 24,768 *A. simplex*, 495 *Hysterothylacium aduncum (Rudolphi, 1892)*, and 24 *Pseudoterranova.decipiens* (Krabbe, 1878) were identified. Another 5,638 nematodes were too damaged for identification. Due to a high degree of morphological similarity between sibling species within the *Anisakis simplex* species complex (Mattiucci & Nascetti, 2008), *A. simplex* are identified as *A. simplex sensu latu* (s.l.). No stomach contents were observed in Atlantic salmon following dissection indicating that feeding had not occurred recently.

Anisakis simplex was prevalent in all three salmon populations (100%) however, *H. aduncum* was less prevalent in Atlantic salmon off the East coast of Scotland (59.4%), in comparison to salmon off the North (73.1%) and West coasts (73.5%) (Table 3.3). Although *P. decipiens* was least prevalent in salmon from the North (7.7%), low prevalences were seen in all populations (14.5%). While mean intensities of *A. simplex* were high within all populations (211.7 ± 162) (mean ± SD), they were significantly different between regions ($F=6.94$, $df=2,114$, $p<0.01$). The intensity of *A. simplex* was significantly higher off the North coast (297.2 ± 151.0) (mean ± SD) compared to the East coast (164.6 ± 140.3) (mean ± SD) ($T=3.62$, $df=1,82$, $p<0.01$). Mean intensities of *H. aduncum* were highest in the East (7.9 ± 14.1), compared to West (4.6 ± 4.2) and North (5.9 ± 3.6) (Table 3.3). However, there was no significant difference between regions ($F=0.13$, $df=2,114$, $p=0.875$). *Pseudoterranova decipiens* mean intensities were low across all populations.

Results of the enzymatic digestion of 10 individual salmon to assess nematode enumeration accuracy resulted in a range of 1-7 nematodes missed, at an average of 3.3

± 1.8 (mean ± SD). As only a small number of nematodes were missed in these samples, compared to the overall nematode burden per fish, the following results can be accepted with a high degree of confidence.

Table 3.3. Prevalence (%) and mean intensities (±SD) of the three nematode species *Aniskais simplex*, *Hysterothylacium aduncum* and *Pseudoterranova decipiens* isolated from populations of Atlantic salmon from East (n = 57), West (n = 34) and North (n = 26) coasts of Scotland.

Species		East (n = 57)	West (n = 34)	North (n = 26)	Total (n = 117)
Anisakis simplex	Prevalence (%)	100	100	100	100
	Mean Intensity ± SD	164.6 ± 140.3	225.2 ± 179.1	297.2 ± 151.0	211.7 ± 162.3
Hysterothylacium aduncum	Prevalence (%)	59.6	73.5	73.1	66.7
	Mean Intensity ± SD	7.9 ± 14.1	4.6 ± 4.2	5.9 ± 3.6	6.4 ± 10.2
Pseudoterranova decipiens	Prevalence (%)	17.5	14.7	7.7	14.5
	Mean Intensity ± SD	1.4 ± 0.7	1.6 ± 0.7	1 ± 0.3	1.4 ± 0.6

Anisakis simplex was present throughout the organs of dissected Atlantic salmon, but was most prevalent in the pyloric caeca (99.2%) and muscle (99.2%). The lowest prevalence was seen in the gills (4.3%) although these are likely to be a result of regurgitation. Prevalence of *A. simplex* was therefore lowest on the surface of the gonad (37.6%) and kidney (41%) within the body cavity (Table 3.4). *Anisakis simplex* intensities were especially prominent within the digestive tract including the stomach (42.3 ± 54.8) and pyloric caeca (31.8 ± 27.8) (mean ± SD) (Table 3.4). High infestation intensities were also observed within vent (83.7 ± 58.7) and muscle regions (33.7 ± 35.5). *Hysterothylacium aduncum* was most prevalent in the digestive tract, specifically within the stomach (20.5%), intestine (32.5%) and pyloric caeca (50%) and had the highest mean intensity in the stomach (5.8 ± 7.4). *Hysterothylacium aduncum* was absent from the kidney and gonad (0%). A small number (10) of *H. aduncum* was recovered in the gills

of one salmon (Table 3.4). *Pseudoterranova decipiens* was also most prevalent within the digestive tract, specifically the pyloric caeca (4.3%) and stomach (4.3%). As only 24 *P. decipiens* were isolated from Atlantic salmon, low mean intensities and prevalence rates were observed throughout.

A significant proportion of isolated nematodes (5,638) did not clear during the identification protocol and therefore, could not be identified. Clearing and the observation of the visceral arrangement however, was only necessary to distinguish between *P. decipiens* and *A. simplex*. As there were very few *P. decipiens* isolated from this study, a significant number of these specimens are highly likely to belong to *A. simplex*. In further analyses, the three identified nematode species plus those unidentified were subsequently pooled for the purposes of this chapter.

Table 3.4. Prevalence (%) and mean intensity (±SD) of *Anisakis simplex*, *Hysterothylacium aduncum* and *Psueudoterranova decipiens* per fish within different organs/regions of pooled Atlantic salmon populations (n = 117).

Infestation Site		*Anisakis simplex*	*Hysterothylacium aduncum*	*Pseudoterranova decipiens*
Muscle	Prevalence (%)	99.2	1.7	0
	Mean Intensity ± SD	33.7 ± 35.5	1 ± 0.13	0
Vent	Prevalence (%)	94	2.56	0
	Mean Intensity ± SD	83.7 ± 58.7	1 ± 0.16	0
Mesenteries	Prevalence (%)	75	2.6	1.7
	Mean Intensity ± SD	10.3 ± 11.9	1.7 ± 0.3	1 ± 0.13
Kidney	Prevalence (%)	41	0	0
	Mean Intensity ± SD	2 ± 1.4	0	0
Liver	Prevalence (%)	88.9	0.9	2.6
	Mean Intensity ± SD	5.4 ± 4.8	1 ± 0.1	1 ± 0.16
Spleen	Prevalence (%)	69.2	2.6	0.9
	Mean Intensity ± SD	5.4 ± 6.7	1 ± 0.16	2 ± 0.2
Gonad	Prevalence (%)	37.6	0	0
	Mean Intensity ± SD	1.9 ± 1.3	0	0
Pyloric caeca	Prevalence (%)	99.2	50	4.3
	Mean Intensity ± SD	31.8 ± 27.8	4.4 ± 4.4	1.4 ± 0.3
Stomach	Prevalence (%)	98.3	20.5	0.9
	Mean Intensity ± SD	42.3 ± 54.8	5.8 ± 7.4	1 ± 0.1
Intestine	Prevalence (%)	94	32.5	4.3
	Mean Intensity ± SD	9.2 ± 11.5	2 ± 1.3	1.8 ± 0.4
Gills	Prevalence (%)	4.3	0.9	0
	Mean Intensity ± SD	1 ± 0.2	10 ± 1	0

3.3.1 Nematode Intensities within Atlantic salmon

Mean infestation intensities per fish were the largest in Atlantic salmon sampled from the North coast of Scotland (371.1 ± 189.2) (mean ± SD). Similarly, Atlantic salmon off the North coast also exhibited the highest mean intensities within the viscera, vent and muscle (173.8 ± 113; 137.8 ± 67.7; 59.4 ± 48.8). The East coast population had the lowest mean intensities per fish (207.8 ± 177.2), in the viscera (99.7 ± 93.9), muscle (37.8 ± 42.1) and vent (70.7 ± 61.5) (Table 3.5). High degrees of variation in infestation intensities however, were observed within samples resulting in large standard deviation values. Nematode intensity per fish showed a significant relationship with both length ($F=4.56$, $df = 1,115$, $p<0.05$) and weight ($F=5.49$, $df =1,115$, $p<0.05$) of Atlantic salmon. Furthermore, nematode intensities in the vent ($F=7.10$, $df =1,115$, $p<0.05$) and the viscera ($F=4.52$, $df =1,115$, $p<0.05$) showed significant relationships with ungutted fish weight. Ungutted fish weight however, showed no significant relationship with nematode intensities in the body (viscera and musculature combined) ($F=0.91$, $df =1,115$, $p=0.342$).

Female salmon showed significantly higher nematode intensities in the vent in comparison to males ($F=5.32$, $df =1,115$, $p<0.05$). However, nematode intensities per fish showed no significant difference between sex ($F=2.85$, $df=1, 115$, $p=0.094$) (Fig 3.10). No significant difference in RVS severity was also observed between sexes (X^2 (3, n = 117) =6.691, $p=0.082$).

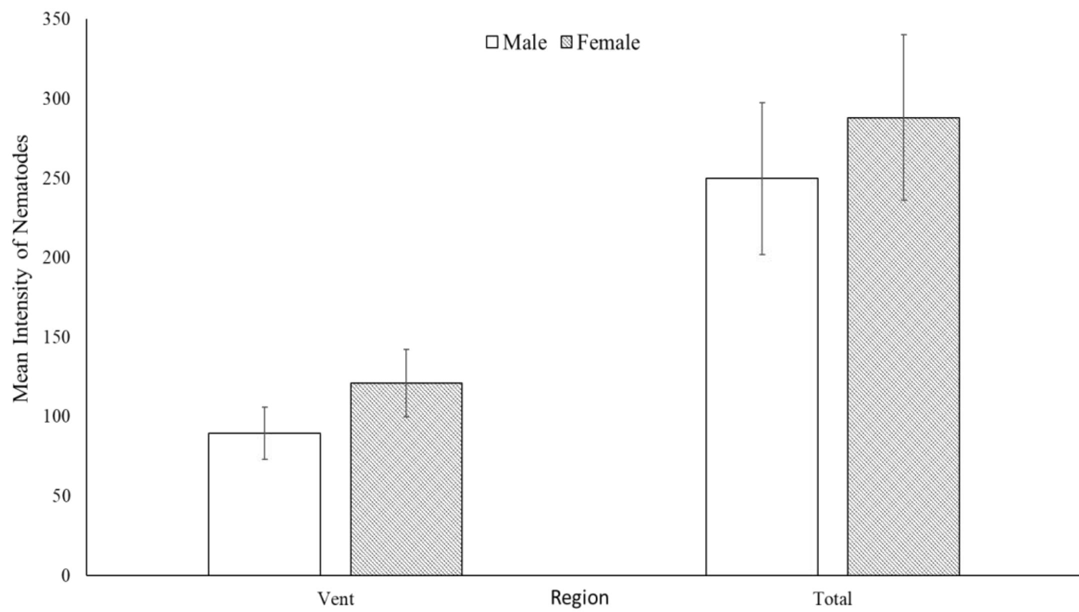

Figure 3.10. Mean intensity of nematodes isolated from the vent region, and per fish, in male (*n* = 72) and female (*n* = 45) Atlantic salmon. (Error bars: 95% confidence intervals).

Table 3.5. Mean intensity (± SD) and percentage (%) of total nematodes isolated from the vent, viscera, muscle and body (viscera and musculature) of Atlantic salmon from the East (n = 57), West (n = 34) and North (n = 26) coasts of Scotland.

Population	Nematodes		Vent		Viscera		Muscle		Body (Viscera and Muscle)
	Total Number	Mean Intensity/per fish ± SD	Mean Intensity ± SD	Percentage of total nematodes (%)	Mean Intensity ± SD	Percentage of total nematodes (%)	Mean Intensity ± SD	Percentage of total nematodes (%)	Mean Intensity ± SD
East Coast (n = 57)	11846	207.8 ± 177.2	79.3 ± 61.6	34.2	99.7 ± 93.9	47.9	37.8 ± 42.1	17.9	136.6 ± 134.0
West Coast (n = 34)	9431	277.4 ± 204.7	125.1 ± 74.7	45.1	121.7 ± 136	43.9	30.6 ± 37.7	11	152.2 ± 169.0
North Coast (n = 26)	9648	371.1 ± 189.2	137.8 ± 67.7	37.1	173.8 ± 113	46.8	59.4 ± 48.8	16.1	233.2 ± 157.8
Pooled Populations (n = 117)	30925	264.3 ± 196.9	107 ± 73	38.4	122.6 ± 1154.5	46.4	40.5 ± 43.4	15.2	162.6 ± 153.8

3.3.2 Nematode Distribution within Atlantic salmon

Nematode distribution throughout muscle portions revealed that the front portion of muscle had the highest prevalence in both left (94%) and right sides (92.3%) (Table 3.6). Ranked by prevalence, both sides showed higher prevalences in front > middle > rear > peduncle proportions. Mean intensities however, revealed a different trend. Although muscle portions on the right hand side showed the same trend as the prevalence, mean intensities on the left hand side were ranked as middle > front > rear > peduncle. Results of the General Linear Model assessing the factor of location on the number of nematodes were significant ($F=21.26$, $df=7, 928$, $p<0.001$). Post hoc pairwise comparisons resulted in significant differences between peduncle and other portions. Furthermore, the mean nematode intensity of the middle left portion was significantly higher than that of the middle and rear portions on the right. In total, nematodes had a prevalence of 100% within each side of muscle, however, mean intensities were slightly higher within the left side (23.2 ± 26.5) than right (17.7 ± 19.2). The distribution of nematodes within the left or right side of muscle tissue showed no significant difference ($F=3.55$, $df=1, 232$, $p=0.061$). The total number of nematodes isolated from the muscle tissue was not related to length ($F=0.08$, $df=1, 115$, $p=0.774$) or weight ($F=0.08$, $df=1, 115$, $p=0.79$) of the salmon host.

Table 3.6. Prevalence and mean intensity (± SD) per fish of nematodes isolated from left and right front, middle, rear and peduncle muscle portions of Atlantic salmon (n = 117).

	Left					Right				
	Front	**Middle**	**Rear**	**Peduncle**	**Total**	**Front**	**Middle**	**Rear**	**Peduncle**	**Total**
Prevalence (%)	94	88	77.8	18.8	100	92.3	82	80.3	7.7	100
Mean Intensity ±SD	8.1 ± 8.6	10.9 ± 12.9	7 ± 8.1	1.7 ± 1.1	23.2 ± 26.5	7.2 ± 8.8	6.7 ± 6.8	6.4 ± 7.3	1 ± 0.27	17.7 ± 19.2

Table 3.7. Summary of P-values from Post-hoc Tukey pairwise comparisons between nematode intensities found within left and right portions of Atlantic salmon muscle tissue

Muscle Portions	Front Right	Middle Right	Rear Right	Peduncle Right
Front Left	0.971	0.393	0.193	**<0.001**
Middle Left	0.073	**<0.01**	**<0.001**	**<0.001**
Rear Left	0.947	1.000	1.000	**<0.001**
Peduncle Left	**<0.001**	**<0.001**	**<0.001**	1.000

Observed nematode distribution within Atlantic salmon revealed that the vent region (*n* = 112) exhibited the highest mean intensity of nematode infestation (Fig. 3.11). On average, 107.1 ± 73 (mean intensity ± SD) nematodes were found in this region. Other high intensity infestation sites were primarily throughout the digestive tract, particularly on the external surface of the pyloric caeca (40 ± 34.6; *n* =117) and encapsulated externally on the stomach wall (48.9 ± 64.8; *n*=116). The liver however, exhibited low mean intensities (6.1 ± 5.6; *n*=109). There were significant differences (F=56.67, df =7, 1160, p<0.005) in nematode distribution between organs/regions of Atlantic salmon

hosts. The results of the Post-hoc Tukey test showed significant differences in mean intensities between the vent, the stomach wall, pyloric caeca and muscle, and the other organs analysed.

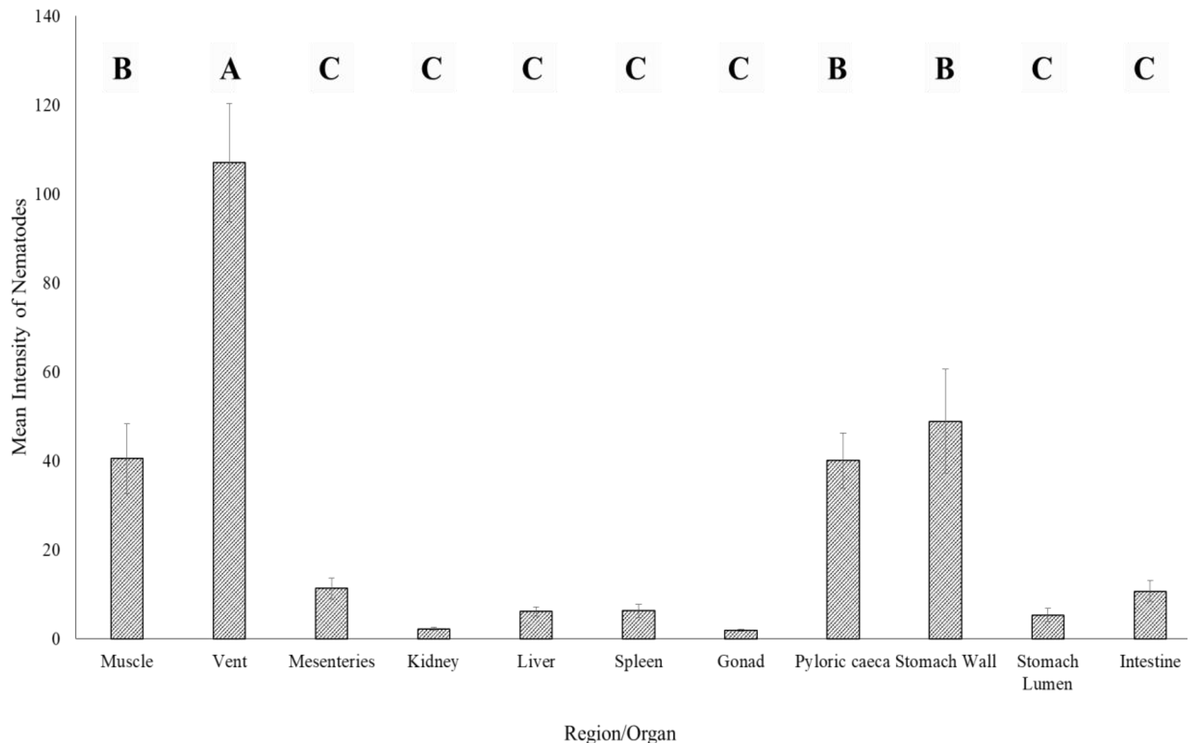

Figure 3.11. Mean nematode intensities per fish isolated from different organs/regions in Atlantic salmon (n = 117). Same letters (A-C) indicate no statistically significant difference (Tukey test p>0.05). (Error bars: 95% confidence intervals)

3.3.3 The Relationship between Nematode Intensities and Larvae Per Gram in the Muscle, Viscera, Body (Viscera and Musculature), and Vent Region of Atlantic salmon.

The comparison of nematode intensities within the vent and the body (viscera and musculature) revealed that the population of Atlantic salmon off the North coast of Scotland had the highest mean infestation intensities in the vent (137.8 ± 67.7) (mean ± SD) and in the body (233.2 ± 157.8). In comparison, populations of Atlantic salmon off the East and West coasts, showed lower intensities of infestation in both the vent (79.3 ± 61.6; 125.1 ± 74.7) and body (136.6 ± 134.0; 152.2 ± 169.0) (Table 3.5). Regression analysis of nematode intensities found exclusively within the vent, and in the rest of the

body of individuals resulted in a significant relationship for pooled data of all three Atlantic salmon populations (F=4.83, df =1,115, p<0.05) (Fig 3.12) No significant relationships however, were observed when salmon was segregated into populations off the East (F=3.80, df =1,55, p=0.057), North (F=1.92, df =1,24, p=0.180) and West coasts (F=1.36, df =1,32, p=0.253) of Scotland. Furthermore, no significant relationships were observed between nematode intensities in the vent and muscle (F=0.80, df =1,115, p=0.373), and the vent and viscera (F=0.64, df =1,115, p=0.420).

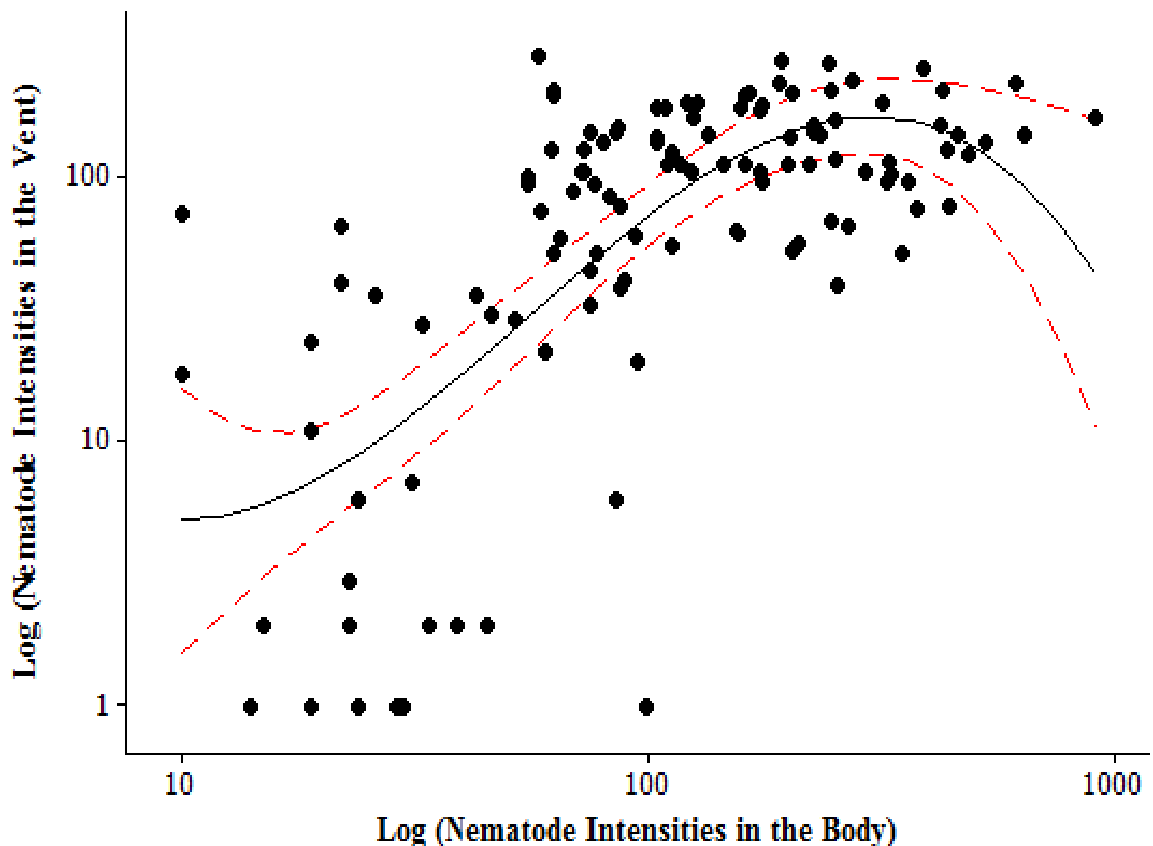

Figure 3.12. Relationship between nematode intensities found within the vent, and the body (viscera and musculature) of pooled Atlantic salmon populations (n = 117) of Scotland. Area between the dotted red line represents the 95% confidence interval for the fitted polynomial curve.

Observed nematode larvae per gram in the muscle (0.06 ± 0.06) (mean ± SD), viscera (1.37 ± 0.87) and vent (15.69 ± 7.50) was highest in Atlantic salmon sampled from the North coast of Scotland (Table 3.8). Lowest larvae per gram in the muscle (0.02 ± 0.03) and viscera (0.78 ± 0.86) was observed in Atlantic salmon sampled from the West coast. With an average of 10.22 ± 8.05 however, Atlantic salmon sampled on the East coast had the lowest larvae per gram in the vent region. Larvae per gram in the body (viscera and musculature) was highest in the West population (0.37 ± 0.74), and lowest in Atlantic salmon from the North (0.20 ± 0.15) (Table 3.8).

Table 3.8. Average nematode larvae per gram (±SD) of muscle, viscera, vent, and body (viscera and musculature) tissue in Atlantic salmon sampled from the East (n = 57), West (n = 34), North (n = 26) coasts of Scotland, and as a pooled population (n = 117).

Population	Nematode Larvae Per Gram (mean ± SD)			
	Muscle	Viscera	Vent	Body (Viscera and Musculature)
East (n = 57)	0.05 ± 0.06	1.02 ± 1.04	10.22 ± 8.05	0.31 ± 0.61
West (n = 34)	0.02 ± 0.03	0.78 ± 0.86	14.89 ± 9.92	0.37 ± 0.74
North (n = 26)	0.06 ± 0.06	1.37 ± 0.87	15.69 ± 7.50	0.20 ± 0.15
Total (n = 117)	0.04 ± 0.06	1.03 ± 0.97	12.86 ± 8.78	0.30 ± 0.59

The relationship between the number of nematode larvae per gram in the body (viscera and musculature), and in the vent was not significant ($F=2.78$, $df = 1, 115$, $p=0.099$) as a pooled population (Fig 3.13). Similarly, no significant relationship was observed when Atlantic salmon were segregated into East ($F=0.51$, $df = 1,55$, $p=0.476$), North ($F=1.98$, $df = 1,24$, $p=0.173$) and West coast populations ($F=2.71$, $df = 1,32$, $p=0.111$). Significant

relationships however, were observed between nematode larvae per gram in the vent and muscle ($F=7.33$, $df =1,115$, $p<0.05$), the vent and viscera ($F=5.85$, $df =1,115$, $p<0.05$). There was no significant relationship between nematode larvae per gram in the vent and the body (viscera and musculature) ($F=2.78$, $df =1,115$, $p=0.099$).

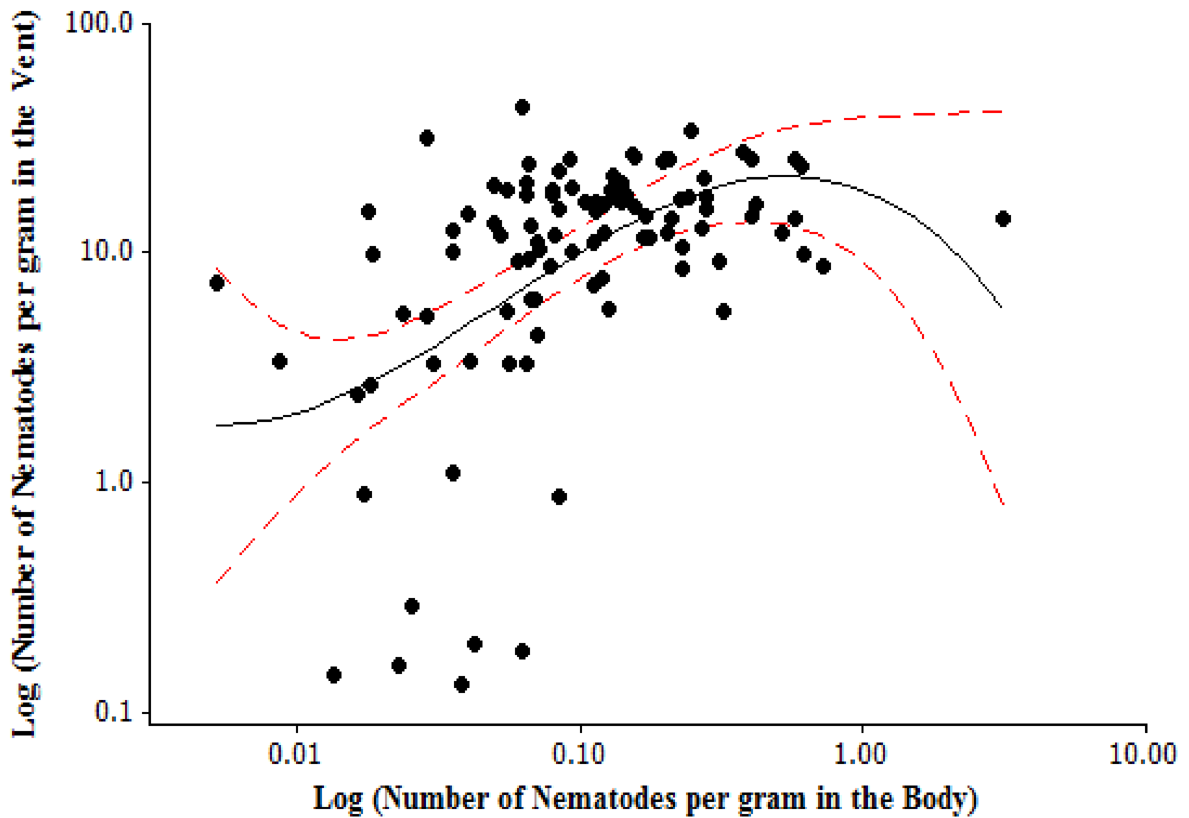

Figure 3.13. Relationship between the number of nematodes per gram found in the vent, and the body (viscera and musculature) of pooled Atlantic salmon populations (n = 117) of Scotland. Area between the dotted red line represents the 95% confidence interval for the fitted polynomial curve.

3.3.4 The Relationship between Nematode Intensities in the Body (Viscera and Musculature), and Vent Region of Atlantic salmon in Relation to RVS Severity

Nematode intensities within the vent differed significantly between the four RVS severities outlined by the fisheries research services (FRS, 2008) (Fig 3.14, Table 3.9). Nematode intensities within the body however, only showed significant differences between salmon without symptoms, and those with moderate and severe severities (Fig 3.14; Table 3.9).

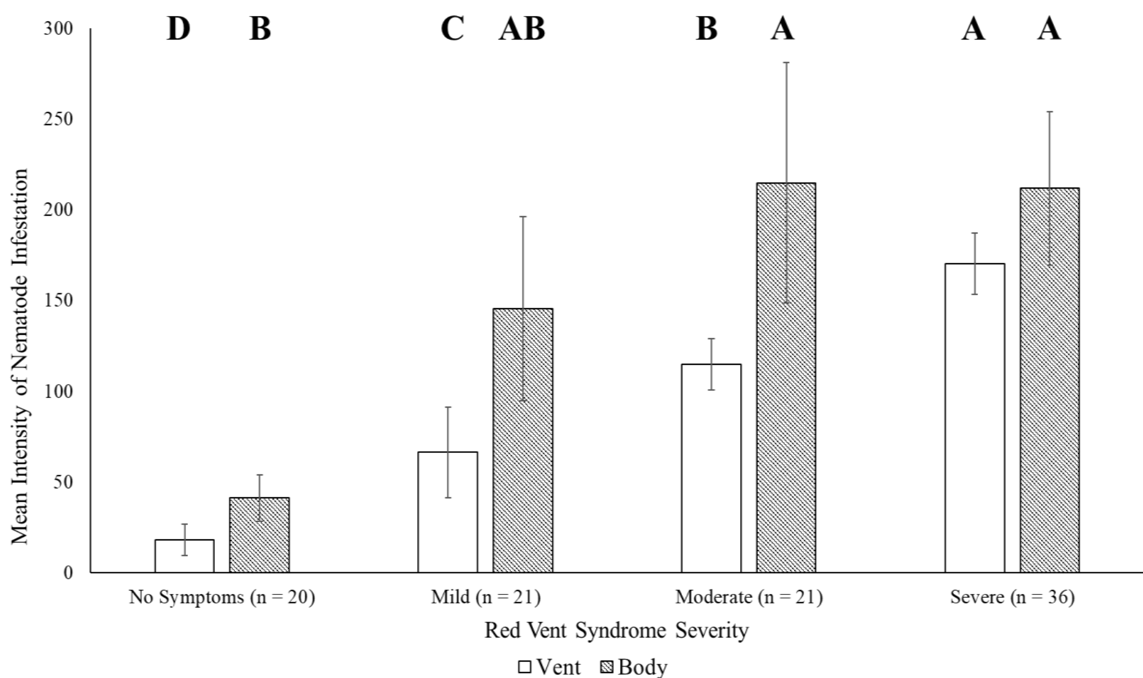

Figure 3.14. Mean nematode intensities per fish isolated from in the body (excl vent), and the vent region of Atlantic salmon within no (n = 20), mild (n = 21), moderate (n = 21) and severe (n = 36) symptoms of Red Vent Syndrome. The same letters between vent (A-D) and body (A-C) regions are not statistically significant (Tukey test p >0.05). (Error bars: 95% confidence intervals).

