

Studies on arteriosclerotic pathologies, haematology, immunology and lipids of captive Atlantic bluefin tuna

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for the degree of Doctor of Philosophy

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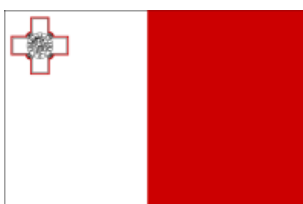
INSTITUTE OF AQUACULTURE



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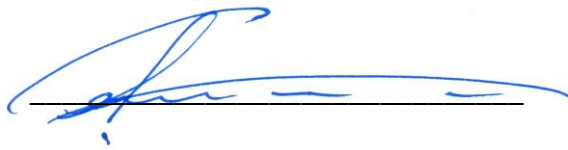
GOOD THINGS COME IN TWOS

... to Emma and Maya

DECLARATION

I declare that this work is original in its entirety and is a result of my own investigations.

All sources of information have been acknowledged and the thesis has not been submitted for any other degree.



Saviour Caruana

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ABBREVIATIONS

×g: multiple of gravity	log ₁₀ : logarithm base 10
µg: microgram	LSWB: low-salt wash buffer
µL: microlitre	m: metre
µm: micrometre	M: molar
Ab: antibody	mAb: monoclonal antibody
ABT: Atlantic bluefin tuna	MCH: mean corpuscular haemoglobin
ALA: actual luminal area	MCHC: mean corpuscular haemoglobin concentration
ANCOVA: analysis of covariance	MCV: mean cell volume
ANOVA: analysis of variance	ME: mucus extract
ARA: arachidonic acid	mg: milligram
BSA: bovine serum albumin	min: minute
cDNA: complementary DNA	mL: millilitre
cm: centimetre	MLT: maximal lesion thickness
CV: coefficient of variation	mm: millimetre
dH ₂ O: distilled water	mM: millimole
DHA: docosahexaenoic acid	MPA: maximal potential luminal area
dNTP: deoxyribonucleotide triphosphate	mt: metric ton
<i>e.g.</i> : example	MUFA: monounsaturated fatty acid
EC: erythrocyte	MW: molecular weight
ELISA: enzyme-linked immunosorbent assay	nm: nanometre
EPA: eicosapentaenoic acid	NTC: non-template control
<i>et al.</i> : and others	OD: optical density
FA: fatty acid	PBS: phosphate buffered saline
FAME: fatty acid methyl esters	PBT: Pacific bluefin tuna
FAO: Food and Agriculture Organization of the United Nations	PLL: potential lumen loss
FL: fork length	PUFA: polyunsaturated fatty acid
g: gram	qPCR: quantitative polymerase chain reaction
h: hour	RNA: ribonucleic acid
Hb: haemoglobin	RT: room temperature
Hct: haematocrit	SBT: southern bluefin tuna
HRP: horseradish peroxidase	SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis
HSWB: high-salt wash buffer	sec: second
<i>i.e.</i> : that is	SFA: saturated fatty acid
ICCAT: International Commission for the Conservation of Atlantic Tunas	spp.: species
IEL: internal elastic lamina	SRBC: sheep red blood cells
Ig: immunoglobulin	TAC: total allowable catch
IgG: immunoglobulin G	TBS: Tris buffered saline
IgM: immunoglobulin M	TL: total lipid
kDa: kilodalton	U: unit of activity
kg: kilogram	v/v: volume/volume
L: litre	V: volt
LC: leucocyte	w/v: weight/volume
LC-PUFA: long-chain PUFA	

ABSTRACT

Commercial capture-based aquaculture of the Atlantic bluefin tuna (ABT), *Thunnus thynnus* (L.), has been prominent in the Mediterranean for over a decade. Owing to several limitations encountered in working with the species, including its high commercial value, there has been little research carried out relating to this species. The objective of this study was to examine several health parameters of captive ABT. These included an examination of coronary artery lesions, haematology, plasma biochemistry, assessment of immune function and changes in fatty acid (FA) flesh content through the on-growing period.

Arteriosclerosis in fish is a pathologic condition of uncertain etiology and involves the main coronary artery in teleosts. Apart from reports of their widespread occurrence in salmonids, they have been described from a restricted number of wild ABT specimens but have not received further attention. This investigation analysed the effect of size and period of net-pen rearing on the prevalence and severity of arteriosclerotic lesions in ABT. Coronary arteries from wild and captive fish were investigated and prevalence was 100 %, but increasing structural degradation was observed with increasing fish size, suggesting that lesions progress throughout the life of the fish. Due to the limited availability of wild specimens, the effect of captivity on arteriosclerosis in ABT could not be adequately quantified, although observations suggest that the farming process has no major effect on arteriosclerotic lesions in ABT.

Studies on the haematology, plasma biochemistry and immunology of ABT are limited. Haematological and plasma biochemical indices are useful in animal health assessment but use of these requires the establishment of species-specific ranges. Blood was collected from captive ABT specimens of varying weight (61-361 kg) and the major haematological ($n = 45$), plasma biochemical ($n = 30$) and immunological parameters ($n = 45$) were quantified. Size-based differences were found in haematological indices between experimental sub-groups including increased erythrocyte number and haemoglobin level in smaller ABT. No differences were found in immunological parameters except for total IgM levels, which were higher in the smaller individuals.

Preliminary investigations indicated that disease prevalence in captive ABT is very low. Epidermal mucus is an important interface between fish and their environment and comprises immunological components which act as a first barrier against pathogen entry or colonisation. Mucus was collected from captive ABT and analysed for innate immune components. The presence of IgM was detected in the mucus of ABT by an enzyme-linked immunosorbent assay and several different enzymes were detected with an API-ZYM kit assay. Zymography experiments confirmed the presence of protease-like enzymes in the mucus, while enzyme assays quantified alkaline phosphatase, protease, esterase and cathepsin B activities. Lysozyme levels were high. The mucus agglutinated sheep erythrocytes but did not demonstrate complement or bacteriolytic activity.

There is restricted information on the fatty acid composition of farmed ABT or how this is influenced when the fish are held under commercial aquaculture conditions. This study investigated the FA composition of farmed ABT, its variation by dorsal muscle region and the correlation between dietary FA composition with that of the fish. Analysis of flesh samples retrieved from farmed ABT did not reveal significant differences in the FA composition of experimental sub-groups irrespective of size, time held in captivity or diet. These results indicate that FA metabolism in ABT is substrate-selective. Gene expression measurements from several organs of ABT showed that expression of *Δfad5* and *elov15*, genes involved in FA metabolism, were highest in the brain followed by the liver but no expression of these genes was detected in the spleen.

The findings of this research address aspects of health evaluation and nutritional status in farmed ABT and are discussed in terms of farming practice. Conclusions from some of these studies suggest that the practice of holding wild-captured stock in cages for periods of up to 18 months does not result in significant impact on ABT.

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CHAPTER 1

GENERAL INTRODUCTION

Tunas comprise several species of highly migratory fish that inhabit many of the world's oceans. Yellowfin and bigeye tunas are found worldwide, the southern bluefin tuna (SBT) inhabit temperate and cold seas while the Atlantic (ABT) and Pacific (PBT) bluefin tunas, although closely related, are restricted to the oceans after which they are named. They are impressively powerful fish, anatomically streamlined and physiologically adapted for speed and long migrations. Their scientific family name, *Thunnus*, originates from the Greek verb '*thuno*' meaning 'rush', a more than adequate description of their pace under the water.

Owing to their large size, ABT and PBT are the most iconic members of the tuna family. The former finds a prominent place in the Mediterranean fishermen's history books and there is evidence of fishing for the species dating back thousands of years (Di Natale, 2012a). Its yearly migrations captured the attention of remarkable poets and philosophers, including Aristotle. It is thought that the ABT was traded in ancient Greece and that it sustained soldiers of the Roman Empire during times of war. More recently it has become intertwined in Mediterranean fishing tradition by way of the '*tonnara*' (Ravazza, 2004), a system of nets designed to trap tunas passing close to the shore.

On the other side of the world, the PBT finds fame in Japan, a country with a long fishing tradition and the Tsukiji fish market, the largest market of its type in existence. It is highly prized for its unique meat quality and is a mainstay of Japanese cuisine. Perhaps this is not more evident than in the annual New Year Tsukiji auction, where traditionally one prized tuna is sold for an astronomical amount of money.

A high demand for the fish in Japan, and to a lesser extent, its popularity in other markets around the world stimulated Mediterranean interest in the culture of the ABT in the late 1990s. At the time, ABT were very abundant and advances in marine aquaculture technology gave opportunity for this activity. Tuna fattening is a form of capture-based aquaculture, where seed is sourced from wild fisheries and farmed in cages to marketable specifications. The attractiveness of the industry was an incentive for the Mediterranean tuna farms to grow and today several are found scattered along the Mediterranean coast.

1.1. Brief systematics of the Atlantic bluefin tuna

Tunas are members of the Scombridae family within the Order Perciformes. The Family Scombridae consists of 15 genera and about 50 species of epipelagic marine fish (Collette *et al.*, 2001). Several members of this family, which apart from tunas includes mackerels and bonitos, are the basis of many commercial fisheries throughout the world and the species contained within possess several morphological and physiological adaptations that make them of interest to evolutionary biologists and physiologists alike.

The larger tuna species belong to the subgenus *Thunnus* (South, 1845) and are considered as the most advanced species within the Family Scombridae (Collette *et al.*, 2001). Species contained in this subgenus are the ABT, *Thunnus thynnus* (L.), the PBT, *T. orientalis* (Temminck et Schlegel), the SBT, *T. maccoyii* (Castelnau), the bigeye tuna, *T. obesus* (Lowe), the yellowfin tuna, *T. albacares* (Bonnaterre), and the albacore tuna, *T. alalunga* (Bonnaterre). The ABT and PBT are morphologically similar but they are treated as separate species based upon differences in their mitochondrial DNA sequences (Collette *et al.*, 2001).

1.2. Biology and physiology of the Atlantic bluefin tuna

The ABT is native to both the eastern and western Atlantic. Based on some biological differences, mostly relating to their reproductive physiology, ABT are treated as two separate stocks – the eastern and western Atlantic – and the two have been conventionally separated by the 45°W meridian. Both ABT populations demonstrate spawning site fidelity with the eastern stock spawning in the Mediterranean and the western population spawning in the Gulf of Mexico. At present it is widely acknowledged that the eastern Atlantic ABT reach reproductive maturity at a significantly earlier age than the ABT in the west (ICCAT, 2011). Although there are more similarities than differences between the two Atlantic populations, the work in this study is based on Mediterranean ABT and so the information presented below puts more emphasis on the eastern stock.

1.2.1. Range, distribution and ecological characteristics

ABT inhabit the pelagic ecosystem of the North Atlantic Ocean and the Mediterranean Sea (Mather *et al.*, 1995). It has the widest geographic distribution amongst tuna species and unique in that it is the only large pelagic fish permanently inhabiting temperate Atlantic waters (Bard *et al.*, 1998; Fromentin & Fonteneau, 2001). Archival tagging has established that it can sustain temperatures as low as 3°C and as high as 30°C whilst maintaining physiological conditions conducive to efficient physiological function (Block *et al.*, 2001). Satellite tag data indicates that the fish primarily resides in surface waters but may dive to depths of 500-1,000 m (Lutcavage *et al.*, 2000; Block *et al.*, 2001). For other tuna species this depth displacement is thought to be linked to foraging behaviour or for body temperature control (Gunn & Block, 2001; Musyl *et al.*, 2003) and it is presumed that ABT exhibits this behaviour for similar purposes (Fromentin & Powers, 2005). The spatial distribution and movement of ABT is thought to be controlled by both temperature gradients and tracts of ocean which offer abundant prey concentrations (Brill *et al.*, 2002), as in the case of other tuna species (Inagake *et al.*, 2001; Fromentin & Powers, 2005).

Over the centuries there has been disagreement regarding the movement of ABT between the Atlantic and the Mediterranean (Fromentin & Fonteneau, 2001). The theory of migration between the two seas gained strength in the 1920s when hooks used in the North Atlantic were found in fish captured in the Mediterranean (Fromentin & Powers, 2005) and confirmed later with the aid of conventional tags (Mather *et al.*, 1995). The movements of ABT have now been documented extensively and it is now known that they display homing behaviour and they migrate to spawn in specific and

well-defined areas (Block *et al.*, 2005; Fromentin & Powers, 2005). Figure 1.1 shows the movement of ABT, as extrapolated from tagging studies, within the Atlantic Ocean.

In contrast to the data available for spawning migrations, little information is available for feeding movements. It has been shown, however, that migration patterns vary to a considerable degree between years, areas and even individuals (Lutcavage *et al.*, 1999; Block *et al.*, 2001; Lutcavage *et al.*, 2001; De Metrio *et al.*, 2002). Ravier and Fromentin (2004) have hypothesised that environmental influences on the spatial dynamics of ABT may have been the cause of the disappearance of the species from established fishing grounds in the past.

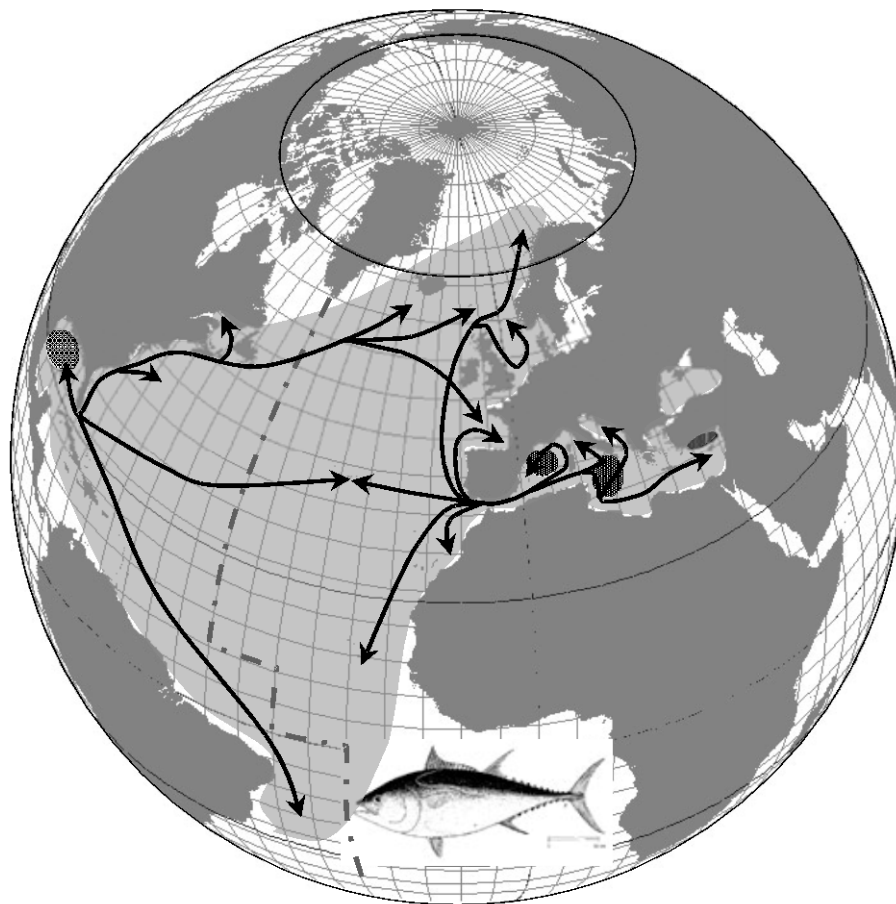


Figure 1.1 Spatial distribution (grey shading) and major migration routes (black arrows) of Atlantic bluefin tuna in the Atlantic Ocean and adjacent seas (from Fromentin & Powers, 2005). The dashed line illustrates the ICCAT delineation between eastern and western Atlantic stocks.

Data from pop-up and electronic tags has shown a higher rate of trans-Atlantic migrations and a larger degree of mixing between the eastern and western ABT stocks (Lutcavage *et al.*, 1999; Block *et al.*, 2001). More recently it has also been proposed that although the two populations are distinct, there is a degree of overlap in their spatial distribution in the North Atlantic (Block *et al.*, 2005).

1.2.2. Tuna physiology and anatomy: distinguishing features

There are several differences between the anatomies and physiologies of tunas and other teleosts, and these are presumably linked to the unique lifestyle of thunnids. Differences exist at the level of the gills, cardiovascular system and muscle aerobic capacity (Korsmeyer & Dewar, 2001).

Tunas are obligate ram ventilators and therefore rely on a constant stream of water over the gills for blood oxygenation (Brown & Muir, 1970). Metabolic measurements indicate that metabolic rates in tunas are elevated compared to those in most other teleosts (Korsmeyer & Dewar, 2001), and the cardiovascular system is capable of delivering oxygen and metabolites at sufficiently high rates to tissues (Brill & Bushnell, 2001). To meet the high oxygen demands of their metabolism, their gills have a large surface area and have additional supporting tissue, fusing lamellae on adjacent filaments to protect them from damage against a high velocity water flow (Muir & Kendall, 1968).

Perhaps the most distinguishing feature of tunas, apart from their endothermic capabilities, are the characteristics of their red and white muscles. The total aerobic capacity of the white muscle tissue is higher than that of the red muscle (Korsmeyer &

Dewar, 2001). While the oxidative red muscle is adapted for sustained aerobic muscle activity during long-distance swimming, the white muscle has a high anaerobic capacity for powerful activity bursts of short duration (Hulbert *et al.*, 1979). Recovery from such strenuous activity during burst swimming is facilitated by the white muscle's aerobic capacity, which is relatively high in tunas, as is lactate dehydrogenase activity (Dickson, 1996; Korsmeyer *et al.*, 1996). Both capillary densities within tuna muscle and myoglobin content are higher than those found in other fish (Mathieu-Costello *et al.*, 1992; Dickson, 1996).

In comparison to other teleosts, tunas have a higher proportion of slow-twitch aerobic red muscles which sustain the requirements of continuous swimming (Graham *et al.*, 1983). Tunas differ from other fish species in that their red muscle is located nearer to the vertebral column and is completely surrounded by white muscle (Graham *et al.*, 1983) and their requirement for constant motion generates metabolic heat within their red muscle. To prevent heat loss through the body surface and the gill-water interface, tunas possess a system of counter-current heat exchangers, termed retia mirabilia (*sing.* rete mirabile), present at critical locations throughout their body. These consist of a parallel arrangement of juxtaposed arterial and venous blood vessels. As the venous and arterial blood meet in the retia mirabilia, heat generated through aerobic metabolism and carried in the former is transferred to the colder arterial blood across a temperature gradient (Graham & Dickson, 2001). Thermal diffusion is rapid and although the retia mirabilia of tuna red muscle contain more arterioles than venules, the latter occupy a larger volume owing to their larger size (Graham, 1975; Graham & Diener, 1979; Graham *et al.*, 1983). Apart from their red muscle, *Thunnus* spp. are also able to maintain

elevated temperatures within their viscera (Carey *et al.*, 1984) and their brains (Graham & Dickson, 2001). This ability to conserve metabolic heat gives tuna species considerable thermal tolerance enabling them to inhabit various environments (Brill, 1994).

Tuna species have anatomies adapted for their active lifestyle and this gives them superior swimming ability amongst teleosts (Magnuson, 1978). Their body has a streamlined form and an anatomical and mechanical build which is specialised for both high performance swimming and migrations over long distances (Westneat & Wainwright, 2001). Their body thickness to length ratio approaches optimal values for minimum drag (Altringham & Shadwick, 2001) while their elliptical profile limits lateral movement and increases stability during vigorous activity (Lighthill, 1970). The paired finlets found before the caudal peduncle aid to maintain flow over the tail, augment lift and reduce drag (Lindsey, 1978; Magnuson, 1978), while their narrow tail reduces loss of kinetic energy (Lighthill, 1970). Their muscle, tendon and bone form a biomechanical system able to support their significant power and differs in several key features from the locomotory mechanisms found in other teleosts (Westneat & Wainwright, 2001). To reduce turbulence, they have smooth skin and relatively small scales (Altringham & Shadwick, 2001).

1.2.3. Reproduction

ABT are oviparous and undergo multiple reproductive cycles throughout their lifetime (Schaefer, 2001). They display asynchronous oocyte development and are multiple batch spawners, with spawning frequency estimated at 1-2 days in the Mediterranean (Medina *et al.*, 2002). Larger females appear to produce a larger number of eggs than smaller specimens and a 5 year old female produces 5 million eggs as compared to the

45 million eggs carried by a 15-20 year old female (Rodríguez-Roda, 1967). Both eastern and western ABT stocks are annual spawners and the eastern stock reaches maturity at a younger age than its western counterpart (Corriero *et al.*, 2005; ICCAT, 2011). They spawn at restricted and specific locations, around the Balearic Islands and the waters off Sicily in the Mediterranean, once water temperatures exceed 24°C (Schaefer, 2001; Heinisch *et al.*, 2008), although there is evidence that spawning within the basin is more dispersed than this (Picinetti & Picinetti Manfrin, 1993; Karakulak *et al.*, 2004; Damalas & Megalofonou, 2012; Koched *et al.*, 2013). Spawning occurs directly in the water column and fertilised eggs hatch following a 2-day incubation period, with hatched larvae being relatively undeveloped, 3-4 mm in size and typically pelagic (Fromentin & Powers, 2005).

1.2.4. Feeding and growth

Characteristic of many marine fish, ABT larvae feed mainly on small zooplankton, primarily copepods and nauplii (Uotani *et al.*, 1990), whilst juveniles and adults are opportunistic feeders. Juveniles tend to prey on crustaceans, small fish and cephalopods while adults feed on fish including herring, anchovy, sardines and mackerels (Ortiz de Zarate & Cort, 1986; Eggleston & Bochenek, 1990; Chase, 2002; Sarà & Sarà, 2007; Karakulak *et al.*, 2009; Battaglia *et al.*, 2013).

The growth of ABT juveniles is about 30 cm year⁻¹ and rapid when compared to the rates observed in other teleost species (Fromentin & Fonteneau, 2001). Remarkably, larvae hatched in June reach a length of 30-40 cm and weigh 1 kg by October, and grow to 4 kg by the following year (Mather *et al.*, 1995). As the fish gets older, growth in length decreases and growth in weight increases, so that elongate juveniles become thicker

and larger with age. At age 10 years, ABT reach a length of 150 cm and weigh 150 kg, 225 kg at 15 years, and at 20 years they will weigh in the region of 400 kg and measure 300 cm (Fromentin & Powers, 2005; Santamaria *et al.*, 2009). ABT are known to grow to larger weights; a fish of weight 685 kg has been reported from an Italian trap (Sara, 1963) while an individual weighing 726 kg was captured in the Gulf of Maine (Bigelow & Schroeder, 1953). The largest fish on record from the farm where this study was based was a male specimen weighing 687 kg and measuring 340 cm (Figure 1.2).



Figure 1.2 The largest Atlantic bluefin tuna specimen on record from the farm where this study was based. The fish measured 340 cm in length and weighed 687 kg.

Mather *et al.* (1995) have suggested that male specimens grow at a faster rate than females and that they are proportionally more frequent in catches of fish of weight in excess of 250 kg. This is also observed in farmed tuna populations where specimens exceeding this size, almost in their entirety, tend to be males. This is thought to be

related to the stress female specimens are subject to during gonadal maturation and spawning, which may lead to an increased rate of predation or natural mortality.

Endothermy requires metabolic energy so that it is expected that growth in ABT is subject to seasonal growth patterns due to greater energy expenditure during colder months. Growth is reported to be rapid and as high as 10 % month⁻¹ in summer and autumn but growth during the colder winter months, in wild specimens, is negligible (Mather *et al.*, 1995; Labelle *et al.*, 1997). Seasonal patterns have also been reported to occur in the SBT (Eggleston & Bochenek, 1990). Inter-annual variability in growth of ABT juveniles in the Mediterranean has been reported by Fromentin (2003) while changes in growth rates over more extended periods of time have been reported in the SBT wild stock (Polacheck *et al.*, 2004; Farley & Gunn, 2007).

1.3. Fisheries and commercial exploitation

ABT have been fished from the seas for centuries and their exploitation has evolved with time. Its recent spike in popularity with consumers worldwide has resulted in increased targeted fishing efforts on the species so that it has evolved from a loosely managed sector into one with very strict control mechanisms. This section briefly describes the history of ABT fisheries, its present commercial exploitation, and its control and management.

1.3.1. A brief history of Atlantic bluefin tuna fisheries

The earliest evidence of fishing for ABT in the Mediterranean dates back to the 7th millennium B.C. in Cyprus and targeted fisheries, mainly hand lines and beach seines, were in place during the time of the Phoenician and the Roman Empires (Doumenge,

1998). From the 16th century, these fishing methods were gradually replaced by traps (Ravier & Fromentin, 2001) throughout the Mediterranean and at several locations around the Straits of Gibraltar (Fromentin & Powers, 2005). These were the first industrial fisheries in these areas and combined yields of these were significant at an average of around 15,000 tonnes year⁻¹ (Ravier & Fromentin, 2002).

Traps remained the main fishing method until the 20th Century but their efficiency decreased possibly due to increased marine traffic and coastal pollution (Ravier & Fromentin, 2001). Progressively, fishing for the species spread to other locations. A hand line fishery targeting ABT juveniles developed in the Bay of Biscay (Bard *et al.*, 1998), and fishing also expanded into the North and Norwegian Seas (Fromentin & Powers, 2005). The development of the purse seine system, an active fishing gear, in the 1930s represented the biggest change in commercial fisheries for this species (Meyer-Waarden, 1959). Nordic purse seine fleets grew rapidly so that by the middle of the century catch volume with this fishing system exceeded that of the traditional trap fisheries (Pursineri *et al.*, 2001), but a sudden decrease in landings in the 1960s caused the collapse of purse seine fleets operating in this area (Fromentin & Powers, 2005).

Around the same period fisheries for ABT juveniles appeared in the western Atlantic Ocean with fishing efforts focused on the continental shelf between Cape Hatteras and Newfoundland, and eventually extending to Cape Cod and Maine (Mather *et al.*, 1995). At the time, there was no demand for the larger fish so that fisheries for such fish sizes were not popular. This changed in the 1960s when the Japanese long line fleet started fishing in the western Atlantic and eventually moved to the Gulf of Mexico to target larger individuals (Fromentin & Powers, 2005). From here onwards there was a

progressive replacement of traditional capture fisheries to active fishing methods, mainly using purse seine and long line methods.

The development of the Japanese sushi and sashimi markets in the 1980s had a great influence on the ABT fishery and has shaped it into the industry it is today. Due to the increase in the profitability of the species, there was a renewed effort to increase fishing capacity and efficiency and this stimulated the modernisation of fishing fleets and the development of new industries, including captive fattening. Exploitation for the species expanded from the traditional North Atlantic and Mediterranean fishing grounds into new areas. This was most evident in the Mediterranean region where purse seine vessels equipped with modern fishing technology moved away from the coast and eventually fished for ABT over the entire Mediterranean basin. Simultaneously the Japanese long line fleets expanded and increased efforts in the central North Atlantic resulted in increased capture yields (Fromentin & Powers, 2005).

1.3.2. ICCAT: Fishery management

The International Commission for the Conservation of Atlantic Tunas (ICCAT) is a convention of signatory member nations and was established in 1969 in the face of increased fishing pressure on tuna species by the fleets of several countries. It is a regional fisheries management organisation and its main responsibility lies in the management of tuna and tuna-like species in the Atlantic Ocean and adjacent seas (Figure 1.3). It is composed of several contracting parties and incorporates the major tuna fishing countries including the USA, Japan and those within the European Union. Its jurisdiction covers several species including ABT and it is responsible for fishery

management including member and fishery compliance, scientific research and stock assessments, the latter being used for adjustments of quota levels.

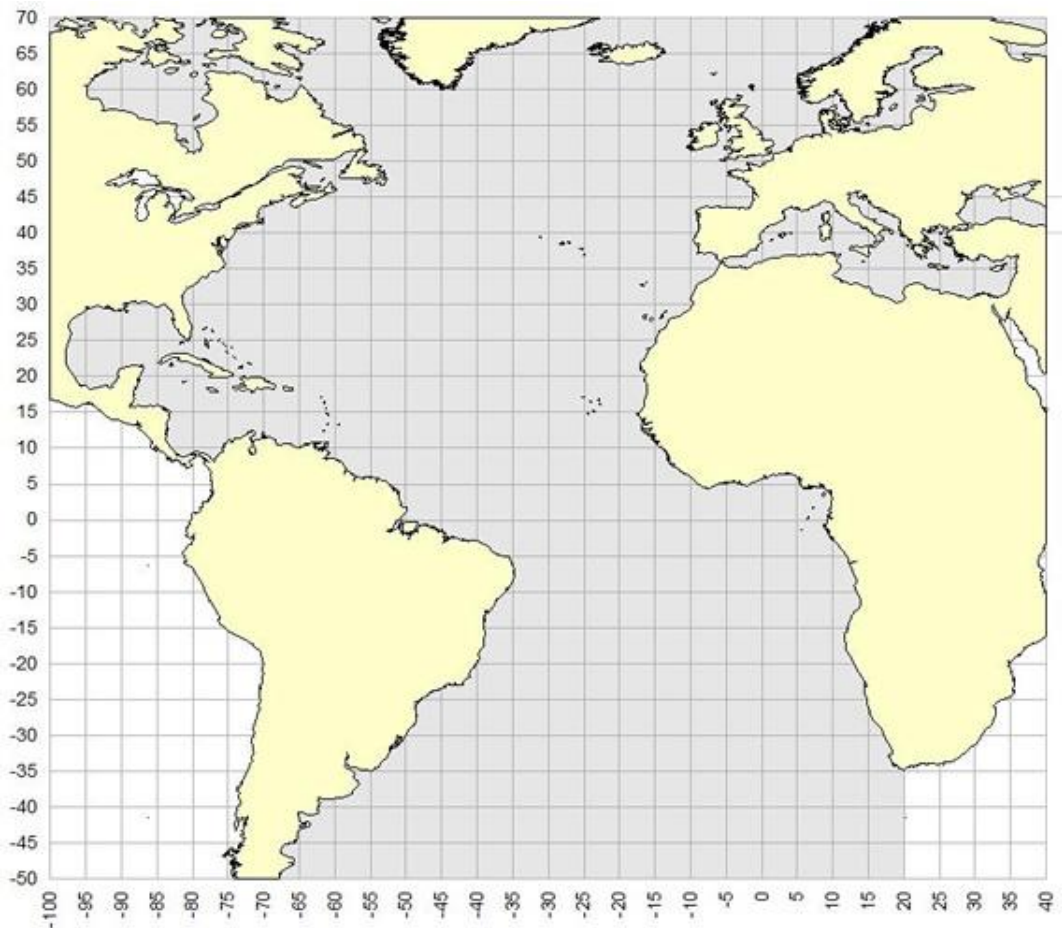


Figure 1.3 The convention area of The International Commission for the Conservation of Atlantic Tunas (from ICCAT, 2006).

1.3.3. The state of Atlantic bluefin tuna stocks

ICCAT has recently been criticised for improper management of ABT fisheries (*e.g.* Longo & Clausen, 2011; Webster, 2011; Longo & Clark, 2012). The major obstacles in the assessment and management of the ABT stocks are a lack of historical data and alleged catch under-reporting in recent periods (ICCAT, 2011). For the last few years, the ICCAT has drastically reduced the total allowable catches (TAC) for ABT to protect the sustainability of the stock (ICCAT, 2002, 2008, 2010b, 2012, 2013) as a consequence of which catches have consistently decreased since 2008 (Figure 1.4).

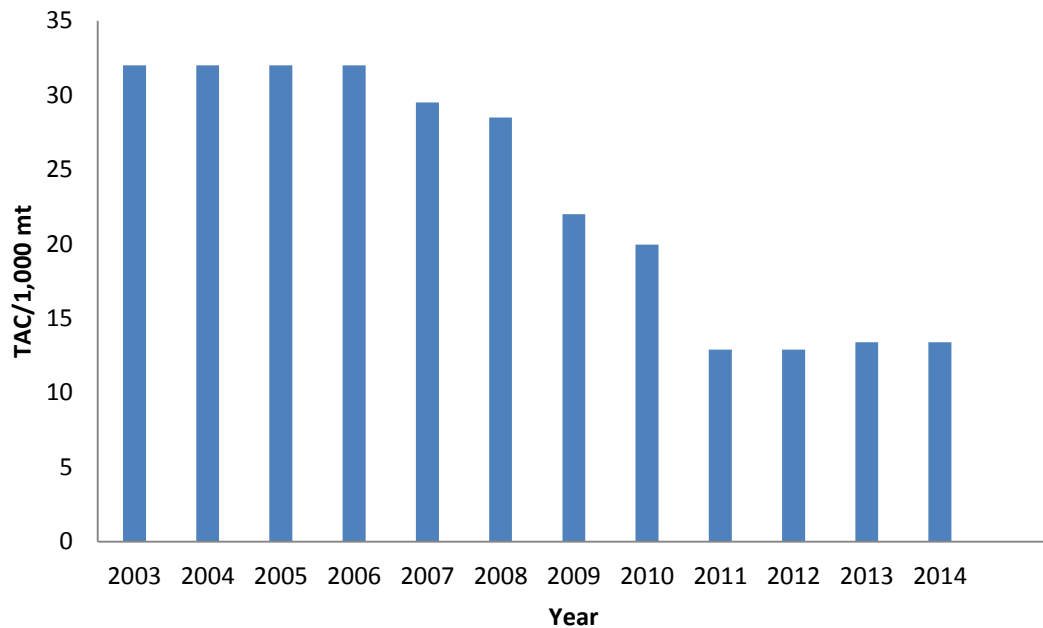


Figure 1.4 Atlantic bluefin tuna total allowable catch (TAC) levels in metric tons (mt) as set by ICCAT throughout the last decade (ICCAT, 2002, 2008, 2010b, 2012, 2013).