Table 3.9. Summary of P-values from Post hoc Tukey comparison analysing nematode intensities within the body (viscera and musculature), and separately within the vent per fish, in relation to RVS severity

Red Vent Syndrome Severity Group Comparison	Adjusted P-value (Body)	Adjusted P-value (Vent)
Mild - No symptoms	0.056	**<0.01**
Moderate - No symptoms	**<0.001**	**<0.001**
Severe - No symptoms	**<0.001**	**<0.001**
Moderate - Mild	0.338	**<0.001**
Severe - Mild	0.315	**<0.001**
Severe - Moderate	1.000	**<0.001**

Analysis of larvae per gram per fish (ungutted weight) correlated strongly with increasing RVS severity ($df= 3, 114$; $F= 105.03$; $p<0.001$). The total number of isolated larvae per gram per fish increased from 0.041 ± 0.034 without RVS, to 0.20 ± 0.09 with severe symptoms (Table 3.10). Within the vent tissue an even more pronounced increase in larvae per gram was observed ($df =3, 102$; $F= 164.46$; $p<0.001$) ranging from 4.1 ± 5.6 without RVS to 20.8 ± 7.3 with severe RVS. Nematode intensities in the viscera followed this trend ($df=3, 114$; $F= 55.52$; $p<0.001$) with 0.33 ± 0.31 to 1.29 ± 0.91 between no and severe RVS classifications. Larvae per gram in muscle tissue strongly correlated with RVS intensities ($df=3, 110$; $F= 24.17$; $p<0.001$). More specifically, between no and moderate severities, increasing intensities were observed from 0.015 ± 0.013 to 0.061 ± 0.071, but intensities decreased to 0.045 ± 0.044 in the severe classification.

Table 3.10. Average nematode larvae per gram (±SD) of muscle, viscera and vent tissues, and total per fish in Atlantic salmon exhibiting no (n = 26), mild (n = 21), moderate (n = 34), and severe (n = 36) symptoms of Red Vent Syndrome

Red Vent Syndrome Severity	Larvae/g			
	Muscle ± SD	**Viscera ± SD**	**Vent ± SD**	**Total Per Fish ± SD**
No Symptoms (n = 26)	0.015 ± 0.014	0.33 ± 0.31	4.1 ± 5.6	0.04 ± 0.03
Mild (n = 21)	0.045 ± 0.065	1.00 ± 0.89	8.9 ± 7.0	0.14 ± 0.11
Moderate (n = 34)	0.061 ± 0.072	1.28 ± 1.15	13.7 ± 5.0	0.18 ± 0.12
Severe (n = 36)	0.045 ± 0.044	1.29 ± 0.91	20.8 ± 7.3	0.20 ± 0.09

3.3.5 Oral Infestation Challenges

Of the 880 nematodes administered orally to 66 Atlantic salmon in the pilot experiment, only four nematodes were recovered from three different salmon. Of those four nematodes three were recovered from the intestine of fish in the encapsulated treatment, and one from the pyloric caeca of fish in the decapsulated treatment.

Of a total of 200 administered *A. simplex* to 15 fishes, the second pilot challenge resulted in two *A. simplex* being recovered from the stomach of one rainbow trout (one alive, one dead), and one dead *A. simplex* recovered from the kidney of one Atlantic salmon. A total of 29 and 15 nematodes were recovered from the nets covering the outflow pipe of the tanks of the rainbow trout and salmon treatments respectively (Fig 3.15). Although the nets were checked until the termination of the trial at 21 days post infestation, these numbers were reached after five and 16 days post infestation, within the salmon and trout treatments respectively (Fig 3.15). All nematodes recovered from outflow pipes were dead.

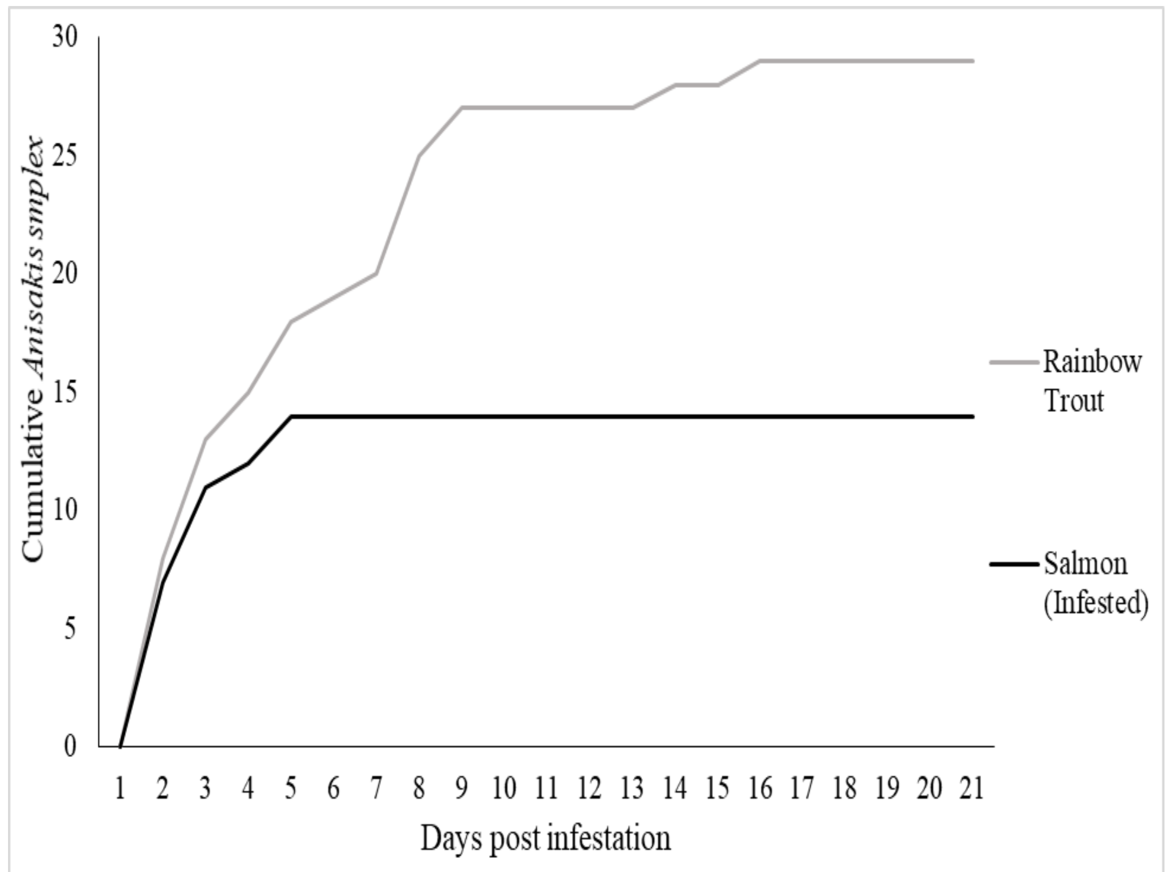

Figure 3.15. Cumulative recovery of *Anisakis simplex* L3 larvae from nets positioned on the outflow pipe of the tanks with orally infested Atlantic salmon, and Rainbow Trout.

3.4 Discussion

Parasitism of Atlantic salmon by *A. simplex* has been reported for over 100 years (Carmichael, 1863), and most infestations have been observed within the musculature and viscera (Strømnes & Andersen, 1998). Coupled with the emergence of RVS in 2005, large numbers of *A. simplex* larvae (L3) have been found in the vent region of Atlantic salmon (Beck *et al*., 2008; Noguera *et al*., 2009; Larrat *et al*., 2013). Although the reason behind this novel site of infestation remains unclear, one hypothesis has suggested that increasing *A. simplex* intensities within the body of Atlantic salmon, is leading to the infestation of the vent region (Senos *et al*., 2013). In this chapter, prevalence, intensity and distribution of nematodes isolated from 117 dissected 1SW Atlantic salmon are presented. The relationship between nematode burdens found in the body and the vent region of Atlantic salmon is also clarified.

Three nematode species, *A. simplex*, *H. aduncum* and *P. decipiens* were isolated from 117 Atlantic salmon with prevalence rates of 100%, 66.7% and 14.5% respectively. Of a total of 30,925 isolated nematodes, 80% were identified as *A. simplex* demonstrating its high prevalence within the North Atlantic and North Sea (Mattiucci & Nascetti, 2008). Only 24 *P. decipiens* were isolated from Atlantic salmon populations within this study. Differences in the life cycle of *P. decipiens* and *A. simplex* are likely to be the cause of these significant differences of intensity within Atlantic salmon. Whilst the second-stage larvae of *A. simplex* possess a cocoon-like cuticle which increases buoyancy of third-stage larvae enabling migration within the water column (Køie, 1995), *P. decipiens* are more restricted to a benthic life cycle (Køie *et al*., 1995; McClelland 2002). Embryonated eggs of *P. decipiens* are passed in seal faeces and settle on the sea bed where they complete development to the third stage larvae (L3) and hatch (Køie, 1995). Newly hatched larvae are still ensheathed in the cuticle of the previous second larval stage (L2),

which is attached to the substrate caudally (Køie *et al.*, 1995; McClelland, 2002). The subsequent ingestion by benthic crustaceans (e.g. amphipods, gammarids, and isopods) serves to enhance transmission to a large variety of benthic macro-invertebrates as second intermediate hosts (Klimpel & Palm, 2011). Atlantic salmon spend over 83% of their time feeding within the upper 10 meters of the water column (Mills, 2003; Strøm *et al.*, 2018). Transmission of *A. simplex* to Atlantic salmon is therefore much more likely, while *P. decipiens* is more abundant in demersal species such as Sculpins (*Myoxocephalus scorpius L.*) (Jensen *et al.*, 1994; Køie, 1995).

High degrees of intra-specific variation of nematode infestation intensity, demonstrated by high standard deviation values, were found within all salmon populations. Intra-specific variability of nematode intensity within host populations is known for Atlantic mackerel and saithe (Priebe *et al.*, 1991; Adroher *et al.*, 1996; Levsen & Midthun, 2007) as well as other species (Manfredi *et al.*, 2000). Variability in the immune response by the host is considered a major factor contributing to those inter- and intra-population differences in parasitic infestations (Bahlool *et al.*, 2012). Saithe for example possess a specific age-related immune response, to reduce infestation immunologically (Priebe *et al.*, 1991; Levsen & Midthun, 2007). Saithe over 5 years old have the ability to produce larger quantities of an antibody in response to an excretory-secretory *Anisakis* sp. antigen resulting in lower abundances and intensities in comparison to younger saithe (Priebe *et al.*, 1991). Intraspecific differences in susceptibility and immunocompetence have also been observed in both rainbow trout and Atlantic salmon in response to *Gyrodactylus salaris* (Malmberg, 1957) infestation (Bakke, 1991; Bakke *et al.*, 1991). Based on parasite survival and infestation pattern, marked heterogeneity in juvenile trout led to the identification of three groups: i) hosts receptive to initial parasite attachment, but unreceptive to parasite establishment and reproduction; (ii) hosts moderately susceptible to parasite establishment and reproduction, but which, after a period of restricted parasite

population growth, responded, recovered and eliminated the parasites; and (iii) hosts very susceptible to parasite infection and reproduction, but which, after a period of significant parasite population growth, responded, recovered and eliminated the parasites. Similarly, only <10% of experimentally infested Norwegian Atlantic salmon possessed the ability to mount an immune response against *Gyrodactylus salaris* (Malmberg, 1957) (Bakke, 1991).

Differences of prevalence and intensity between populations were observed for *H. aduncum* which was less prevalent in Atlantic salmon off the East coast of Scotland in comparison to West and North coast populations. *Hysterothylacium aduncum* exhibits low host specificity and is widespread in the North Atlantic and North Sea (Palm *et al*., 1999, Klimpel *et al*., 2001). Hydrographic conditions such as fronts and and increased primary and secondary production caused by stratified waters have been attributed to differences in prevalence of *H. aduncum* within the North Sea (Klimpel & Rückert, 2005). Similar factors are likely to have contributed to differences in *A. simplex* infestation intensity, which was higher in Atlantic salmon off the North coast in comparison to the East and West coasts.

Abundances of *A. simplex* at local and regional scales are controlled by a number of abiotic and biotic factors akin to *H. aduncum* (Kuhn *et al*., 2016). Geographic differences in infestation intensity of *A. simplex* are well documented (Konishi & Sakurai, 2002). Parasitic nematodes such as *A. simplex* and *H. aduncum* are transmitted through ingestion of prey (Klimpel & Rückert, 2005; Audicana & Kennedy, 2008). Therefore, the availability and species of potential dietary inputs is often key to prevailing infestation intensities within intermediate hosts such as Atlantic salmon. Stratified waters result in increased abundances of suitable hosts for *H. aduncum* (hyperiids) in the North Sea (Klimpel & Rückert, 2005). Subsequently, high infestation intensities have been observed

in haddock and whiting (Klimpel & Rückert, 2005). Similarly, geographical infestation differences with *A. simplex* have been observed in grey gurnard in the North Sea (Levsen & Karl, 2014), and Atlantic salmon in the North West Atlantic (Dufour *et al*., 2010).

Variations in local abundances of the lesser sandeel (*Ammodytes marinus,* Raitt, 1934) (Levsen & Karl, 2014), and capelin (Dufour *et al*., 2010; Mouritsen *et al*., 2010) in the North Sea and North West Atlantic respectively, were correlated with increased infestation intensities of *A. simplex* within grey gurnard (Levsen & Karl, 2014), Greenland cod (*Gadus ogac* Richardson, 1836) (Mouritsen *et al*., 2010) and Atlantic salmon (Larrat *et al*., 2013). However, reasons for observed infestation differences in Atlantic salmon populations remain unclear. A number of tagging studies have resulted in the hypothesis that the majority of European Atlantic salmon populations migrate to the Norwegian Sea and wider Arctic Sea (Holst, 2011). Feeding during the spawning migration is restricted to opportunistic feeding (Kjellman, 2015), and there is little dietary input during the final leg of this migration (Kjellman, 2015). Therefore, differences in infestation intensity must be derived from feeding grounds. Intra-population variation in horizontal migration of Atlantic salmon in Norway north of the Arctic Circle have already been observed (Strøm *et al*., 2018). Furthermore, proportions of arachidonic acid (ARA; 20:4*n*-6) in total lipid of spawning Baltic salmon show significant negative correlations with feeding grounds in the Baltic Sea basin (Torniainen *et al*., 2017). Further investigation using stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ values therefore, will provide additional clarification on differences in diet and feeding grounds (Chapter 4).

With a contribution of 97.9% to all identifiable nematodes, it is perhaps unsurprising that *A. simplex* was present throughout the entire body of Atlantic salmon. *Hysterothylacium aduncum* however, was mainly present within the digestive tract corroborating with similar studies (Bristow *et al*., 1996; Navone *et al*., 1998). The last two moulting stages

of *H. aduncum* usually occur in the digestive tract of their definitive host species (usually Gadidae) (Navone *et al*., 1998). Thus, the observation of *H. aduncum* exclusively in/on visceral organs (Levsen & Karl, 2014) is not unexpected. A small number (5) of *H. aduncum* was found in the muscle and vent region. However, the inability of these nematodes to migrate into the fish hosts' body musculature (Levsen & Karl, 2014) means that *H. aduncum* found in this region could be an artifact. These nematodes could have been misplaced from the body cavity or intestine potentially during the dissection process or regurgitation, respectively (Staniland *et al*., 2001). The present study observed lower prevalence rates of *H. aduncum* in Atlantic salmon than a similar study in Tanafjorden, Norway where 95% of Atlantic salmon was infested (Bristow *et al*., 1996). The higher prevalence rates recorded in northern Norway however, may be a result of higher abundances of potential definitive hosts such as the Norwegian pollock (*Theragra finnmarchica* Koefoed, 1956) (Privalikhin & Norvillo, 2010) in this region, in comparison to coastal waters surrounding Scotland.

In the present study, there was no significant difference in nematode infestation intensity per fish between male and female Atlantic salmon. Females however, did have significantly higher nematode infestation intensitites in the vent in comparison to males ($p<0.05$). Although this didn't result in observed differences in susceptibility to RVS aetiology, the observation of higher nematode intensities in females is not uncommon and has been observed in hake (*Merluccius gayi Guichenot, 1848)* (Carvajal & Cattan, 1985) and Atlantic horse mackerel (*Trachurus trachurus L.)* (Eissa *et al*., 2018). Sexual differences in *Anisakis* spp prevalence and intensitiy have been associated with hormonal changes (Kubokawa *et al*., 2001). Mature spawning females exhibit significantly higher levels of stress hormones (corticosterols) hich can act as an immunosuppressant (Kubokawa *et al*., 2001) in comparison to males leaving them susceptible to parasitic infestation (Eissa *et al*., 2018).

3.4.1 Nematode Distribution within Muscle Tissue

Overall, a total of 4,698 nematodes were recovered from muscle tissue portions (excl vent) of Atlantic salmon. This total accounted for 15.2% of the total nematodes recovered from all samples. This result falls within the observed ranges seen in Atlantic salmon sampled in Norwegian rivers (25.4%) (Senos *et al*., 2013), and Atlantic salmon sampled off the North coast of Scotland in 2009 (8.02%) (Noguera *et al*., 2015). Historically, high proportions of larvae have been found in the musculature of Atlantic salmon (Beverley-Burton & Pippy, 1978), and maturing Pacific salmon *Oncorhynchus* spp. (Karl *et al*., 2011). However, high levels of variability observed between studies can be attributed to migratory behaviour of *A. simplex* (Young, 1972; Smith, 1984; Strømnes & Anderson, 1998), and the studied host species (Haarder *et al*., 2013).

Although no differences in muscle portions were observed in previous studies (Noguera *et al*., 2015), results in this study revealed that the front and middle portions showed the highest nematode prevalences and intensities, respectively. These portions encompass much of the digestive tract and would offer the shortest migratory routes for *A. simplex* larvae. The results support the suggestion that migratory distance could be a factor, if migration is non-random (Young, 1972). The optimal migratory distance hypothesis by Young (1972) suggests that smaller fish would exhibit higher infestation intensities in the muscle tissue, but the results in this study showed no statistical relationship between nematode intensities within the musculature and fish length or weight, which is in concurrence with previous observations (Strømnes & Andersen 1998, Larrat *et al*., 2013; Noguera *et al*., 2015).

Substantial research has been conducted into *A. simplex* infestation within muscle tissue of commercial fish species (Levsen & Lunestad, 2010; Levsen & Karl, 2014). Due to the associated human health risks (Audicana & Kennedy, 2008) however, much of this

research has been restricted to the comparison of *A. simplex* burdens within epaxial or hypaxial muscle. Norwegian spring spawning herring (Levsen & Lunestad, 2010), Atlantic cod (Milligan, 2008) and grey gurnard in the North Sea for example, have shown significantly higher abundances of *A. simplex* in the hypaxial muscle. Commonly referred to as the 'belly flaps', 80% of all isolated nematodes from maturing Pacific salmon have been reported within these regions (Karl *et al*., 2011). Chemical cues are involved in *A. simplex* distribution within a host (Bahlool *et al*., 2012). Greater lipid reserves in muscle of the redfish were proposed as a reason for the more frequent occurrence of L3 *A. simplex* within this region in comparison to Atlantic cod, or saithe (Strømnes & Anderson, 1998). The largest percentages of lipid in farmed Atlantic salmon muscle are found anteriorly from the dorsal fin, dorsally of the lateral line, and posteriorly of the dorsal fin, ventral to the lateral line (Do, 2013). With the higher intensities of L3 *A. simplex* not found in these muscle portions, it is unlikely lipophilic behaviour can explain these results and therefore, require further investigation.

The contiguity or arrangement of visceral organs has been suggested to affect whether nematodes pass into the left or right side of the muscle (Smith & Hemmingson, 2003; Berland, 2006), but there were no significant differences in nematode distribution between left and right side in the current study. These results agree with those reported for Atlantic salmon in Scotland (Noguera *et al*. 2015), and chum salmon (*Oncorhynchus keta* Walbaum*, 1792*) (Novotny & Uzmann, 1960) in the US, which makes any preferential selection between left and right sides of muscle by *A. simplex* larvae in Atlantic salmon unlikely.

3.4.2 Nematode Distribution within the Viscera

With mean intensities of 40 ± 34.6 and 48.9 ± 64.8 (mean ± SD), the pyloric caeca and stomach lumen exhibited significantly higher intensities of nematodes than other organs within the body cavity. As endoparasitic nematodes are transmitted through the ingestion of prey (Audicana & Kennedy, 2008), the digestive tract is a common site of infestation (Haarder *et al*., 2013), and has been identified as a preferred microhabitat for *A. simplex* within rainbow trout and Atlantic salmon (Haarder *et al*., 2013). Furthermore, the pyloric caeca, have been suggested as the optimum route to the peritoneal cavity in Pacific herring (Hauck & May, 1977). The fact that similarly high proportions of nematode larvae were found within front portions of muscle (which encompass the pyloric caeca), support the suggestion that this may provide a preferable migratory route within Atlantic salmon.

In total, 94% of *A. simplex* were found encapsulated externally on the stomach wall. The majority of nematodes found within the lumen of the stomach were *H. aduncum,* which are unable to migrate through tissue (Levsen & Karl, 2014). With the feeding of Ascaridid nematodes believed to involve the sucking action of the oesophagus drawing 'nutrient' soup towards the opening of their mouth (Berland, 2006), the observed high abundances of *H. aduncum* within the stomach lumen may be attributable to the availability of dietary resources. When hosts are fasting or starved in aquaria for example, nematodes are lost from the gut of their hosts due to reductions in available digestive mucus and fluid (Möller, 1976; Berland, 2006). *Anisakis simplex* has been observed to penetrate the mucosa of the stomach and intestine earlier in Baltic salmon compared to rainbow trout and brown trout (Haarder *et al*., 2013). Only opportunistic feeding occurs during the final leg of the spawning migration (Kjellman, 2015), and there was a lack of dietary items present in analysed Atlantic salmon. The observation of large numbers of encapsulated

A. simplex externally on the stomach wall is unlikely to be due to recent transmission events.

The liver is known for high infestation intensities of the 'hepatotropic' *A. simplex* (Smith & Hemmingsen, 2003; Horbowy *et al*., 2016), but significantly lower intensities were seen in the liver compared to the pyloric caeca and stomach of the dissected Atlantic salmon. Low infestation intensities of *A. simplex* in the liver have been observed in fasting Atlantic cod with smaller liver lobes (Berland & Hemmingson, 1991). Although nutritional stress is common within the spawning migration (Doucett *et al*., 1999a), surplus energy (not used for maintenance, activity, and processing of food) is employed to sustain the maturation processes of Atlantic salmon, until the fish become anorexic in autumn (Kadri *et al*., 1995; 1996). It is after this period when short-term energy stores are used, which is indicated through decreasing hepatosomatic indices (HSI) (Jonsson & Jonsson, 2011). The average HSI of 1.4 ± 0.4 (see Chapter 4.3) recorded from Atlantic salmon sampled in the current study during the early summer are within the range seen in healthy farmed post-smolt Atlantic salmon (1.37-1.60) (Hemre *et al*., 2007), and maturing and pre-vitellogenic female Chinook salmon (*Oncorhynchus tshawytscha* Walbaum, 1792) (1.35 – 1.69) (Unwin *et al*., 2004). It is therefore unlikely that decreases in the mass of the liver lobes in relation to the soma had begun to occur. Therefore, the liver should be of sufficient size to sustain *A. simplex* infestation.

The heteroxenous life cycle employed by *A. simplex* often leads to an accumulation of larvae in an intermediate host as they age (Levsen & Lunestad, 2010). Transmitted larvae encapsulate and await transmission to their definitive hosts (Smith, 1984). As the intermediate host continues to feed, greater numbers of *A. simplex* larvae are transmitted resulting in high infestation intensities seen in species such as Norwegian spring spawning herring (Levsen & Lunestad, 2010), and Atlantic cod (Mouritsen *et al*., 2010).

Therefore, it is of no surprise that total nematode larval intensity was positively correlated with length and weight of Atlantic salmon. The significant positive relationship between ungutted weight and nematode intensity was also reflected in the vent, and the viscera of Atlantic salmon.

The interpretation of nematode distributions in the viscera and musculature must be used with slight caution. Much of the research into nematode distribution has been focussed on edible portions of commercial fish. Post-mortem migration of *A. simplex* between the body cavity and muscle of *A. simplex* in particular has studied in a number of fish hosts including whiting, blue whiting (*Micromesistius poutassou* A. Risso, 1827), (Smith, 1984) and Alaskan pollock (*Theragra chalcogramma*, Pallas, 1814) (Arthur *et al.*, 1982), when kept on ice over a period of time. Furthermore, evidence of post-mortem migration within the viscera has been observed as well (Karl *et al.*, 2011). Although Atlantic salmon were frozen as soon as possible, the location of commercial fisheries in respect to Edinburgh Napier University meant that samples were kept on ice for the duration of the 2-7 hr journey before they were frozen at -20 °C meaning post-mortem migration of *A. simplex* cannot be ruled out entirely.

3.4.3 The Relationship between Nematode Intensities and Larvae per gram in the Body (Viscera and Musculature), and the Vent: the 'Hyper-Infestation' Hypothesis.

In the present study, no significant positive relationship was observed between nematode intensities in the vent and viscera. Furthermore, no significant relationship was also observed between nematode intensities within the body (viscera and musculature) and the vent region in salmon populations from the North, East and West coasts of Scotland. However, when coastal Atlantic salmon populations were pooled, a significant positive relationship was observed ($p<0.05$). When nematode intensity was analysed by larvae per gram of tissue weight, significant positive relationships between the vent and viscera

($p<0.05$) and the vent and muscle ($p<0.05$) were observed. The results of the present study contradict those of Noguera *et al*. (2015) who suggested that total larval loads relative to tissue weights in the vent region were independent of *A. simplex* intensity in the viscera (Noguera *et al*., 2015). Moreover, these results suggest that the levels of infestation intensity within the vent region are related to nematode intensities in other regions of the fish including within the body as proposed by Senos *et al*. (2013).

Haarder *et al*. (2013) suggested that *A. simplex* larvae may preferentially choose to travel down the intestinal tract before migrating directly from the hindgut out into the terminal portion of the body cavity and vent due to potential fitness benefits of these regions. *Anisakis simplex* larvae in the vent region however, were on average smaller than in the viscera (Noguera *et al*., 2015) potentially indicating poorer growth. It is therefore unlikely that this migration is beneficial to larvae and preferentially chosen (Noguera *et al*., 2015). The observation of smaller larvae however, could be a result of the time of sampling. As larvae are accumulated within the digestive tract resulting in chronic inflammation and large recruitment of granulocytes to internal tissues of helminth-infested salmonids (Ferguson, 2006), the 'inhospitable' environment created (Murphy *et al*., 2010; Senos *et al.,* 2013) could result in recently ingested larvae to migrate directly to the vent in these high intensity scenarios. Therefore. larvae in the vent could potentially have had less time to develop in this region in comparison to those in the viscera resulting in their smaller size (Noguera *et al*., 2015). It is possible however, that the vent region has been historically overlooked during analyses (Noguera *et al*., 2015), and that anisakid nematodes were present within the vent region prior to the emergence of RVS but were not reported.

The presence of *A. simplex* within the vent increases the likelihood of RVS (Larrat *et al*., 2013), but it has not been attributed as the sole cause of RVS within Atlantic salmon. In

this study nematode intensities in the vent, and the body (viscera and musculature) in relation to RVS severity was analysed. Results for the nematodes in the body were significantly different between Atlantic salmon with no symptoms, and salmon with moderate and severe symptoms of RVS. In contrast, nematode intensities found in the vent showed significant differences in between each severity, and higher intensities with increasing RVS severity. Furthermore, when numbers of larvae were analysed per gram of tissue by RVS severity, increased symptoms were strongly positively correlated with higher numbers of perianal larvae corroborating with previous reports (Larrat *et al*., 2013; Noguera *et al*., 2015). These results support the observation that increasing infestation intensities of *A. simplex* within the vent region increase the likelihood of RVS (Larrat *et al*., 2013).

The historical comparisons of nematode infestation by Senos *et al*. (2013) showed considerable increases in *A. simplex* infestation intensity from the late 1970's to the present day (Wootten & Waddell, 1977; Beverley-Burton & Pippy, 1978). Similar increases have also been noted in Atlantic herring (Levsen & Lunestad, 2010) around Norway, and further afield in adult chum salmon (*Oncorynchus keta,* Walbaum, 1792) in the North Pacific (Urawa & Fujisaki, 2006) during the period when RVS first emerged. Noguera *et al*. (2015) found mean nematode intensities of 63.6 ± 31.9 (mean ± SD) per fish in Atlantic salmon sampled in 2009. Within the vent region, mean nematode intensities were enumerated as 31.7 ± 21.34. In the present study Atlantic salmon sampled in 2015, mean nematode intensities per fish were 264.3 ± 196.9, and 107 ± 73 in the vent. These results support the theory that nematode abundances of Atlantic salmon in the North Sea, and the North Atlantic are generally increasing (Senos *et al*., 2013).

Observed ocean-scale warming has been especially pronounced in the North Atlantic with sea-surface temperatures (SST) rising between 0.5 to 1.5 °C per decade^{-1} since the 1990's

(Todd *et al*., 2008). In an area encompassing common foraging areas of wild salmon (Todd *et al*., 2008), potential alterations in dietary inputs therefore, are likely (Dufour *et al.,* 2010; Mouritsen *et al.,* 2010).

Increases in SST can drive changes in ecosystem dynamics including; marine fish prey resources, species interactions, community composition, food availability and food web structure (Pörtner, 2002; Pörtner & Peck, 2010). Changes in the availability of dietary inputs have previously been suggested to be the cause of increased of parasitic transmission observed in grey gurnard (Levsen & Karl, 2014), and Atlantic salmon in Canada (Larrat *et al*., 2013). The fact that common intermediate hosts for *Anisakis* spp. including warm water copepods are undergoing large-scale northward shifts (Beaugrand *et al*., 2002), could result in similar direct effects on the levels of parasitic transmission to Atlantic salmon in Scotland. Furthermore, marine mammal communities in the North-West of Scotland have experienced marked changes in community structure (MacLeod *et al*., 2005). On the one hand there have been declines of cold water species such as the killer whale (*Orcinus orca* L.), long-finned pilot whale, and the northern bottle nose whale (*Hyperoodon ampullatus* Forster, 1770) since 1980 (MacLeod *et al*., 2005). On the other hand, there has been an increase in occurrence of new warm water species including the striped dolphin, Fraser's dolphin (*Lagenodelphis hosei* Fraser 1956), and pygmy sperm whale (*Kogia breviceps* Blainville, 1838). The environmentally driven range shifts of potential intermediate hosts, coupled with changes in marine mammal communities as potential definitive hosts, are likely to affect the abundance of *Anisakis* sp. in Scottish coastal waters.

3.4.4 Oral Infestation Trial

The pilot infestation trial resulted in the recovery of only 0.45% of the administered *A. simplex*. Unfortunately, such a small recovery rate means that these data could not be

analysed for any infestation trends or patterns. There could be several reasons why this experiment was not successful and led to the following adjustments to be made in the subsequent re-trial:

- Reduced time between *A. simplex* isolation and infestation – *A. simplex* L3 larvae were isolated from Atlantic herring and administered to Atlantic salmon within 48 hours in the pilot challenge. This interval however, may have adversely affected the viability of *A. simplex* to re-infest a new host. In the second pilot, *A. simplex* was isolated and transmitted to new hosts within 12 hours.
- Installation of a net over the outflow pipe of the tank - As very low numbers of *A. simplex* larvae were recovered from Atlantic salmon during the pilot experiment, one logical assumption was that larvae had either been regurgitated or passed through the host and subsequently lost through the outflow of the tank. Nets were installed to control for any larvae that were expelled by the fish permaturely.
- Rainbow trout as a positive control – in the oral trial run by Haarder *et al*. (2013), rainbow trout was used in addition to other salmonids and exhibited similarly high recovery rates of administered *A. simplex* larvae to that of Atlantic salmon. This treatment group was included to investigate whether successful *A. simplex* infestation could be initiated within this species.

Following the termination of the second pilot challenge, the results proved the challenge to be similarly unsuccessful to the first pilot experiment. In total, only 1.5% of *A. simplex* larvae administered were recovered from the body of the host, however, an additional 22% of larvae were recovered from the net covering the outflow. The majority of passed larvae were found within faecal pellets suggesting an element of digestion.