ABT stock assessments are carried out periodically with the latest analysis being done in 2012 (ICCAT, 2014). Stock outlook in the last analysis was more positive than previous assessments and ABT appears to be on its way to recovery. Indicators from several Mediterranean and eastern Atlantic fishery sectors have also corroborated this improvement in stock status and these are probably indicative of positive outcomes from stricter management and control of the ABT fishery.

1.3.4. Historical data and present state of Atlantic bluefin tuna fisheries

The majority of ABT is captured in the Mediterranean although commercial fisheries for the species operate in both the eastern and western Atlantic, with high seas fisheries being prominent also in the west (Figure 1.5).

In coastal western Atlantic fisheries, ABT is captured mostly by rod and reel methods. This type of fishing activity extends along most of the Atlantic coast of the USA and the

fish is generally air freighted to Japan where it is sold at auction. Japanese large-scale long line vessels target ABT in the western North Atlantic. On these the fish are gilled, gutted and frozen on-board the fishing vessels for transport to major markets, generally Japan.

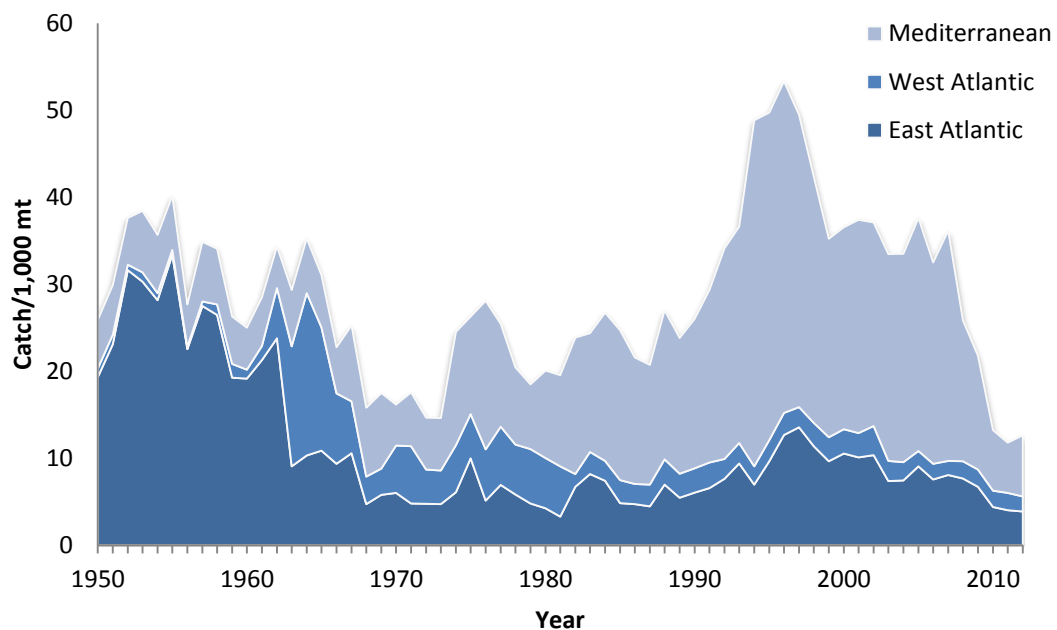


Figure 1.5 Atlantic bluefin tuna capture production in metric tons (mt) by region (ICCAT, 2010a).

In the eastern Atlantic, a minor fishery operates in the Bay of Biscay, where hand lines are used to fish for small ABT. The fish captured here is destined for fresh sale in local markets. Within the Mediterranean there is a contribution to ABT landings by artisanal long line vessels, which generally fish in traditional fishing grounds not far away from the coast, but by far the species is fished by trap net and purse seine systems (Figure 1.6).

Trap nets are the most ancient fishing industry known to date (Di Natale, 2012b). They were traditionally found along several Mediterranean coasts and the Gibraltar strait but have decreased in number in recent times. Within the Mediterranean itself, only two

trap nets in Sardinia were operational in 2013. A trap net system operates in Portugal although recruitment rates, and consequently catch volumes, are low. Several trap nets remain in operation on either side of the Gibraltar Strait, along the southern Atlantic Spanish and northern Atlantic Moroccan coasts, targeting fish entering the Mediterranean from the Atlantic. Catch volumes in this region are significant and the traps consistently reach their allocated quotas. Traps consist of a compartmentalised system of nets anchored to the seabed. Differences in designs between different traps are minor, but their arrangement is such that it directs fish passing close to the shore into a labyrinth of nets leading to an enclosed chamber from which the fish are harvested (Figure 1.7). The systems in this region target larger ABT and the capture of smaller specimens is uncommon (Abid *et al.*, 2012; Cort *et al.*, 2012).

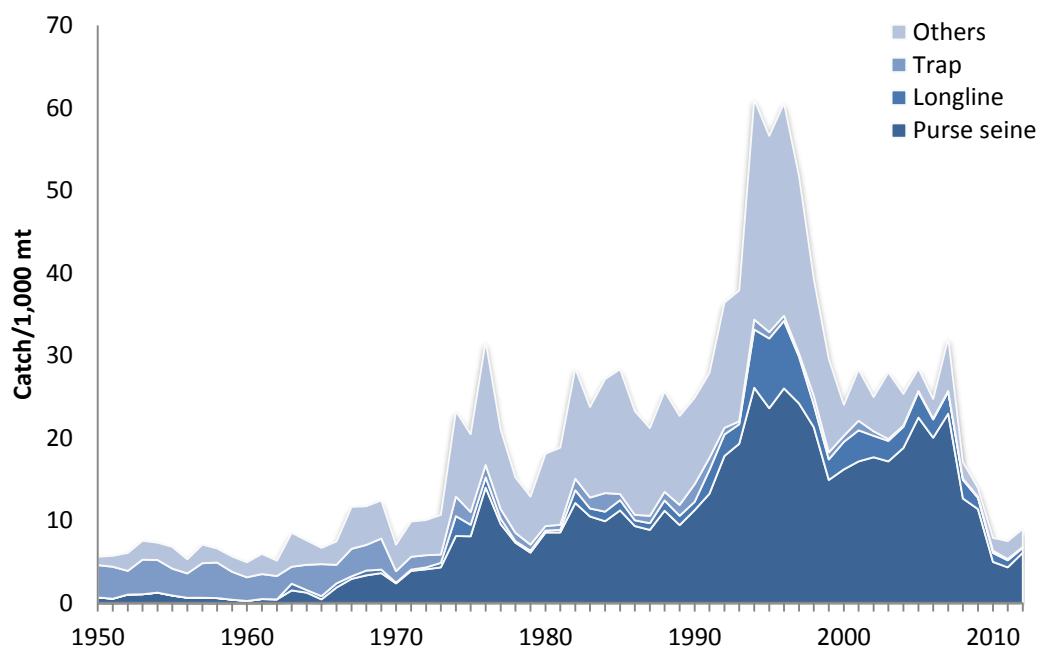


Figure 1.6 Mediterranean Atlantic bluefin tuna capture production in metric tons (mt) in the Mediterranean Sea by gear type (ICCAT, 2010a).



Figure 1.7 A traditional tuna trap in Bonagia, Sicily. Tunas are enclosed in a chamber prior to the start of the ‘*mattanza*’ (left) and being hauled aboard (from Ravazza, 2004).

As mentioned previously, the development of the Asian sushi and sashimi markets towards the end of the last decade, resulted in the development and modernisation of a purse seine fishing fleet in the Mediterranean. Figure 1.8 shows an example of a modern purse seine vessel working in the Mediterranean ABT fishery. The catches were at first landed on the high seas into reefer vessels until new aquaculture technologies allowed for the placing of tunas in cages. Purse seining is an active fishing method whereby aggregated schools of fish are encircled using nets and find widespread use in targeted fisheries for several species. It is now the major fishing gear type in use for large-scale ABT fisheries and is the only method suitable for the farming of large tunas.



Figure 1.8 A typical purse seine fishing vessel used in the exploitation of Atlantic bluefin tuna in the Mediterranean.

1.4. Farming of the Atlantic bluefin tuna

Research on tuna aquaculture started in the 1970s when Japanese government-backed research attempted to grow PBT juveniles to marketability in marine cages (Ottolenghi *et al.*, 2004). The first attempts at the commercial farming of the ABT in floating pens took place in Nova Scotia, Canada, and sourced tunas from mackerel traps (Farwell, 2001). The objectives of this were the same as those of modern tuna farms; to grow and improve the quality and value of the fish, and to market the product at times of low commodity availability and a consequent high demand (Miyake *et al.*, 2003). This operation, however, was abandoned following several years of low catch volumes.

Around the world, all three bluefin tuna species are farmed and at present all are stocked through wild-capture fisheries. Farms in Japan and Mexico grow the PBT, the SBT is ranched in Australia and ABT is farmed in the Mediterranean. Of the three, the Mediterranean ABT industry has the largest production volume.

1.4.1. Origins of Atlantic bluefin tuna farming in the Mediterranean

ABT farming in the Mediterranean has its origins in Ceuta, Spain, in the late 1970s. Post-spawned lean tuna returning to the Atlantic were captured in traps and transferred to grow-out cages and marketed after they regained their fat content (Miyake *et al.*, 2003). Annual production levels at the site were low however, and rarely surpassed 200 metric tons (mt).

ABT in the Mediterranean were highly abundant and purse seine captures were on the increase (Figure 1.5), but despite the availability of wild seed technological limitations restricted the transfer and towage of tunas from high seas to farming sites.

Developments in aquaculture engineering allowed for the construction of durable cages that were of adequate dimensions to accommodate large ABT and were resistant to forces acting on cage structures during towing. Mediterranean ABT farming in its present form was pioneered in Spain and Croatia in 1996 (Miyake *et al.*, 2003). Farming operations quickly spread across many Mediterranean countries including Tunisia, Italy, Greece, Turkey and Malta, the latter being located in close proximity to major fishing grounds. The increase in the number of tuna farms also stimulated growth in the Mediterranean purse seine fishing fleet. The industry went through a period of rapid growth until recent reduction in ABT TAC resulted in a significant decrease in both the numbers of farms and purse seine vessels. Only 15 Mediterranean tuna farms were operational in 2013 and only 4 establishments have annual productions exceeding 1,000 mt, with major outputs coming from Malta and Spain. Figure 1.9 shows a typical Mediterranean ABT farm.



Figure 1.9 The Atlantic bluefin tuna farm, located off the southern coast of Malta, where this study was based.

1.4.2. Atlantic bluefin tuna farming: catch to market

The premises and methods of tuna farming are similar across different tuna species. As in the aquaculture of other fish species, it is a three-step sequential process starting with stocking, followed by farming or fattening, and ending with the marketing of the fish.

1.4.2.1. Capture and transfer to cage

The Mediterranean purse seine tuna fishing season runs from the 26 May to the 24 June every year (ICCAT, 2013). Vessels leave port the previous night and navigate to fishing grounds. There are several of these in the Mediterranean with the major ones found around the Balearic Islands, in the Tyrrhenian Sea, the Medina Embankment off Malta, and off the Libyan coast. Vessels locate schooling tunas (Figure 1.10) with scanning sonars and aggregate them by distributing bait on the sea surface. Once the fish are sufficiently aggregated a purse seine net is deployed to encircle and capture the fish, which are then transferred to tow cages through a common opening (Figure 1.11).



Figure 1.10 A single migrating tuna school may extend over larger areas and sardines are supplied to the fish to aggregate them prior to fishing manoeuvres.



Figure 1.11 A fishing operation for Atlantic bluefin tuna in the Mediterranean (top) and subsequent transfer of fish from a purse seine into a tow cage (bottom).

Estimates of catch weights are formulated from analysis of an underwater video of the transfer operation, weighing of the transfer mortalities and professional observation. ABT do not school according to size or age and consequently populations transferred to cages reflect this in having wide-ranging size distributions (40-500 kg). Proper care for a

few days after catch is crucial for eliminating mortality and acclimatising fish to domestication so it is essential that cages are properly set up to allow for free movement of the fish contained within. Once cages have reached their holding capacity, they start their way back to the farm. Towing speeds are in the region of 1 knot (speeds beyond this will induce high mortalities) and such voyages may take as long as 3 weeks to reach their destination, depending on the distance between the capture and farm sites. Once at their destination, the fish are transferred into a farm cage in a manner similar to that described above.

1.4.2.2. Farming the fish

Feeding regimes are implemented after a short period of acclimation within farm cages. Tunas are fattened with a mixture of fish – mackerels, sardines and herrings – and feed lipid levels range from 8 to 25 %. Some operations also supply squid and cuttlefish species to the stock, although this is done in minute proportions because of the costs involved. Figure 1.12 shows ABT feeding from a feeding net in a commercial ABT farm. Feeding regimes vary across farms and this serves to adjust the quality of the fish to the different markets and their respective preferences. As is expected, feeding rates vary with mean fish weight and sea temperature and generally range from 4-10 % biomass day⁻¹ in summer and 1-4 % biomass day⁻¹ in winter.

Growth of smaller tunas is remarkable and with careful husbandry, they may double their weight and beyond within a year (ICCAT, 2009). Originally, the primary aim of tuna farming was to increase the fat content of the fish rather than to increase biomass (Miyake *et al.*, 2003; Ottolenghi *et al.*, 2004; Aguado-Giménez & García-García, 2005) but recent changes in markets have seen this pattern change and several operations

take advantage of fast growth rates and retain their smaller stock for on-growing and harvest in subsequent years.



Figure 1.12 Atlantic bluefin tuna are fattened on a controlled combination of mackerel, sardine and herring species.

1.4.2.3. Marketing

Tunas are marketed in fresh or frozen forms once the quality of their flesh meets market specifications and harvest generally commences after a 5-8 month farming period. Harvesting is fast-paced to ensure the fish retain their meat quality. Fish destined for the frozen market are processed aboard reefer vessels and on these the fish are transformed into product and blast frozen at -60°C . Fish marketed in fresh form are harvested in a similar manner but gilled and gutted, cooled in ice slurry, packaged and air-freighted to market within 24 hours.

Figure 1.13 shows a tuna processing vessel and processing operations aboard it, and frozen or fresh forms of marketable ABT product.



Figure 1.13 A tuna processing and freezing vessel (top) and tuna processing aboard such vessels (middle). Tunas are either marketed frozen (bottom, left) or auctioned in fresh form at Japanese markets such as the Tsukiji fish market in Tokyo, Japan (bottom, right).

1.4.2.4. Disease

The ABT is a resistant species and to date disease has presented no major problems to the Mediterranean industry. Tuna seem to be only slightly affected by bacterial and viral diseases, even when subject to considerable stress (Munday *et al.*, 2003), but little is known about disease in farmed tuna (Nowak, 2004; Ottolenghi *et al.*, 2004).

Diseases caused by parasites appear to have the most significant effect on the culture bluefin tunas (Colquitt *et al.*, 2001; Nowak, 2004; Deveney *et al.*, 2005; Aiken *et al.*, 2006). Encephalitis caused by the scuticociliate parasite *Uronema nigricans* is one of the most problematic infections in SBT and on occasion has resulted in mass mortalities (Munday *et al.*, 1997). A risk analysis conducted on farmed SBT in Australia identified two parasitic species as deserving attention; the sanguinicolid blood fluke, *Cardicola forsteri*, and an unidentified capsalid monogenean gill fluke (Nowak, 2004), but the latter was not observed in a subsequent parasite survey (Deveney *et al.*, 2005). In addition to these, Metazoan gill parasites, including *Pseudocycnus appendiculatus* and the crustacean copepod *Euryphorus brachypterus*, are also commonly encountered in farmed SBT but pose little problem to the welfare of the species (Hayward *et al.*, 2007, 2008). Monogenean and sea lice infections have also been described in cultured SBT (Deveney *et al.*, 2005; Hayward *et al.*, 2009, 2010).

There are few reports of disease in cultured ABT and in their entirety originate from the Croatian industry. Wild ABT captures for purposes of farm stocking are subject to a 30 kg minimum size limit, except for the Adriatic where derogation decreases the minimum catch weight to 10 kg, so that this may partly explain this pattern. An outbreak of *Photobacterium damsela* subsp. *piscicida* is the only report detailing mass mortality in

farmed ABT (Mladineo *et al.*, 2006) and the bacterial fish pathogen was also detected in a separate study, although the fish were unaffected (Kapetanović *et al.*, 2006). Similarly, the only description of digenean parasites in farmed ABT originates from Croatian-farmed tuna (Mladineo & Tudor, 2004) while separate research has also identified the occurrence of *Didymocystis wedli* on the gills of Croatian cage-reared ABT (Mladineo *et al.*, 2008). A pan-steatitis outbreak has been reported from captive ABT in Croatia, and it was believed to be caused by a fatty acid imbalance in the feed (Roberts & Agius, 2008).

1.4.3. Hatchery production of Atlantic bluefin tuna juveniles

The reliance of the tuna farming industry on wild seed has prompted institutions worldwide to attempt to close the life-cycle of the three bluefin tuna species. Of the three, only the PBT has been successfully reared from eggs to mature spawning adults (Sawada *et al.*, 2005). It has not yet reached the commercial stage due to a number of complications including high mortality rates post hatch, larval and juvenile cannibalism, issues in swim bladder inflation and collision trauma (Sawada *et al.*, 2005; Ishibashi *et al.*, 2013; Kurata *et al.*, 2013; Satoh *et al.*, 2013). In addition, high mortality rates are also observed at transfer to sea cages (Tsuda *et al.*, 2012; Okada *et al.*, 2014). Progress in the other two bluefin species lags behind and it is likely that these will need to overcome identical problems to achieve commercial viability (Woolley *et al.*, 2013).

In the case of the ABT, research relies on the collection of fertilised eggs from captive mature adults held in sea cages. Captive fish generally spawn without intervention towards the end of June although there is a degree of inter-annual variation. Hormones have also proven to be effective in the stimulation of the reproductive endocrine system

as well as spawning induction in captive ABT (Corriero *et al.*, 2009; De Metrio *et al.*, 2010; Aranda *et al.*, 2011; Rosenfeld *et al.*, 2012). Eggs are collected with plankton nets or similar methods but larval survival rates have been reported to be low (de la Gándara *et al.*, 2010).

1.5. Regulatory and control measures in the Atlantic bluefin tuna industry

In recent years, the ICCAT has strengthened the enforcement of its regulations in the ABT industry and these are revised periodically (ICCAT, 2012, 2013). ABT fisheries are now amongst the most tightly controlled in the world, where every operation is documented and each harvested fish is traceable to catch. A brief review of present regulations is given below.

1.5.1. Control measures at catch

Purse seine and towing vessels involved in the ABT fishery are tightly controlled at several levels to ensure adherence to ICCAT regulations. Both classes of vessels require authorisation to operate and this is only obtained once they satisfy stringent standards and controls. Purse seine vessels have individual allocated catch quotas, and these may be caught during the open fishing season which runs annually for four weeks. No fishing is allowed after season closure, irrespective of whether catch quotas have been fulfilled by the fleets. The activities of the fleets are monitored with vessel monitoring systems, and regularly verified by at-sea inspections and aerial surveillance.

Similarly, transfers from purse seine nets to farm tow cages are rigorously controlled. Such transfers require fishing vessel masters to seek authorisation from their flag state

prior to transfer, and only after the approval of the national authority can such transfers go ahead. Fish transfers are monitored by time-stamped underwater video which is then used by at-sea ICCAT observers to verify the amounts declared by fishing masters, a measure to tackle under-reporting of catch quantities. Following verification and the completion of an ICCAT bluefin tuna catch document – essentially a quota document – the fish may start being towed back to farm under observation by national authority representatives aboard the tugboat.

1.5.2. Control measures at farm

From a regulatory point of view, ABT farms are considered as part of the ABT fishery and thus subject to the same controls as fishing vessels. Simultaneous controls by national and international authorities are affected during both caging and harvesting operations. Verifications of the origins of the fish starts whilst the fish are still under tow and a few days prior to arrival at the farm. Once verification procedures are completed and the regulatory criteria satisfied, caging procedures are authorised to go ahead. On arrival of fish to the farm, the tow and farm cages are joined and the fish transferred. Again this transfer is monitored by time-stamped video as well as a stereoscopic camera system operated by national authority officials, with transfer footage from the latter being used by regulators to verify the amounts of tuna declared (Figure 1.14). Once biomass quantities are confirmed correct, the quota documents are approved and validated, and the farming process is allowed to commence. In cases where fish biomass is deemed to be in excess of the quota held, the surplus quantity is released from the farm back into the sea. In addition to national authority officials, caging operations are attended in their entirety by ICCAT observers.

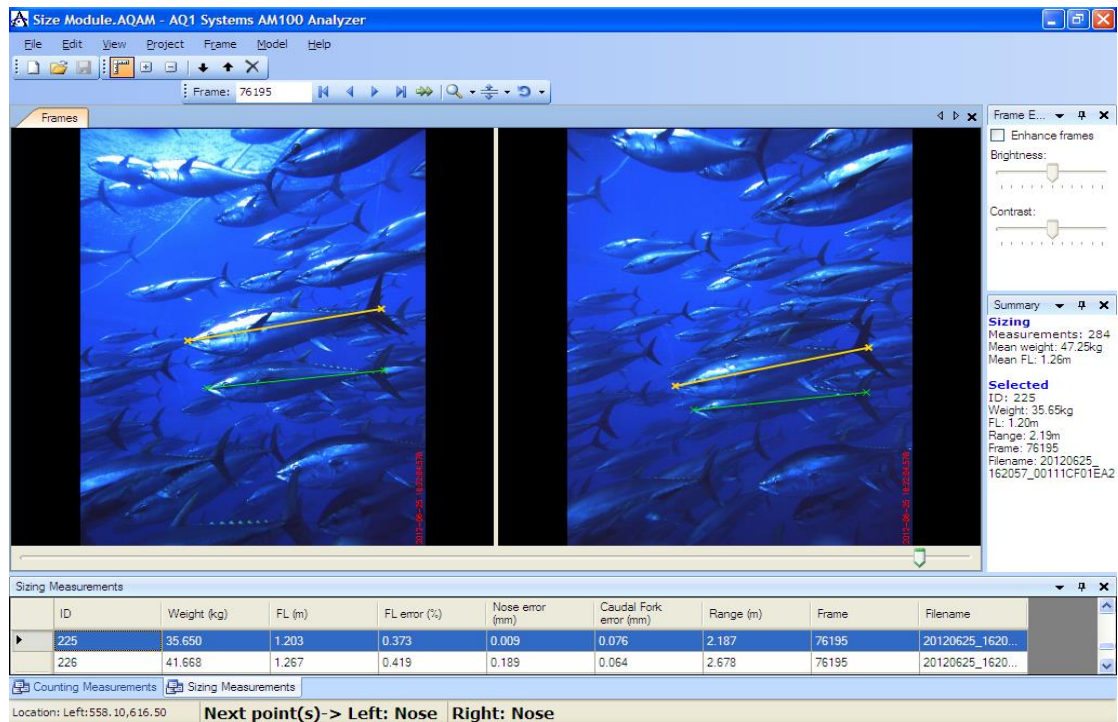


Figure 1.14 Software analysis of size composition of caged Atlantic bluefin tuna.

Control procedures extend to harvesting operations from ABT farms and monitoring by national authority representatives and ICCAT observers is mandatory, with controls focusing on numbers and weights of fish harvested. This ensures that harvests do not exceed a growth-adjusted weight threshold and/or the numbers of fish declared on the quota documents. As a final regulatory measure, processed product weights are measured prior to export and compared against relevant validated harvest quantities. The countries receiving fish control their imports by comparing validated harvest quantities against an online database carried by ICCAT.

1.6. Practical limitations in Atlantic bluefin tuna research

There are several practical limitations encountered in research work with ABT and this contributes to a general lack of knowledge on the species. In fact much of what is known on the biology of tuna species mainly originates from work on smaller tuna species such as the yellowfin and bigeye tuna.

A major obstacle is the availability of specimens to work with. This is very evident in the sourcing of wild specimens, which are generally fished at offshore locations so that sample retrieval is logistically complicated and sometimes impossible due to safety regulations. For this reason, sample collection from wild ABT samples was not available in most studies in the present work.

Most of the studies on ABT now rely on fish sourced from traps or farms. This entails limited flexibility in study design and owing to the commercial nature of both sources, sampling tends to be opportunistic and needs to conform to commercial harvest practices or requirements. Commercial commitments and the considerable cost of each specimen makes farms reluctant to experiment with the stock they hold in cages. Furthermore, when specimen sampling is permitted post-harvest treatment is time-critical and retrieval needs to be quick since delays will deteriorate the quality of the fish, resulting in substantial depreciation in the product's value and a consequent decrease in return.

Apart from commercial reasons that limit study design, other factors limit the scope of research with the ABT. For example tagging trials on small fish have resulted in significant mortalities (Tičina *et al.*, 2004). In addition, regulations now require the presence of ICCAT observers during the harvest of any quantity of fish from ABT farms and costs for these are significant and borne by the farms themselves. Since tunas are costly, these two reasons make studies investigating temporal changes difficult to undertake.

Finally, sampling at offshore locations lacking laboratory equipment limit the downstream processes that can be performed on retrieved samples and this may

restrict the amount of information that can be gained from research on ABT. This, in conjunction with the restricted sample availability confines laboratory procedures to those which are well established and easy to modify.

1.7. Research objectives

Preliminary work and parasite surveying performed during the initial period of study showed a very low prevalence of disease in captive ABT. Considering this, research objectives were adjusted to investigate changes as a result of the domestication process and the mechanisms behind the absence of disease in farmed ABT. Changes in the ICCAT regulatory framework brought into force in 2010 required 100 % observer coverage for harvests irrespective of quantity, substantially increasing the cost of periodical harvests.

Given these reasons, the scope of the research was adjusted to investigate several aspects of ABT farming while trying to maintain focus on the health and immunological characteristics of captive ABT. There is a general lack of data on ABT and contributions made by parts of this work may aid in the development of monitoring procedures for farmed ABT.

The research focused on four areas. The first chapter investigates the effects of farming on arteriosclerotic lesions within the main coronary artery of ABT and draws on the availability of wild specimens for comparison between fish of different origins. The second chapter examines the haematological and plasma biochemical parameters as well as important immunological properties of the blood of captive ABT, and compares indices across different size ranges. The third section analyses the immunological

properties of ABT epidermal mucus as a first line of defence against pathogenic infection while the final section studies several aspects of lipids in farmed ABT.

**IT IS EMPHASISED THAT ALL ATLANTIC BLUEFIN TUNA SPECIMENS USED
IN THIS STUDY WERE CAUGHT, FARMED AND HARVESTED ETHICALLY
AND IN LINE WITH ALL ICCAT RECOMMENDATIONS, AND EUROPEAN
UNION AND NATIONAL LEGISLATION**

CHAPTER 2

CORONARY ARTERIOSCLEROSIS IN WILD AND CAPTIVE ATLANTIC BLUEFIN TUNA

2.1. Introduction

Arteriosclerosis in fish was first described by Robertson and Wexler (1960) in several salmon species including the chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). The changes detected in these fish were linked to Cushing's syndrome – a disorder caused by prolonged exposure to the stress hormone cortisol in humans – and included degradation in the salmon's cardiovascular system, more specifically degenerative changes featuring myointimal hyperplasia (Saunders & Farrell, 1988) within the arteries supplying the cardiac system. Early works attributed these changes to senescence in salmon (Robertson & Wexler, 1960; Robertson *et al.*, 1961a, b; Robertson & Wexler, 1962) while others indicated that the lesions regressed after spawning (*e.g.* Van Citters & Watson, 1968; Maneche *et al.*, 1972; Schmidt & House, 1979). The latter stimulated interest in the use of salmon as models for human atherosclerosis, principally since it was proposed that the arterial lesions regress after spawning. Later studies disproved this (*e.g.* Saunders & Farrell, 1988; Farrell *et al.*, 1990a; Kubasch & Rourke, 1990) and it is now accepted that lesions do not regress but are considered a progressive condition.

Most research on arteriosclerosis has focused on salmonids and on the main coronary artery lying on the bulbus arteriosus due to its accessibility, and also because lesions appear to be more prevalent within this vessel (Farrell, 2002). In salmonid species lesions have been described in Pacific salmonids, notably *Oncorhynchus* spp. (Robertson & Wexler, 1960; Moore *et al.*, 1976c), in Atlantic salmon, *Salmo salar* L. (see Saunders *et al.*, 1992; Seierstad *et al.*, 2005a) and in rainbow trout, *O. mykiss* (Walbaum) (see McKenzie *et al.*, 1978; House & Benditt, 1981). Lesions, however, have also been reported in several other fish species. Eastman (1969) and Durán *et al.* (2010), for example, described the presence of lesions in the carp, *Cyprinus carpio* L., and in the rabbitfish, *Chimaera monstrosa* L., respectively. Arterial lesions have been reported also from several elasmobranch species including the lesser spotted dogfish (*syn.* small-spotted catshark), *Scyliorhinus canicula* (L.) (see Muñoz-Chápuli *et al.*, 1991; García-Garrido *et al.*, 1993), and several shark species including thresher, *Alopias vulpinus* (Bonaterre) and blue sharks, *Prionace glauca* (L.) (see Borucinska *et al.*, 2012).

The occurrence of arteriosclerotic lesions in the ABT was reported by Vastesaegeer *et al.* (1962). Working on small ABT of age 3-4 years, they reported widespread diffuse fibrosis within the intima of the main coronary artery in all of the ten specimens they examined. The intimal lesions reported here were described as consisting mainly of rounded nodules of fibrous tissue with a fragmented elastic lamina, containing considerable collagen and a large number of histiocytes. Additionally, and in contrast to findings in other fish species, lipid deposits were identified in the more severe lesions. To date, no further research has been carried out on arteriosclerosis in ABT.

Tuna hearts are s-shaped and as in other teleosts consist of four chambers; the sinus venosus, atrium, ventricle and bulbus arteriosus arranged in a caudal to rostral direction (Bone & Moore, 2008). Tuna ventricles are morphologically designed to produce high ventricular pressures and are large, thick-walled and assume a pyramidal form (Tota, 1983; Farrell & Jones, 1992). Deoxygenated blood is driven forwards from the ventricle to the passive and compliant bulbus arteriosus which in tunas swells prominently proximal to the ventricle and tapers towards the ventral aorta. The bulbus contains no cardiac muscle and is not actively contractile (Braun *et al.*, 2003) and acts as a passive pressure reservoir, dampening cyclic variations in pressure caused by ventricular contractions and thus maintaining constant flow in the ventral aorta (Bone & Moore, 2008) which is required for efficient gas exchange.

The coronary artery forms as a branch of the efferent brachial arteries (Farrell & Jones, 1992), and generally lies as an unbranched vessel on the bulbus arteriosus (Farrell, 2002). The orientation of the heart in tuna species differs from that in salmonids so that the coronary artery runs along the dorsal surface of the bulbus in skipjack tuna, *Katsuwonus pelamis* (L.) (see Farrell & Jones, 1992), and in ABT (personal observation) as opposed to its ventral location on the bulbus of the former. In teleosts possessing a coronary circulation, it is morphologically associated with the compact myocardium and serves to deliver a supplemental supply of oxygen to cardiac muscle (Farrell, 2002) and remove waste (Jones & Braun, 2011). Given the high proportion of compact myocardium in skipjack tuna (*i.e.* 60 % as compared with 30-40 % in salmonids), Farrell *et al.* (1992) suggest that skipjack tuna might have an obligate dependence on the supply of oxygen to cardiac muscle through the coronary artery. This is not the case for

other fish species, including rainbow trout and chinook salmon, where surgical removal of the coronary artery affected cardiac performance but not specimen survival (Daxboeck, 1982; Farrell & Steffensen, 1987; Farrell *et al.*, 1990b; Gamperl *et al.*, 1994).

Histologically the structure of the fish coronary artery follows closely that of mammals and is composed of an endothelium, an internal elastic membrane (*syn.* lamina) lying on a media consisting of circular or spirally arranged vascular smooth muscle and elastic fibres, and an outer layer of connective tissue referred to as the adventitia (Jones & Braun, 2011). The thinner media in the fish coronary artery, as compared to that found in mammals, is likely due to the lower coronary arterial blood pressure in the former (Farrell, 2002; Jones & Braun, 2011).

Typically, lesions in salmonids manifest themselves as intimal proliferations of vascular smooth muscle (*i.e.* myointimal hyperplasia) and commonly feature a thickened, duplicated, disrupted or altogether absent internal elastic lamina (IEL; Maneche *et al.*, 1972; Moore *et al.*, 1976a; Farrell *et al.*, 1986; Kubasch & Rourke, 1990; Farrell, 2002; Seierstad *et al.*, 2005b) through which proliferated smooth muscle cells enter the intima to form focal lesions which, at later stages, may merge to form more severe lesions that may occlude the lumen by as much as 50 % (Saunders & Farrell, 1988). Several authors (Moss & Benditt, 1968; Ross & Glomset, 1973; Saunders & Farrell, 1988; Kubasch & Rourke, 1990; Farrell, 2002) have stated that arteriosclerotic lesions in fish resemble the early forms of mammalian atherosclerosis. Although these lesions possess characteristics of smooth muscle cells, they also contain varying amounts of collagen and elastin, mucopolysaccharides and other amorphous material (McKenzie *et al.*,

1978). Eaton *et al.* (1984) described the presence of degenerative, as opposed to proliferative, lesions in the coronary arteries of chinook salmon.