In total, 16 *A. simplex* larvae were recovered from Atlantic salmon, with 15 being recovered from the net covering the outflow after 5 days post infestation. Nematodes in

the rainbow trout tank however, were passed after 16 days post infestation. Whether differences in the rate of passing of nematodes between rainbow trout and Atlantic salmon is an indication of inter-species differences in susceptibility and immune response to *A. simplex* infestation (Bahlool *et al*., 2012) however, is unknown. The recovery rates in both experiments were significantly lower than those of similar studies (Shih *et al*., 2010; Quiazon *et al*., 2011; Haarder *et al*., 2013).

Reasons for the failure of the two infestation challenges presented in this study remain unclear. In this study and previously published studies, live *A. simplex* L3 larvae were obtained from the viscera of another intermediate host (Atlantic herring and Atlantic mackerel). It is therefore unlikely that the 'personal history' of *A. simplex* larvae affecting their ability to migrate through tissue within this study, and other published examples differed greatly. Additionally, the viability test followed the protocol in Haarder *et al*. (2013), to minimise the chances of 'exhausted' larvae to be used (Smith, 1984). The lack of *in vivo* migration seen within treatment groups however, suggests this may not be a valid assessment of nematode viability, with their inability to penetrate and migrate out of the digestive tract leaving them susceptible to digestion and subsequent excretion.

Administration methods of *A. simplex* larvae into the stomach of hosts however, differed between studies. Although both Haarder *et al*. (2013) and Shih *et al*. (2010) used forceps to directly place live larvae within the stomach of the hosts, Quiazon *et al*. (2011) placed larvae within feed pellets, which were subsequently fed to the hosts. The use of the oral gavage within this study was seen as a more effective method than using forceps to directly apply larvae within the stomach of a host and minimise potential damage to larvae. Although damage to larvae cannot be completely ruled out, the majority of larvae recovered from outflow pipes was alive and showed no indication of damage. Although a number of protocols were put in place to determine larvae viability following the

published examples of similar studies, questions remain whether these protocols are effective methods in assessing larval condition.

This study recommends the future use of larvae found within Crustacea and not within other intermediate fish hosts. This will ensure larvae have increased potential for migration as they will not have previously reached an intermediate host such as Atlantic herring or mackerel. Whether the time taken for Crustacea or small intermediate fish hosts to be digested is an important factor for the successful transmission of anisakid larvae can only be speculated however, mirroring the natural mode of transmission within the marine environment (Køie *et al.*, 1995), can only improve the ecological validity of such challenges.

The results reported in this study support the presence of a significant relationship between nematode intensities in the body of Atlantic salmon, and the vent. Nematode intensities in Atlantic salmon showed increased levels compared to studies conducted in between the 1970's and today (Senos et *al*., 2013; Noguera *et al*., 2015). This suggests that the observation of *A. simplex* within the vent and emergence of Red Vent Syndrome may be in response to overall increasing burdens within Atlantic salmon (Larrat *et al*., 2013). Increasing numbers have also been observed in other species such as herring (Levsen & Lunestad, 2010), or cod (Horbowy *et al*., 2016) amongst others, but these other fish species did not show any signs of RVS so far. High affinity to the epaxial muscle shown here and in other studies, along with the high prevalences in front and middle muscle portions suggest that migration is not completely random. The *in vivo* behaviour of *A. simplex* within a host however, remains complex and multi-factorial. Experimental challenges as implemented by Shih *et al*. (2010), Quiazon *et al*. (2011), and Haarder *et al*. (2013), remain the most appropriate method to elucidate the migratory behaviour of *A. simplex* within a host. Care however, must be taken in the experimental design of these

studies, which ideally, should replicate the natural cycle of *A. simplex* transmission to Atlantic salmon. Potentially, the ingestion of infested Crustacea and small intermediate fish hosts could be used to replicate the dietary composition of wild MSW and 1SW Atlantic salmon where possible.

Chapter 4

Identification of potentially different migratory routes of Atlantic salmon populations of Scotland using stable isotopes and parasite component communities

4.1 Introduction

Anisakis simplex is transmitted to Atlantic salmon through the ingestion of Crustacea or small intermediate fish hosts (Audicana & Kennedy, 2008). The significant differences in nematode intensities observed between Atlantic salmon populations in Scottish coastal waters (Chapter 3) therefore, may be a result of differences in migratory behaviour, or the use of different feeding grounds.

Tracing movement and migratory behaviour of organisms, such as Atlantic salmon, between different geographical areas is a fundamental aspect of population ecology, whether this is on daily or seasonal timescales, and/or local or regional geographical scale (Hobson, 1999). The assessments of an organism's population identity, location and the extent of mixing between different populations are critical to understand the linkages between a species, and geographical regions within a marine ecosystem (MacKenzie *et al*., 2011). Historically, much of the assessment of these factors have been biased towards conspicuous larger species such as marine mammals including for example the blue whale (*Balaenoptera musculus* L.) (Mate *et al*., 1999). Studies on the behaviour and movement of fish in the marine environment have previously been restricted to tagging studies using either electronic or satellite data tags (Wilson *et al*., 2005; Wright *et al*., 2006). In addition to both being prohibitively expensive for large-scale studies (MacKenzie *et al*., 2011), these methods are either reliant on the transmission of data back to researchers (Block *et al*., 2011), or the recapture of tagged animals. With the large geographical scales encountered in the marine environment, and high levels of mortality of organisms (MacKenzie *et al*., 2011), low recapture rates using these methods are common (ICES, 2009b).

In recent decades however, technological advances and the use of novel methods have dramatically improved the ability to trace an individual's movement and understand migratory behaviour in the marine environment without relying on tagging studies. The use of genetic sequence divergence within DNA for instance, has been shown to enable the geographical separation of populations of the same species (Bernatchez *et al*., 1992; Wenink *et al*., 1994). Furthermore, the composition of fatty acid profiles (Torniainen *et al*., 2017), stable isotopes (Fry, 1981), and acoustic transmitters (ICES, 2017) have all shown to be useful in tracing movement and behaviour within specific geographical areas (Hobson, 1999).

The understanding of movements and behaviour of highly migratory pelagic species such as the Atlantic salmon has been improved through tagging studies, and the use of more novel approaches (Torniainen *et al*., 2017). Two large-scale migrations within the Atlantic salmon's life cycle have been clarified. The first is the migration from riverine systems to marine feeding grounds, also known as the smolt migration (Holm *et al*., 2000). Atlantic salmon commonly remain between 1-5 winters at sea, before embarking upon the return migration to their natal rivers in the summer also known as the spawning migration (Doucett *et al*., 1999a). Little is known about the details of these migrations, except that these movements are associated with very high rates of mortality (Friedland *et al*., 2000; Lefèvre *et al*., 2012; 2013; Skoglund & Barlaup, 2016). This lack of knowledge hinders the effective implementation of management practices, resulting in ineffective conservation of populations that are declining (Myers *et al*., 1987; MacKenzie *et al*., 2011).

4.1.1 Smoltification, Post-Smolt Migration and Marine Feeding

Prior to the smolt migration, a transformational process known as smolting, resulting in behavioural, morphological and physiological changes in preparation for marine life

occurs (Lefèvre *et al*., 2012, 2013). The migration from freshwater to the marine environment is instigated by environmental cues such as photoperiod and water temperature (Lothian *et al*., 2017). The subsequent direction of migration upon entering the marine environment however, is strongly influenced by wind direction and the prevailing currents (Thorstad *et al*., 2004; Økland *et al*., 2006; Lefèvre, *et al*., 2012; Martin *et al*., 2013). The geographic distribution of post-smolts around the United Kingdom conforms well to the main surface current patterns in the area, particularly with the strong slope currents present in the West and North of Scotland (Fig 4.1) (Holm *et al*., 2000). The influence of these northward flowing currents coming from the European continental shelf edge result in the majority of Atlantic salmon populations of Scotland utilising nursery and feeding grounds within the Norwegian Sea, and the Arctic Ocean (Hansen & Jacobsen, 2003; Booker *et al*., 2008) (Fig 4.1). Some southern European populations of Atlantic salmon including those from Spain and France however, have been known to travel as far as the coast of West Greenland (Hansen & Quinn, 1998; Reddin & Friedland, 1999).

In these nutrient rich feeding grounds Atlantic salmon spend between 1-5 years feeding on zooplankton and crustaceans as well as smaller fish such as Atlantic herring (*Clupea harengus L.* 1761) and Norway pout (*Trisopterus esmarkii*, Nilsson, 1855) (Jutila *et al*., 2003; Mills, 2003). Atlantic salmon that spend one winter at sea before returning to spawn are termed grilse, or one sea-winter (1SW) fish, while individuals that spend two or more winters at sea are termed multi sea-winter (MSW) fish. This period is crucial for Atlantic salmon, as the lipids stored during this period will be exhausted due to the energetic cost of the spawning migration, and for the significant maternal investment required for high quality eggs and successful spawning (Jonsson *et al*., 1997; Torniainen *et al*, 2017).

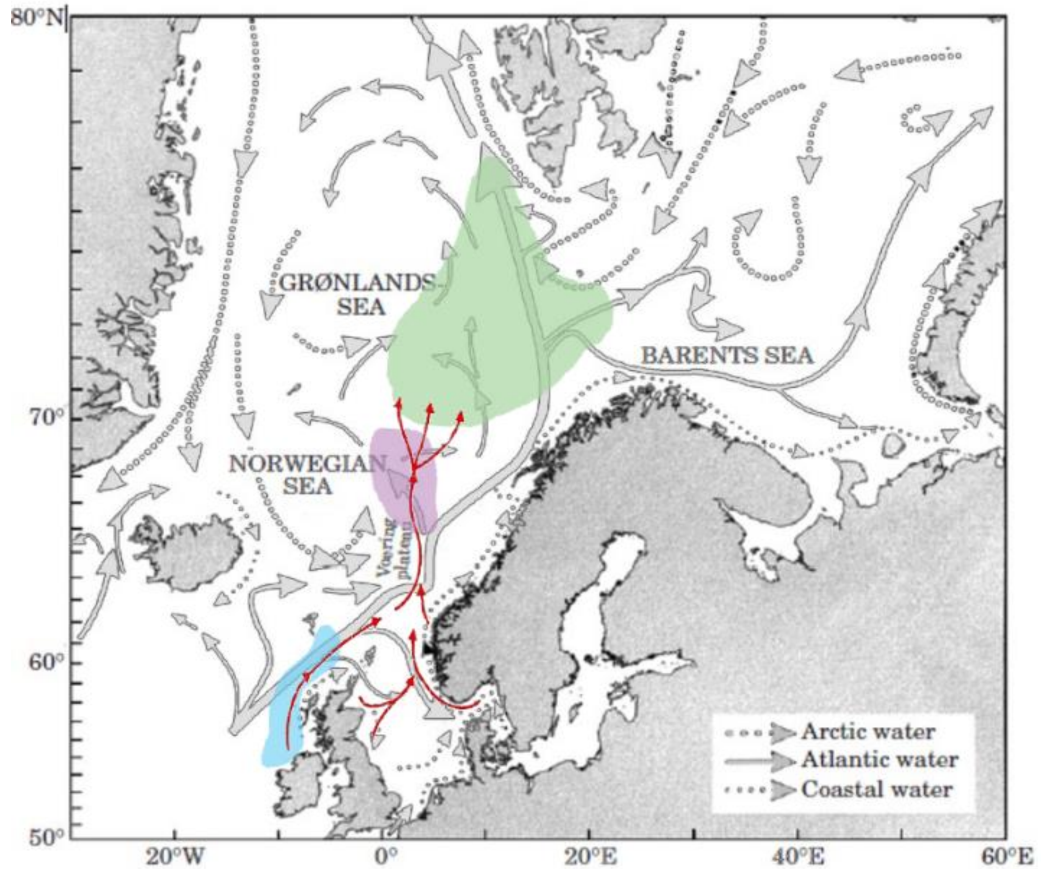

Figure 4.1. The dominating surface currents in waters around the United Kingdom. Current vectors, strength and water types are represented by arrows of different width (the wider the arrow, the stronger the current) and appearance. Atlantic salmon migratory routes indicated by red arrows, with shaded areas indicating common feeding grounds in the East of Atlantic (blue), Norwegian sea (purple) and Arctic (green) (Adapted from Holm *et al*., 2000 and the Atlantic Salmon Trust).

4.1.2 Spawning Migration

As Atlantic salmon return to natal rivers to spawn, the sexual maturation of an individual is central to the timing of the return migration. For successful maturation to occur, a minimum energy storage threshold is required (Jonsson & Jonsson 2003; 2004). This is reached through the separation of metabolic energy towards lipid storage rather than protein production, which is stimulated through warming temperatures (Jonsson & Jonsson 2004; 2005). Subsequently, cooling temperatures acts as the cue for returning

southward migrations (Turrell & Shelton, 1993). As northern waters cool, grilse in particular are often observed to move southward earlier than MSW salmon due to lower tolerances to cooling waters (Turrell & Shelton, 1993).

The migration itself is believed to consist of two distinct phases. The first phase involves the migration from feeding grounds to coastal regions, and the second, more precise navigation towards their natal rivers (Hansen *et al.*, 1993; Ulvan *et al*., 2017). Although fasting does not occur until entering the freshwater environment (Doucett *et al*., 1999a), feeding is believed to be only opportunistic during the spawning migration (MacKenzie *et al*., 2012). During the final stages of this migration however, a large proportion of salmon was reported with empty stomachs, or highly digested items in the stomach content, suggesting feeding had occurred some time ago (Kjellman, 2015).

Although the use of novel approaches has greatly improved our understanding of both the smolt and spawning migration, much of the marine phase of the Atlantic salmon's life cycle remain cryptic. The use of stable isotope analysis, and the use of other spatially related macro ecological patterns such as parasitic component communities are two modern methods that can further investigate this element (Mattiucci, 2006; Poulin, 2007; Dempson *et al*., 2010; MacKenzie *et al*., 2011).

4.1.3 Stable Isotope Analysis (SIA)

Within any environment, there are over 300 known naturally occurring non-radioactive isotopes of elements, which occur in predictable proportions throughout the environment (Högberg, 1997). The relative abundance of stable isotopes is expressed in δ notation as parts per thousand (‰) deviation from a standard. The international standard for carbon (C) is the Vienna Pee Dee Belemnite, and Air for nitrogen (N) (Högberg, 1997). In any given environment, a wide range of stable isotopes is available. The use of particular elements has been found to be more appropriate for certain analyses covering a number

of different purposes (Michener & Lajtha, 2008). In food web and dietary interaction studies, the use of ratios of $^{15}N/^{14}N$ and/or $^{13}C/^{12}C$ are the most common (Perkins *et al*., 2014). They are primarily used for their predictability of isotopic enrichment between the proteins of the dietary item, and that of the consumer, which has been measured at approximately 3.4 per mil in $\delta^{15}N$ and 1 per mil in $\delta^{13}C$ per trophic level (DeNiro & Epstein 1978; 1981). This predictability of enrichment, provides a rapid, cost effective method for the clarification of animal migrations (Hobson, 1999), dietary composition (Vandermerwe & Vogel 1978; Phillips, 2001), niche shifts (Post, 2002) and trophic structure (Ponsard & Arditi, 2000; Post *et al*., 2000) that does not suffer from any observer bias associated with conventional methods e.g. gut contents analysis. Levels of enrichment within an organism however, can be highly variable. The type of species and tissue analysed, growth rate, diet and nutritional status are all factors that have significant effects on the results and must be taken into consideration during analyses (Högberg, 1997; Vanderklift & Ponsard, 2003; Post *et al*., 2007).

In marine ecosystems, $\delta^{15}N$ values are set by the levels of primary productivity in a food web. $\delta^{15}N$ values in dissolved inorganic nitrogen (DIN) increase with decreasing nitrate concentrations. Macroalgae and phytoplankton show a preferential uptake of the lighter isotope $^{14}NO_3$- (Fry, 2006). Preferential feeding in areas containing large populations of phytoplankton can lead to significant depletion of $^{14}NO_3$-, and subsequently leads to the uptake of the heavier isotope $^{15}NO_3$- (Fry, 2006). As a result, phytoplankton exhibit elevated $\delta^{15}N$ values, which are subsequently passed up the trophic food web (Satterfield & Finney, 2002). Nitrogenous nutrient concentrations are also dependent on temperature, pollution, coastal proximity, land use, and trophic level effects (Cabana & Rasmussen, 1996; Cole *et al.,* 2004). Basal $\delta^{15}N$ values are harder to establish and interpret in comparison to $\delta^{13}C$. Unlike Carbon, $\delta^{15}N$ values are passed on from the food source to consumers with a typical enrichment between 2-5 ‰ (DeNiro & Epstein, 1978). The

levels of enrichment enable the quantification of dietary inputs and they are also used to study food web interactions (Post *et al.*, 2000), food chain lengths and trophic structures (Post, 2002; Perkins *et al.*, 2014).

Similarly to $\delta^{15}N$, $\delta^{13}C$ signifies the level of primary productivity found at the base of these food webs (Wada *et al.*, 1991). Differences in $\delta^{13}C$ isotopic composition have been observed between freshwater and marine ecosystems, and also between inshore and offshore environments (Miller *et al.*, 2008), which make it a useful marker for tracing animal movements. General observations have revealed that primary producers in marine environments are enriched with heavier stable isotopes (Doucett *et al.*, 1999b), leading marine organisms to be enriched by 7 ‰ with the heavy isotope $\delta^{13}C$ (Bearhop *et al.*, 1999) in comparison to freshwater. Coastal and offshore marine ecosystems can also be differentiated due to differences in dietary carbon sources (Miller *et al.*, 2008) as a result of coastal regions containining additional allochthonous terrestrial inputs of dietary carbon sources e.g. leaves (Doucett *et al.* 1996). However, in all open marine systems the isotopic composition of organisms is solely linked to the autochthonous primary productivity of the regions (Eby, 2004).

In open marine systems, $\delta^{13}C$ values are strongly linked to temperature (Graham *et al.*, 2010). Higher uptake of the lighter isotope ^{12}C by phytoplankton is observed in cooler waters containing higher levels of CO_2 and dissolved nutrients. In warmer and nutrient limited waters, the opposite is seen (Hofmann *et al.*, 2000; Kamykowski & Zentara, 2005). Furthermore, increased plankton cell growth rates are linked to higher temperatures (Hofmann *et al.*, 2000). With the uptake of both heavy and light isotopes used to maintain higher growth rates in these conditions (Trueman & Moore, 2007), enrichment of ^{13}C commonly leads to a reported latitudinal gradient of $\delta^{13}C$ values (Lorrain *et al.* 2009). These factors contributing to regional variations in $\delta^{13}C$ values,

result in clearly observable changes in $\delta^{13}C$ values of zooplankton, between different regions of productivity, and temperature (Fig 4.2).

Fry (1981) was among the first scientists to use stable isotope analysis to demonstrate that isotopic changes in tissues of brown shrimp (*Farfantepenaeus aztecus* Ives, 1891) could be used to demonstrate migratory behaviour between three estuarine and one offshore feeding location off the South of Texas and the Gulf of Mexico. Utilising $\delta^{13}C$ values, Fry (1981) was not only able to differentiate between these locations, but also between seasons in these locations in relation to obtained values. There have been clear indications that $\delta^{13}C$ can be used to define separate populations of Atlantic salmon on a spatial scale (Dempson *et al*., 2010; MacKenzie *et al*., 2011). $\delta^{13}C$ values of Atlantic salmon populations caught in Newfoundland, Quebec and northwest Ireland demonstrated sufficient differences to classify them as separate populations (Dempson *et al*., 2010). Similarly, Atlantic salmon populations from the River Frome and from the North East Coast of England could also be separated (MacKenzie *et al*., 2011). $\delta^{13}C$ has also been used successfully to separate and identify different populations of Pacific salmon (Satterfield & Finney, 2002). Although Stable Isotope Analysis (SIA) has been commonly used to examine the nutritional status of migrating Atlantic salmon (Doucett *et al*., 1999b) and to differentiate between wild and farmed Atlantic salmon (Dempson & Power, 2004), there is a paucity of research into the marine phase of their life cycle.

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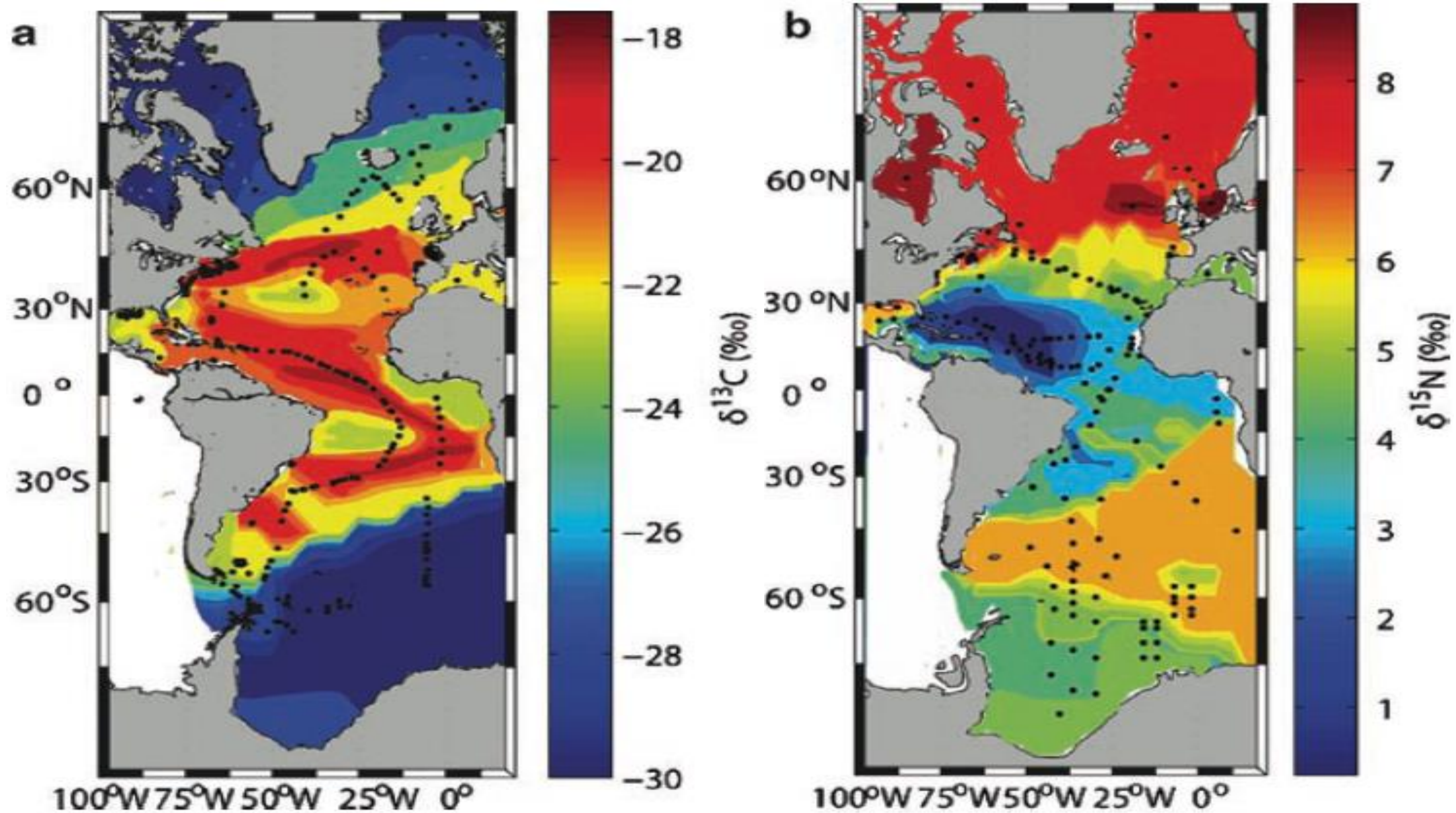

Figure 4.2. Contour plots of isotope values in the Atlantic Ocean from a meta-analysis of published data. (a) δ ^{13}C values of plankton from the upper ocean (0–500 m; n = 425) and (b) δ ^{15}N values of zooplankton, primarily calanoid copepods, from the upper ocean (0–500 m; n = 198). *Black dots* indicate sample locations taken from McMahon *et al*. (2013) (Adapted from Graham *et al*., 2010).

4.1.4 Parasite Component Communities and their use as Biological Markers

In recent decades, another method, which has found prominence in the identification of geographically separate populations, is the use of parasitic species and component communities as biological markers (MacKenzie, 2002; Catalano *et al*., 2014). Although single parasite species were first used as biological markers over 70 years ago, the utilisation of this approach has increased only recently (MacKenzie, 2002; Catalano *et al*., 2014). To use a single or multiple parasitic species as biological markers, strict criteria have to be applied and fulfilled to assure the viability of such parasites as markers (Garcia *et al*., 2010). The three criteria currently used are i) infestation levels need to be different among the host sampling areas, (ii) the parasite/s should be detectable in the host throughout the time-scale of the study, and (iii) the parasite/s should not be pathogenic to the fish host (Garcia *et al*., 2010). One species group that meets these criteria, are the sibling species of anisakid nematodes (MacKenzie, 2002). With recent identification improvements between sibling species (Mattiucci & Nascetti, 2008), species of the *A. simplex* species complex have been useful markers for stock discrimination of both marine fishes and cephalopods (MacKenzie, 2002). In addition to presence and absence data, geographical differences in *A. simplex* abundance can also be used to discriminate between populations (Abaunza *et al*., 1995). Significant differences in abundance of *A. simplex* in horse mackerel (*Tracharus tracharus L.*) have been reported for populations resident in Gallician waters (ICES divisions: VIIIe West and IXa North), and the Cantabrian Sea (VIIIe East) in Northern regions of Spain (Abaunza *et al*., 1995).

As part of a multi-disciplinary approach, the use of multivariate statistical analyses on parasite community data for stock assessment purposes is increasing (MacKenzie, 2002; Oliva, 2013). Parasitic component communities can be spatially and temporally variable within the same host species (Poulin & Valtonen, 2002). This can often be attributed to

regional differences in parasitic community. Within these parasitic species pools, the effects at local, regional and global scale affect their species richness and abundance (Simková *et al.,* 2001). At a local scale, increasing intermediate or definitive host abundance or density will readily sustain increased abundances of parasitic species (Simková *et al*., 2001). This has been observed in Denmark for example, where increasing grey seal populations have led to increased abundances of *Contracaecum osculatum* (Rudolphi, 1802) within intermediate hosts (Haarder *et al*., 2014). At a regional and global scale, reliance on the vagility of paratenic hosts of some parasite species for dispersal (Poulin, 2003), coupled with biological gradients within hosts and the environment (Timi *et al*., 2010), restrict parasite assemblages. These factors lead to a decrease in the proportion of species shared by two host populations with increasing geographical distance, also known as the distance decay of similarity (Poulin, 2003). The prevailing parasite species richness in a host however, is also affected by a number of parameters within a host.

The infra-community of a host is formed by all different parasitic species present in the same individual at a specific time (Holmes & Price, 1986). Infra-communities however, are often short lived. In addition to positive and negative ecological interactions between parasite species that determine their composition (Holmes, 1973; Pietrock & Marcogliese, 2003), there is a constant turnover of parasite species with new parasites being recruited and old ones dying out (Poulin, 1997). Due to these factors, infra-communities are assembled from a pool of currently and locally available species (Poulin, 1997). This pool consists of all parasite species exploiting the host population at one point in time. These species form what is known as the component community (Holmes & Price, 1986).

Although a number of factors such as host density, diet, behaviour, and various life-history traits (Sasal *et al*., 1997; Morand & Poulin, 1998) can affect a host's parasite

species richness, host species with a large geographical range such as Atlantic salmon, are likely to encounter and harbour an increasing parasite diversity in their component community (Poulin, 1996; Kennedy & Guégan, 1996).

Most analyses of parasite component communities have focussed on commercially valuable wild species including flounder (*Platichthys flesus L.*) (Gibson, 1972), horse mackerel (Abaunza *et al*., 1995) and narrow-barred Spanish mackerel (*Scomberomorus commerson,* Lacepède, 1800) (Lester *et al*., 2001). Although of significant commercial value (Morton *et al*., 2016), wild populations of Atlantic salmon have not received the same attention within the marine environment.

Two large-scale migrations occur in the life cycle of Atlantic salmon, which follow the surface currents in the surrounding areas (Holm *et al*., 2000). Atlantic salmon populations embarking on these migrations are likely to feed in different local and regional trophic webs and interact and harbour a range of different regional pools of parasite species in different localities (Strøm *et al*., 2018). In light of the currently cryptic behaviour of the marine phase of Atlantic salmon's life cycle, the study presented in this chapter will try to elucidate differences in migratory route and feeding ground employed by populations of Atlantic salmon sampled from the different coasts of Scotland.

To assess migratory route and feeding ground, this chapter retrospectively constructed a temporal and spatial window of the feeding grounds and dietary inputs of Atlantic salmon using both $\delta^{15}N$ and $\delta^{13}C$ values. The presence of differences in either $\delta^{15}N$ or $\delta^{13}C$ values will suggest the use of different migratory routes and trophic webs. Atlantic salmon was also assessed for differences in parasite component communities between coastal populations. Highly migratory species are expected to encounter different regional pools of parasite communities. Differences in these component communities would strengthen the support for the use of different feeding grounds or migratory routes.

4.2 Materials and Methods

4.2.1 Salmon Aging

A total of 117 Atlantic salmon was sampled from wild capture inshore net fisheries from the East (Usan, Angus) (56°40'55.7"N 2°27'02.5"W; n = 57), North (Armadale, Sutherland) (58° 32′ 59.64″ N, 4° 5′ 22.2″ W; n = 26) and West (Snadyhills, Dumfries and Galloway) (54°52'45.7"N 3°43'46.9"W; n = 34) coasts of Scotland (See Fig. 3.5). Approximately five scales were removed from around the dorsal fin of each Atlantic salmon and cleaned using distilled water. The scale that was in the best condition was photographed using an Olympus D25 camera mounted on a Leica – MDG41 microscope. Scale images were imported into Image Pro Premier V 9.1 software package. A transect was drawn along the 360° axis from the focus of the scale recording both fresh and marine growth zones as reported in Smolyar & Bromage (2004) and Friedland *et al*. (2008) (Fig. 4.3).

The interval between circuli within the marine phase was automatically measured using the calliper tool of the software. Atlantic salmon are aged based on the numbers of winter spent at sea during their marine phase (Friedland *et al*., 2008). Winters at sea are defined by a slower growth period, characterised by closer banding of circuli. The distances obtained using the calliper tool, coupled with traditional scale reading, made it easy to identify closer banding of circuli indicating one ‘winter’.

Figure 4.3. Salmon scale with overlay of the 360° transect. Length data of river life and growth data (A1-A40) from the marine phase determined using the calliper window of the Image-Pro Premier. Summer at sea, winter at sea and a period of 'closing' are clearly visible based on band width.

4.2.2 Parasite Isolation, Fixation and Identification

Sampling of the 117 Atlantic salmon from East, West and North coasts of Scotland and fish dissection were carried out as outlined in Chapter 3. All parasites were removed from organs and tissue samples with forceps and placed in petri dishes containing 0.9% saline solution. Samples were fixed in Davidsons' AFA fixative solution (1.5L = glacial acetic acid (250ml), formaldehyde (500ml), and Alcohol (750ml) and subsequently stored in 75% ethanol (Sigma Aldrich, Irvine, UK). Parasite species were all identified using morphological features outlined in taxonomic keys and original literature for Nematoda (Arai & Smith, 2016), Digenea (Gibson, 1996; Gibson & Bray, 1986; Bray *et al.*, 2008),

Copepoda (Kabata, 1969; Pike & Wadsworth, 1999) and Acanthocephala (Margolis & Kabata, 1989).

Prior to observing morphological features using a dissecting microscope, the following preparatory procedures were carried out to enable internal organs to be visible:

Digenea - Samples were stained using carmine solution (equal parts carmine and alcohol), and destained using hydrochloric acid (HCL) to an appropriate translucency. Stained Digenea were washed in 70% ethanol and fixed onto object slides using glycerol jelly with the coverslip applied using gentle pressure.

Nematoda - were placed for clearing in a solution of equal parts 70% ethanol (Sigma-Aldrich, Irvine, UK) and glycerol (Sigma-Aldrich, Irvine, UK). The alcohol evaporated leaving the nematodes in pure glycerol. Care was taken not to expose the nematodes too quickly to the pure glycerol to avoid potential collapse.