The precise aetiology of coronary arteriosclerosis in salmonids is not known (Farrell, 2002). In early investigations, it was believed that sexual maturation and the physiology of upstream migration (Robertson *et al.*, 1961b) were the primary causes of arteriosclerosis in salmonids but later studies indicated that well developed coronary artery lesions are present also in juveniles of several salmon species (Moore *et al.*, 1976a; McKenzie *et al.*, 1978; House *et al.*, 1979; Farrell *et al.*, 1986; Kubasch & Rourke, 1990; Saunders *et al.*, 1992). Although sexual maturation might play a role, since the injection of sex hormones has been shown to promote an increase in arteriosclerotic lesions in rainbow trout (House *et al.*, 1979), it is now perceived to be a secondary rather than primary factor in the lesion aetiology. Conflicting evidence is available as to the role of polyunsaturated fatty acids (PUFAs) in arterial lesion progression; Farrell (2002) suggested that PUFAs and their metabolites stimulate coronary artery vascular smooth muscle proliferation and may be significant in the promotion of arterial lesions but other research has found no effect of dietary fatty acid (FA) composition on lesion development in salmon (Saunders *et al.*, 1992; Seierstad *et al.*, 2008). Dietary cholesterol, more specifically the low-density lipoprotein (LDL) fraction, are central causative agents of certain types of artery disease in mammals (Brown & Goldstein, 1984), and dietary cholesterol supplements have been shown to increase coronary lesions in brook trout, *Salvelinus fontinalis* (Mitchill) (see Farrell & Munt, 1983) while Eaton *et al.* (1984) have shown that lesion proliferation follows the natural variation in blood plasma LDL levels of maturing chinook salmon. It is now widely believed that over-

distension of the bulbus arteriosus during strenuous activity is the most significant initiating cause of arterial lesions in fish, and proliferation of the medial vascular smooth muscle leads to lesion formation (Farrell, 2002).

The limited information there is on arteriosclerotic lesions in ABT indicates that these are widespread in the species (Vastesaeger *et al.*, 1962). The main objective of this study was to investigate coronary artery lesions in ABT specimens of varying age and origin, and to use a semi-automated image analysis method to quantify lesion severity. Comparison of lesion severity between wild and farmed fish may also reveal the influence, if any, of the farming process on the prevalence and severity of coronary artery lesions in farmed ABT.

2.2. Materials and Methods

2.2.1. Study populations

2.2.1.1. Investigating arteriosclerosis in Atlantic bluefin tuna

The main study compared arteriosclerotic lesions in coronary arteries collected from two separate populations of farmed ABT and a third population consisting of wild-caught specimens.

The first ABT population (Group 1) had been held under commercial culture conditions for a period of 6 months. Approximately 1,550 wild ABT individuals were captured in June 2011 in the Tyrrhenian Sea by ICCAT authorised purse seine fishing vessels (FAO Statistical Area 10; 39°41'N, 014°41'E) and towed to a farm site located off the southern coast of Malta (35°49'N, 014°35'E). The fish were harvested after a 6-month fattening period in November 2011.

A second ABT population (Group 2) consisted of approximately 1,600 wild ABT individuals caught by purse seine fishing vessels in June 2010 off the Libyan coast (FAO Statistical Area 21; 33°31'N, 013°58'E). They were towed to the same farm site (35°49'N, 014°35'E) and harvested after an 18-month farming period.

Seawater temperatures during the culture period ranged from 14°C in winter to 26°C during the summer period, oxygen was always at or near saturation and average current speeds were 0.45 m s⁻¹. The fish were contained in 150 m circumference cages at stocking densities of approximately 1.55 kg m⁻³.

The ABT were fed *ad libitum* on a mixture of sardine, mackerel and herring at an average daily rate of 5 % kg biomass⁻¹ day⁻¹. Crude fat content of the feed ranged from 8 to 28 %. Mortalities during the farming period were negligible for both populations.

Coronary arteries from wild ABT (Group 3) were collected to examine the occurrence of lesions in wild populations. The specimens were fished by ICCAT authorised long line vessels in the Adriatic Sea (FAO Statistical Area 17; 42°59'N, 014°12'E) in November 2011.

Table 2.1 gives individual data for specimens used in this study and Figure 2.1 shows the FAO statistical areas for the Mediterranean Sea, where the three ABT populations used in this study were caught.

2.2.1.2. Investigating the effect of ventricle size on arteriosclerotic lesion severity

Coronary arteries were collected from ABT of varying size to analyse for any correlation between ventricle size and severity of arteriosclerotic lesions. To reduce the effect of

differing environmental conditions, artery specimens were collected from fish captured in a single fishing operation and kept in the same cage until they were harvested. The fish were caught in the Mediterranean Sea (FAO Statistical Area 14 [Figure 2.1]; 35°10'N, 014°46'E) in June 2012, towed to farm, and harvested in December 2012. Husbandry conditions were as those described above.

Table 2.1 Individual data for the Atlantic bluefin tuna specimens used to investigate the occurrence of arteriosclerotic lesions (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$; Group 3, wild, $n = 12$).

Group 1			Group 2		
Weight (kg)	Length (cm)	Sex	Weight (kg)	Length (cm)	Sex
46	125	Male	68	141	Male
48	123	Male	70	153	Female
53	147	Female	83	151	Female
59	134	Male	99	181	Male
66	137	Female	103	174	Female
77	157	Male	103	176	Female
94	174	Male	112	168	Male
101	172	Male	117	183	Male
107	180	Female	126	188	Female
108	187	Female	147	223	Male
131	197	Female	166	215	Female
138	197	Male	184	217	Female
173	224	Female	186	224	Male
187	229	Male	191	218	Male
190	220	Female	220	249	Male
210	227	Male	241	247	Female
228	236	Female	Group 3		
251	265	Female	23	112	Male
260	259	Female	29	113	Female
273	245	Male	30	115	Male
311	248	Female	32	115	Female
314	274	Female	32	117	Male
340	263	Male	34	114	Female
403	282	Male	36	118	Male
442	287	Male	38	133	Female
476	289	Male	38	118	Male
			41	121	Female
			43	135	Male
			49	124	Female



Figure 2.1 FAO Mediterranean statistical areas. This study was based on Atlantic bluefin tuna specimens captured by purse seine vessels in Statistical Areas 10, 14, 17 and 21. Blue shading highlights areas of capture and red shading indicates location of farm site off Malta (adapted from GFCM, 2014).

2.2.2. Sample collection

Samples were collected from farmed fish during commercial tuna harvests and the size of individual fish that were processed varied according to commercial demand. Fish were fasted for one day prior to harvest. They were killed by a shot to the head in the cage net following standard industry procedures and landed on a harvest boat after which they were weighed and their fork length measured. Samples from wild long-line specimens were collected opportunistically as the line was being brought aboard ship. Since both the farmed and wild fish are commercialised on a whole round basis, the heart including the intact bulbus arteriosus and the ventricle (Figure 2.2) was excised from the fish through a small incision made beneath the operculum and at the base of the gill arches. Samples were collected according to availability and efforts were made to collect samples discriminately to balance the representation of different weight classes and specimen sex.

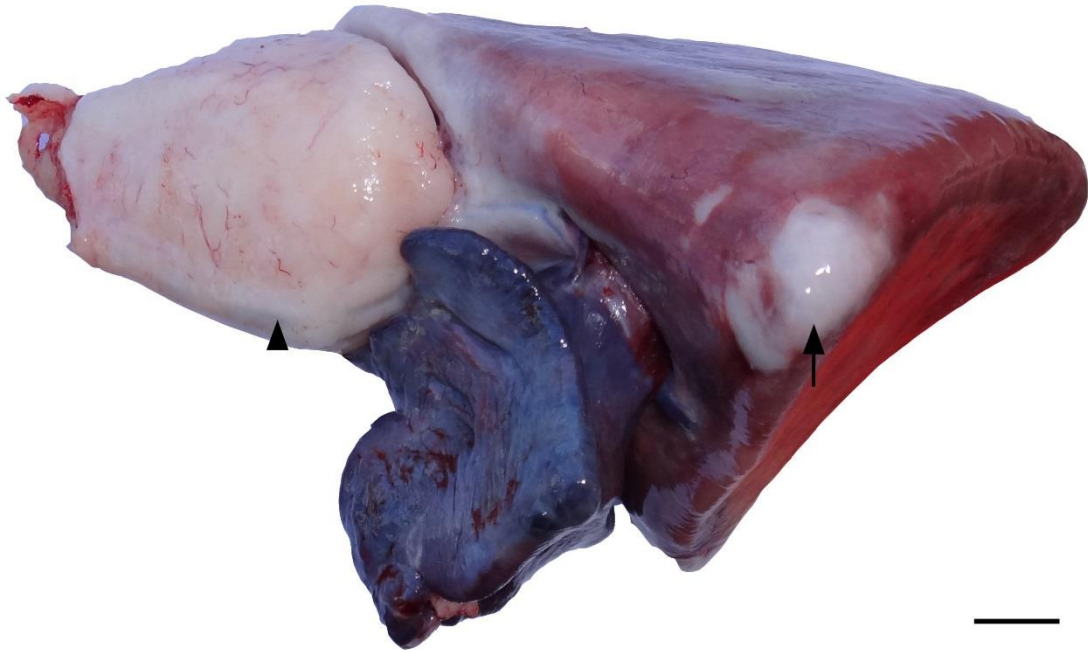


Figure 2.2 The entire heart as excised through a sub-opercular incision from the Atlantic bluefin tuna (ABT) for collection of the main coronary artery. The arrowhead shows the location of the coronary artery on which this study was based. The white lipidic deposits (arrow) on the surface of the ventricle are frequently encountered in the ABT (bar = 25 mm).

The main objective of this study was to investigate lesion occurrence in the main coronary artery (Figure 2.3), which lies on the dorsal surface of the bulbus arteriosus. Since the size of the bulbus did not allow for its entire fixation, the entire main coronary artery (from the distal end of the bulbus up to a location after the major bifurcation) was excised from the intact bulbus and fixed in 10 % neutral buffered formalin (25 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 46 mM Na_2HPO_4 , 10 % formaldehyde, pH 7.0). Time between specimen mortality and fixation of the arterial sample was less than 15 min.

Sampling procedures were identical for both parts of the study except that the ventricle was separated from the intact heart and weighed for studying the effect of ventricle size on lesion severity (**Section 2.2.1.2**).

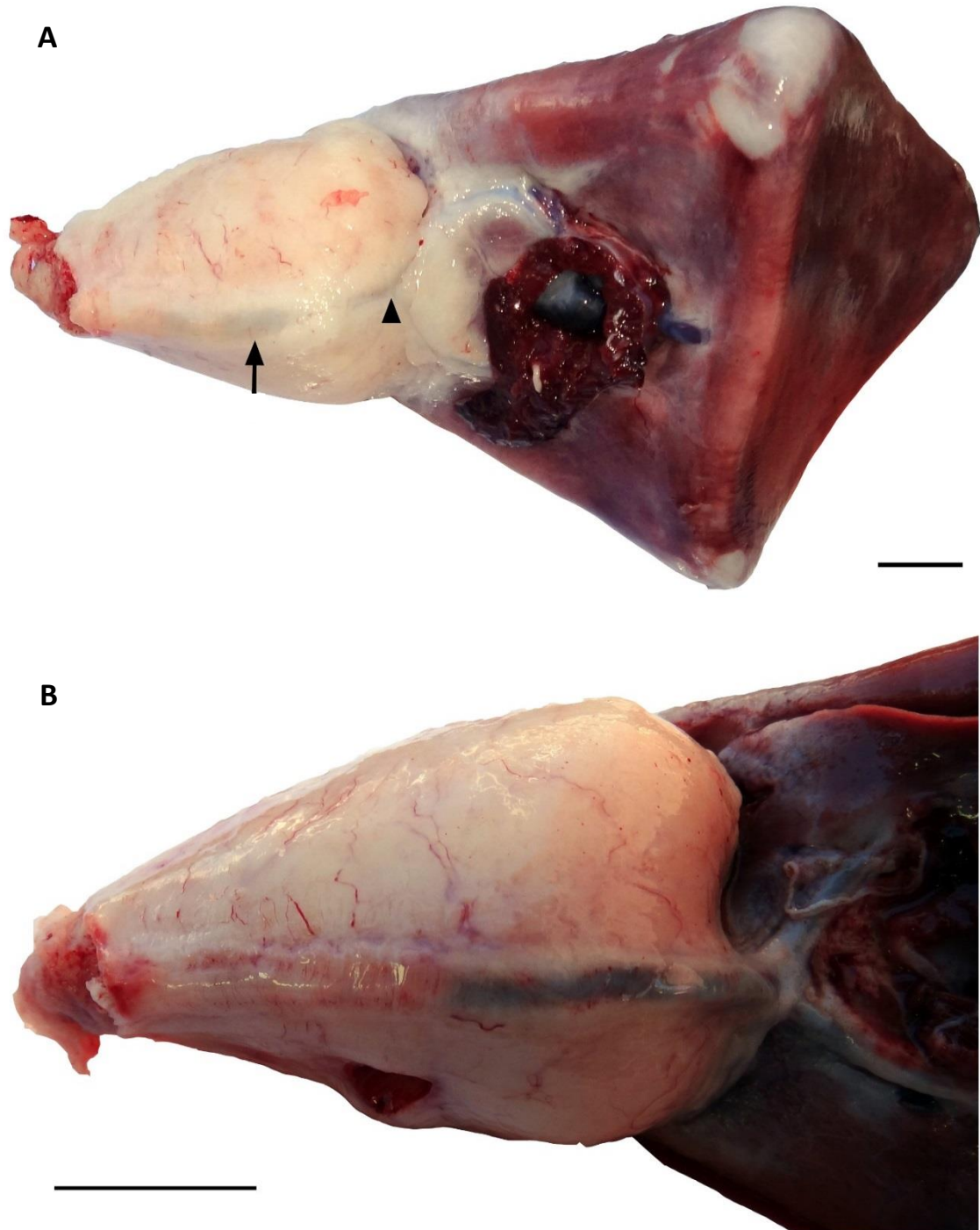


Figure 2.3 A, dorsal view of the Atlantic bluefin tuna heart with the atrioventricular sulcus removed. The main coronary artery lies on the surface of the bulbus arteriosus (arrow) and bifurcates into two daughter branches (arrowhead) at a point close to the ventricle (bar = 25 mm); B, macro view of the main coronary artery lying on the dorsal surface of the bulbus arteriosus (bar = 25 mm).

2.2.3. Histological processing

Studies on arteriosclerotic lesions in mature ABT species are very limited to date. The main coronary artery lies on the ventral surface of the bulbus arteriosus (Figure 2.3).

The first branching point of the artery occurs at a location close to the ventricle and is known as the major bifurcation. The two daughter branches branch at several other points to form a network of vessels which deliver oxygenated blood to the ventricle. To investigate the occurrence and prevalence of such lesions along different sections of the main coronary artery (*i.e.* prior to the major bifurcation), the fixed vessel was arbitrarily separated into three regions according to distance away from the ventricle (Figure 2.4) and cut into nine segments (*i.e.* three segments from each region) of equivalent thickness. Where possible tissue segments through the major bifurcation and daughter branches were also made but orientation of the latter was difficult due to a lack of supporting tissue and consequent distortion during embedding.

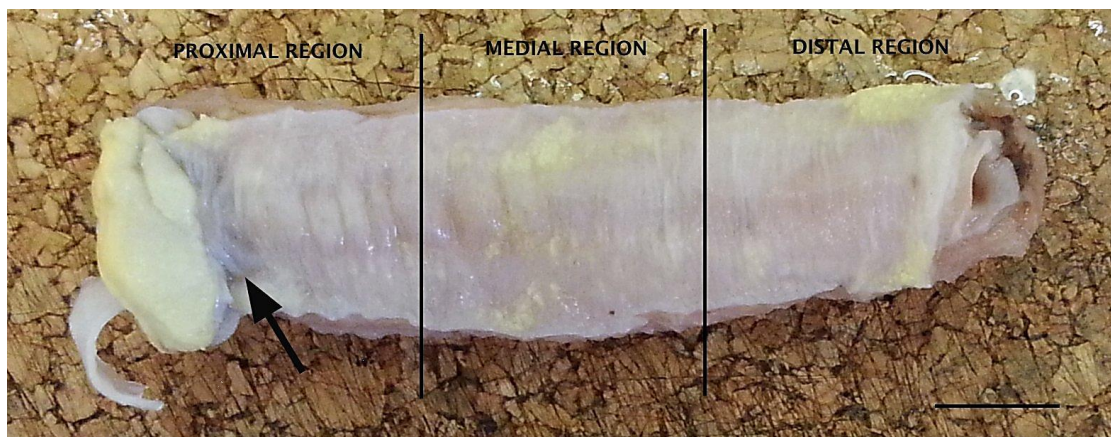


Figure 2.4 Excised formalin-fixed artery prior to cassetting for routine processing. The artery was divided into three regions (proximal, medial and distal) and sections were taken from multiple areas within these regions. The arrow marks the location where the coronary artery bifurcates into left and right daughter branches (bar = 10 mm).

The tissue samples were subsequently loaded onto an automated tissue processor (Shandon Citadel 1000, Thermo Scientific, Loughborough, UK), passed through an ethanol series and cleared using chloroform, wax impregnated and embedded to obtain transverse section sequences through the main artery axis. The artery sections were decalcified for 15 min to reduce brittleness (RDC Rapid Decalcifier, Cellpath, Powys, UK) and 5 μ m thick sections were cut at close locations from each of the tissue blocks on a

rotary microtome (Shandon Finesse, Thermo Scientific). The sections were left to dry at 60°C for 2 h prior to staining.

Histology on the arteries collected to examine the correlation between ventricle size and lesion severity (**Section 2.2.1.2**) were processed as above except that six tissue blocks – two each from the proximal, medial and distal regions – were prepared from each of the artery specimens.

2.2.3.1. Haematoxylin and eosin staining

Sections were stained with a routine haematoxylin and eosin stain. Briefly, the sections were deparaffinised through two 2 min changes of xylene, hydrated with absolute ethanol and methylated spirit respectively for 2 min and washed in running tap water for 5 min. They were then stained for 5 min in haematoxylin (Haematoxylin Z, Cellpath), washed in running water, dipped twice in 1 % acid alcohol, washed in running water, differentiated in Scott's tap water substitute (Cellpath) for 30 sec and placed in water for 2 min. The sections were stained in eosin (Putt's Eosin, Cellpath) for 5 min, washed briefly in water, and dehydrated through methylated spirit (30 sec), and two 2 min changes of absolute alcohol. They were cleared in xylene for 5 min and cover-slipped with mounting medium (Pertex, Cellpath).

2.2.3.2. Verhoeff-van Gieson staining

Additional sections were stained with a Verhoeff-van Gieson method adapted from Woods and Ellis (1994) for demonstration of elastic fibres. They were dewaxed and rehydrated as above. The sections were placed in Lugol's iodine (1 % I₂, 2 % KI, dH₂O) for 5 min and washed in water. They were treated with 5 % sodium thiosulphate for 2

min or until the brown colouration imparted to the section by Lugol's iodine receded. They were washed in water and stained in Verhoeff's haematoxylin (2.5[5 % alcoholic haematoxylin, 100 % EtOH]:1[10 % FeCl₃, dH₂O]:1[Verhoeff's iodine – 2 % I₂, 4 % KI, dH₂O]) for 18 min and rewashed in water to remove excess stain. They were differentiated in ferric chloride (2 % FeCl₃, dH₂O) for 25 sec, washed in water followed by 95 % alcohol for 1 min to remove iodine staining. The alcohol was rinsed off in water and the sections counterstained with van Gieson's solution (1[1 % acid fuchsin, dH₂O]:10[saturated aqueous picric acid]) for 45 sec. They were dehydrated quickly through methylated spirits and two changes of absolute alcohol, cleared for 5 min in xylene and cover-slipped.

2.2.3.3. Masson's trichrome staining

A Masson's trichrome stain was used for differentiation of collagen. Slides were dewaxed and rehydrated to water as described previously. Nuclei were stained with celestine blue (0.5 % celestine blue B, 5 % ammonium iron(III) sulphate [NH₄Fe(SO₄)₂.12H₂O], 14 % glycerol, dH₂O) for 5 min, rinsed in water and placed in haematoxylin (Haematoxylin Z, Cellpath) for 5 min. The sections were rinsed in water and the nuclear stain differentiated in acid alcohol. The slides were left in a cytoplasmic stain for 1 h (2[1 % ponceau 2, 1 % acetic acid, dH₂O]:1[acid fuchsin, 1 % acetic acid, dH₂O]), washed in water and differentiated in 1 % phosphomolybdic acid (H₃[P(Mo₃O₁₀)₄]). The slides were washed in distilled water and collagen was stained in light green (2 % light green, 1 % acetic acid, dH₂O) for 2 min followed by washing in 1 % acetic acid. Slides were rinsed in water, dehydrated, cleared and mounted as described above.

2.2.3.4. Lipid fixation and staining

Although arteriosclerotic lesions in teleost fish species lack lipid inclusions as compared to similar lesions in mammals (Moore *et al.*, 1976c; McKenzie *et al.*, 1978; House & Benditt, 1981; Seierstad *et al.*, 2008) the presence of lipid inclusions from lesions in ABT has been reported by Vastesaege *et al.* (1962).

To examine for the presence of lipids in lesions on the tuna, formalin-fixed artery specimens were pre-processed for lipid fixation according to the procedure described in Tracey and Walia (2002). Briefly, coronary arteries were cut at thicknesses of 1 mm or less and placed in a 70 % polyethylene glycol solution containing 1 % linoleic acid and 0.4 % soy lecithin (Holland and Barrett Retail Limited, Nuneaton, UK) for 72 h at 56°C. They were then rinsed in several changes of 70 % ethanol for 8 h, washed for 8 h in distilled water and placed in 2 % chromic acid for 24 h at 4°C. Subsequently they were rinsed in distilled water for 24 h, treated with 5 % sodium bicarbonate for 24 h, rinsed in water for a further 8 h and processed routinely to obtain paraffin-embedded tissue.

The slides were sectioned as described above and stained for fat with an oil red O procedure adapted from Woods and Ellis (1994). Slides were deparaffinised and rehydrated to water. Sections were rinsed in a 60 % iso-propanol solution for 2 min and stained for lipids by immersion in a solution of 60 % iso-propanol containing 0.25 % oil red O for 10 min. They were rewashed in 60 % iso-propanol followed by water. Counterstaining was in haematoxylin (Haematoxylin Z, Cellpath) for 2 min. The sections were differentiated in 1 % acid alcohol, washed in water and mounted using a polyvinylpyrrolidone aqueous mounting medium (Kiernan, 2008). Positive control was formalin-fixed liver tissue from ABT.

2.2.4. Light microscopy and image capture for morphometric measurements

The images were examined for lesion occurrence and morphology under an Optika B-600 light microscope (Optika, Bergamo, Italy) equipped with a C-mounted Optikam PRO5 (Optika) still-image capture device. 8-bit RGB images (2592 × 1944 pixels) of the entire artery cross-section were recorded at a magnification of ×40 for image morphometric measurements.

2.2.5. Morphometric evaluation: quantification of lesion areas by a semi-automated method

The cross-sectional micrographs of the arteries were used for the evaluation of lesion size and severity in ImageJ (v 1.47; U.S. National Institutes of Health, Bethesda MD, USA), an image processing software package, using methods adapted from Seierstad *et al.* (2005a). The software was calibrated for measurements against a digital image of a stage micrometer taken at the same magnification of the micrograph.

To quantify the degree of intimal lesions, area measurements were made to evaluate the loss of luminal area resulting from the protrusion of arteriosclerotic lesions into the lumen. The images were loaded into ImageJ and the different components of the artery – the tunica externa, tunica media, tunica intima – were identified. A line was drawn around the circumferential edge of the tunica intima and the area measured (μm^2) by a software command. This area was defined as the maximal potential luminal area (MPA; Figure 2.5) and represented the area of the lumen in the absence of intimal lesions. To measure the actual luminal area (ALA), the same procedure was followed but a line was traced along the actual luminal border of the intimal lesions. In some sections there were disruptions in the intimal layer and in such instances the line was drawn along a

presumed path between the two points. The difference between the MPA and the ALA was termed the percentage lumen loss (PLL) and was expressed as a percentage of the MPA. Increasing PLL values represented intimal lesions of increasing severity.

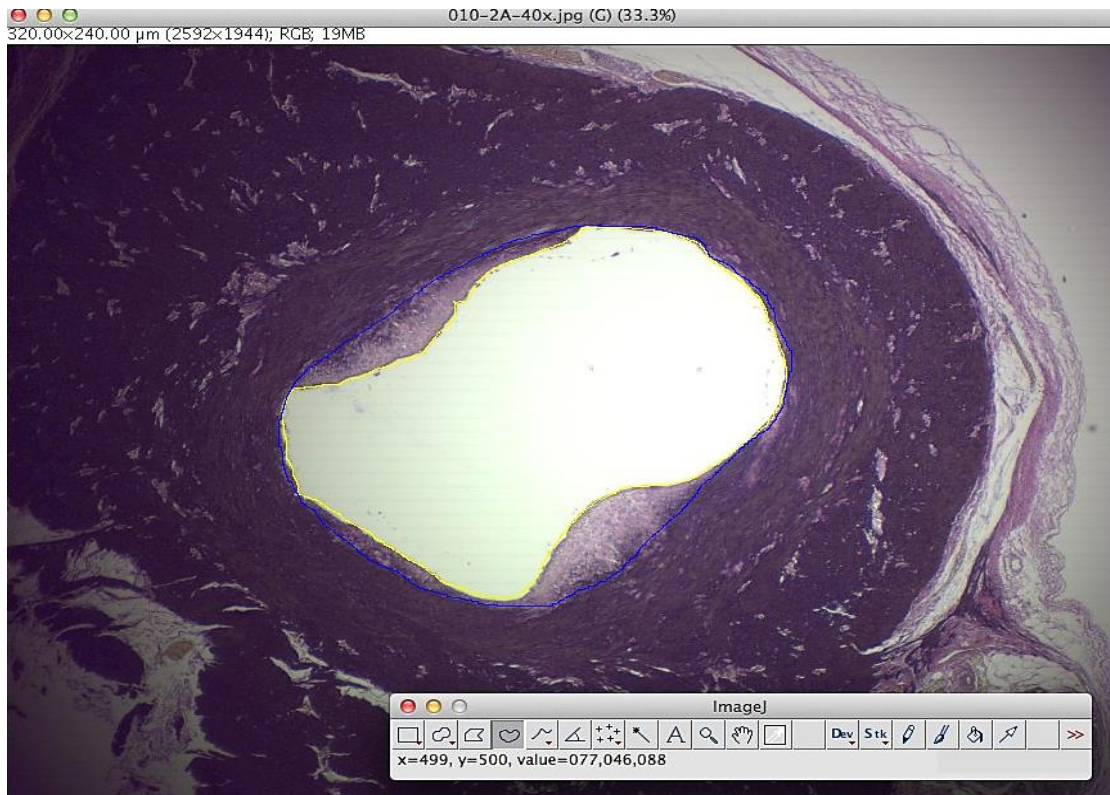


Figure 2.5 Morphometric measurement for percentage lumen loss (PLL) with ImageJ. Maximal potential luminal area (MPA) is denoted by the blue boundary while the area enclosed with the yellow boundary represents the actual luminal area. PLL was calculated as the difference between the two indices and expressed as a percentage of the MPA.

Area measurements were repeated for all the cross-sections from the main coronary artery of each fish. To obtain a better understanding of the severity of these proliferations along the entire length of the artery, the nine sections were arbitrarily categorised into three sequential groups according to their position from the major bifurcation (*i.e.* the point closest to the ventricle attachment). The 'proximal region' consisted of the three sections occurring immediately prior to the major bifurcation, the second set was classified as the 'medial region' and the three sections furthest away from the major bifurcation termed the 'distal region' (Figure 2.4).

In an attempt to quantify the lesions in a different dimension, ImageJ was used to measure the point of maximal lesion thickness (MLT; Seierstad *et al.*, 2005a). The location where endothelial proliferation extended furthest into the lumen was visually identified and a straight line was drawn from the intimal border to the luminal end of the lesion (Figure 2.6). The MLT was recorded in μm and measurements were performed for all the arterial cross sections except for those through the major bifurcation and daughter branches.

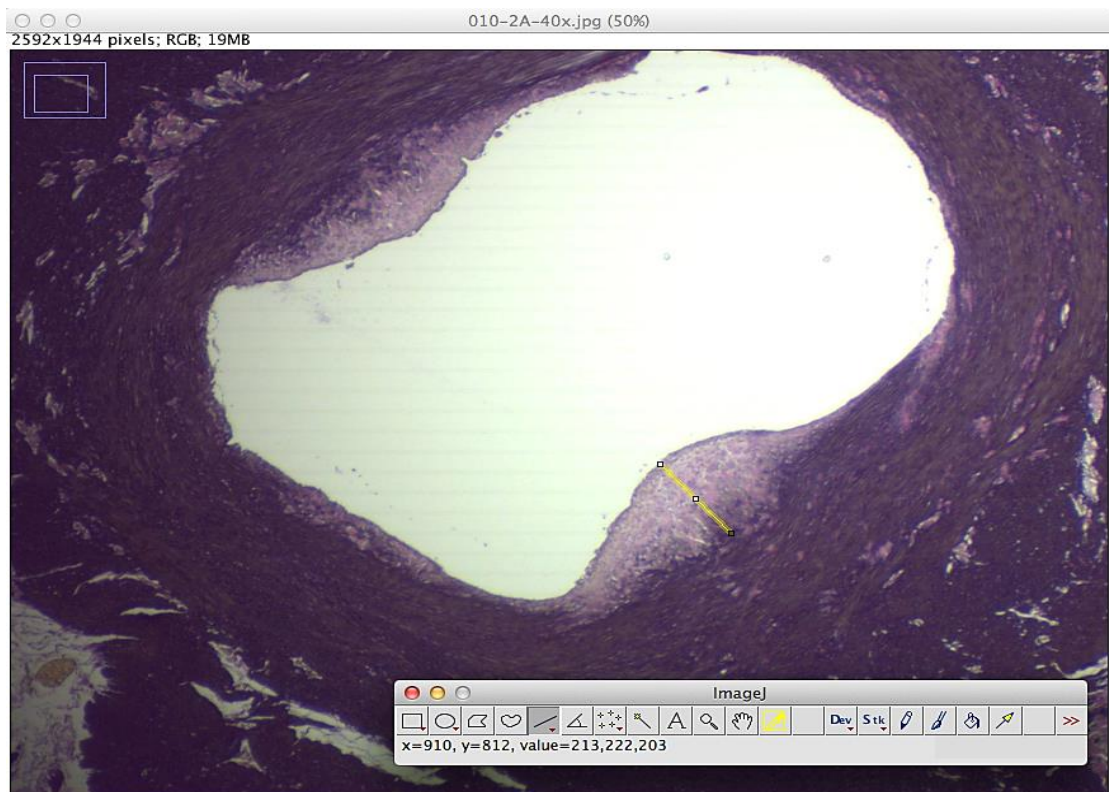


Figure 2.6 Morphometric measurement for maximal lesion thickness with ImageJ. The point at which the lesion showed the largest protrusion into the lumen was visually identified and measured.

2.2.6. Statistical analysis

Data were analysed statistically for the effects of weight, sex and origin on the PLL and MLT. All statistical tests were performed two-tailed with significance value of 5 % ($p = 0.05$). Continuous variables were tested for normality using a non-parametric one-sample Kolmogorov-Smirnov test. PLL and MLT values were logarithmically (\log_{10}) transformed where distribution deviated from normality. Within individual sample groups, one-way analysis of variance (ANOVA) was used to analyse for the effects of artery region and sex on PLL and MLT while Pearson correlation tests were used to analyse for possible associations between specimen length and weight on PLL and MLT. Analysis of covariance (ANCOVA) on pooled data from all experimental sub-groups was used to analyse for the effects of origin and sex on PLL and MLT. Weight of the fish was used as a covariate in this analysis. A Pearson correlation test was used to examine the effect of ventricle weight on PLL and MLT indices. All statistical tests were performed with SPSS Statistics 20 (IBM Corp., New York, USA).

2.3. Results

2.3.1. Investigating arteriosclerosis in Atlantic bluefin tuna: the study populations

Descriptive data for fish used in this study are listed in Table 2.2. Males and females were equally represented within the individual groups. Recent reports indicate that ABT reach maturity at a weight of approximately 25 kg and a fork length (FL) between 97-120 cm (Corriero *et al.*, 2005; ICCAT, 2011, 2014). On the basis of the measured FLs, it could not be ascertained if the wild ABT samples had reached maturity, and only two wild specimens had FLs exceeding 130 cm. Only one fish, however, weighed less than 25 kg, so that it was likely that all specimens collected came from fish which were

mature or close to maturity. With the exception of three specimens from Group 1, all individuals sourced from the farm had FLs exceeding 140 cm. None of the fish were thought to be in an active stage of their reproductive cycle since spawning of the eastern Atlantic stock is known to occur during the period June to July (Mather *et al.*, 1995). This was corroborated through visual examination of the gonads where possible.