Acanthocephala – Samples were stored in distilled water at 4 °C for 24 hours until the proboscis had everted. Samples were transferred into a series of 30%, 50% and 70% ethanol for 15 minutes respectively, and then stained using carmine solution. They were then destained using hydrochloric acid (HCL) to an appropriate translucency before being washed in 70% ethanol and fixed onto object slides using glycerol jelly with the coverslip applied using gentle pressure.

Crustacea – Samples were cleaned in 70% ethanol before being fixed onto object slides with the coverslip applied using gentle pressure.

Examples of each identified species were photographed using an Olympus D25 camera mounted on a Leica – MDG41 microscope.

4.2.3 Fulton's condition Factor K and Hepatosomatic index (HSI)

Fish condition was assessed with Fulton's condition factor K:

$K = 100 * TW/L^3$ where TW = total weight, and L = length (Froese, 2006).

Hepatosomatic indices were also calculated to assess energy reserves for the sample of Atlantic salmon using the following formula:

HSI = LW/TW*100 where LW = liver weight and TW = total weight.

4.2.4 Muscle and Scale Isotopic Preparation and Analysis

Dorsal muscle tissue samples (116) and scales (88) were dried in an oven at 60 °C for 48 hours. The dried samples were ground with a sterile pestle and mortar that were sterilised with 70% ethanol (Sigma-Aldrich, Irvine, UK) between each sample. On a Sartorius ENTRIS124-1S lab balance, 0.6 - 0.8 mg of each sample were weighed and placed in pressed tin capsules (5 mm x 3.5 mm) (Elemental Microanalysis, Okehampton, UK) which were enclosed using forceps, and stored in a glass desiccator until processing.

Additionally, a sub-sample of 45 ground dorsal muscle tissue samples underwent a lipid removal procedure. 1 ml of chloroform/methanol (2:1, v/v) was combined with ~4 mg of sample in a 1.5 ml polypropylene micro centrifuge tube following a modified Folch method (Folch *et al.*, 1957). Samples were sonicated for 10 minutes and centrifuged (3000 RPM) at 21 °C for 5 minutes. Supernatants were removed, and the process was repeated twice using 1ml chloroform/methanol (1:1, v/v) mix. Samples were dried at 60 °C for 48 hrs and reground.

All stable isotope analyses were carried out using the Elementar Pyrocube elemental analyser, and the Thermo Fisher Scientific - Delta Plus X mass spectrometer at the Scottish Universities Environmental Research Centre (SUERC) (East Kilbride, Glasgow, Scotland). Three in-house standards were run every 10–12 samples, and four USGS40 isotopic reference samples were run per plate. Results were reported in δ notation as the deviation from standards in parts per thousand (‰). A MANOVA was used to assess overall differences between the combination of $\delta^{13}C$ and $\delta^{15}N$ values from different

coastal populations of Atlantic salmon. One-way ANOVAs were conducted for $\delta^{13}C$ and $\delta^{15}N$ values to identify differences between the coastal populations. Regression analysis was performed to investigate the relationship between trophic level ($\delta^{15}N$ signature) and nematode intensity per fish. Full exploration of best linear fits using linear, quadratic and cubic terms on the data was performed prior to analysis. The standard error of the regression, S values and R^2 values were used to assess the curve fitting effectiveness of the different models. All statistical analyses were carried out using Minitab 17 Statistical Software (2010) (Minitab Ltd, Coventry, UK).

4.2.5 Analyses of Geographical Variability of Parasite Component Communities

Differences in parasite component communities of Atlantic salmon populations were tested with a 1-way permutational multivariate analysis of variance (PERMANOVA) using a Bray–Curtis similarity matrix involving 9999 permutations. Nonmetric multidimensional scaling (nMDS) was used to graphically represent differences in parasite component community structure among salmon from each site. The Bray–Curtis similarity matrix was used to generate a two-dimensional graphical solution that arranges parasite communities such that the distance between two points is inversely proportional to their similarity. The relative contribution of individual species to differences between coastal populations was determined using a test for similarity percentages (SIMPER). All statistical analysis and non-metric dimensional scaling were carried out using the statistical software PAST3 (Øyvind Hammer, Natural History Museum, University of Oslo).

4.3 Results

Dorsal scale analysis revealed that the scales sampled from all 117 Atlantic salmon exhibited one period of slow growth indicating that they had spent one winter at sea. All fish specimens are classed as 1 sea-winter salmon or grilse.

Results of Fulton's condition factor K calculations indicated that all populations were in similar condition (Table 4.1). Atlantic salmon sampled from the East coast however, had the lowest values (mean ± SD) 0.89 ± 0.09 in comparison to those sampled off the West (0.98 ± 0.08) and North (0.97 ± 0.08) coasts (Table 4.1). Hepatosomatic indices were also lowest in Atlantic salmon off the East coast (1.3 ± 0.45), with West (1.5 ± 0.17) and North (1.5 ± 0.50) recorded with similar condition.

Table 4.1. Average Fulton's condition factor K and hepatosomatic index (HSI) (Mean ± SD) of Atlantic salmon sampled from East, West and North coasts of Scotland

Population	Fulton's Condition Factor K ± SD	Hepatosomatic Index (HSI) (%) ± SD
East (n = 56)	0.89 ± 0.09	1.3 ± 0.45
West (n = 34)	0.98 ± 0.08	1.5 ± 0.17
North (n =26)	0.97 ± 0.08	1.5 ± 0.50
Total (n = 117)	0.93 ± 0.10	1.4 ± 0.41

4.3.1 Description of Isolated Parasite Species

A total of 13 metazoan parasite species were recovered from 117 Atlantic salmon. Metazoan parasites comprised of six species belonging to the taxon Digenea, three species belonging to Nematoda, three species belonging to Copepoda, and one species from the Acanthocephala.

Digenea:

A total of 801 adult *Derogenes varicus (Müller, 1784)* were isolated from the digestive tract (pyloric caeca (16), stomach (778), intestine (7)). The identification is based on the ventral sucker that is positioned in the mid portion of the body, and twice the size of the oral sucker (Fig 4.4). Testes are round and symmetrical. A smooth, almost spherical ovary sits between testes and vitellarium. Eggs are spread throughout the body and are thick shelled and operculated (Gibson & Bray, 1986). *Derogenes varicus* is widely distributed and has been reported in over 100 marine fish species (Marcogliese & Price, 1997).

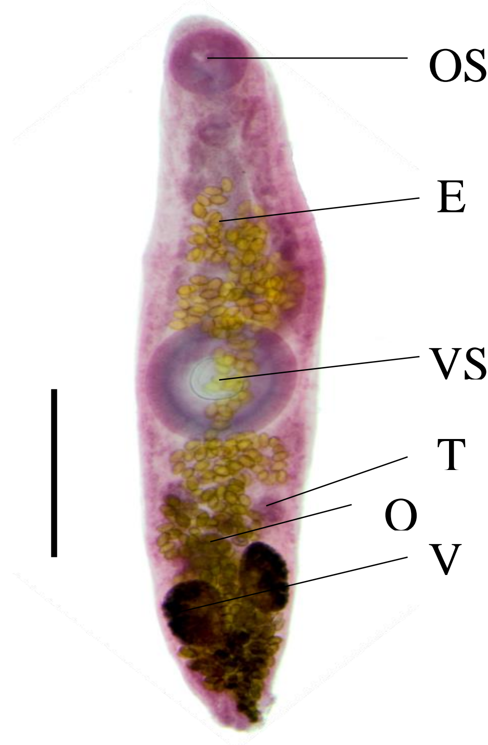

Figure 4.4. Morphology of *Derogenes varicus* with oral sucker (OS), eggs (E), ventral sucker (VS), teste (T), ovary (O) and vitellaria (V). Scale bar: 300µm

A total of 39 adult *Hemiurus communis* (Odhner, 1905) were isolated and identified based on the body shape that is elongate and fusiform, with the length greatly dependent on the extent to which the ecsoma is extruded. The ventral sucker is roughly 1.5x larger than the oral sucker and the vitellarium is composed of two large, symmetrical and oval or lobed masses, which occur posteriorly to the ovary (Fig 4.5) (Gibson & Bray, 1986). The two oval testes occur obliquely in the anterior half of the hind body, usually halfway between the ovary and ventral sucker (Gibson & Bray, 1986). All specimens were isolated from the stomach content, which is the most common site of infestation for this species (Gibson & Bray, 1986). It is a common parasite of non-clupeid fishes in the boreal region of the North-East Atlantic with a preference for shallow waters (Køie, 1995).

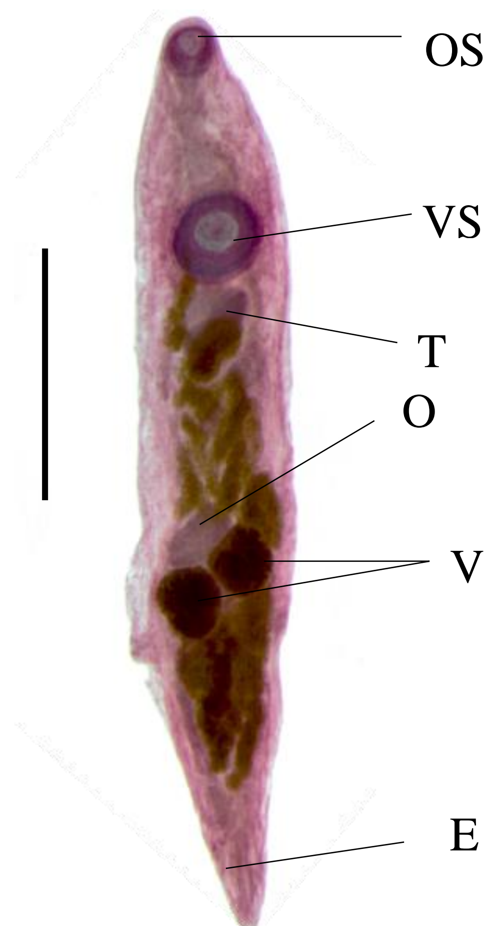

Figure 4.5. Morphology of *Hemiurus communis* with oral sucker (OS), ventral sucker (VS), vitellarium (V), testes (T), ovary (O) and ecsoma (E). Scale bar: 300µm

In total 130 adult *Hemiurus luehei* (Odhner, 1905) were isolated and identified based on morphological characteristics including a cylindrical body with the posterior end usually broader than the anterior. The ventral sucker is situated close to the oral sucker and is roughly 1-1.5 times larger in size (Fig. 4.6) (Gibson & Bray, 1986). The oval ovary is present between the testes and the posterior end of the soma and is separated from the testes by uterine coils filled with eggs (Gibson & Bray, 1986). The vitellarium is composed of two entire or slightly lobed masses, which occur immediately posteriorly to the ovary (Gibson & Bray, 1986). 125 specimens were isolated from the stomach, a common site of infestation. Only 5 specimens were isolated from another common site of infestation, the intestines (Gibson & Bray, 1986). *Hemiurus luehei* is prevalent in the North East Atlantic and North Sea, commonly infecting species such as brown trout, sprat (*Sprattus sprattus* L.) and Atlantic herring (Gibson & Bray, 1986).

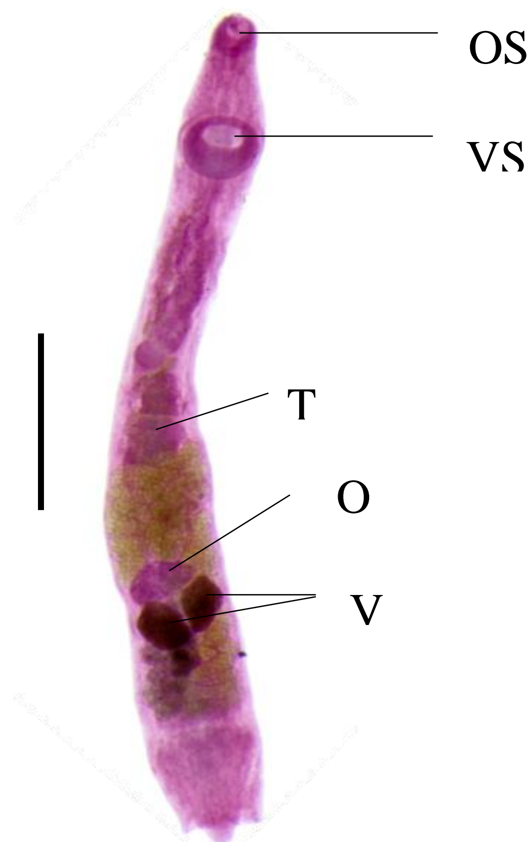

Figure 4.6. Morphology of *Hemiurus luehei* with oral sucker (OS), ventral sucker (VS), testes (T), ovary (O) and vitellaria (V). Scale bar: 300µm

A total of six adult *Brachyphallus crenatus* (Linstow, 1873) were isolated from the dissected fish. Specimens were identified based on morphological characteristics including a prominent pharynx, a ventral sucker that is similar in size or slightly larger than the oral sucker and two large lobed flower shaped vitellaria, which are positioned immediately posteriorly to the ovary. Two oval testes occur obliquely a short, but distinct, distance posterior to the ventral sucker (Fig.4.7) (Gibson & Bray, 1986). All specimens were isolated from the stomach where it is frequently found in marine and migratory teleost species (Gibson & Bray, 1986). This species is more common in deeper waters (Køie, 1995).

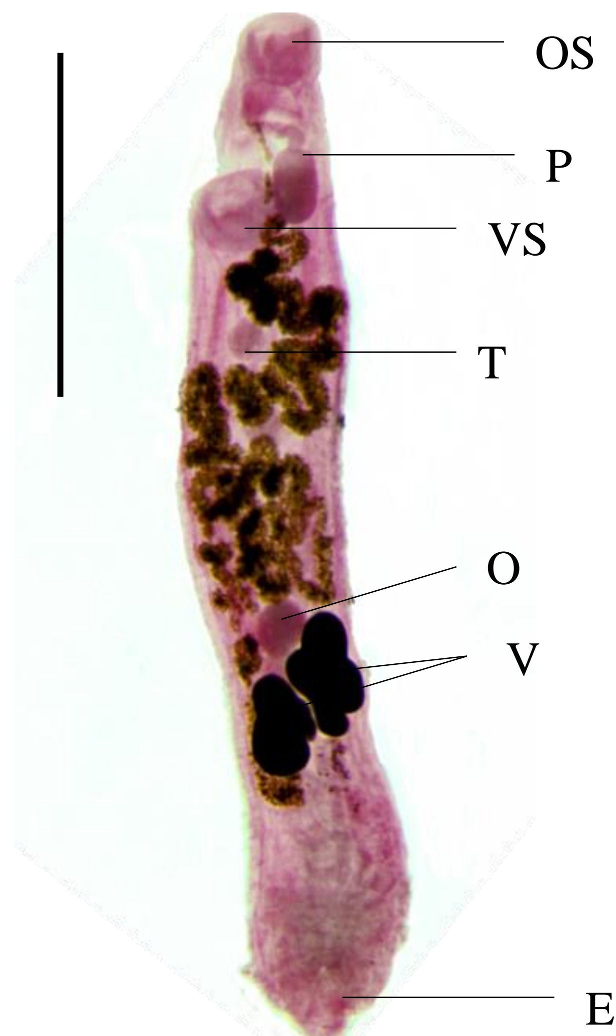

Figure 4.7. Morphology of *Brachyphallus crenatus* with oral sucker (OS), ventral sucker (VS), pharynx (P), vitellaria (V), ovary (O), testes (T) and ecsoma (E). Scale bar: 300µm

In total 53 adult *Lecithaster confusus* (Odhner, 1905) were isolated and identified based on morphological characteristics including a vitellarium that comprises of usually seven globular to slightly elongated lobes. Oral and ventral suckers well developed. Ventral sucker normally in anterior half of the body (Gibson, 1996). Two testes which are usually oval, but occasionally lobed, and a four-lobed ovary (Fig. 4.8) (Bray *et al*., 2008). All *L. confusus* were isolated from the digestive tract (pyloric caeca (39), stomach (4) and intestine (10)). *Lecithaster confusus* are distributed throughout the Atlantic, North Sea and East Arctic oceans and are commonly found in Atlantic herring amongst others (McDonald & Margolis, 1995).

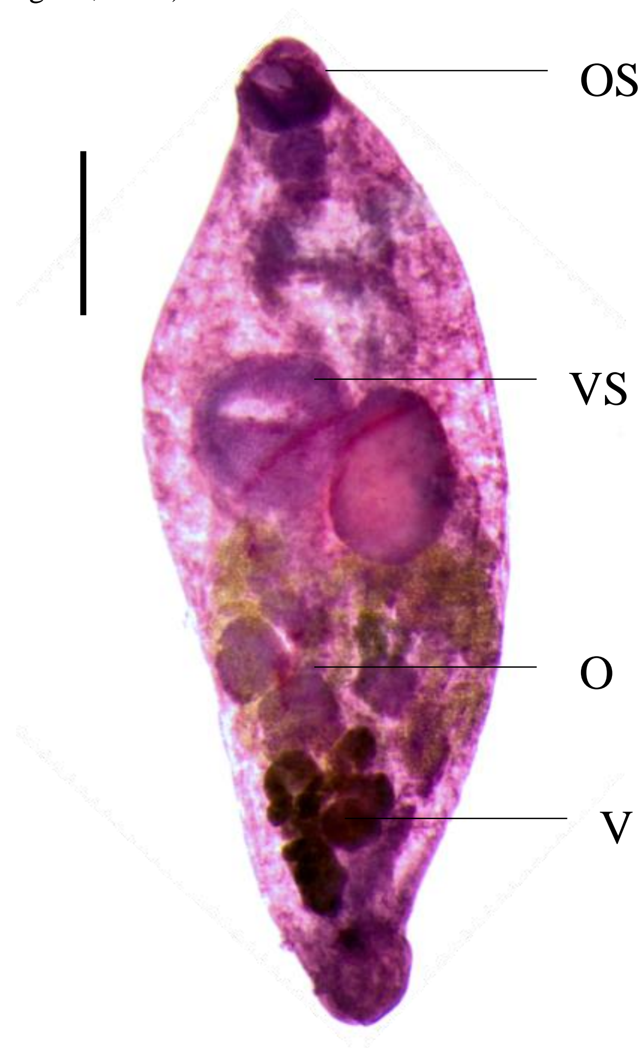

Figure 4.8. Morphology of *Lecithaster confusus* with oral sucker (OS), ventral sucker (VS), ovary (O) and vitellarium (V). Scale bar: 300µm

A total of 131 adult *Lecithaster gibbosus* (Rudolphi, 1802) were isolated and identified using the morphological feature of the lobes of the vitellarium being longer than wide in contrast to *L. confusus*. Two testes which are usually oval, but occasionally lobed, a four-lobed ovary, and uncollapsed eggs normally >19 µm in length (Fig 4.9). Oral and ventral suckers well developed. Ventral sucker normally in anterior half of the body (Gibson, 1996). All *L. gibbosus* were isolated from regions in the digestive tract (pyloric caeca (92), stomach (1) and intestine (38)), common sites of infestation. *Lecithaster gibbosus* is a circum-boreal species, occurring in northern Atlantic and north-eastern Pacific regions (Bray *et al*., 2016). It is commonly found in species such as saithe, Atlantic cod, and Atlantic herring.

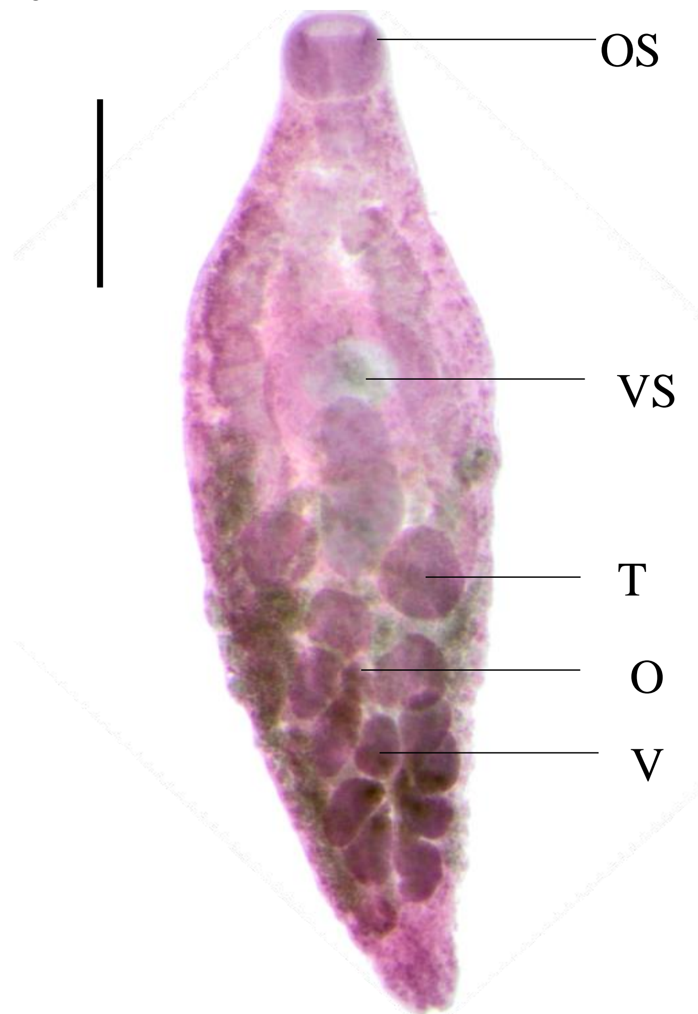

Figure 4.9. Morphology of *Lecithaster gibbosus* with oral sucker (OS), ventral sucker (VS), teste (T), ovary (O), and vitellarium (V). Scale bar: 300µm

Nematoda

A total of 24,768 *A. simplex* L3 larvae were isolated from 117 Atlantic salmon. The dark ventriculus clearly separates the intestine (I) and oesophagus (O) (Fig.4.10), a typical characteristic for *A. simplex*. They were found throughout the viscera and musculature, with higher infestations found particularly in the vent, stomach lumen, and pyloric caeca. *A. simplex* are globally distributed (Mattiucci & Nascetti, 2006) and infest over 200 species of fish and 75 cephalopod species (Pozio, 2013).

Figure 4.10. Arrangement of the ventriculus (V), intestine (I) and oesophagus (O) of *Anisakis simplex*. Scale bar: 2 mm

A total of 495 *Hysterothylacium aduncum* L4 larvae were isolated from Atlantic salmon. In *H. aduncum*, the intestinal caecum slightly exceeds the ventriculus (V) anteriorly, with a ventricular appendix also present (VA) (Fig. 4.11a). The posterior end has a conical tail (CT) without a mucron. Males also possess two spicules (S), which are used for copulation during reproducton (Fig 4.11b). Additionally, the mouth possesses three distinct lips. Most of the isolated *H. aduncum* (471) were isolated from the pyloric caeca (256), stomach lumen (140) and intestine (75). Although commonly found within the digestive tract of fish hosts, they can be found throughout the viscera and in the musculature. *Hysterothylacium aduncum* has a circumpolar distribution in the Northern Hemisphere, and is found mainly in marine teleosts in temperate and cold waters (Berland, 1991) e.g. Rainbow trout (Arai & Smith, 2016).

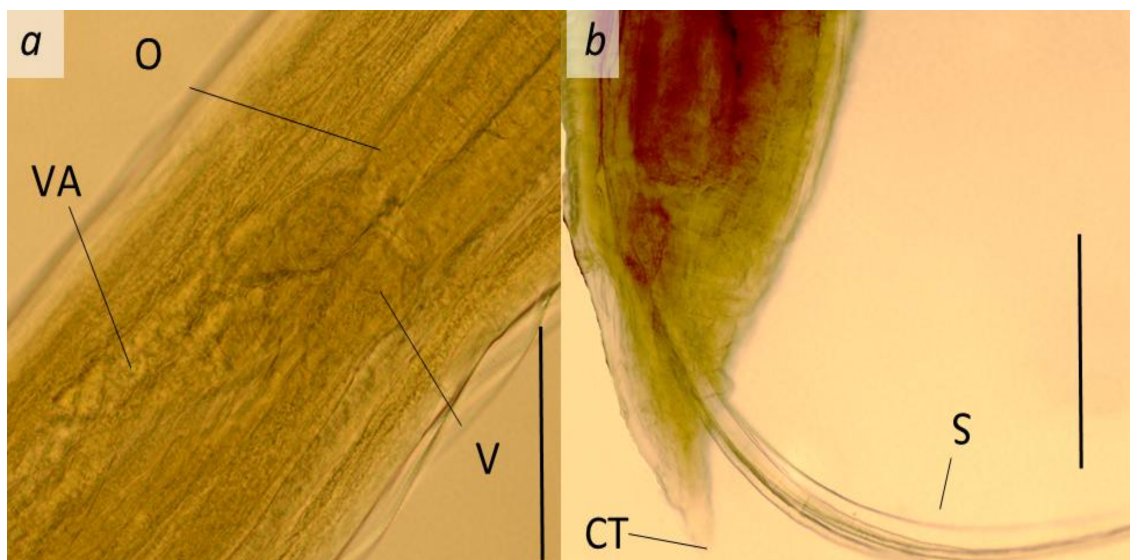

Figure 4.11. Morphology of *Hysterothylacium aduncum:* (*a*) arrangement of intestinal structures including the ventricular appendix (VA), ventriculus (V) and oesophagus (O) and, (*b*) the male posterior end with conical tail (CT) and spicule (S). Scale bar: 1 mm

A total of 24 *Pseudoterranova decipiens* L3 larvae were isolated from Atlantic salmon. One of the defining morphological features is the intestinal caecum that exceeds the ventriculus (Fig 4.12). The boring tooth at the anterior end is smaller than that of *A. simplex* (Arai & Smith, 2016). *Pseudoterranova decipiens* were found mainly in the intestine (9) and pyloric caeca (7) however, they were also isolated from the stomach (1), liver (3), spleen (2) and body cavity (2). *Pseudoterranova* spp. are known to infest 70 fish species in the North Atlantic alone including Atlantic cod (Pozio, 2013). They are more associated with demersal host species due to their inability to swim in the free living stages (Palm, 1999).

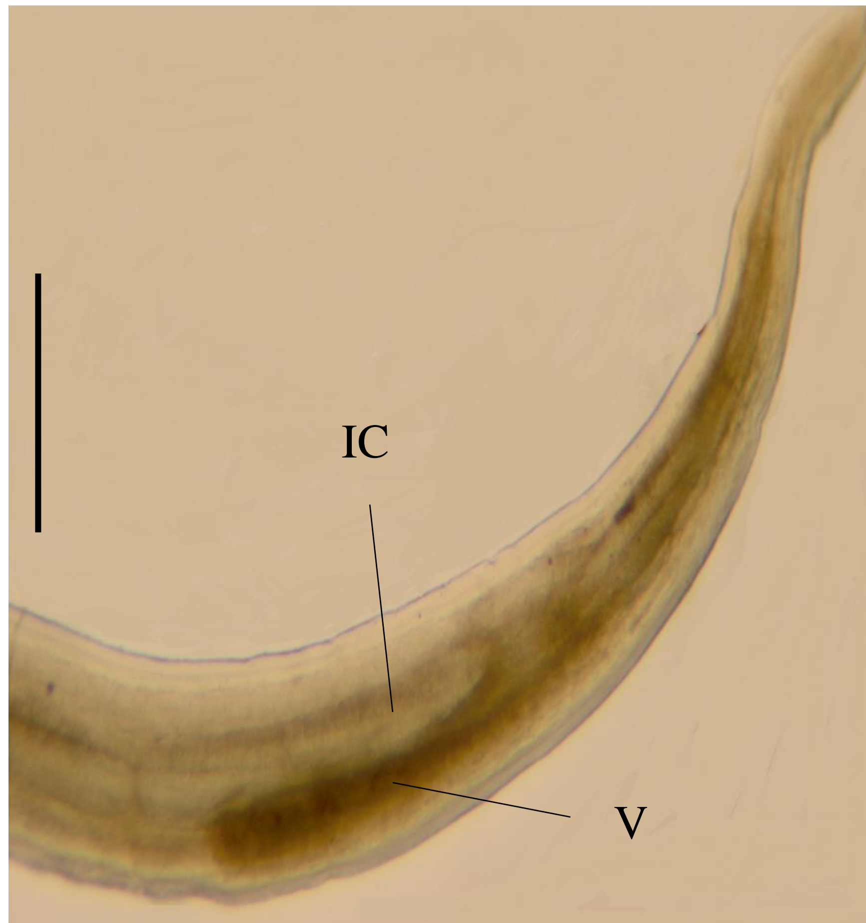

Figure 4.12. Morphology of L3 *Pseudoterranova decipiens*, arrangement of the intestinal caecum (IC) and ventriculus (v) Scale bar: 1 mm

Acanthocephala

One acanthocephalan species was isolated from Atlantic salmon from Scotland. In total four specimens of *Echinorhynchus gadi* (Müller, 1776) were isolated and identified mainly based on the cylindrical proboscis armed with 11-13 hooks in a row, with 5-6 hooks in the ring (Sobecka *et al*., 2012) (Fig 4.13). Although commonly found in the host's intestines, specimens isolated within this study were encysted within the pyloric caeca. They are commonly found in numerous marine teleost species including Atlantic cod (Wayland *et al*., 2005; Khan, 2008), and haddock (Wayland *et al*., 2005).

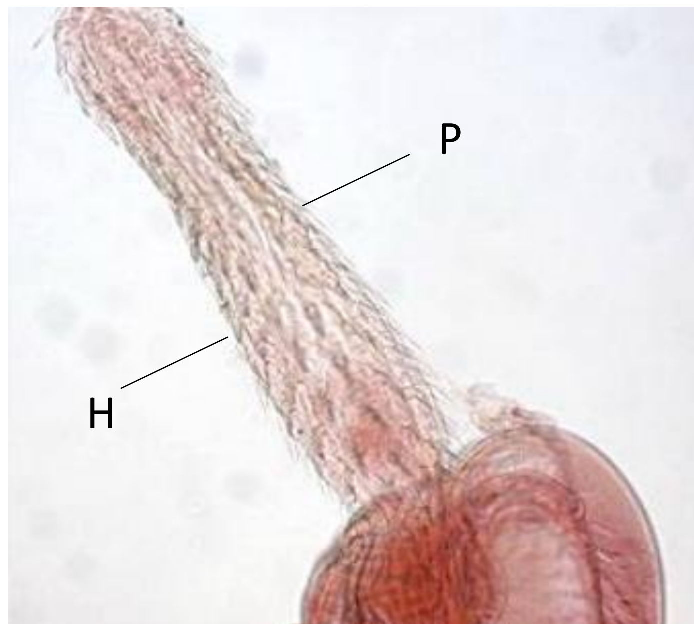

Figure 4.13. The proboscis (P) and hooks (H) of the Acanthocephalan *Echinorhynchus gadi* (Banner, 2016)

Copepoda

In total 743 *Caligus elongatus* (von Nordmann, 1832) were collected from the epidermis of Atlantic salmon. Morphologically, they appear golden-brown or yellow in colour and have a cephalothorax with disc-shaped lunules near the anterior end of the frontal plate, a thoracic segment, genital complex and uropoda (Hogans & Trudeau, 1989). Both the smaller size (6 - 8 mm), and presence of lunules are key morphological features distinguishing them from *Lepeophtheirus salmonis* (Kroyer, 1837) (Hogans & Trudeau, 1989). All *C. elongatus* were in the attached parasitic stages (chalimus 1-4 to adult stages) (Fig 4.14). *Caligus elongatus* is a cosmopolitan species found in most regions of the world (WORMS, 2018), and has been reported from more than 80 species of marine fishes, with salmonid fishes often being infected (Hogans & Trudeau, 1989).