Table 2.2 Descriptive data of Atlantic bluefin tuna specimens used in the study. Values represent mean \pm standard deviation and figures in brackets show upper and lower ranges.

Origin	Weight (kg)	Length (cm)
Farm, 6 months (Group 1; FAO 10; $n = 26$)	196 \pm 124 (46-476)	211 \pm 52 (123-289)
Farm, 18 months (Group 2; FAO 21; $n = 16$)	139 \pm 52 (68-241)	194 \pm 33 (141-249)
Wild (Group 3; FAO 17; $n = 12$)	35 \pm 7 (23-49)	120 \pm 7 (112-135)

2.3.2. Histology of the normal coronary artery

All of the artery sections collected from the three different ABT populations were contained at least one identifiable lesion (*i.e.* lesion prevalence was 100 %). Several regions of the artery sections, however, appeared to be free of proliferations and this was presumed to approach, or indeed represent, the histological arrangement of a coronary artery free of lesions. Figure 2.7 shows the general structure of a coronary artery from a wild fish.

Figure 2.8 shows the different layers of the coronary arteries of the ABT. The tunica adventitia (*syn.* tunica externa) takes the form of numerous thick elastic lamellae, which extend around the peripheral circumference of the artery, and appeared to contain amounts of collagenous material. In most cases there appeared to be minimal distinction between the adventitial layer of the artery and the adjoining tissues, the latter appearing to be composed almost entirely of fibrous components.

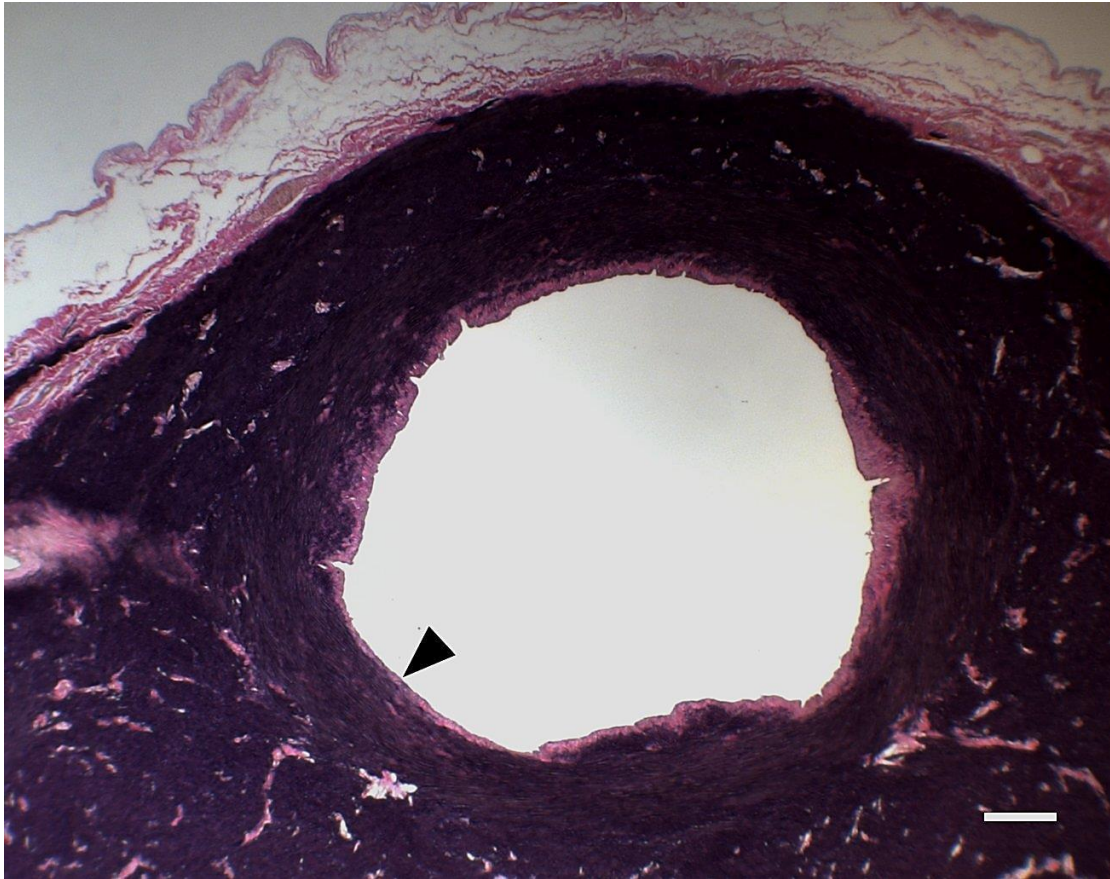


Figure 2.7 Low power view of an Atlantic bluefin tuna coronary artery cross-section. Arrowhead refers to locations on the artery which were classed as being free of lesions (Verhoeff-van Gieson; bar = 200 μ m).

The tunica media was thick and composed principally of thick elastic fibres with interspersed collagenous and smooth muscle components around its outer periphery. Smooth muscle was present towards the inner circumference just before the intima. The intima was often difficult to distinguish in the main coronary artery and appeared as a layer of cells resting on a moderately convoluted IEL. Sections through daughter branches were similar in structure although distinctive in that they had a visibly smaller amount of elastic fibres than found in the media of sections from the main coronary artery trunk, these being substituted by smooth muscle cells.

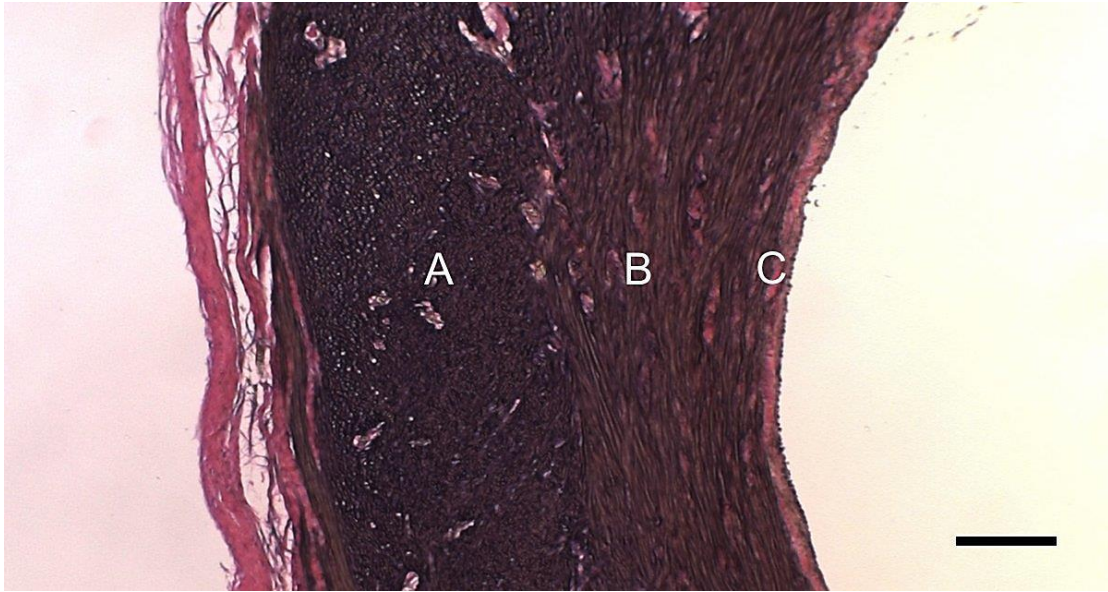


Figure 2.8 View of an artery cross-section showing the three layers of the Atlantic bluefin tuna coronary artery (Verhoeff-van Gieson; A, adventitia; B, media; C, intima; bar = 100 μ m).

2.3.3. Pathology of coronary artery lesions

All coronary artery sections had one or more lesions present so that prevalence was equivalent to 100 %. Lesions ranged from focal proliferations to growths around the entire luminal surface of the vessels, were diverse in morphology and for the most part appeared to increase in severity with increasing fish weight. The structural arrangement of the artery also appeared to deteriorate with increasing size, and the arterial layers were more difficult to distinguish with increasing specimen weight. Frequently, lesions continued between subsequent sections indicating that they spanned the entire distance between tissue blocks. Generally they were composed of fibrous and collagenous components and proliferated endothelial and smooth muscle cells towards the luminal margin. In most sections, the latter three were oriented transversely to the main artery axis so that lesion areas contrasted heavily with the circular organisation found in normal artery structures. Microscopic evaluation also revealed that in the majority of lesions the IEL was thinned, fragmented or altogether absent. Figure 2.9 shows a mild lesion from the coronary artery of a farmed ABT. The media is thinned and

appears to be losing structural organisation, endothelial cells have proliferated at locations close to the lumen and the IEL is absent. Masson's trichrome staining indicated widespread collagenous deposition within the arteriosclerotic lesions.

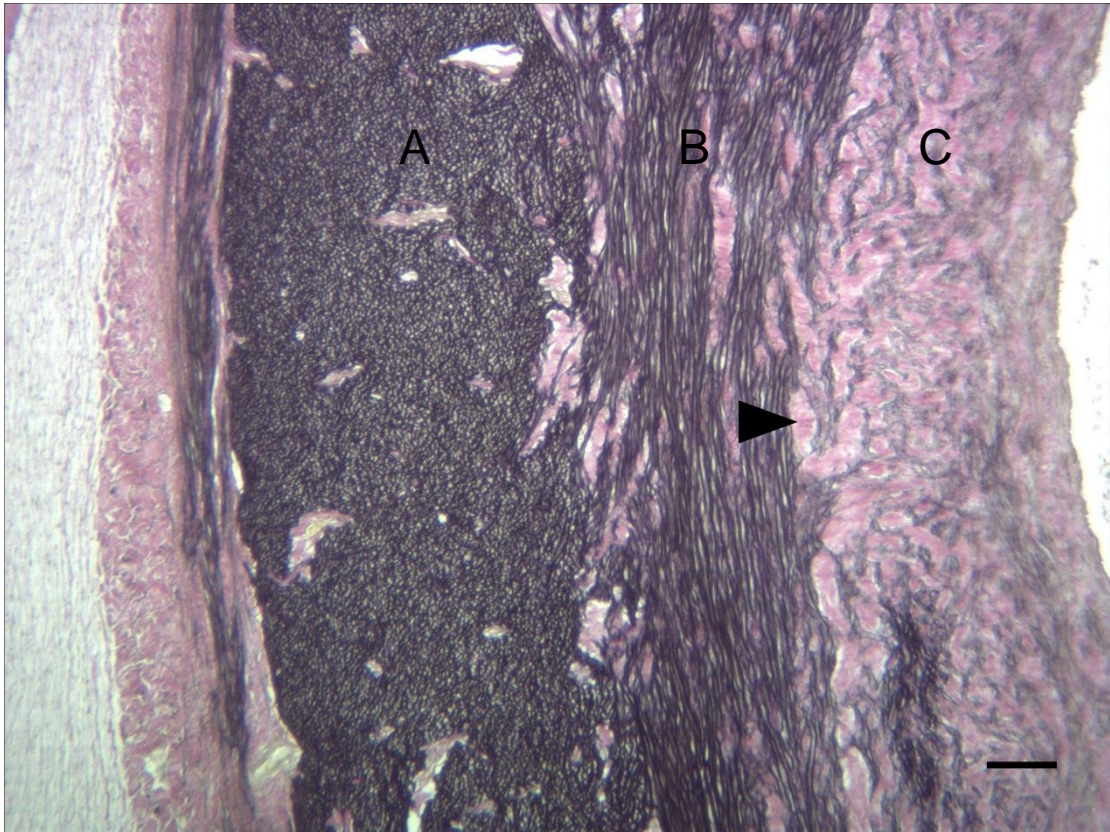


Figure 2.9 Lesion from the coronary artery of a farmed 250 kg Atlantic bluefin tuna. Arrowhead shows location where the media thins and loses structural arrangement and smooth muscle cells proliferate at right angles to the medial fibres, giving rise to an arterial lesion (Verhoeff-van Gieson; A, adventitia; B, media; C, intima; bar = 150 μ m).

More pronounced lesions were characterised by a much higher proportion of proliferated endothelial cells and a thinned or completely disrupted medial layer. As was the case for the smaller lesions, Verhoeff-van Gieson and Masson's trichrome staining revealed the presence of collagen and fibrous structures within these larger proliferations. Lesions of this type were more frequently encountered within the larger fish and Figure 2.10 shows one of the most severe lesions encountered during analysis. Additionally, some of the cross sections showed lesions which were composed entirely of thick elastic fibres.

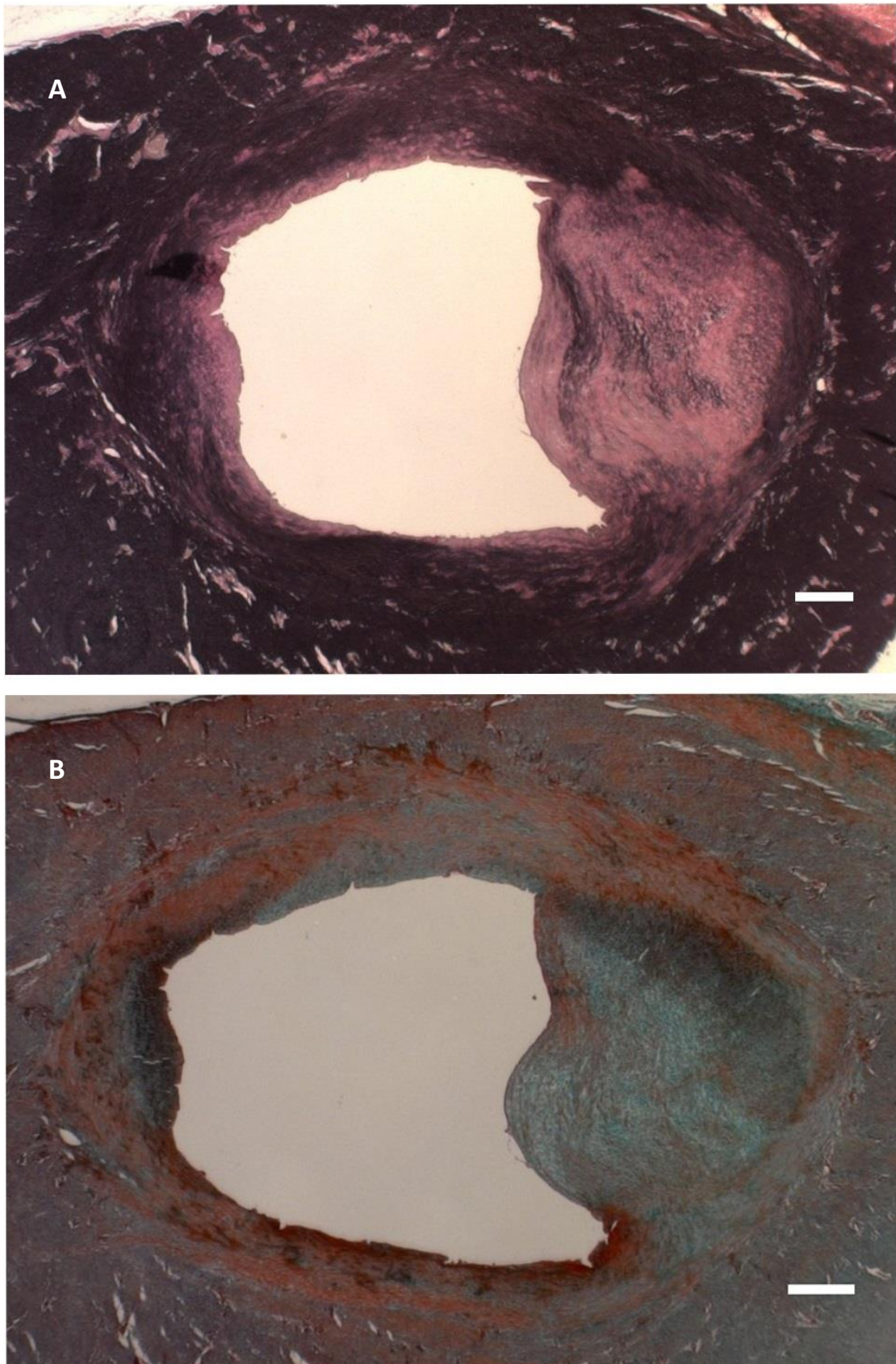


Figure 2.10 A, severe lesion from the main coronary artery of a 245 kg farmed Atlantic bluefin tuna (ABT), resulting in a potential lumen loss of more than 40 % (Verhoeff-van Gieson; bar = 300 μ m); B, coronary artery lesion adjacent to A. The intense green staining within the proliferation indicates the widespread presence of collagen within arteriosclerotic lesions in ABT (Masson's trichrome; bar = 300 μ m).