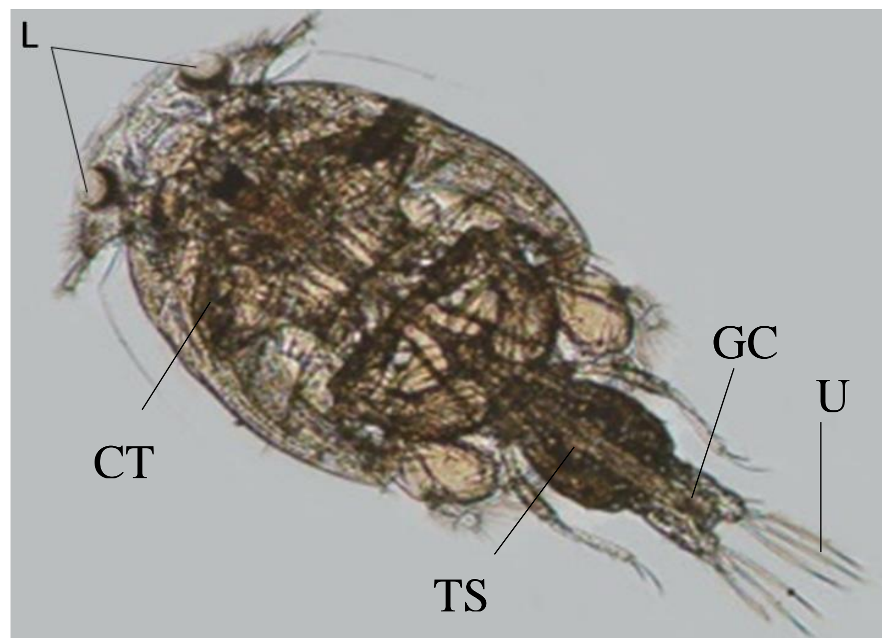

Figure 4.14. Morphology of *Caligus elongatus*. The cephalothorax (CT) has lunules (L) present at the anterior end. The posterior end is formed by the thoracic segment (TS), gentical complex (GC) and uropoda (U) (Bryant, 2017).

A total of 665 *L. salmonis* were collected and identified based on the absence of lunnule at the anterior end of the cephalothorax. *Lepeophtheirus salmonis* is approximately twice the size of *C. elongatus*. The body consists of the cephalothorax, which acts like a suction cup holding the louse on the fish, a leg-bearing segment, genital complex, and abdomen (Johnson & Albright, 1991). Life stages were not differentiated for this species, but all collected *L. salmonis* were in the attached parasitic stages (chalimus 1-2 to adult stages) (Hamre *et al*., 2013) (Fig 4.15). *Lepeophtheirus salmonis* is commonly found in marine and brackish waters, and have been found world-wide in areas of Atlantic salmon aquaculture (Hamre *et al*., 2013)

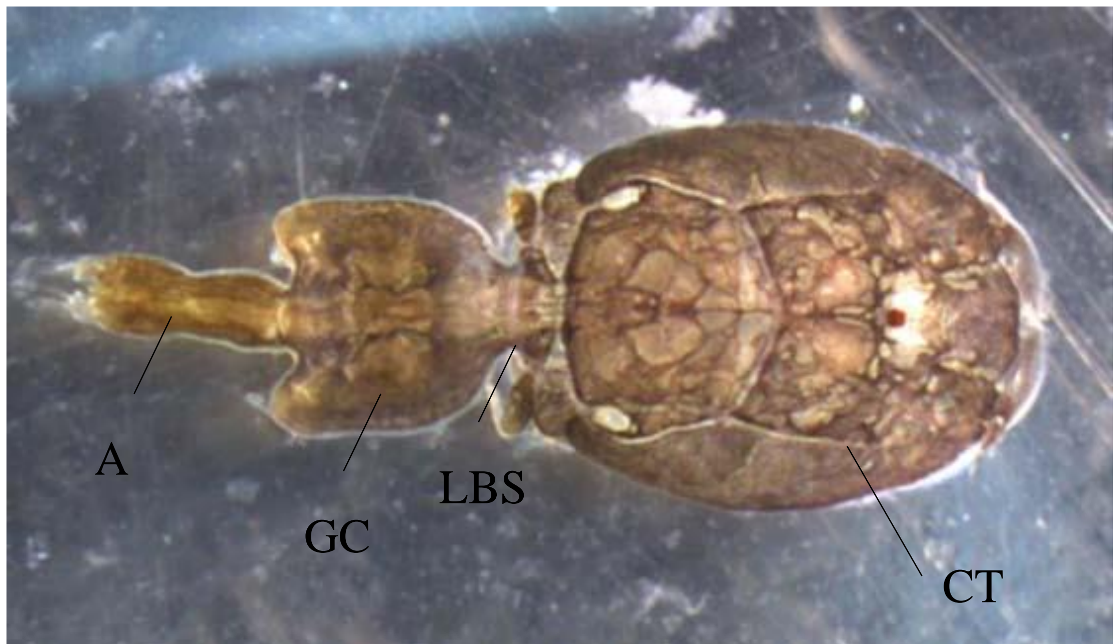

Figure 4.14. Morphology of an adult female sea louse *Lepeophtheirus salmonis*. Dorsal view of the cephalothorax (CT) with no lunules present anteriorly, the leg-bearing segment (LBS), genital complex (GC) and abdomen (A) (BCCAHS, 2011).

Eight *Salmincola salmoneus* (Wilson, 1915) were isolated from the gill of one Atlantic salmon. All were attached to the outfacing primary gill lamella. The 'gill maggot' is defined by a well-developed claw on the maximiliped (MP) (Fig 4.15) (Kabata, 1969). The modified mouth part known as the bulla (B) is inserted into gill tissues attaching the copepod permanently to the fish host. Egg sacks (E) can also be visible. The genus *Salmincola has a circumpolar distribution and* occurs in freshwater environments across North America and Europe (Bruno *et al*., 2013). Although other species such as *S. californiensis* and and *S. edwardsii* infect a number of salmonids, *S. salmoneus* is only found on Atlantic salmon (Conley, 1994).

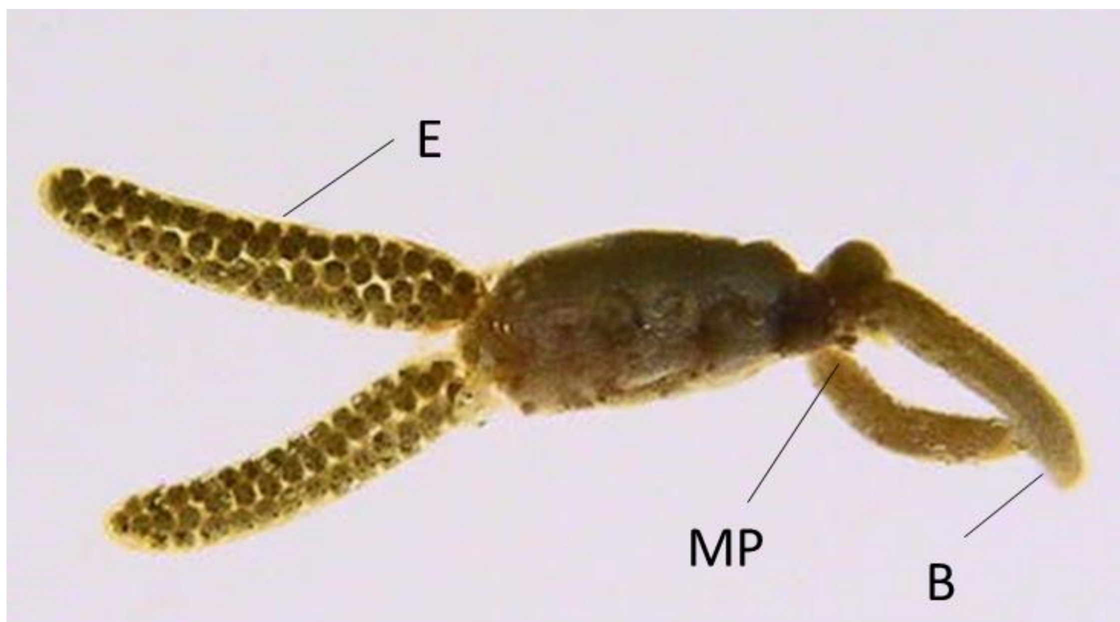

Figure 4.15. Lernaean copepod *Salmincola salmoneus* showing the maximiliped (MP), bulla (B) and egg sacks (E) (Bruno *et al*., 2013)

Key descriptors of quantitative parasitology including prevalence and mean intensity were calculated for the 13 metazoan parasites isolated from salmon populations.

Table 4.2. Prevalence (%) and mean intensity (± SD) data of parasitic species isolated from Atlantic salmon (n = 117).

Taxa	Species		East (*n*=57)	West (*n*= 34)	North (*n*= 26)
Digenea	***Derogenes varicus***	Prevalence (%)	82.5	73.5	69.2
		Mean Intensity ± SD	10.9 ± 21.2	8.5 ± 23.6	4.3 ± 3.3
	Hemiurus communis	Prevalence (%)	10.5	35.3	3.8
		Mean Intensity ± SD	1.5 ± 0.5	2.4 ± 2.6	1 ± 0.2
	Hemiurus luehei	Prevalence (%)	21.1	8.8	11.5
		Mean Intensity ± SD	9.7 ± 9.8	1.7 ± 10.4	3 ± 1.1
	Lecithaster confusus	Prevalence (%)	21.1	5.9	3.8
		Mean Intensity ± SD	4 ± 2.9	2 ± 3.5	1 ± 0.2
	Lecithaster gibbosus	Prevalence (%)	14	8.8	30.8
		Mean Intensity ± SD	9.4 ± 4.7	9.7 ± 4.3	3.4 ± 2
	Brachyphallus crenatus	Prevalence (%)	7	0	3.8
		Mean Intensity ± SD	1.3 ± 0.3	0	1 ± 0.2
Nematoda	***Anisakis simplex***	Prevalence (%)	100	100	100
		Mean Intensity ± SD	164.6 ± 140.3	225.2 ± 174.1	297.2 ± 151
	Hysterothylacium. aduncum	Prevalence (%)	59.6	73.5	73.1
		Mean Intensity ± SD	7.9 ± 14.1	4.6 ± 4.0	5.9 ± 3.6
	Pseudoterranova decipiens	Prevalence (%)	17.5	14.7	7.7
		Mean Intensity ± SD	1.4 ± 0.7	1.6 ± 2.7	1 ± 0.3
Acanthocephala	***Echinorhynchus gadi***	Prevalence (%)	0	0	3.8
		Mean Intensity ± SD	0	0	4 ± 0.8
Copepoda	***Caligus elongatus***	Prevalence (%)	91.2	91.2	96.2
		Mean Intensity ± SD	5.8 ± 4.6	11.6 ± 16.4	3.2 ± 2.2
	Lepeophtheirus salmonis	Prevalence (%)	87.7	94.1	96.2
		Mean Intensity ± SD	4.7 ± 3.6	8.2 ± 13.4	6.6 ± 4.3
	Salmincola salmoneus	Prevalence (%)	1.8	0	0
		Mean Intensity ± SD	8 ± 1.1	0	0

Of the Digenea, *D. varicus* showed the highest prevalence rates (82.5, 73.5, 69.2%) and mean intensities (10.9 ± 21.2, 8.5 ± 23.6 and 4.3 ± 3.3) for East, West and North populations respectively (Table 4.2). *Hemiurus luehei* had a higher prevalence and mean intensity (21.1%; 9.7 ± 9.8) in the salmon population off the East coast, in comparison to those in the North (11.5%; 3 ± 1.1) and West (8.8%; 1.7 ± 10.4) (Table 4.2). Conversely, *H. communis* was more prevalent in West coast salmon (35.3%) than in either North (3.8%) or East (10.5%). *Brachyphallus crenatus* was noticeably absent from Atlantic salmon off the West coast, and prevalences in East (7%) and North (3.8%) populations were also very low (Table 4.2).

The nematode *A. simplex* was present in all Atlantic salmon (100% prevalence) from East, West and North regions. They also showed the highest mean intensities of all metazoan parasites isolated with 164.6 ± 140.3, 225.2 ± 174.1 and 297.2 ± 151 found in Atlantic salmon from East, West and North regions respectively. Low prevalence rates (17.5, 14.7 and 7.7%) and mean intensities (1.4 ± 0.7, 1.6 ± 2.7 and 1 ± 0.3) of *P. decipiens* were observed across East, West and North salmon populations. *Hysterothylacium aduncum* prevalence was lowest within the East population (59.6%) in comparison to the West (73.5%) and North (73.1%). Mean intensity however, was highest in the East population (7.9 ± 14.1) compared to 4.6 ± 4.0 and 5.9 ± 3.6 in the West and North respectively.

High prevalence rates (87.7 - 96.6%) of both sea lice species (*C. elongatus* and *L. salmonis*), were observed in all populations. In contrast the copepod *S. salmoneus* and the acanthocephalan *E. gadi* were only observed in a single fish from the West and North coast populations respectively. Many of the isolated parasite species exhibited high levels of variability in mean intensities. Although mean intensities of *A. simplex* for example were 164.6, 225.2 and 297.2 within East, West and North coast salmon populations

respectively, reported standard deviation values of 140.3, 174.1 and 151 indicate the high levels of intra-population variability in infestation intensity.

4.3.2 The Parasite Component Communities of Atlantic salmon Populations in Scotland

Analysis of multidimensional scaling of Bray-Curtis similarities revealed that Atlantic salmon from the West coast exhibited the least variability of Bray-Curtis dissimilarity data points (Fig 4.17), with increasing variability in the East and North populations. PERMANOVA analyses of populations resulted in significant differences of Bray-Curtis similarities between the East and North coast populations ($F=5.007$, $df=2$, $p<0.001$). Subsequent SIMPER analysis revealed that dissimilarities between the East and North coast populations, were primarily driven by relative abundances of *A. simplex* (SIMPER; 41.42%), with *D. varicus* the next highest value explaining 2.14% of observed differences.

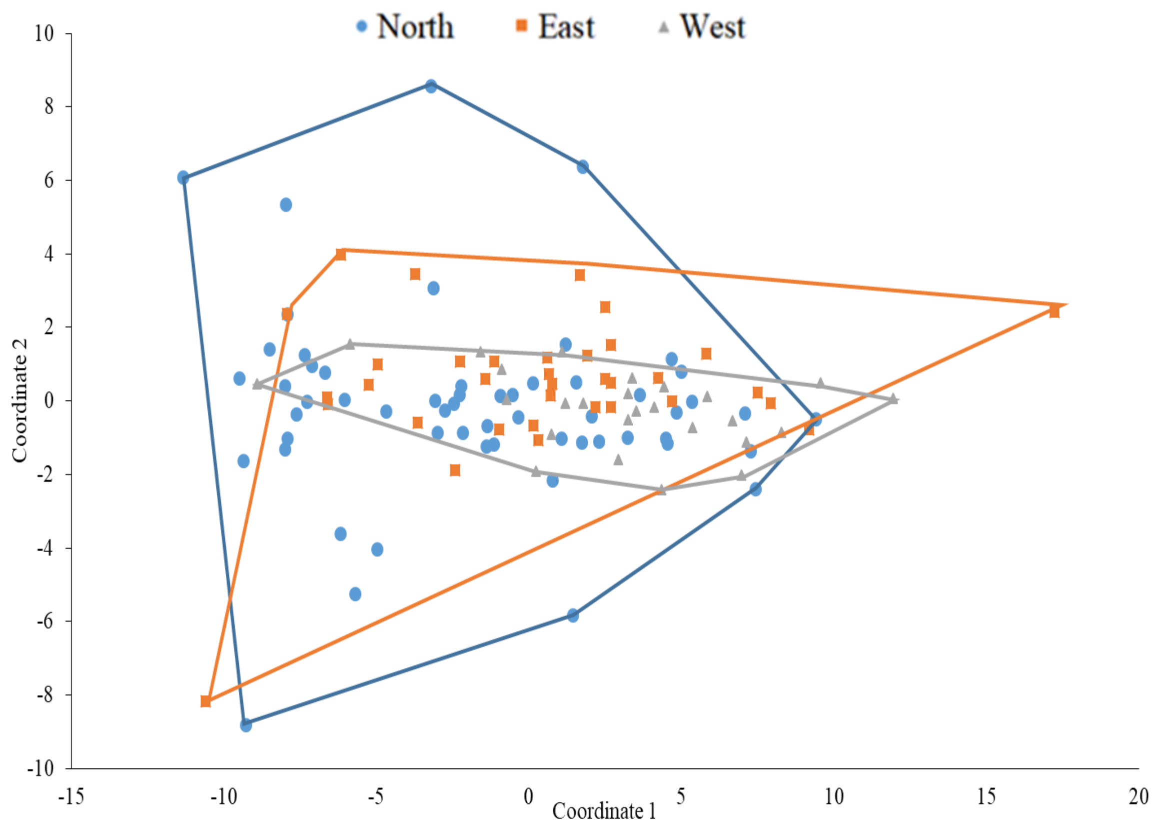

Figure 4.17. Parasite component communities from East (□) (n = 57), West (Δ) (n = 34) and North (○) (n = 26) coast Atlantic salmon populations. Non-metric dimensional scaling (nMDS) plot estimated by zero-adjusted Bray-Curtis similarities

4.3.3 Stable Isotope Analysis

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ signatures from dorsal muscle tissue of Atlantic salmon, resulted in a $\delta^{13}C$ range of -24.74‰ and -19.01‰, and a $\delta^{15}N$ range of 10.53‰ and 13.44‰ (n = 116) respectively. When divided into separate coastal populations, $\delta^{13}C$ values ranged from -24.74‰ to -19.52‰ (n = 25), -23.93‰ to -19.64‰ to (n = 35), and -24.57‰ to -19.01‰ (n = 56) in North, West and East coast populations respectively. $\delta^{15}N$ values ranged from 10.53‰ to 12.43‰, 10.82‰ to 12.81‰, and 10.55‰ to 13.44‰ in North, West and East coast populations respectively. These results revealed that the largest ranges for both $\delta^{13}C$ and $\delta^{15}N$ signatures were seen in the Eastern population, -5.56‰ and 2.28‰ respectively. Convex hulls constructed using isotopic signatures of North, East and West coast populations showed a large degree of overlap of $\delta^{13}C$ and $\delta^{15}N$ signatures (Fig 4.18a) and there was no significant difference between populations ($F=1.57$, $p=0.182$, Wilk's $\Lambda=0.946$). Further analyses using one-way ANOVAs also resulted in no differences in $\delta^{13}C$ ($F=1.86$, df = 2,115, $p=0.160$), and $\delta^{15}N$ ($F=1.15$, df = 2,115, $p=0.32$) signatures between populations.

Stable isotope analysis of $\delta^{13}C$ and $\delta^{15}N$ signatures from dorsal scales of Atlantic salmon, demonstrated a $\delta^{13}C$ range of -22.89‰ to -16.49‰, and a $\delta^{15}N$ range between 8.98‰ and 11.88‰ (n = 88), respectively. When divided into separate coastal populations, $\delta^{13}C$ values ranged from -22.89‰ to -16.49‰ (n = 20), -21.36‰ to -16.88‰ (n = 34), and -19.8‰ to -16.8‰ (n = 34) in North, West and East coast populations respectively. $\delta^{15}N$ values ranged from 8.98‰ to 11.35‰, 9.52‰ to 11.88‰, and 9.73‰ to 11.02‰ in North, West and East coast populations respectively. Convex hulls constructed using isotopic signatures of North, East and West coast populations showed a large degree of overlap of $\delta^{13}C$ and $\delta^{15}N$ signatures from scales (Fig 4.18b), and no significant difference between populations ($F=0.63$, $p=0.641$, Wilk's $\Lambda=0.951$).

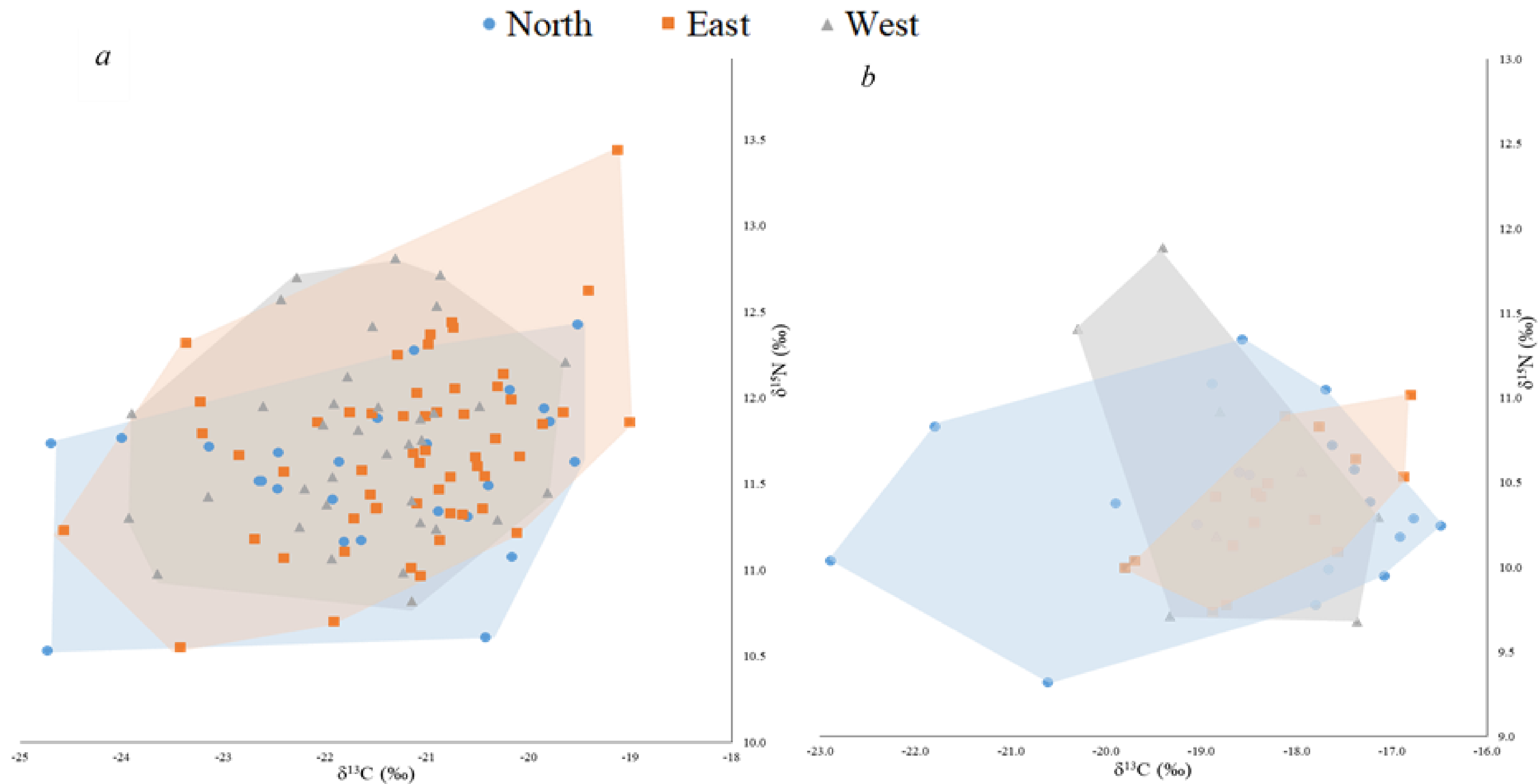

Figure 4.18. Convex hulls encompassing $\delta^{13}C$ and $\delta^{15}N$ signatures from (*a*) dorsal muscle tissue, and (*b*) scales of Atlantic salmon sampled from the East (□) (n = 56), West (Δ) (n = 35), and North (○) (n = 25) coasts of Scotland

Analysis of dorsal muscle tissue sub-samples post lipid removal showed a $\delta^{13}C$ range of -20.79‰ to -18.69‰, and a $\delta^{15}N$ range between 11.40‰ to 13.24‰ (*n* = 45) respectively. When divided into separate coastal populations, $\delta^{13}C$ values ranged from -20.79‰ to -19.03‰ to (*n* = 14), -20.07‰ to -18.69‰ (*n* = 16), and -19.94‰ to -18.74‰ (*n* = 15) within North, West and East coast populations respectively. In comparison to isotopic signatures pre-lipid removal, $\delta^{13}C$ shifted to lower values in all populations. However, similar average Carbon shift sizes of -2.15‰, -2.40‰ and -1.86‰ in North, West and East populations resulted in a large degree of overlap. $\delta^{15}N$ values ranged from 11.40‰ to 12.59‰, 11.56‰ to 13.24‰, and 11.64‰ to 12.73‰ in North, West and East coast populations respectively. There was no significant difference in either $\delta^{13}C$ and $\delta^{15}N$ signatures between populations from dorsal muscle tissue post lipid removal. Convex hulls constructed using isotopic signatures of North, East and West coast populations showed a large degree of overlap of $\delta^{13}C$ and $\delta^{15}N$ signatures from the scales and no significant difference between coastal populations (Fig 4.19).

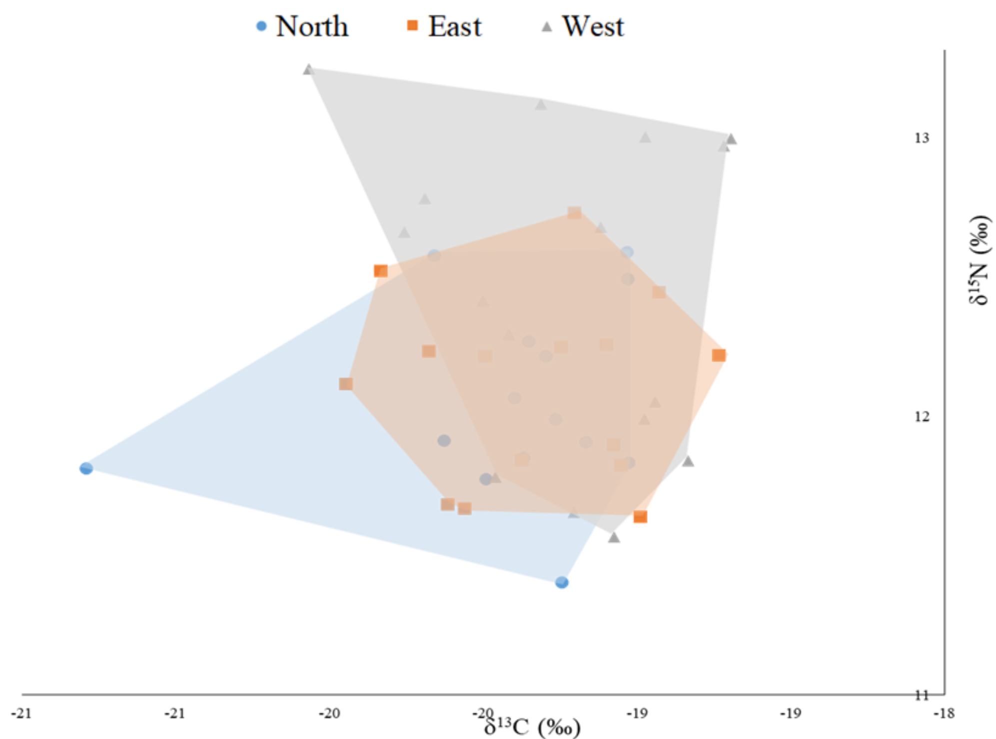

Figure 4.19. Convex hulls encompassing $\delta^{13}C$ and $\delta^{15}N$ signatures from dorsal muscle with lipid removed sampled from the East (□) (n = 15), West (Δ) (n = 16), and North (○) (n = 14) coasts of Scotland

A linear regression of $\delta^{15}N$ signature of dorsal muscle tissue from Atlantic salmon in comparison to nematode intensities showed no significant relationship (Fig 4.20) ($F=1.08$, $df = 88,111$, $p=0.431$).

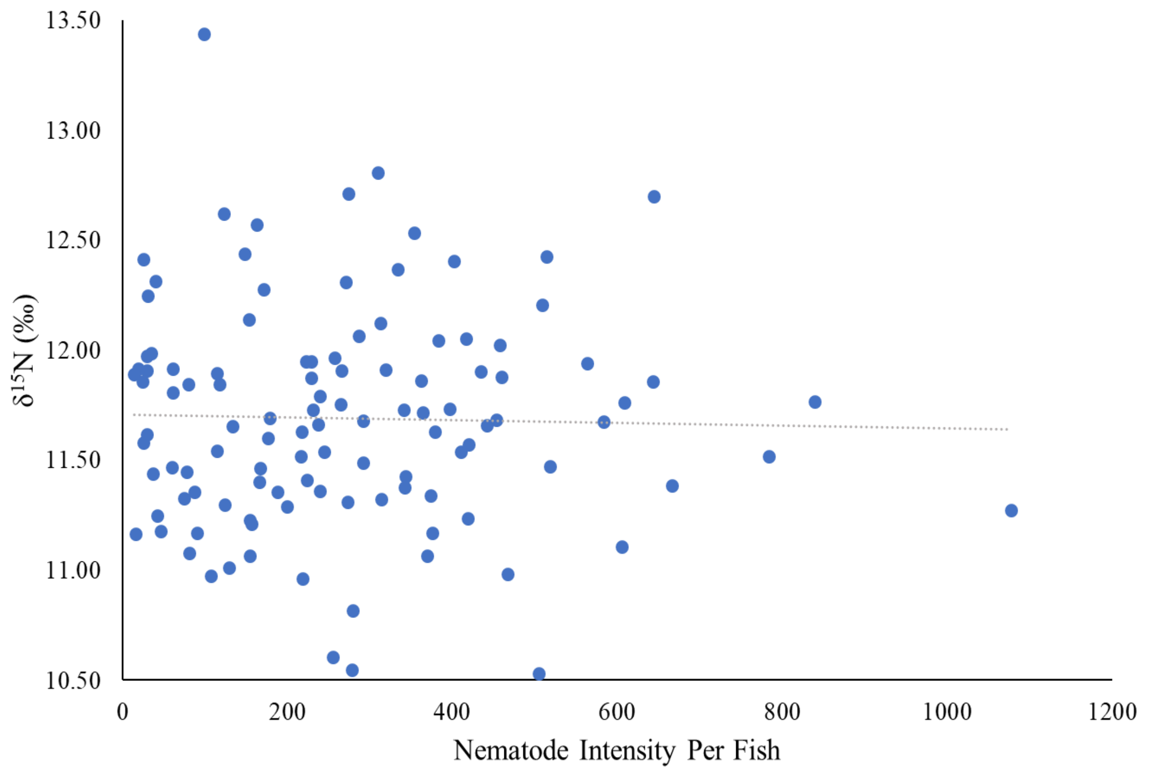

Figure 4.20. Relationship between nematode intensities of Atlantic salmon (n = 106) and $\delta^{15}N$ signatures from dorsal muscle tissue.

4.4 Discussion

The use of modern approaches in recent decades has enabled assessments of migratory route and feeding ground and the behaviour of some species such as Atlantic salmon, in the vast spatial and temporal scales encountered in the marine environment. Although considerable improvement in our understanding of the migratory and feeding behaviour of Atlantic salmon has been made, much of their behaviour within the marine phase of their life cycle remains cryptic. In this study, modern approaches such as stable isotope analysis, and parasite component communities were used to investigate whether differences in either isotopic signature, or parasite assemblages, could provide support that the three Atlantic salmon populations from the North, East and West coast of Scotland use different migratory routes and/or feeding grounds.

4.4.1 Stable Isotope Analysis

Stable isotope analysis resulted in no significant differences between $\delta^{13}C$ and $\delta^{15}N$ signatures from Atlantic salmon populations around Scotland. $\delta^{13}C$ signatures reflect the isotopic values of primary productivity levels at the base of the food chain (Wada *et al*., 1991) and as such, can be used as a proxy between different water bodies. Although conspicuous changes in $\delta^{13}C$ values are common in feeding location changes between freshwater, coastal and offshore regions (MacKenzie *et al*., 2012), the majority of European Atlantic salmon feed in the Norwegian Sea in offshore environments where potentially only latitudinal differences could be observed in the open ocean (Graham *et al*., 2010). Results of tagging studies have suggested that populations of Atlantic salmon around Scotland migrate northwards following major surface currents (Holm *et al*., 2000; Holst, 2011). The $\delta^{13}C$ results add to the evidence that the different Scottish salmon populations are likely using the same trophic food webs.

Although there is evidence of a strong association between trophic level and the level of aggregation of aquatic metazoan parasites (Lester & McVinish, 2016; Shaw & Dobson, 1995), in the present study trophically linked $\delta^{15}N$ signatures of Atlantic salmon showed no significant relationship to nematode intensity per fish. Furthermore, no differences in $\delta^{15}N$ signature was observed between sampled populations in this study.

Regional differences in dietary composition of Atlantic salmon however, have been previously demonstrated through gut content analysis of Atlantic salmon from oceanic areas of the Northwest Atlantic and the Eastern Atlantic (Reddin *et al*., 1988; Hislop & Shelton, 1993). The diet of the former consists of higher proportions of fish (Reddin *et al.*, 1988; Hislop & Shelton, 1993) and the diet of the latter of greater proportions of amphipods, krill, mesopelagic shrimp, and squid (Hislop & Youngson, 1984; Hansen & Pethon, 1985; Jacobsen & Hansen, 2001). For pre-adult and adult salmon, mesopelagic fishes continue to make up a large proportion of the diet, but opportunistic feeding on other fish species e.g. Atlantic cod that frequent the area is also common (Haugland *et al*., 2006). In comparison to the large spatial difference between the East and West Atlantic, geographical distance between populations around Scotland is relatively small. Furthermore, there is also a limited isoscape within these regions shown by isotopic signatures of zooplankton (Fig 4.2). Therefore, exposure to populations of common crustacean and fish communities within this limited isoscape along the migratory routes around Scotland and in Arctic waters will produce similar isotopic signatures in the Atlantic salmon (Johnsen *et al*., 2002; ICES, 2006). Species such as Atlantic herring constitute significant proportions (64%) of the diet for post-smolts in these areas (Haugland *et al*., 2006). Although the distribution of Atlantic herring in the North Sea is dependent on ontogeny (ICES, 2006), their high abundance, and wide distribution throughout the North Sea (Johnsen *et al*., 2002), is likely to negate any impact that ontogenetic driven differences in distribution may have on Atlantic salmon diet.

Furthermore, the similarities in isotopic signatures of Atlantic salmon returning to natal rivers in different Scottish regions support the suggestion that all populations are feeding within the same geographical area. Some European populations of Atlantic salmon have been recorded towards the western Atlantic (Hansen & Quinn, 1998). Distinct differences in dietary composition were reported for Atlantic salmon from feeding grounds in the West Atlantic, compared to the East Atlantic (Hislop & Shelton, 1993; Jacobsen & Hansen, 2001). Thus, based on the similar isotopic signatures of Atlantic salmon in this study, it is highly unlikely that any salmon made this migration.

Recent evidence of significant intra-population differences in horizontal migratory route within an Atlantic salmon population from the same natal river catchment has been reported in Norwegian Atlantic salmon (Strøm *et al*., 2018) and may also explain the variability in isotopic signature within salmon populations. Norwegian Atlantic salmon showed a strong affinity to the North of the Norwegian Sea. However, individuals also utilised feeding grounds in the Barents Sea in the east, in the Svalbard archipelago in the north, and Jan Mayen Island in the west (Strøm *et al*., 2018). The use of partially distinct oceanographic regions could lead to differential isotopic signatures of Atlantic salmon within the same population. If similar intra-population differences are present within Atlantic salmon returning to natal rivers in different Scottish regions, any differences in feeding ground utilisation is unlikely to be discriminated using stable isotope analysis, due to intra-population isotopic variability.

SIA is an extremely useful tool, but there are a number of biological implications that can cause difficulties in the analysis of particularly $\delta^{15}N$ values in highly migratory pelagic fish species (Richert *et al*., 2015). Dietary inputs in Atlantic salmon are limited and depend on the capture ability and size of prey (Brodeur, 1991; Andreassen *et al*., 2001; Jacobsen & Hansen, 2001; Hansen *et al*. 2003). Higher incidences of fish found in the stomachs of larger MSW salmon support this theory, which has been corroborated

through observed increases in $\delta^{15}N$ values and consequently trophic level (MacKenzie *et al*., 2012). As all sampled Atlantic salmon were grilse, biological limitations such as inferior acceleration and smaller gape size could restrict dietary inputs to a wide variety of slower invertebrates of lower trophic levels (Christensen, 1996; Lundvall *et al*., 1999; Scharf *et al*., 2000). Feeding from a wider trophic niche, or on dietary inputs with variable $\delta^{15}N$ values, should be reflected in prevailing $\delta^{15}N$ signatures of Atlantic salmon (MacKenzie *et al*., 2012).

Furthermore, physiological changes are likely to affect the levels of carbon within tissues (Fry & Arnold, 1982; Thorpe, 1988). During the marine phase of the life cycle, Atlantic salmon reach their peak growth rate and gain significant quantities of lipid and therefore, carbon (Thorpe, 1988). As significant changes in metabolic rate and cessation of feeding occur during their migration (Doucett *et al*., 1999a), the increased metabolism and loss of stored carbon within tissue is again likely to directly impact prevailing isotopic signatures (Fry & Arnold, 1982). Differences in remaining lipid in muscle tissue have already been shown to be problematic during isotopic analysis (Post *et al*., 2007). Even with the lipid removal from the muscle tissue in this study, there was no significant difference. It can be surmised that populations of Atlantic salmon around Scotland have either minimal differences in choice of dietary inputs and feeding grounds, or factors associated with the salmon samples in this study e.g. age, have resulted in high levels of variability in both $\delta^{13}C$ and $\delta^{15}N$ values potentially masking any isotopic differences within these populations.

4.4.2 Parasite Component Communities

Analysis of parasite component communities resulted in significant differences between Atlantic salmon from the East and North coast of Scotland. On further analysis, this was primarily driven by differences in *A. simplex* intensities (SIMPER: 41.42%). The secondary driver *D. varicus* accounted for only 2.14%. Although $\delta^{13}C$ and $\delta^{15}N$ signatures

between these populations were not significantly different, observed differences in parasite component community may suggest that migratory routes or feeding grounds for these populations are not uniform.

Atlantic salmon populations often travel with the prevailing surface currents during the smolt migration (Holm *et al*., 2000; Holst, 2011). It has been proposed that Atlantic salmon populations off the East coast of the United Kingdom including Scotland enter the Dooley current, and migrate across the North Sea to travel northward along the Norwegian coast with the Norwegian coastal current (Holm *et al*., 2000; Todd *et al*., 2008; Dadswell *et al*., 2010). Populations on the West coast however, follow the strong northerly North Ocean Atlantic Current towards the Norwegian Sea (Holm *et al*., 2000). It is believed that all populations of Atlantic salmon would congregate along the North coast before returning to rivers on the East and West coasts respectively (Shearer, 1992), but the route along Norway by East coast populations, may be mirrored during the spawning migration. Although active feeding during the spawning migration is not common (Kjellman, 2015), opportunistic feeding during this period could be reflected in differences in parasite component community reported in this study.

Anisakis simplex intensities were the primary drivers in observed differences in component communities between populations of Atlantic salmon off the North and East coast of Scotland. Transmission of *A. simplex* to intermediate hosts occurs through the ingestion of dietary items (Audicana & Kennedy, 2008). Therefore, dietary input, are a key factor in prevailing *A. simplex* intensities. Effects of changing dietary inputs have previously been seen in Canada. There was a decrease in less infested intermediate hosts such as krill, and a rise in more densely infected paratenic hosts, such as Icelandic capelin (*Mallotus villosus* Müller, 1776) and herring in the Gulf of St. Lawrence (Dufour *et al*., 2010). These changes were deemed responsible for higher anisakid infestations in Atlantic salmon (Larrat *et al*., 2013), and Greenland cod (Mouritsen *et al*., 2010).

Prevailing $\delta^{13}C$ and $\delta^{15}N$ signatures of Atlantic salmon populations in the current study were very similar, suggesting that all populations utilise the same dietary inputs. Regional differences in *A. simplex* abundance are well documented (Abaunza *et al.*, 1995; Konishi & Sakurai, 2002). It is therefore likely, that local and regional biotic and abiotic factors (Kuhn *et al.*, 2016) are causing higher intensities of *A. simplex* as seen in Atlantic salmon off the North coast of Scotland.

Seasonality is affecting both the hatching time of *A. simplex* eggs (Højgaard, 1998) and the presence of definitive cetacean hosts (Strømnes & Andersen, 2000). Temporal changes in migration of Atlantic salmon, could result in observed regional differences in nematode burdens. The smoltification rate is generally controlled by photoperiod and feeding opportunity, often leading to a latitudinal effect where populations at higher latitudes take additional years to reach maturation (Marschall *et al.*, 1998). Not only will this affect the timing of the migration, but slower growing post-smolts may be forced to remain in less favourable areas until they are large enough to actively migrate to areas with more optimal temperatures and feeding opportunities (Friedland *et al.*, 1999; Friedland *et al.*, 2000). Poorer foraging conditions are likely to delay sexual maturation, and achievement of the necessary energy threshold to begin the spawning migration, leading to temporal changes in migration (ICES, 2017).

Alternatively, low nematode burdens in Atlantic salmon off the East coast could be due to geographic distance between feeding grounds and natal rivers. The total migratory distances to the natal catchments are likely to be higher on the East coast. The lower Fulton's condition factor K and HSI within this population could be a product of increased nutritional demand by this longer migration (Doucett *et al.*, 1999a; Jonsson & Jonsson, 2005). During this period of fasting there is potential for the loss of a number of *A. simplex* from the gut (Möller, 1976; Berland, 2006), meaning the increased distance and resulting poorer condition, could have led to the lower burdens within this population.

Stable isotope analysis resulted in no significant differences between 1SW Atlantic salmon populations in Scotland, but significant differences of *A. simplex* intensity between North and East coast populations suggests the presence of variable ecological conditions between these populations. The lower condition factors and HSI indices observed in the East could potentially support this theory. Further study into the migratory route and feeding grounds of Atlantic salmon populations in Scotland is required. Using SIA on MSW Atlantic salmon from Scotland instead of 1SW seems to be the next logical step to further clarify migratory behaviour. Significant differences in horizontal migratory routes however, have been observed in an Atlantic salmon population from the same natal river catchment in Norway (Strøm *et al.*, 2018). The use of isotopic signatures to discriminate differences in migratory route and feeding ground between Atlantic salmon populations from separate natal river catchments therefore, may be confounded by these intra-population differences.

Chapter 5

Expression levels of the cytokine TNF-α1 in muscle tissue with and without symptoms of Red Vent Syndrome

5.1 Introduction

On the phylogenetic spectrum, the infraclass Teleostei is situated in a key evolutionary position (Whyte, 2007) between species heavily dependent on adaptive immunity (mammals), and species solely possessing an innate immunity (i.e. invertebrates) (Tort *et al*., 2003; Workenhe *et al*., 2010). Therefore, Teleostei represent one of the earliest vertebrate groups to possess both adaptive and innate components within an immune system (Kum & Sekkin, 2011; Uribe *et al*., 2011). This evolutionary position of Teleostei, has resulted in considerable attention into their immune relevant genes to improve understanding of both fish immunology and the evolution of immune systems. Although historically categorised as two separate immune systems, advances in our understanding of immune response has shown that these are in fact, combinational systems for organisms including teleosts (Whyte, 2007) (Fig 5.1).

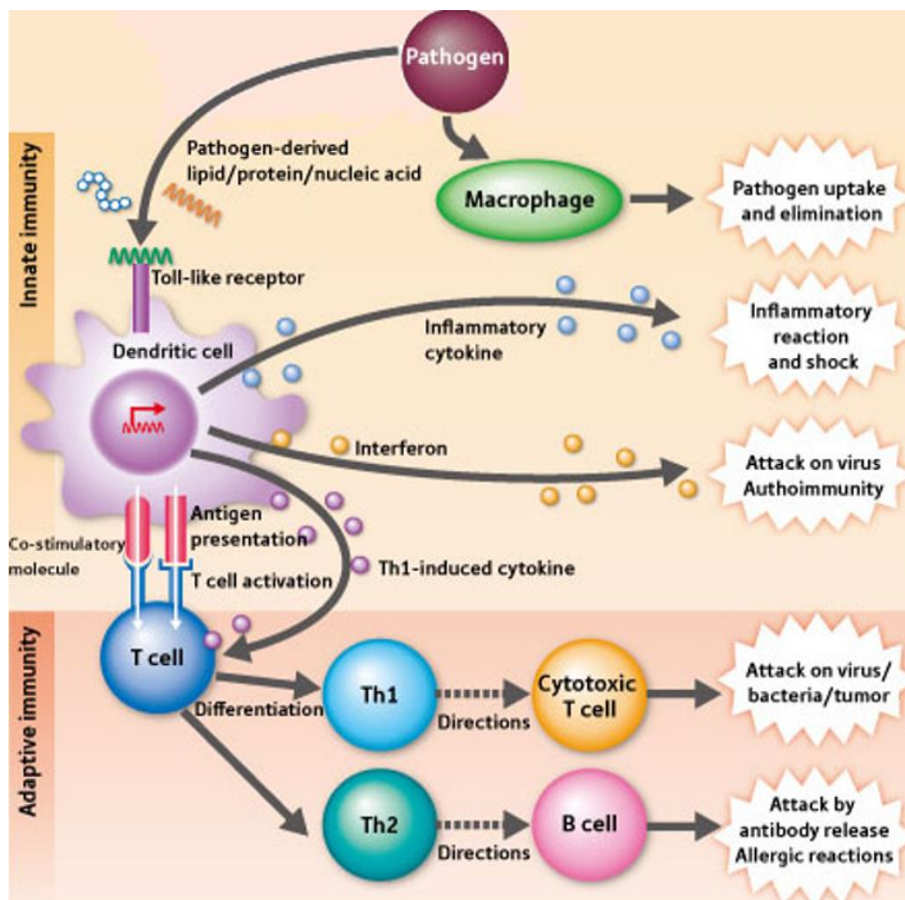

Figure 5.1. Diagrammatic view of the innate and adaptive immune systems and their combinational system (from Kum & Sekkin, 2011)

The innate response generally precedes that of the adaptive system, but subsequently activates and co-ordinates the nature of the adaptive response (Fearon & Locksley, 1996; Magnodóttir, 2010). Each system however, provides markedly different functions of the immune response.

5.1.1 Adaptive Immune Response

The adaptive immune response plays an important role in the protection against recurrent infections. This immunological system is composed of a complex network of antibodies, specialised cells, proteins, genes and biochemical messages (Uribe *et al*., 2011). Through the generation of 'memory cells' and specific soluble and membrane bound receptors such as immunoglobulins (IgM) and T-cell receptors, the adaptive system's main function is to provide a long-term memory to specific pathogens (Holland & Lambris, 2002; Uribe *et al*., 2011). This long-term memory of specific antigens with high specificity and affinity results in the fast and efficient elimination of the specific pathogen (Holland & Lambris, 2002; Uribe *et al*., 2011).

Specific antibodies used within the adaptive immunity in fishes are generated within the skin (Cain *et al*., 2000), intestine, (Jones *et al*., 1999), gills (Lumsden *et al*., 1993) the most common of which, is the IgM class (Jones, 2001). Other immunoglobulin antibodies however, are also present such as IgT which is specialized in gut mucosal immune responses (Zhang *et al*.,2011). Other cells that are used within an adaptive immune response include lymphocytes such as B cells, natural killer (NK) cells, and T cells, with the latter composed of Cytotoxic T-Lymphocytes (CTLs), T-helper cells (Th) and regulatory T-cells (T-reg). All lymphocytes are produced by the lymphoid organs, the thymus, spleen and kidneys (Buchmann, 2012). Due to the functionality of the adaptive immune system, a number of vaccines have been developed and utilised within the aquaculture industry to prevent pathogenic infection from bacteria such as *Vibrio* sp. and *A. salmonicida* (Furunculosis), and viruses e.g. birnavirus, which causes infectious

pancreatic necrosis virus (IPNV) (Holland & Lambris, 2002; Sommerset *et al.*, 2005). Although parasitic diseases such as amoebic gill disease (AGD) and proliferative kidney disease (PKD) are common problems in aquaculture, there are no vaccines commercially available (Sommerset *et al.*, 2005).

5.1.2 Innate Immune Response

Whether against bacterial and viral infection, or parasitic infestation, the innate immune system plays a greater role in resistance than the adaptive immune system in teleosts in comparison to other homeothermic vertebrates (Anderson, 1992; Yano, 1996). Offering an almost immediate non-specific response to a pathogen (Whyte, 2007), the innate system enables teleosts to overcome the constraints of the adaptive immune system, which includes low antibody titres caused by their poikilothermic nature, and the slow proliferation of lymphocytes, which can initially take between 4-6 weeks (Ellis, 2001).

The innate system itself is formed of three compartments; the epithelial/mucosal barrier, cellular components and humoral parameters (Uribe *et al.* 2011). The provision of physical barriers such as skin mucus, and gills are imperative to the prevention of pathogen entry, as these organs are constantly in direct contact with the environment (Jones, 2001; Ellis, 2001). The cellular component of the innate system is made up of phagocytic cells, including granulocytes (neutrophils) and monocytes/macrophages and non-specific cytotoxic cells (Frøystad *et al.*, 1998; Evans *et al.*, 2001; Neumann *et al.*, 2001).

Humoral parameters are a diverse array of both proteins and other molecules, which are classified based on their ability to recognise specific pathogens, or their effector functions in killing and/or preventing the growth and spread of pathogens (Magnodóttir, 2010; Kum & Sekkin, 2011) (Table 5.1).

Table 5.1. Non-specific humoral molecules and their complement system/mode of action in fish (from Kum & Sekkin, 2011).

Humoral components	Composition	Mode of action
Antibacterial peptides e.g. histone H2B, cecropin P1, pleurocidin, parasin, hipposin, SAMP H1	Protein	Constitutive and inducible innate defence mechanism, active against bacteria, defence before development of the specific immune response in the larval fish.
Antiproteases e.g. α1-anti-protease, α2-anti-plasmin, α2-macroglobulin	--	Restricts the ability of bacteria to invade and growth *in vivo*, active against bacteria.
Ceruloplasmin	Protein	Copper binding
Complement system e.g. C3, C4, C5, C7, C8, C9 and their isoforms, B- and D-factors	Protein	Promote binding of microbes to phagocytes, promote inflammation, and cause osmotic lysis or apoptotic death.
Interferons (IFNs)/Myxovirus (Mx)-proteins e.g. IFN-αβ, IFN-γ	Glycoprotein /or Protein	Aids in resistance to viral infection, inhibit virus replication, inducible IFN-stimulated genes.
Lectins e.g. legume and cereal lectins, mannose-binding lectin, C-type lectins, intelectin, ladder lectin	Glycoprotein and/or specific sugar binding protein	Induce precipitation and agglutination reactions, recognition, promote binding of different carbohydrates in the presence of Ca^{+2} ions, active complement system, opsonin activity and phagocytosis.
Lytic enzymes e.g. lysozyme, chitinase, chitobiase	Catalytic proteins lysozyme, Complement components	Change the surface charge of microbes to facilitate phagocytosis, haemolytic and antibacterial and/or anti-virucidal, anti-parasitical effects, opsonic activity, inactivation of bacterial endotoxin(s)
Natural antibodies	--	Recognition and removal of senescent and apoptotic cells and other self-antigens, control and coordinate the innate and acquired immune response, activity against haptenated proteins.
Pentaxins e.g. C-reactive protein, serum amyloid P	Protein	Opsonisation or activation of complement, promote binding of polysaccharide structures in the presence of Ca+2 ions, induce cytokine release, coast microbes for phagocytosis by macrophage.
Proteases e.g. cathepsine L and B, trypsin-like	--	Defence against bacteria, activity against *Vibrio Anguillarum.*
Transferrin/Lactoferrin	Glycoprotein	Iron binding, acts as growth inhibitors of bacteria, activates macrophage.

Proteins such as transferrin can act as growth inhibitors of bacteria (Langston *et al*., 1998), as well as an acute phase protein invoked during an inflammatory response to remove iron from damaged tissue (Bayne & Gerwick, 2001). Other proteins involved in the humoral response include various lytic enzymes (e.g. Lysozymes and chitinase) (Manson *et al*., 1992), pentraxins (lectins) (Bayne & Garwick, 2001), lysozymes (Kum & Sekkin, 2011) and even natural antibodies (Gonzalez *et al*., 1989). They also comprise cytokines, which have recently been described in a number of fish and generated significant interest in recent years.

5.1.3 Cytokines

Cytokines in teleosts form an integral part of the non-specific innate immune response. Cytokines are soluble mediators involved in initiating, maintaining, and regulating amplification of the immune response (Kum & Sekkin, 2011). Genome projects of the Fugu (*Takifugu rubripes*, Temminck & Schlegel, 1850) (Aparicio *et al*., 2002) and zebrafish (*Danio rerio*, Hamilton, 1822) (Howe *et al*., 2013) have resulted in significant progress in our knowledge of the role of cytokines. A number of interleukin cytokines have been cloned in fish species e.g. interleukin-1β, IL-2, IL-4, IL-6, IL-10, IL-11, IL-12 (Zou *et al*., 1999; Fujiki *et al*., 2000; Lutfalla *et al*., 2003; Savan *et al*., 2003; Yoshiura *et al*., 2003; Zou *et al*., 2003a; Bird *et al*., 2005a; b; Inoue *et al*., 2005; Wang *et al*., 2005; Corripiyo-miyar *et al*., 2007; Li *et al*., 2007), potentially offering future applications for the development of vaccines and or immuno-stimulants within aquaculture (Savan & Sakai, 2006).

A number of B-jellyroll cytokines, referred to as the Tumour Necrosis Factor superfamily, have also been described from fish including TNF-α1, TNF-α2 and TNF-α3 isoforms amongst others (Savan & Sakai, 2004; Savan *et al*., 2005; Hong *et al*., 2013; Zou & Secombes, 2016). Although TNF-α is the closest family member to TNF-β lymphotoxin

found in mammals (Hong *et al*., 2013), TNF-β lympthotoxin has yet to be isolated in teleost fish species.

TNF-α and TNF-β lympthotoxin possess some overlapping functions (Calmon-Hamaty *et al*., 2011), but their roles within the immune system are quite distinct, partly due to their differential expression (Bodmer *et al*., 2002; Ware, 2005). The absence of TNF-β lympthotoxin in teleost fish, has led to the suggestion of a more prominent role of TNF-α (Whyte, 2007), or compensation through an expansion of TNF-α isoforms such as type-II TNF-α in trout (Hong *et al*., 2013).

Comprising part of the 'cascade' of pro-inflammatory cytokines released at an early stage of infection in teleosts, TNF-α triggers expression of a number of other immune genes associated with inflammation including IL-1β, IL-8, IL-17C, TNF-α and COX-2 (Zou & Secombes, 2016). Host resistance to some parasitic diseases such as *Toxoplasma gondii* (Johnson, 1992; Yap *et al*., 1998; Grigg *et al*., 2001), *Leishmania major* (Chakour *et al*., 2003) and *Trypanosoma* spp. (Castaños-Velez *et al*., 1998; Iraqi *et al*., 2001) has been achieved through manipulating the duration of the inflammatory process and thus controlling pathogen multiplication (Derouich-Guergour *et al.*, 2001). TNF-α has been implicated as playing a key role in the response to these parasitic diseases. Its role in the immune response to *A. simplex* however, remains unclear. While *A. simplex* is known to infest over 200 fish species worldwide (Klimpel *et al*., 2004), the infestation of Atlantic salmon by *A. simplex* has received significant attention since the observation of Red Vent Syndrome (RVS) caused by this parasite (Beck *et al*., 2008).

The cellular component responds to endo-parasitic helminths such as *A. simplex*, with a biphasic wave of neutrophils followed by the observation of monocytes/macrophages and degranulating eosinophils in the digestive tract of teleostean fish (Reite & Evensen, 2006). Within RVS affected vent tissue of Atlantic salmon, degranulating eosinophils and

occasionally melano-macrophages have also been observed (Dezfuli *et al*., 2007; Beck *et al*., 2008; Noguera *et al*., 2009).

In mammalian hosts however, both innate and adaptive responses (humoral and cellular) are prominent actors in antihelminthic responses (Buchmann, 2012). A skewing of the Th1 lymphocyte response towards a Th2 type is a characteristic element in mammalian hosts (Buchmann, 2012). Many elements found within mammalian Th1 and Th2 find counterparts in fish e.g. CD4+ and CD8+ T cells (Olsen *et al*., 2011), and Cytokines IL-4/IL-13 and their receptors (Wang *et al*., 2011). Both type I and type II TNF-α genes have been described in Atlantic salmon (Hong *et al*., 2013) and are produced by CD4+ cells associated with both Th1 reactions, and Th17 cells (Buchmann, 2012). In some teleosts however, e.g. Atlantic cod (*Gadus morhua* L.), the genome does not possess either MHCII and CD4 genes (Star *et al*. 2011) challenging the notion that Th2 type responses occur in this species which evidently shows a clear encapsulation response towards tissue penetrating nematodes.

To date, although histological analyses of vent tissue have been previously carried out (Beck *et al*., 2008; Noguera *et al*., 2009), there has been no assessment of gene expression within the region. Furthermore, there has been no evaluation of any cytokine in the exhibition of RVS symptoms. The results of this study therefore, provide the first assessment of the levels of expression of TNF-α1, within RVS affected tissue.

As a part of the Th1 response, TNF-α1 functions as a pro-inflammatory cytokine, which has been shown to mediate the proliferation of epithelial cells in mammals (Ip *et al*., 1992). It is also involved in rapid recruitment of phagocytic granulocytes to regions of tissue damage similar to those seen within the vent region during RVS (Garcia-Castillo *et al*., 2004 Roca *et al*., 2008). This chapter hypothesised that the abundance of this cytokine will be increased in the regions of RVS inflammation.

5.2 Materials and Methods

The vent region of 23 Atlantic salmon sampled from wild capture fisheries on the East (Usan, Angus) (56°40'55.7"N 2°27'02.5"W; n = 8), North (Armadale, Sutherland) (58° 32′ 59.64″ N, 4° 5′ 22.2″ W; n = 8) and West (Sandyhills, Dumfries and Galloway) (54°52'45.7"N 3°43'46.9"W; n = 7) coasts of Scotland (Chapter 3.2.1) were assessed for Red Vent Syndrome severity using the Fisheries Research Services (Now Marine Scotland) guidelines (FRS, 2008) (Chapter 3.2.1). Fish samples were sub-divided into groups showing 1 – no symptoms (n = 11), 2- mild symptoms (n = 5), 3-moderate symptoms (n = 7), and 4-severe (n = 7). Fish samples were dissected following the protocol in Chapter 3.2.2 and parasitic nematode larvae were isolated and enumerated from vent muscle tissue. Muscle tissue samples (0.5 cm^3, ~25-30 mg) were excised from the vent region as soon as samples had been caught and immediately stored in RNAlater® (Thermo Fisher Scientific, Loughborough, UK) at -20 °C until processing. Additionally, five samples of vent muscle tissue taken from Atlantic salmon from the Sandbank hatchery of the River Spey (57°21'0.001"N 3°18'21.188"W) were included in this study. These fish samples had signs of *Saprolegnia* sp. an oomycete infection and were chosen for comparative purposes.

5.2.1 RNA Extraction

Total RNA was extracted from vent muscle tissue using TRIzol™ reagent (Invitrogen™, (Thermo Fisher Scientific, Loughborough, UK). Muscle tissue was homogenized using a sterile pestle in an RNase free microcentrifuge tube (Thermo Fisher Scientific, Loughborough, UK) with 300 µl of a Buffer RLT (Qiagen, Manchester, UK)-β-mercaptoethanol (β–ME) solution consisting of 10 µl of β–ME per 1 ml of Buffer RLT. Once homogenised, the solution was mixed with 1 ml of TRIzol™ reagent and re-homogenised with a pipette. After incubation for 5 minutes at room temperature to permit

complete dissociation of the nucleoproteins complex, 200 µl of chloroform (Thermo Fisher Scientific, Loughborough, UK) was added to the solution, and mixed by hand vigorously for 15 seconds. After incubation for 15 minutes at room temperature, this solution was centrifuged for 15 minutes at 12,000 g at 4 °C. The resulting aqueous phase containing the RNA was transferred to a new RNase free microcentrifuge tube and mixed by inversion with 500 µl of isopropyl alcohol. After further incubation for 10 minutes at room temperature, samples were centrifuged again for 10 minutes at 12,000 g and 4 °C. The subsequent supernatant was discarded, and samples were washed with 1 ml of 75% ethanol solution (molecular grade ethanol diluted with RNase free water) and centrifuged for 10 minutes at 12,000 g and 4 °C. The process of washing RNA with isopropyl alcohol and 75% ethanol was repeated twice. RNA pellets were then air dried at room temperature for 10 minutes.

A DNase treatment was performed using the MasterPure™ RNA purification kit (Epicentre, Madison, USA). RNA pellets were re-suspended in a 200 µl DNase I solution made by diluting 5 µl of RNase-Free DNase 1 enzyme up to 200 µl with 1x DNase buffer. This solution was incubated at 37 °C for 20 minutes. Following incubation, 200 µl of 2x T and C lysis solution was added and vortexed for 5 seconds. A further 200 µl of MPC protein precipitation reagent was added and vortexed for 10 seconds and subsequently incubated on ice for 5 minutes. Debris was pelleted by centrifugation for 10 minutes at 12,000 g and 4 °C. The supernatant containing RNA was transferred to a new RNase free microcentrifuge tube and washed with isopropyl alcohol and 75% ethanol as outlined during RNA extraction.

RNA quality was assessed using the NanoDrop2000 spectrophotometer (Nanodrop Technologies, Wilmington, USA). Pure nucleic acids typically yield a 260/280 ratio of ~2.0 when assessed by spectrometry (Desjardins & Conklin, 2010). RNA samples had found to have $OD_{260/280}$ nm values ranging from 1.7–2.0 and concentrations (average of

two results) between 25.1 – 398 ng/µl. Integrity of RNA was assessed by electrophoresis on a 1% (w/v) agarose gel using the stain GelRed™ along with a 1 kb plus DNA Ladder GeneRuler™ (Thermo Fisher Scientific, Loughborough, UK) (Fig 5.3). With RNA extractions only yielding low concentrations of RNA and with already small sample size, all RNA was diluted to a standardised concentration of 20 ng/µl prior to cDNA synthesis to maximise available sample size. RNA samples were stored at -80 °C until further processing

5.2.2 Complementary (c)DNA Synthesis

cDNA synthesis was performed with extracted total RNA using Precision NanoScript™ 2 (Primerdesign, Southampton, UK). A total of 20 ng (1 µl) of RNA template was added to 1 µl of Oligo-dT primers and made up to 10 µl with RNAse-free water (Thermo Fisher Scientific, Loughborough, UK). Each sample was heated to 65 °C for 5 minutes, and immediately cooled on ice. The 10 µl solution was mixed with a 10 µl solution containing: 5 µl NanoScript2 4x buffer, 1 µl dNTP mix 10 mM, 1 µl NanoScript2 enzyme and 3 µl DNAse/RNAse free water. The solution was briefly vortexed and incubated at 42 °C for 20 minutes, followed by heat inactivation at 75 °C for 10 minutes and stored at -20 °C until further processing.

5.2.3 Reference Gene Selection and TNF-α1 Probe

In quantitative (q)RT-PCR, expression levels of the target gene (TNF- α1 in this instance) are quantified relative to endogenous controls (Olsvik *et al.,* 2005). As genes are differently expressed between tissues and the particular condition of an individual e.g. smoltification (Olsvik *et al*., 2005), the selection of a stable housekeeper gene is a critical consideration for the reliable relative quantification of TNF-α1 expression. Therefore, to select an appropriate endogenous control for this experiment, six genes were tested using a GeNorm kit (PrimerDesign, Southampton, UK). The genes used within the GeNorm kit included a selection of targets including; 18SrRNA (Ribosomal RNA), GAPDH

(glyceraldhyde-3P-dehydrogenase – a catalytic enzyme in the glycolytic pathway), EEF1A2 (eukaryotic elongation factor-1α-2), which plays an important role in protein translation (Olsvik *et al*., 2005), B2M (Beta-2 microglobulin which is the Beta chain of the major histocompatibility complex I (MHC) (Kales *et al*., 2006), YWHAZ (Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Zeta which is a component of the mitochondrial import stimulation factor (Tang *et al*., 2007) and EiF3EA (eukaryotic translation initiation factor 3 subunit E, which is also involved in protein synthesis (Żarski *et al*., 2017). These potential reference genes were selected to include a variety of cellular functions and follow previous use in other validation studies (Olsvik *et al*., 2005) (Table 5.2).

Table 5.2. Oligonucleotide sequences, amplicon sizes, and corresponding GenBank accession numbers of primers tested in the GeNorm Kit.

Transcript Target	Forward or Reverse	Oligonucleotide sequence (5'-3')	Amplicon size (bp)	GenBank Accession Number
18s rRNA	F	CCGTAATTGGAATGAGTACACTTTAAA	97	AJ427629
	R	CGCTATTGGAGCTGGAATTACC		
B2M	F	TCCTGAAGAATGGTGTGGAGATC	83	NM_00124591
	R	CTTGGTGAGGTGGAACTGC		
YWHAZ	F	CAAGCCTATCTTGATAGCCTTATGC	139	XM_01413162
	R	CAACCTCCTTACACAACTTACATTAGG		
EiF3EA	F	CCTCAACGCCATTCAGACCA	124	NM_00114169
	R	CTGCTGTATGACCTTGACCAGA		
EEF1A2	F	GCATCAACAGCAACAGAATTCCTA	100	NM_00113975
	R	GGCGAGTGCATCTTCAGAGG		
GAPDH	F	CGTCTGGTGACCCGTGC	119	NM_00112356
	R	CCGTGGGTGGAGTCATACTTG		

qPCR assays of candidate endogenous controls were tested in duplicate with 15 individual cDNA samples from Atlantic salmon encompassing all RVS severity sub-groups. For each reaction, 5 ng (5 µl) of cDNA template was added to 15 µl PrecisionPLUS Mastermix with SYBR green (PrimerDesign, Southampton, UK) in two 96-well optical plates using the StepOnePlus Real-Time PCR System (Applied Biosystems Ltd, Warrington, UK). Plates were setup according to the manufacturer's recommendation including two negative controls (RNase-free water – no template controls) per candidate gene. Samples were run as part of a qPCR program comprising an enzyme activation step at 95 °C (2 minutes), followed by 50 cycles of a denaturation step at 95 °C (10 seconds) and data collection step at 60 °C (60 seconds). A post-PCR melt curve analysis was also included to determine the specificity of primers.

Threshold cycle (C_T) values were uploaded into Qbase+ software (Biogazelle, Gent, Belgium), with the stability value (M-value) used to assess the expression stability of candidate genes. The gene-stability measure M is defined as the average expression stability value of remaining reference genes at each step during stepwise exclusion of the least stable reference gene (Vandesompele *et al*., 2002). Further details of calculations involved can be found in Vandesompele *et al*. (2002). None of the six candidate reference genes selected were suitable to be used as endogenous controls individually, with GeNorm M-values below the 0.5 required for use in normalization of qPCR data (Fig 5.2) (Hellemans *et al*., 2007). Furthermore, the GeNorm V values defined as the pairwise variation V between two sequential normalization factors containing an increasing number of genes, did not fall within the acceptable range of <0.15. Due to both time and economic limitations however, the combination of the three most stably expressed candidate genes (0.523 – V2/3) (Hellemans *et al*., 2007) (YWHAZ, B2M, and EiF3EA), were selected to be used as endogenous controls.

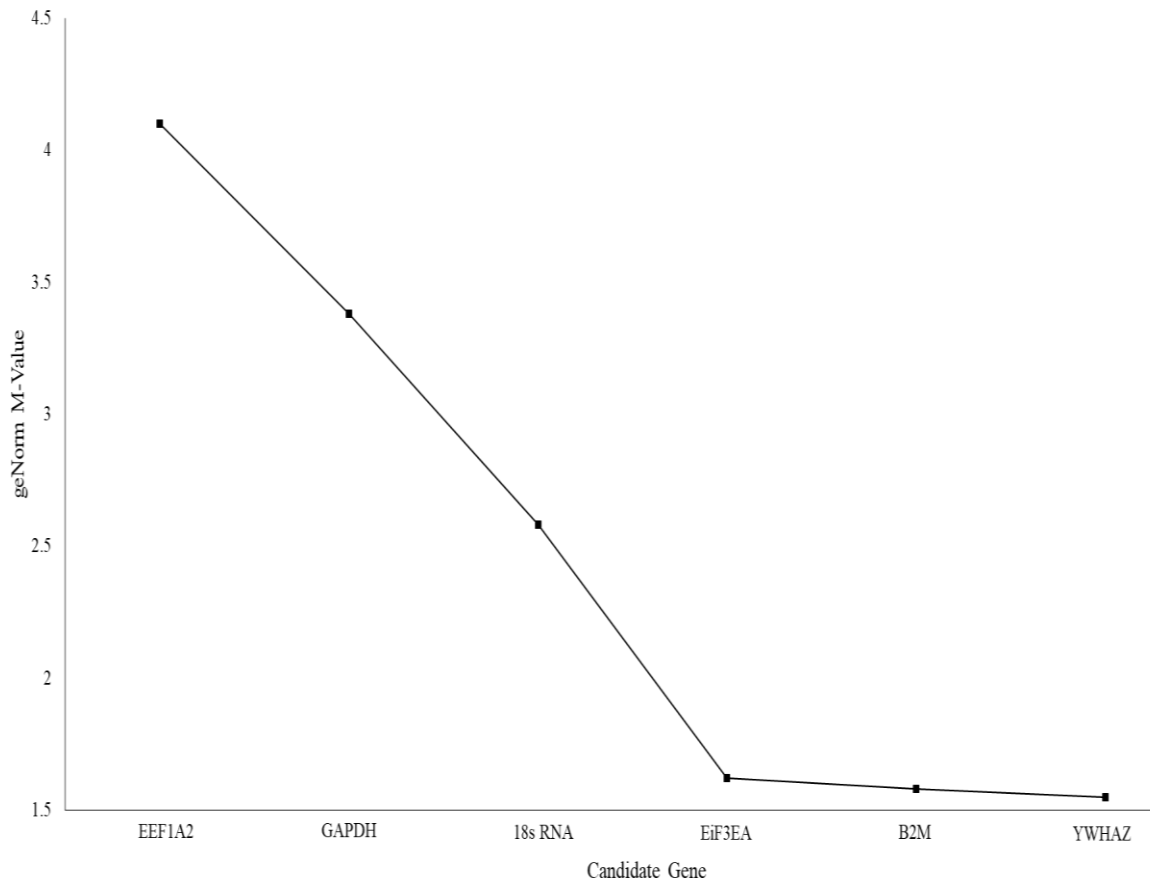

Figure 5.2. Candidate genes ranked according to their stability (expressed in GeNorm M values) from most unstable transcripts on the left (high M value) to the most stable reference targets on the right (low M value)

5.2.4 Quantitative (q)PCR assays

Efficiencies of all primer/probe sets were evaluated on a series of five, two-fold serial dilutions of cDNA (100 to 6.25 ng/µl). Using the efficiency (E) calculations according to the equation $E = 10^{(1/\text{-slope})}$, all assays demonstrated acceptable efficiencies (1.96-2.00). Singleplex qPCR assays were conducted in 96-well optical plates using the StepOnePlus Real-Time PCR System (Applied Biosystems). Taqman 6-FAM primer probe mixes paired with the BHQ-1 quencher for selected endogenous controls (YWHAZ, B2M and EiF3EA) and target gene TNF-α1 were designed by Primerdesign Ltd (Southampton, UK) (Table 5.3). Primers and probes were diluted to a working concentration of 300 nM in a 20 µl reaction, and were mixed with PrecisionPLUS Mastermix (PrimerDesign, Southampton, UK). In total, 5 ng (5 µl) of cDNA templates obtained from 28 individual

Atlantic salmon samples, was mixed with 15 µl Mastermix/Primer solution and analysed in triplicates using plates which included both non-RT controls (excluding reverse transcriptase) (n = 3) and no template controls (RNAse free water in place of template) (n = 3) per plate. A qPCR program comprising an enzyme activation step at 95 °C (2 minutes), followed by 50 cycles of a denaturation step at 95 °C (10 seconds) and data collection at 60 °C (60 seconds) was used.

Table 5.3. Oligonucleotide sequences, amplicon sizes and corresponding Genbank accession numbers of Taqman primer/probes used in (q)RT-PCR assays.

Transcript Target	Primer Type	Oligonucleotide sequence (5'-3')	Amplicon Size (bp)	GenBank Accession Number
	Forward	ACTGCCACCAAGAACCAAG		
TNF-α1	Reverse	GCTGTCACCGTTGTCATGTAC	119	NM_00112358
	Probe	TCTCCATGTCGCCAGTTGTCATCGCAT		
YWHAZ	Probe	ACTTGCTTACACACTCGTCGTGGGCA	139	XM_01413162
B2M	Probe	CAGACGCCAAGCAGACAGACCTGG	83	NM_00124591
EiF3EA	Probe	AGGACCTGCCGCCGCTTACGAAC	124	NM_00114169

5.2.5 Data and Statistical Analysis

Relative expression of TNF-α1 was calculated using the $2^{-\Delta\Delta C_T}$ method and equations outlined in Livak & Schmittgen (2001). Statistical analyses were performed using Minitab 17 Statistical Software (2010) (Minitab Ltd, Coventry, UK). All data were checked for normality and homogeneity of variances. When assumptions of normality were not met, the data were log10 or log10 (x+1 transformed). A General Linear Model was used to test gene expression differences between the four RVS severities. In the case where the models were significant, Tukey's HSD Post-hoc test was used to determine significant differences and grouping. Regression analysis was performed to investigate the relationship between ΔC_T values and nematode intensity within the vent of Atlantic

salmon. Full exploration of best linear fits using linear, quadratic and cubic terms on the data was performed prior to analysis. The standard error of the regression, S values and R^2 values, were used to assess the curve fitting effectiveness of the different models.

5.3 Results

Electrophoresed RNA samples showed 18S and 28S bands (Fig 5.3) of sufficient integrity for further analyses.

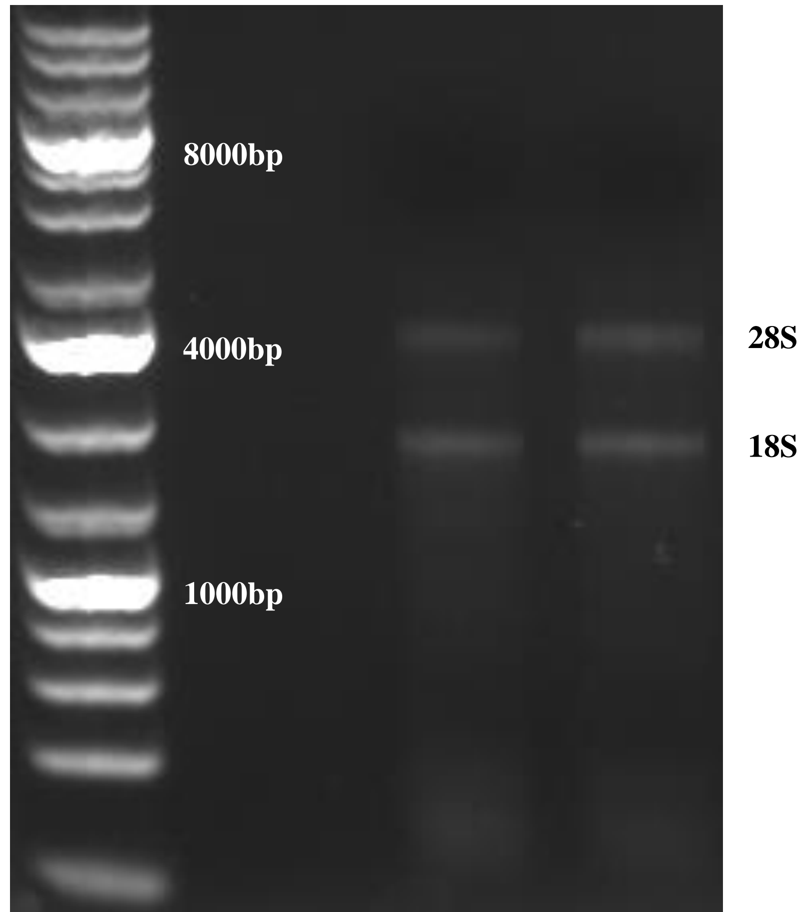

Figure 5.3. Agarose gel electrophoresis image showing two exemplarily electrophoresed extracted RNA samples. The 18S and 28S are visible, correlating to the small and large cytoplasmic ribosomal (r)RNA subunits.

Results from (q)RT-PCR assays showed that cytokine TNF-α1 expression in vent muscle tissue is highly variable (Fig 5.4). In Atlantic salmon with RVS, TNF-α1 expression was correlated with RVS severity, demonstrated by increased fold-changes of 0.36 ± 0.39, 0.83 ± 0.39, and 0.80 ± 0.59 (mean ± SE) in mild (2), moderate (3) and severe (4) cases respectively (Fig 5.4). The highest fold change in expression, was seen in the moderate RVS severity sub-group (0.83 ± 0.39). This sub-group however, contained lower mean

intensities of parasitic nematode (127.4 ± 42.9; mean ± SD) than those with severe symptoms (145.6 ± 60.3) (Table 5.4). Atlantic salmon sampled from the Sandbank hatchery on the River Spey had the greatest change in gene expression (67.1 ± 18.7 fold change). Nematode intensities were moderate (32.2 ± 15.5), but they showed signs of a *Saprolegnia* sp. infection.

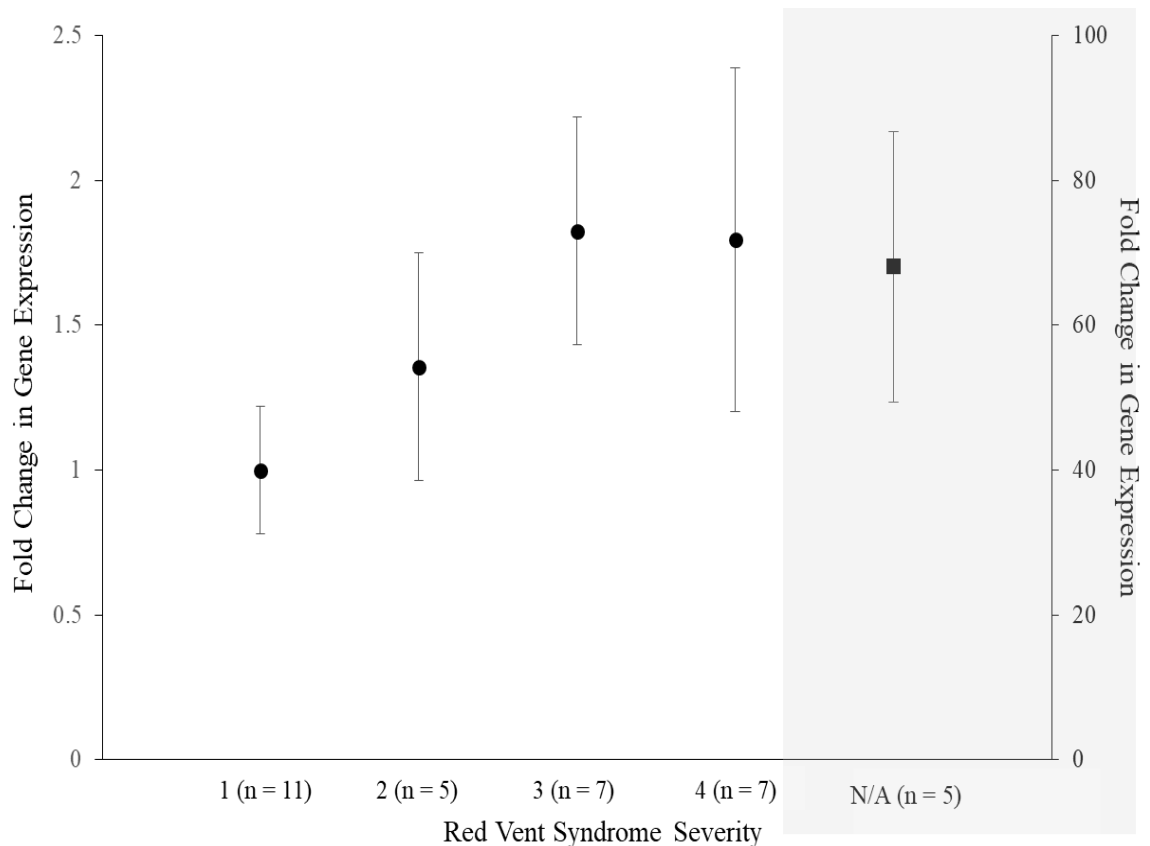

Figure 5.4. Average fold change in TNF-α1 expression in vent muscle tissue of Atlantic salmon with no (1) (n = 11), mild (2) (n = 5), moderate (3) (n = 7) and severe (4) (n = 7) symptoms of Red Vent Syndrome. Shaded area: Atlantic salmon from the Sandbank hatchery (n = 5) (N/A). (Error bars = standard error of mean fold change).

Table 5.4. Results of the $2^{-\Delta\Delta C_T}$ equations leading to average fold-change values (±SE) obtained from Red Vent Syndrome (RVS) treatment groups 1 (control) (n = 11), 2 (mild) (n = 5), 3 (moderate) (n = 7), 4 (severe) (n = 7), and from hatchery salmon expressing no RVS symptoms (n = 5).

RVS Severity (Number of fish)	Mean Nematode Intensity in the Vent ± SD	Fold Change ± SE
4 (*n* = 7)	145.6 ± 60.3	1.80 ± 0.59
3 (*n* = 7)	127.4 ± 42.9	1.83 ± 0.39
2 (*n* = 5)	93.9 ± 91.2	1.36 ± 0.39
1 (*n* = 11)	27.9 ± 30.5	1.00 ± 0.22
Hatchery (N/A) (*n* = 5)	32.2 ± 15.5	68.10 ± 18.67

Results of the General Linear Model showed that observed increases in TNF-α1 expression between different groups were significant ($F=12.58$, $df=4,30$, $p<0.001$). However further analysis using a Tukey's HSD post-hoc test showed that the significance was solely driven by the hatchery group. There were no significant differences between any of the RVS severity groups (Table 5.5). Further analysis of ΔC_T values in comparison to nematode intensity in the vent corroborated with fold changes between severities (Fig 5.5).

Table 5.5. Summary of P-values from Post-hoc Tukey comparison analysing average ΔC_T values, in relation to Red Vent Syndrome severity (subgroups 1-4), or with S*aprolegnia* sp. infection (Hatchery)

RVS Severity Subgroups	**Adjusted P-value**
2 (Mild) –1 (No symptoms)	0.986
3 (Moderate) –1 (No symptoms)	0.892
4 (Severe) – 1 (No symptoms)	0.821
Hatchery - 1 (No symptoms)	**<0.001**
3 (Moderate - 2 (Mild)	0.991
4 (Severe) - 2 (Mild)	0.986
Hatchery - 2 (Mild)	**<0.001**
4 (Severe) - 3 (Moderate)	1.000
Hatchery - 3 (Moderate)	**<0.001**
Hatchery - 4 (Severe)	**<0.001**

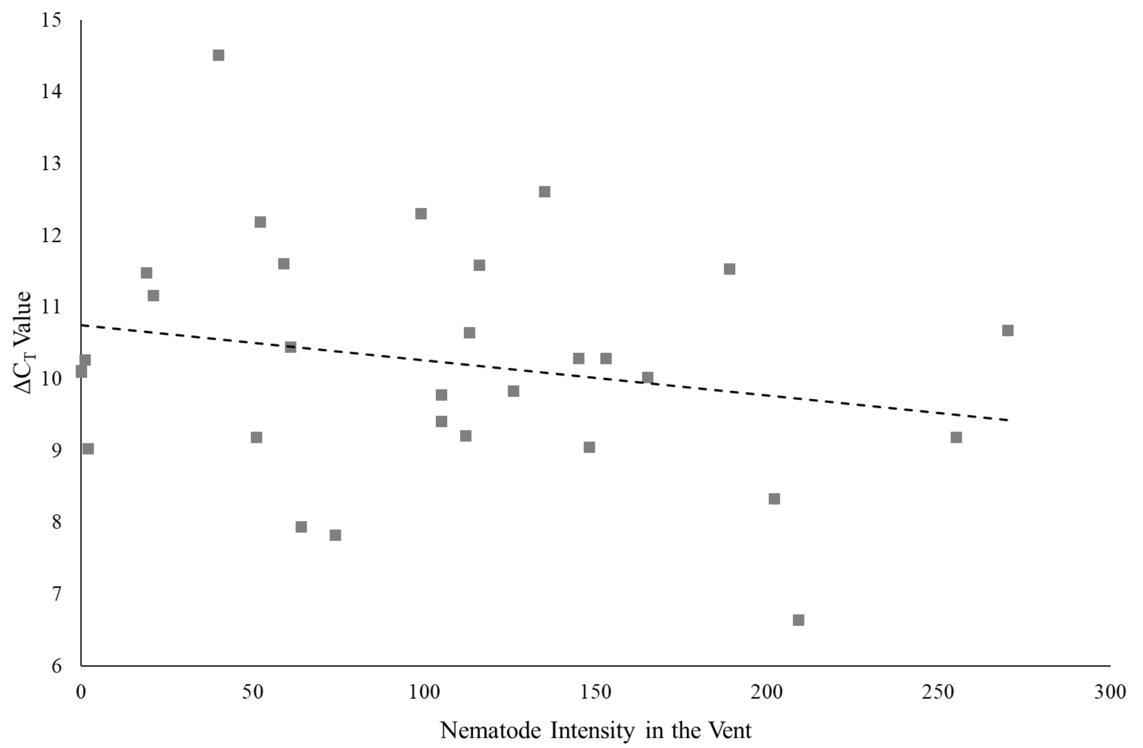

Figure 5.5. Relationship between ΔC_T values (lower values indicate higher TNF-α1 expression) in comparison to nematode intensities within the vent region of Atlantic salmon (*n* = 30)

5.4 Discussion

This study represents the first investigation into the expression of the pro-inflammatory cytokine TNF-α1 in relation to anisakid infestation of the vent and exhibition of RVS symptoms. As TNF-α triggers the expression of a number of other immune genes associated with inflammation, this study hypothesized that expression of TNF-α1 would be upregulated in response to both increasing nematode intensity, and the severity of RVS symptoms.

In accordance with the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001), ΔC_T values of subgroups with RVS symptoms were normalised through subtraction of the ΔC_T calibrator. To establish a baseline of TNF-α1 expression, and avoid potential interference of TNF-α1 constitutive expression present within some tissue types (Garcia-Castillo *et al*., 2002; Praveen *et al*., 2006), ΔC_T values obtained from the vent muscle tissue of subgroup 1 (no symptoms) were used as the ΔC_T calibrator. Although nematodes were still present within the vent region of this subgroup (27.9 ± 30.5), nematode intensities were much lower than in those fish with mild, moderate and severe RVS symptoms (93.9 ± 91.2; 127.4 ± 42.9 and 145.6 ± 60.3). Although high intra-group variability of expression was observed, increases in TNF-α1, generally correlated with increasing nematode intensities in the vent region (Table 5.4). Expression of TNF-α1 increased by 36%, 83%, and 80% for mild, moderate and severe symptoms of RVS respectively. While the presence of *A. simplex* within the vent region seems to increase the likelihood of the exhibition of RVS symptoms (Larrat *et al*., 2013), the new results do suggest a potential link between *A. simplex* intensity, and the expression of TNF-α1. The fact that larvae were present within the vent tissue without RVS symptoms suggests that their may be a threshold required to induce RVS, or their presence is not the only factor causing this condition.

The largest increase in expression of TNF-α1 however, was observed in the moderate severity subgroup. This subgroup was infested with lower average intensities of nematodes (127.4 ± 42.9) in comparison to salmon with severe cases of RVS (145.6 ± 60.3). The factors driving the upregulation in this sub-sample remain unclear however, there is the potential for physiological stress during the spawning migration to be involved in TNF-α1 expression in Atlantic salmon.

A histological study has revealed large numbers of eosinophilic granule cells (EGC's) present in RVS affected tissues (Noguera *et al*., 2009). The presence of EGC's however, may not solely be in response to *Anisakis* infestation. Although significant recruitment of EGC's has been observed during chronic inflammation within intestinal tissue of helminth-infested salmonids (Ferguson, 2006), degranulation of EGC's followed by acute inflammatory reactions can be induced through injection of hydrocortisone (Reite, 1997). The dominant EGC reaction in the vent tissue of Atlantic salmon from sea and estuarine waters has been observed to recede in samples from freshwater (Noguera *et al*., 2009). The EGC's presence, similarly to TNF-α1 expression, which is controlled by stress related hormones expression in gilthead seabream (*Sparus aurata L.)* (Castillo *et al*., 2009), may be due to stress of the reproductive migration, rather than a specific reaction to the parasite.

The lack of significant increase of TNF-α1 expression in the vent region of RVS fish could be attributed to the absence of cells with the capacity to produce TNF-α1 as suggested by Morrison *et al*. (2007) in relation to infection with *Neoparamoeba perurans* and amoebic gill disease. This however, can not be attributed in this study as TNF-α1 expression in Atlantic salmon from the Sandbank hatchery was significantly higher (Fig 5.4). Therefore, TNF-α1 can be either highly expressed, or migrate in high numbers to the vent region. It is possible that *A. simplex* does not sufficiently stimulate the intracellular signalling required for significant induction of the TNF-α1 gene (Morrison

et al., 2007). Atlantic salmon from the Sandbank hatchery were only infested with a moderate number of *A. simplex* (32.2 ± 15.5) in the vent region. The significantly increased expression of the TNF-α1 gene (+67.1 ± 18.7) in this subgroup was more likely in response to the *Saprolegnia* sp. oomycete infections they were suffering, as TNF-α is released by macrophages in response to β-glucan found in fungal cell walls in mammals (Romani, 2004; Olson *et al*., 1996).

Differences in immune response are commonly observed between different species (Bahlool *et al*., 2012), but can also be seen between individuals of the same species (Reite & Evensen, 2006). In this study high levels of intra-group variability of TNF-α1 expression was observed within all subgroups. Although the low sample sizes within salmon subgroups will account towards the high variability of TNF-α1 expression, temporal variability (Kutyrev *et al*., 2016), and external factors such as temperature and light (Magnadóttir, 2006, 2010) could potentially affect teleostean immune response.

Expression of TNF-α1 has been shown to be temporally variable in response to *Flavobacterium psychrophilum* in rainbow trout with an upregulated expression after six hours, and decreased expression at 144 hours (Kutyrev *et al*., 2016). The lack of significant expression differences in TNF-α1 may be a product of the timing of sampling.

During the inflammatory process, mast-cell derived TNF-α in mice, promotes the migration of cells such as neutrophils and monocytes to gastrointestinal (GI) tissues in response to GI infection by helminths (Ierna *et al*., 2008). As part of an immediate non-specific innate immune response, significant TNF-α1 expression may therefore, only be present during the initial migration of *A. simplex* larvae to the vent region. The majority of *A. simplex* larvae in the vent region were encapsulated when isolated. If the immuno-stimulation is caused by the mechanical influence of the moving larvae (Larsen *et al*., 2002), it would not have been present at the time of sampling.

The differences of TNF-α1 expression in Atlantic salmon with similar RVS symptom severity in this study offers another example of intra-specific variability in immune response, which could explain differences in *A. simplex* infestation intensities within host populations (Adroher *et al.*, 1996). Other teleosts such as Atlantic mackerel and saithe utilise a specific age-related immune response to reduce infestation immunologically. Older fishes have significantly lower intensities of *A. simplex* in comparison to younger fish (Levsen & Midthun, 2007; Priebe *et al.*, 1991). In addition to differences in TNF-α1 expression, differences in other parts of the innate immune response are very likely in Atlantic salmon.

Red Vent Syndrome has only been reported in Atlantic salmon with the exception of one known case in brown trout (Noguera *et al.*, 2009). Previous *in vivo* studies have shown an increased susceptibility of Baltic salmon populations to *A. simplex* in comparison to brown trout and rainbow trout. Class II molecules of the major histocompatibility complex ($MHCII^{+}$), which play an important role in the encapsulation of *A. simplex,* have been observed around infective nematodes in other salmonids, but are conspicuously absent in Baltic salmon (Bahlool *et al.*, 2012). Vastly different immune responses to the monogenean *Gyrodactylus derjavini* (Mikailov, 1975) exist between Atlantic salmon in the Baltic, and other populations. Baltic salmon from the River Ume up-regulated Serum Amyloid A (SAA) and the antibody IgM during elimination of this parasite. The more susceptible East-Atlantic salmon (Buchmann, 2012) however, initiates a sustained inflammatory reaction without effect (Kania *et al.*, 2010).

In addition to intra-specific differences, specific physiological properties of different fish host species also translate into inter-specific differences in immune response (Levsen & Berland, 2012), and can be crucial in the efficacy of the response towards specific parasites. This leads to the inference that these inter-specific differences in immune response are the main reason for the restriction of RVS symptoms to Atlantic salmon.

With the baseline ΔC_T calibrator still containing nematode larvae, although the results of this study suggest that TNF-α1 is not significantly expressed within muscle tissue of the vent region, the inclusion of a control without *A. simplex* larvae is require before this can be confirmed. Due to the variability in immune response (Bahlool *et al*., 2012) however, and the isoform driven differentiation between different tissues (Morrison *et al*., 2007), a significant expression of TNF-α1 in other tissue types of specimens with RVS symptoms can also not be ruled out. The immune function of cytokines such as TNF-α1 overlaps with other cytokines e.g. IL-β (Zou & Secombes, 2016). Therefore, the localised response within muscle tissue may be dominated by another cytokine. With high infestation intensities throughout RVS affected Atlantic salmon (Chapter 3), there is the potential of a systemic response to *A. simplex* infestation, which has been outlined by Larsen *et al.* (2002). In brown trout fry a systemic immune response was induced through the experimental infestation with endoparasites (*Anisakis* sp.) (Larsen *et al*., 2002). The subsequent activation of macrophages caused by *Anisakis* sp. infestation of brown trout resulted in significant decreases in the ectoparasitic monogenean *Gyrodactylus derjavini* (Mikailov, 1975) during the following three weeks.

In future studies of the immune response involved in Red Vent Syndrome symptoms, a number of factors must be considered. Firstly, the use of different tissues e.g. head kidney, a major lymphoid tissue (Press & Evensen, 1999; Castillo *et al*., 2009) should be included to assess the presence of a systemic response. Secondly, the use of relevant markers within the TNF-α1 pathway e.g. the downstream effects on members of the RTS-11 cell line as seen in rainbow trout (Zou *et al*., 2003b) should be included to assess temporal changes in the immune response to *A. simplex* infestation and clarify signaling systems involved. Finally, the inclusion of appropriate endogenous controls is essential to quantify the relative expression of single genes (Olsvik *et al*., 2005). Unfortunately, the best controls possible were not able to be identified within the time frame of this study.

Although multiple endogenous controls were selected, the observed GeNorm V value of 0.523 was far higher than the recommended value of <0.15 and might have resulted in inaccurate assessments of TNF-α1 expression levels. The identification and inclusion of a more appropriate endogenous control(s) are imperative for future accurate relative gene quantification studies.

Chapter 6

General Discussion

The emergence of Red Vent Syndrome (RVS) in 2005 prompted several studies to investigate the underlying cause(s) of RVS, and the novel infestation site of the vent region by *Anisakis simplex* L3 larvae (Beck *et al*., 2008; Noguera *et al*., 2009; Mo *et al*., 2010; Larrat *et al*., 2013). The definitive cause/causes of both however, have remained unclear. This study represents a continuation of previous work and provides further insights into the relationship between *A. simplex* and Atlantic salmon (*Salmo salar* L.) populations in Scottish coastal waters.

6. 1 The Novel Infestation Site of the Vent Region

Large numbers of nematodes in the vent region have not been reported for Atlantic salmon nor any other fish host of anisakid larvae prior to the emergence of RVS (Noguera *et al*., 2009). The presence of *A. simplex* in this novel infestation site led to the suggestion that larvae in the vent region may represent a new genetic strain of higher pathogenicity (Noguera *et al*., 2009). The analyses of anisakids isolated from Atlantic salmon from the North and East coasts of Scotland could not identify any genetic differences based on sequence analysis of the nuclear ribosomal Internal Transcribed Spacer region (ITS) between anisakids in the body cavity, and the vent (Noguera *et al*., 2009). In addition to anisakids isolated from the vent region and body cavity of Atlantic salmon from the East and North coast (Noguera *et al*., 2009), Atlantic salmon from the West coast of Scotland was also analysed. Phylogenetic analyses showed no genetic differences between the two infestation sites (Chapter 2.3). The phylogenetic tree showed that the sequences of the four anisakid nematodes isolated from Atlantic salmon obtained from Douglas Hall wild capture fisheries on the West coast of Scotland and from the Sandbank salmon hatchery on the River Spey on the East coast, were closely related to *A. simplex sensu stricto* in both maximum-likelihood (Chapter 2.3.1) and Bayesian analyses (Chapter 2.3.2). Although one *Anisakis* specimen from the body cavity presented an example of C/T heterogeneity, it was not at a position used as a fixed diagnostic marker and therefore, it

is highly unlikely to be an example of any speciation event. Therefore, these isolates can be identified as *A. simplex sensu stricto* and corroborate with larger scale studies carried out in Scotland (Noguera *et al*., 2009), and Norway (Mo *et al*., 2010). Furthermore, the results provide the first new sequence data since 2007, which suggest that there has been no *A. simplex* speciation event during this intervening period. The available data would strongly suggest that speciation of *A. simplex* is not the cause behind the mass infestation of the vent region.

Because of the nominal sample size of anisakids from the Atlantic salmon population off the West coast of Scotland however, this study is not fully representative for the whole Scottish salmon population. Analyses of anisakids from multiple Atlantic salmon in this population should therefore be conducted to fully assess the possibility of anisakid speciation.

Introgression or introgressive hybridisation describes the permanent incorporation of gene(s) from one species into the gene pool of another through repeated mating between an interspecific hybrid with one of its parent species (Mastrantonio *et al*., 2016). Mitochondrial introgression between *Anisakis pegreffii* and *A. simplex* (s.s.) has been observed off the South west coast of England in mackerel, horse mackerel, and blue whiting (Abattouy *et al*., 2016). Anisakid speciation or hybridisation could lead to genetically driven phenotypic changes in resulting species/hybrids. Changes in phenotype can result in improved avoidance of recognition and resistance from a hosts' adaptive immune system, leading to increased pathogenicity (Gandon *et al*., 2002; Ebert & Bull, 2003), which could pose a threat to the host organisms. Due to phenotypic changes, the amphibian zoosporic fungus *Batrachochytrium* did become hyper virulent to many hosts (Farrer *et al*., 2011), causing dramatic outbreaks of the emergent infectious disease chytridiomycosis (King *et al*., 2015). The potential for similar phenotypic changes to occur in anisakid species/hybrids, could possibly have significant detrimental impacts on

hosts in UK waters, including Atlantic salmon. Hybridisation or introgression generates patterns of genetic variation, and the use of a single character type (e.g. DNA sequence variation) does not have the power to identify these events (Mattiucci *et al*., 2016). The use of parental, taxa specific diagnostic markers at nuclear loci, such as allozymes and sequence analysis of multiple genes has the potential to discriminate any hybridisation between *Anisakis* sibling species (Mattiucci *et al*., 2016) and should be utilised in any further study into *Anisakis* speciation to allow full analysis of genetic interaction between *Anisakis* sibling species.

The presence of large numbers of *A. simplex* larvae in the vent region, also known as 'hyper-infestation' (Noguera *et al*., 2009), prompted Senos *et al*. (2013) to suggest that this was a consequence of increasing nematode burdens in the body cavity of Atlantic salmon. Senos *et al.* (2013) outlined a ten-fold increase in average nematode intensities in the viscera with averages increasing from 5.3 to 54.5 between 1978-2013 (Beverley-Burton & Pippy, 1978; Senos *et al*., 2013). A six-fold increase from 3.6 to 22.8 in mean nematode intensity was also observed in the musculature (Beverley-Burton & Pippy, 1978; Senos *et al*., 2013). Data from this study confirm the trend of increasing nematode burdens in Atlantic salmon. The results in Chapter 3 reveal a four-fold increase of nematodes per fish (264.3 ± 196.9) (Table 3.5), in comparison to nematode numbers of Atlantic salmon sampled in coastal waters of Scotland in 2009 (63.6 ± 31.9) (Noguera *et al*., 2009). In comparison to nematode intensities observed in 1978 (Beverley-Burton & Pippy, 1978), the results present a 50-fold increase per fish. The theory that increasing nematode intensities within the body (viscera and musculature) of Atlantic salmon (Senos *et al*., 2013), also referred to as the 'hyper-infestation hypothesis within this study, is supported by the results presented in this thesis. Significant positive relationships were observed between nematode intensities in the body (viscera and musculature) and the vent when coastal populations of Atlantic salmon were pooled ($p<0.05$). Additionally, when

nematode larvae were analysed per gram of tissue weight, significant relationships were also observed between nematode larvae per gram in the vent and muscle ($p<0.05$), and the vent and viscera ($p<0.05$). The increasingly severe immune responses to larger nematode intensities in gastrointestinal (GI) tissues may be leading to an inhospitable environment for later arriving nematodes (Ferguson, 2006). Consequently, nematodes may be forced to migrate further down the digestive tract and thus, could also be driving the infestation of the vent region of Atlantic salmon. In addition to supporting the hypothesis that infestation of the vent is being driven by increasing nematode intensities in body of Atlantic salmon (Senos *et al*., 2013), these results also suggests the dramatic increase of nematode intensities in Atlantic salmon over the last 50 years (Chapter 3) have played a significant role in the emergence of RVS and its continued prevalence which was recorded at 87% in 2017 (Armstrong *et al*., 2018).

Increasing infestation of Atlantic salmon by anisakids however, also poses a major problem for the fishing industry (Noguera *et al*., 2009). Anisakids are known to reduce the quality of edible tissue (Noguera *et al*., 2009). Furthermore, they can cause Anisakiasis in humans when raw or undercooked fish is consumed (Lymbery & Cheah, 2007; Audicana & Kennedy, 2008), and also a range of gastro-allergic (Mattiucci *et al*., 2013) and hypersensitivity reactions (Heffler *et al*., 2016). In the current study, a substantial proportion of the total nematodes recovered from Atlantic salmon were in the edible portions of the fish such as the fillets (15.2%) (Chapter 3). The presence of a substantial number of *Anisakis* sp. in the edible tissue of Atlantic salmon is likely to further degrade muscle tissue and exacerbate the risk of Anisakiasis and associated allergic reactions for the public (Audicana & Kennedy, 2008; Mattiucci *et al*., 2013; Heffler *et al*., 2016).

Increasing cases of Anisakiasis in Japan have prompted the Japanese government to release statements of the dangers posed by consumption of improperly cooked fish

containing anisakid larvae (Osumi, 2017). Following an article in the British Medical Journal Case Reports (Carmo *et al*., 2017), the British Broadcasting Corporation (BBC) released a news article "Sushi Lovers Warned of Parasites Danger in Raw Fish" [Retrieved from http://www.bbc.com/news/health-39882381] to raise the general public's awareness in the United Kingdom of the dangers posed by consuming raw or undercooked wild caught fish. However, even if awareness of the appropriate storage (below -20 °C for at least a day), and cooking instructions (>1 min in temperatures exceeding 60 °C) for fish is raised, the threat of associated allergic and severe hypersensitivity reactions to public health, which are likely to increase, will remain.

To date, 13 allergens have been described from *A. simplex* (s.s.), many of which are heat and pepsin resistant (Moneo *et al*., 2005; Caballero *et al*., 2008). These allergens can be transmitted through food-borne, airborne, or skin contact routes, even if the nematode is dead (Baird *et al*., 2014). Clinical symptoms induced by these allergens can include urticaria, rhinitis, bronco-constriction, cough, and/or gastrointestinal responses (Audicana & Kennedy, 2008; Hochberg *et al*., 2010). Moreover, cases of severe hypersensitivity have been reported, which can lead to anaphylaxis in a worst-case scenario, being a lot more dangerous than Anisakiasis itself (Heffler *et al*., 2016).

6.2 The Cause/Causes of Red Vent Syndrome

6.2.1 The Role of Increasing Nematode Intensities in the Emergence of Red Vent Syndrome

Since its emergence in 2005, RVS has consistently been observed in UK rivers (ICES, 2017). The inflammatory and haemorrhagic symptoms of RVS, have been pathognomonically associated with the presence of *A. simplex* larvae in the vent region (Beck *et al*., 2008; Noguera *et al*., 2009). The present study documents increasing and significantly different mean nematode intensities 18 ± 11.8, 66 ± 58.3, 115 ± 43.0 and

170 ± 52.0 (mean ± SD) in relation to increasing RVS severity from no, to mild, moderate and severe symptoms respectively ($p<0.05$). These results support the hypothesis that the presence of *A. simplex* larvae in the vent region increases the likelihood of RVS symptoms (Larrat *et al*., 2013). The results of the current study support the central concept of the 'hyper-infestation hypothesis', that increasing nematode intensities within the body of Atlantic salmon are reflected in the vent region. The emergence of RVS in 2005 therefore, may be a product of the increasing presence of *A. simplex* in the vent region, ultimately being driven by increasing nematode intensities in Atlantic salmon as a whole.

Increases of recorded sea surface temperatures (SST) of approximately between 0.5 - 1.5 °C since 1901 (IPCC, 2013; NOAA, 2016) throughout Atlantic salmon's natural range, have been attributed to causing marked changes in the distribution of intermediate and definitive hosts, as well as other biological characteristics of marine organisms associated with the life cycles of *Anisakis* sp. Consequently, these changes could be a significant factor in the regional abundance of *A. simplex* and infestation intensities observed in Atlantic salmon.

European populations constitute the majority of Atlantic salmon that primarily feed in the Norwegian Sea with minorities from West Greenland and the USA populations (Hansen *et al*., 1993) where *Calanus finmarchicus* (Gunnerus, 1770), which is an important dietary input of Atlantic herring (*Clupea harengus L.* 1761) and Icelandic capelin (*Mallotus villosus* Müller, 1776) (O'Driscoll *et al*., 2001; Prokopchuk & Sentyabov, 2006), dominates small zooplankton biomass (<2 mm) during May (Kaartvedt, 2000; ICES, 2017). Between 1996 - 2016 warming waters are likely to have had a significant role in the declining biomass of this species in the Norwegian Sea (Buren *et al*., 2014; ICES, 2017). Consequently, the declining biomass of *C. finmarchicus* in the Norwegian Sea has led to changes in the distribution of Icelandic capelin (Carscadden *et al*., 2013) and Atlantic herring (Sissener & Bjørndal, 2005) in the Arctic. More specifically, Icelandic

capelin have been observed migrating further northwest than previously recorded (Carscadden *et al*., 2013; ICES, 2017). Similarly, although adult Atlantic herring have been recorded in their common feeding grounds in the central and eastern Norwegian Sea since 1987, they are also migrating to feeding grounds off the east of Iceland, and further northwest towards Greenland (Sissener & Bjørndal, 2005; ICES, 2017).

As an opportunistic feeder (Rikardsen & Dempson, 2011), changes in the availability and distribution of common dietary inputs is likely to have resulted in a change of dietary composition for Atlantic salmon. Historically, changes in dietary composition have been reflected in changes in nematode intensity (Mouritsen *et al*., 2010; Larrat *et al*., 2013). For example, in the Gulf of St. Lawrence, Canada, (Dufour *et al*., 2010), decreases in the abundance of krill (low nematode infestation) and increases in the abundance of paratenic hosts such as capelin and herring (high nematode infestation), were attributed to increases in nematode burden seen in Atlantic salmon, Atlantic cod (*Gadus morhua* L.), and Greenland cod (*Gadus ogac* Richardson, 1836) (Mouritsen *et al*., 2010; Larrat *et al*., 2013).

Changing climate has also resulted in shifts in the community structure of potential definitive hosts of *Anisakis* sp. in the northwest of Scotland (MacLeod *et al*., 2005). Cold water species such as the killer whale (*Orcinus orca* L.) and long-finned pilot whale (*Globicephala melas* Traill, 1809) are declining, while new warm water species including the striped dolphin (*Stenella coeruleoalba* Meyen, 1833), Fraser's dolphin (*Lagenodelphis hosei* Fraser, 1956), and pygmy sperm whale (*Kogia breviceps* Blainville, 1838) have been increasing since 1980 (MacLeod *et al*., 2005). The presence of definitive hosts in regions e.g. fjords of the Faroe Islands (Harrison & King, 1965) and coastal waters of central Norway (Strømnes & Andersen, 2000), have been attributed to substantial increases in nematode infestations in Atlantic cod around the Faroe plateau (Platt, 1975), and in saithe (*Pollachius virens* L.), cod, and redfish (*Sebastes marinus* L.)

during spring (Strømnes & Andersen, 2000), respectively. Another example is increasing grey seal populations (*Halichoerus grypus, Nilsson, 1820*) in the Baltic Sea that have led to increased infestations of Baltic cod with *Contracaecum osculatum* (Haarder *et al*., 2014).

6.2.2. Differences in Dietary Composition are Driving Regional Differences in Nematode Intensity

Regional differences in nematode infestations were also revealed in the present study. Average nematode intensities of Atlantic salmon sampled from the North were much higher (297.2 ± 151), than in the East (164.6 ± 140) (Chapter 4.3.1) and were the primary driver (SIMPER: 41.42%) of significant differences in parasite component communities between Atlantic salmon populations ($p<0.001$) (Chapter 4.3.2). Observed differences in nematode burden between regions however, is only representative of a single year's data. Multiple datasets over a number of years are required to negate the potential impact of annual fluctuations in nematode burdens within Atlantic salmon populations.

The absence of differences in $\delta^{13}C$ and $\delta^{15}N$ signatures of dorsal muscle tissue and scales however, suggests that variances in prevailing nematode intensity are not a result of differential use of feeding grounds, or dietary composition. Although these results suggest that changes in the abundance and community structure of marine mammals play a significant role in the variability of nematode intensities between Atlantic salmon populations, the inability to discriminate differences in $\delta^{13}C$ and $\delta^{15}N$ signatures, may be a result of the physiological limitations of the feeding behaviour of 1SW salmon.

Dietary inputs of 1SW salmon encompass a wide variety of slower invertebrates such as crustaceans (amphipods and euphausiids), and cephalopods (armhook squid) from lower trophic levels due to limitations such as poor acceleration and small gape size (ICES, 2017). The resulting diverse dietary composition leads to highly variable isotopic signatures (Christensen, 1996; Lundvall *et al*., 1999; Scharf *et al*., 2000). All populations

analysed in the present study exhibited highly variable isotopic signatures and consequently, any differences in isotopic signature were harder to be identified. Further investigations using stable isotope analyses on Multi-Sea-Winter (MSW) salmon would increase the likelihood of distinguishing any preferences or availability in diet. MSW salmon possess a larger gape size, and increased acceleration, and therefore, are not physiologically restricted to ingesting slower moving invertebrates (MacKenzie *et al*., 2012). This results in increased preferential feeding on specific dietary inputs, commonly from higher trophic levels such as capelin and Atlantic herring (MacKenzie *et al*., 2012). Consequently, conserved isotopic signatures would enable the identification of any differences in feeding ground or diet choice between populations.

6.2.3 Assessment of the Immune Response Involved in Anisakis sp. infestation

Although the rising intensities of *A. simplex* in the vent region increase the likelihood of more severe RVS symptoms, their presence in the vent of Atlantic salmon is not guaranteed to be symptomatically evident (Larrat *et al*., 2013).

To assess the localised immune response in vent muscle tissue to *Anisakis* infestation, (q)RT-PCR was used to investigate the expression levels of the pro-inflammatory cytokine TNF-α1 in relation to the inflammatory symptoms associated with RVS. Although TNF-α1 expression increased by 36%, 83%, and 80% in cases of mild, moderate and severe RVS in comparison to the ΔC_T calibrator (no RVS symptoms), there was no significant difference in its expression between severities (Chapter 5.3.1). The expression of TNF-α1 is therefore, not significantly up-regulated with increasing presence of *A. simplex* in the vent region. This would suggest that other elements of the immune response are involved, or the presence of *A. simplex* in the vent alone is not the trigger of RVS symptoms (e.g. haemorrhaging around the vent) (Larrat *et al*., 2013). The expression of TNF-α1 however, can be temporally variable (Kutyrev *et al*., 2016), and therefore, the lack of significant expression may be a product of the timing of sampling.

During their life cycle, hormonal changes in salmonids result in periods of immundeficiency (Harris & Bird, 2000). During sexual maturation and freshwater migration, salmonids display high plasma levels of the gonadal steroids, oestradiol, testosterone, 11-ketotestosterone and androstenedione (Maule *et al*., 1996; Harris & Bird, 2000). During these periods, both sexually mature male and female salmonids demonstrate an inability to produce isohaemagglutinins, antibodies that are produced in immature fish (Ridgeway, 1962) and are subject to an increased frequency of ectoparasitic infestations, particularly males (Pickering & Christie, 1980). In the present study, significantly higher nematode infestation intensities in females were observed and may be a potential result these hormonal factors. Previous studies investigating the immune response involved in RVS affected tissues have identified an immune response dominated by eosinophilic granule cells (EGC) (Noguera *et al*., 2009). This dominant EGC reaction however, receded in samples from freshwater (Noguera *et al*., 2009). Although receding EGC's may be due to the host having encapsulated anisakids and therefore, no subsequent inflammatory response being generated, stress related hormones have also been identified as playing a key role in the abundance of EGC's and similarly to TNF-α1 expression in other teleosts e.g. gilted seabream (Castillo *et al*., 2009). Reite (1997) showed that EGC degranulation and subsequent acute inflammatory reactions can also be induced through the injection of stress hormones e.g. hydrocortisone. The association between the presence of stress hormone controlled EGC's and the expression of RVS symptoms therefore suggests that the physiological stress involved during the spawning migration (Jonsson *et al*., 1997) may also be a factor in RVS' aetiology (Noguera *et al*., 2009).

6.2.4 The Role of Physiological Stress in RVS Emergence

The spawning migration requires substantial energetic resources and is a period of large-scale morphological (Kacem *et al*. 2000), and physiological (Miller *et al*., 2009) transformation. The inception of physiological stress first occurs within the marine

environment (Miller *et al*., 2009). Sockeye salmon (*Oncorhynchus nerka*, Walbaum 1792) for example, cease feeding approximately 850 km from natal rivers (Miller *et al*., 2009). The initial physiological responses to the onset of starvation include enhanced protein turnover, reduced transcription of actin, muscle contractile and heme-related proteins (Miller *et al*., 2009). As little or no gut contents were observed in Atlantic salmon in coastal waters during their spawning migration (Kjellman, 2015), the cessation of feeding might occur at similar distances to their natal rivers as for sockeye salmon. The spawning migration and associated physiological stress however, has always been a fundamental feature of the anadromous life cycle of Atlantic salmon. Are other physiological factors resulting in greater nutritional stress experienced by Atlantic salmon during the spawning migration potentially playing a role in the emergence of RVS in 2005? In recent years, populations of Atlantic salmon have declined in mean size and mass (Todd *et al*., 2012; Bal *et al*., 2017) as a result of poorer growth during the marine phase (Todd *et al*., 2012; ICES, 2016; Bal *et al*., 2017). Poor growth has been subsequently attributed to warming SST's (Todd *et al*., 2008, 2012) and fluctuations in teleconnection patterns (climate forcing indices), e.g. the North Atlantic Oscillation (NAO) and the Atlantic Multidecadal Oscillation (AMO) (Friedland *et al*., 2014) which have directly affected the availability, distribution and nutritional quality, of several common dietary items of Atlantic salmon.

The distribution and migratory behaviour of common dietary inputs such as Icelandic capelin (Carscadden *et al*., 2013), and Atlantic herring (Sissener & Bjørndal, 2005) has changed in the Arctic. Individual lesser sandeels (*Ammodytes marinus,* Raitt, 1934) in the North Sea, a dietary item for post-smolts (Holm *et al*., 2000) commonly infected by *Anisakis* sp (Levsen & Karl, 2014), have declined in average size over the last 30 years (Wanless *et al*., 2004), which has been approximated to a reduction in energy content of 40% (Wanless *et al*., 2004). Their embryonic development is strongly affected by

environmental cues e.g. temperature and salinity and changes in these cues have been attributed to cause early and late larval hatching and consequently, declines in size and energy content (Wanless *et al*., 2004). Furthermore, the strict association of lesser sandeels with coarse sandy sediments severely restricts their ability to adapt their distribution to compensate for warming sea temperatures (Heath *et al*., 2012). Thus, populations will likely suffer further declines in the future.

The change in distribution and declining availability of common prey (e.g. Icelandic capelin and Atlantic herring), and the declining nutritional values of other inputs (e.g. lesser sandeel) have the potential to be driving opportunistic feeding on organisms of poorer nutritional value (Renkawitz *et al*., 2015). The poorer overall condition of Atlantic salmon will consequently lead to increased stress levels due to the nutritional demands of this migration (Jonsson *et al*., 1997). To date, there has been no study investigating the presence of RVS symptoms in Atlantic salmon in feeding grounds of the Norwegian Sea before the initiation of the spawning migration. RVS symptoms therefore, have only been observed in coastal waters once initial physiological responses to the onset of starvation have occurred. The role of physiological stress its role in the expression of RVS therefore, cannot be rejected.

6.3 Future Threats to Atlantic salmon

The current status of Atlantic salmon remains a huge concern (Gibson, 2017). All populations across the North Atlantic have exhibited multi-decadal declines (Friedland *et al*., 2009). Climate change is the most detrimental stressor to the restoration and rehabilitation of wild Atlantic salmon (ICES, 2017), having significant impacts on wild populations of Atlantic salmon in both freshwater and marine phases of their life cycle (Jensen *et al*., 1991; Handeland *et al*., 2008; Mills *et al*., 2013; ICES, 2017). It will also have a profound impact on the spread of parasites and diseases in aquatic ecosystems (Marcogliese, 2001; Harvell *et al*., 2002).

Climate change will reduce the immune-competence of Atlantic salmon, which can be severely affected by external factors including light, water quality, salinity and other stress inducers (Marcogliese, 2008; Magnadóttir, 2010). Furthermore, alterations in seasonal dynamics of transmission, parasite development and survival in marine environments will also cause numerical changes that lead to increased rates of amplification and emergence of parasite populations (Brooks & Hoberg, 2007; Marcogliese, 2008) subsequently triggering increasing occurrence of disease outbreaks (Marcogliese, 2001; Hudson *et al*., 2006).

The functional responses e.g. the distribution and range of parasites (Harvell *et al*., 2002) and host assemblages (Dobson & Carper, 1992, Marcogliese, 2001), will also be affected and result in novel host and geographic colonisation (Brooks & Hoberg, 2007). The co-invasion of alien host assemblages and their associated viral and parasitic fauna, are often considered to be important causes of disease emergence, resulting in high morbidity and mortality in native hosts (Peeler *et al*., 2011; Lymbery *et al*., 2014). Ecological isolation caused by alterations in distribution (Dobson & Foufopoulos, 2001) fosters micro-evolutionary responses of parasites including local adaptation, and changes in gene frequencies through mutation (Brooks & Hoberg, 2007). The functional response coupled with these micro-evolutionary adaptations of parasites therefore, pose a severe risk to Atlantic salmon populations through increased pathogenicity, or the acquisition of novel parasite species. The monogenean parasite *Gyrodactylus salaris* (Malmberg, 1957) for example, underwent at least one single hybridisation event leading to a permanent host switch from grayling (*Thymallus thymallus L.*) to Baltic salmon (Meinilä *et al*., 2004; Kuusela *et al*., 2007). Baltic salmon however, are considered to be less susceptible to *G. salaris* in comparison to Eastern Atlantic salmon (Gilbey *et al*., 2006). The introduction of *G. salaris* to Norway in the 1970's led to catastrophic losses of Atlantic salmon parr

(FRS, 2003). By 2002, 44 Norwegian rivers had been infected, and their salmon populations decimated (FRS, 2003).

Current parasites of Atlantic salmon such as sea lice (*Lepeophtheirus. salmonis* and *Caligus elongatus*) will also continue to be a problem. Studies in Norway, Scotland and Ireland, reported survival to recruitment was reduced by 0.6 – 39% when infested by sea lice (Krkošek *et al.*, 2013; Vollset *et al.*, 2016). The development rate of sea lice is strongly dependent on temperature (Costello, 2006). Increasing mean sea temperatures could increase infestation pressure on both cultured and wild Atlantic salmon (Costello, 2006). Moreover, sub-lethal infestation of Atlantic salmon by sea lice is associated with physiological stress, reduced appetite and behavioural changes (Dawson *et al*., 1999; Finstad *et al*., 2000), which have resulted in poorer growth and slower maturity rates (Vollset *et al.*, 2014). These two factors will further reduce the size of Atlantic salmon populations through increased mortality and poorer recruitment to the extent that certain populations will possibly become critically endangered or lost (Forseth *et al*., 2017).

6.4 Recommendations for Further Work

This study presents significant progression in our understanding of the relationship between *A. simplex*, Atlantic salmon and the exhibition of RVS. However, many aspects of the current study require improvement and further study:

6.4.1 Anisakis simplex Speciation in Atlantic salmon

The presence of *A. simplex* speciation in Atlantic salmon sampled from the West coast of Scotland was assessed using only a small sample size, which is not sufficient to be able to fully reject the hypothesis concerning its presence and as cause of RVS in this population. Although similar studies suggest that *A. simplex* speciation is unlikely (Noguera *et al*., 2009; Larrat *et al*., 2013), a further assessment using a more representative sample size of multiple Atlantic salmon hosts of the West coast population would be necessary to reject this hypothesis with confidence. Furthermore, the presence

of introgressive hybridisation of *Anisakis* in Atlantic salmon in these regions has not been studied. Multi-allozyme markers have the potential to discriminate any hybridisation between *Anisakis* sibling species (Mattiucci *et al*., 2016) and should be utilised in any further study into *Anisakis* speciation to allow full analysis of genetic interaction between *Anisakis* sibling species.

6.4.2 Assessments of the Link between Immune Response and RVS Symptoms

Assessments of the immune response within the vent region remains integral to understanding the exhibition of RVS symptoms in Atlantic salmon. The use of immunohistochemical (IHC) techniques and (q)RT-PCR will aid further clarification of the underlying immunological reasons behind RVS associated symptoms in vent regions of Atlantic salmon where *A. simplex* are still present. However, with lower *A. simplex* intensities observed in no symptom sub-groups, there remains the possibility of a potential threshold intensity that induces RVS symptoms. In the current study, the absence of a ΔC_T calibrator where no larvae were present means the absence of significant differences in TNF-α1 expression between RVS severities can not be definitively confirmed. Furthermore, as this was the first study in this area, the best suitable endogenous controls were not able to be identified to fully ascertain its expression. Using solely vent muscle tissue did not allow investigation into the presence of a wider systemic response involved in RVS as seen in endo- and ectoparasites in brown trout (Larsen *et al*., 2002). The hypothesis that an inhospitable environment within the alimentary canal in response to high nematode intensities is the driver behind the presence of *A. simplex* in the vent region can not be accepted. The expression of TNF-α1 in vent muscle tissue should be re-assessed with the inclusion of more suitable endogenous controls. Additionally, a suite of cytokine genes and other elements of the immune response would allow further discrimination of the localised immune response in this tissue. To investigate the immune response within the alimentary canal and presence of a wider

systemic response involved in RVS, other tissues should be included into any immunological study. The inclusion of tissues, which are associated with immune response e.g. head kidney, and GI tissues, would help to answer these remaining questions.

6.4.3 The Use of Stable Isotopes to Discriminate Dietary Composition or Feeding Ground

The reasons behind significant differences in *A. simplex* intensity between Atlantic salmon populations sampled off the North and East coasts of Scotland remain unclarified. The use of stable isotope signatures of MSW salmon instead of 1SW would potentially allow to discriminate any differences in dietary inputs or feeding grounds between these regional populations. Such results could clarify the cause of differences in *A. simplex* intensities between regions, allowing populations to be ranked by their risk of RVS.

6.4.4 The Future Use of In Vivo Experimental Infestation Challenges

Two *in vivo* experimental infestation challenges were unsuccessful in this study (Chapter 3.3.5). Expelled or digested *A. simplex* larvae by Atlantic salmon during the experiment suggest that no migration had occurred. The use of motility as an indicator of a larva's viability and migration potential may not be sufficient, even though a successful challenge was conducted by Haarder *et al*. (2013) using a similar protocol. Sampling *Anisakis* specimens from other teleost species (herring and mackerel), a potentially longer 'personal history' e.g. an increased number of intermediate hosts, may have reduced their migration potential (Smith & Hemmingson, 2003). A future challenge design should mirror the natural cycle of *A. simplex;* e.g. feeding Atlantic salmon with *A. simplex* infested Crustacea should ensure larvae have the migration potential for successful challenges. Furthermore, as warming sea surface temperatures are likely to suppress the efficacy of the Atlantic salmon's immune response to *A. simplex* and cause additional stress, the manipulation of temperature as one abiotic variable in an *in vivo* infestation challenge will allow to investigate its role in RVS and help assess future risk with

changing environments. *In vivo* experimental challenges will most likely be the method of choice to fully clarify the mechanisms of anisakid migration and RVS symptom development. If RVS symptoms could be induced under experimental conditions, a much clearer understanding of the factors underlying RVS could be achieved.

Chapter 7

General Conclusion

In this study the potential causes for the infestation of the vent by *A. simplex* and the expression of Red Vent Syndrome (RVS) were investigated. This study focused on: i) genetic speciation of *Anisakis* sp. in the vent, ii) location and intensity of nematodes and their relationship with RVS severity, iii) dietary composition and parasite burdens of different populations of Atlantic salmon, and finally, iv) the immune response in the vent region in response to *A. simplex* infestation.

No genetic differences based on the complete ITS region (ITS1, 5.8S rDNA gene and ITS2) between *A. simplex* specimens in the vent and the body cavity were found in this study, providing further evidence that the infestation is unlikely to be a result of a micro-evolutionary event. Using a multi-marker approach instead of the ITS region alone would allow a more detailed/fine-scale investigation to clarify whether this 'novel' infestation is a result of hybridisation, micro-evolution or genetic drift within this species.

The significant positive relationship between nematode intensities in the body (viscera and musculature), and in the vent, a four-fold increase in nematode intensity compared to a similar study ten years ago, as well as a fifty-fold increase since 1978, indicate that the infestation of the vent region is most likely a response to the increasing nematode burdens in Atlantic salmon. Nematode intensity is strongly correlated with RVS severity, which corroborates with other studies (Larrat *et al*., 2013) and underpins that their presence increases the likelihood of RVS. In the present study however, there was a degree of overalap of nematode intensities in the vent regions of Atlantic salmon with no and mild RVS severities. Furthermore, as nematodes have also been found in vents of Atlantic salmon with no symptoms, *A. simplex* cannot be the sole cause for inducing RVS symptoms.

Stable isotope analysis of 1SW Atlantic salmon from different coastal regions of Scotland showed no significant differences. Consequently, Atlantic salmon in coastal waters of Scotland seem to have a similar dietary composition and feeding grounds. Significant

differences in parasite component communities however, were identified between populations of Atlantic salmon from the North coast in comparison to the East coast of Scotland. These differences were primarily driven by much higher intensities of *A. simplex* in the Northern population. Therefore, regional differences in *A. simplex* abundances may place specific populations at higher risk of RVS. Further SIA analysis should be conducted using MSW salmon. Increased selective feeding at higher trophic levels than 1SW salmon resulting in a narrower isotopic niche will increase the ability to discriminate differences in dietary input/feeding ground between coastal populations of Atlantic salmon.

Finally, the expression of the pro-inflammatory cytokine TNF-α1 did not significantly increase in response to greater nematode intensities in the localised vent region and does not seem to play a role in the exhibition of increasing RVS severity. The induction of the immune response leading to inflammation of the vent region may be observed in other tissues such as the head kidney, which generates cytokine-producing lymphoid cells. Assessments of these tissues would help to explore the presence of a larger systemic response to the infestation of the vent region by *A. simplex*, and subsequent expression of RVS symptoms.

The present study used different approaches to deepen the understanding of the relationship between *A. simplex* and Atlantic salmon. While some factors were identified in playing a role in *A. simplex* infestation and RVS symptom expression, the causes of RVS remain complex. The use of *in vivo* infestation challenges remain the most powerful method to analyse the interaction between *A. simplex* and Atlantic salmon under specific scenarios e.g. warmer waters, hyper-infestation, and poor nutritional condition of Atlantic salmon.

The results of this study show that the emergence of RVS in 2005 seems to be multifactorial, and not due to one single cause. The interaction between *A. simplex* and

Atlantic salmon is central to its aetiology. However, regional factors including the effects of increasing SST's on the wider trophic web throughout the natural range of Atlantic salmon are likely to have influenced its emergence. The true cause(s) of RVS may only become clear, if the associated symptoms could be induced in an experimental set up. However, as climate conditions in the North Atlantic, North Sea and Arctic continue to change, the emergence of RVS in Atlantic salmon will not be the last observation of a novel disease.

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