Although focal lesions were occasionally observed in the main artery trunk, lesions generally assumed larger, more diffuse forms and extended over larger lengths of the luminal margin. Figure 2.11 shows a lesion from the coronary artery of a farmed ABT; not all lesions expanded over such areas but often were found to be present over most, or the entire flank, of the artery. There was also a trend for arteriosclerotic lesions to be more common along the lateral and top edges (*i.e.* the luminal surface away from the bulbus arteriosus). Lesions stemming from the edge attached to the bulbus, however, generally consisted of a deeper cell mass and extended farther into the lumen. There were also isolated cases, generally in the larger fish, where the lesion extended over the entire luminal surface (Figure 2.12). In addition, the arterial lesions generally had a smooth and continuous boundary but there were cases in which focal hyperplastic nodules were scattered along the entire IEL (Figure 2.13).

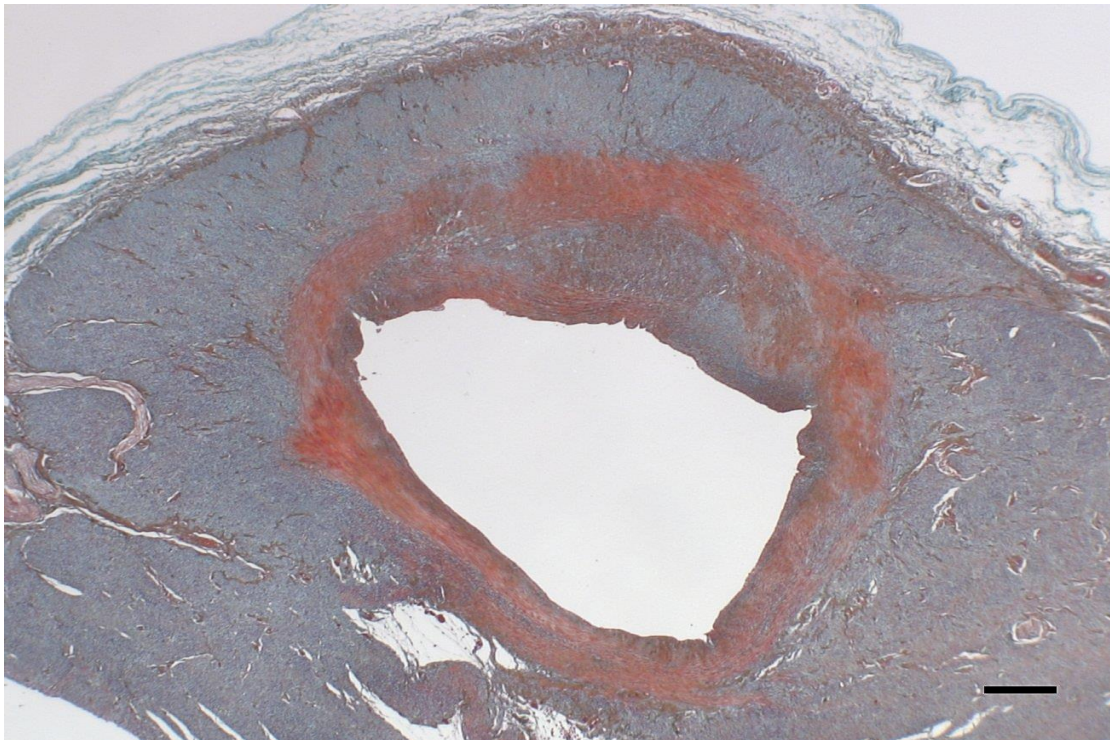


Figure 2.11 Additional section through coronary artery of a farmed Atlantic bluefin tuna demonstrating amounts of collagen within the coronary lesion and in the surrounding bulbus arteriosus parenchyma (Masson's trichrome; bar = 300 μ m).

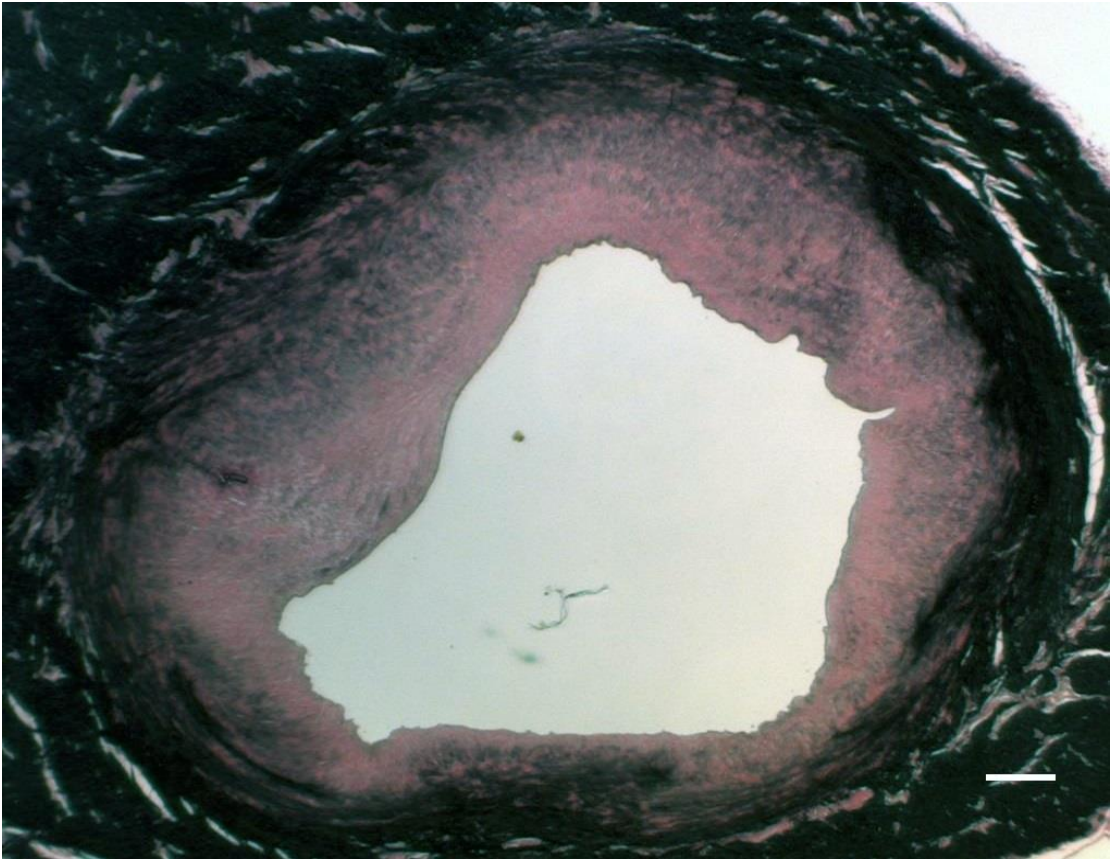


Figure 2.12 Coronary artery from a farmed Atlantic bluefin tuna specimen weighing 440 kg. Lesion extends continuously along lateral and dorsal periphery of the artery (Verhoeff-van Gieson, bar = 200 μ m).

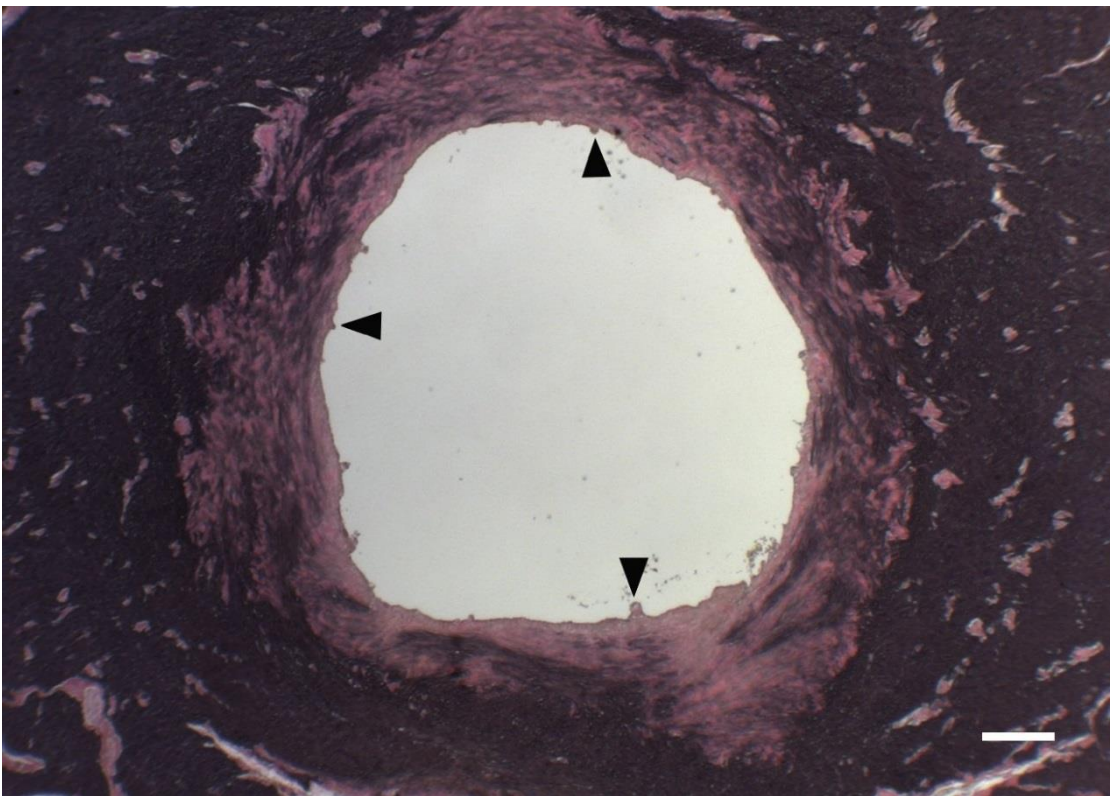


Figure 2.13 Focal myointimal proliferations (arrowheads) were present in a small proportion of the coronary arteries of Atlantic bluefin tuna (Verhoeff-van Gieson; bar = 300 μ m).

Some sections exhibited disruptions in the arterial walls suggesting past trauma to the arterial structure, with some extending from the lumen to the adventitia. In these sections collagen bridged an abrupt break between the elastic fibres (Figure 2.14) but some also contained smooth muscle components between the two points.

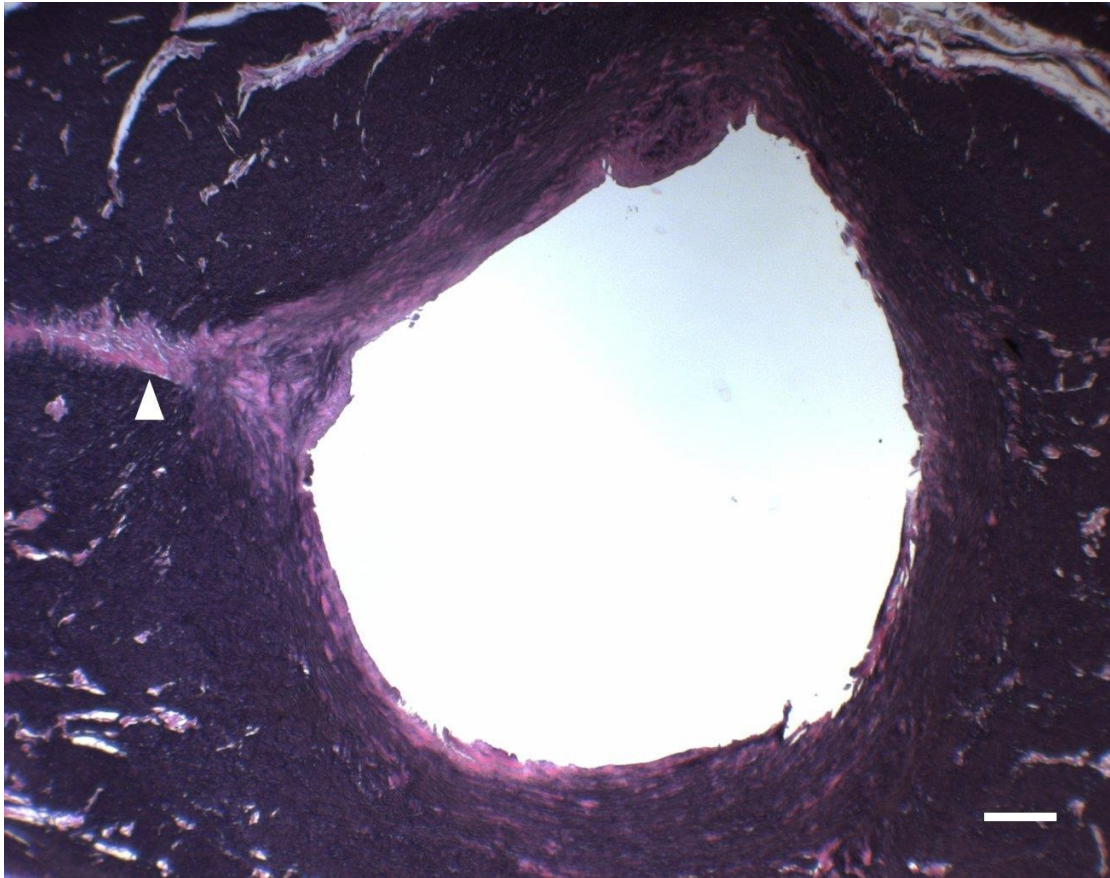


Figure 2.14 Artery section showing collagen deposition at point of disruption (arrowhead) in the arterial wall of a coronary artery from an Atlantic bluefin tuna (Verhoeff-van Gieson; bar = 100 μ m).

Under high-power magnification the lesions exhibited varying morphology and although consisting of smooth muscle and fibrous structures, the proportion of these within the proliferations varied greatly between different lesions. In their majority, the lesions consisted of proliferated smooth muscle but in a minor proportion of pathologies several fibrous elements were observed to be present within the lesions to differing degrees. High power micrographs of representative lesions are shown below (Figures 2.15, 2.16, 2.17, 2.18, 2.19, 2.20).

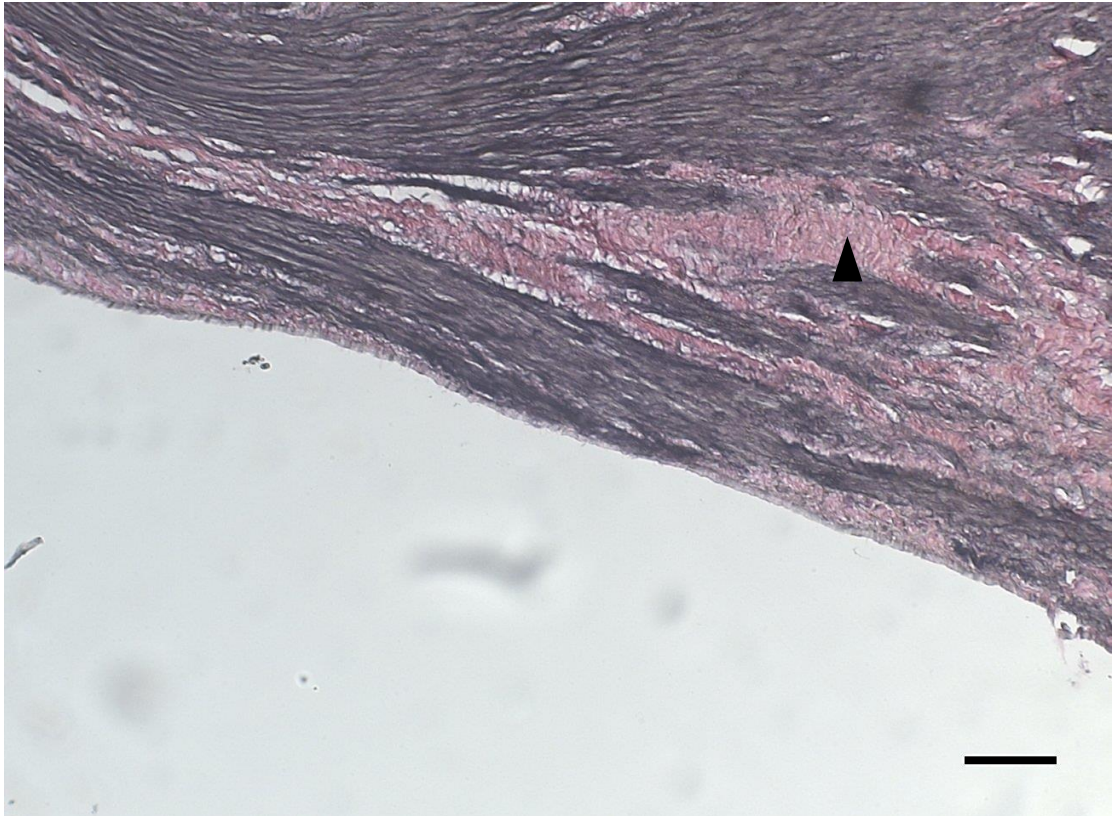


Figure 2.15 Smooth muscle cell proliferations (arrowhead) in the coronary artery of an Atlantic bluefin tuna. They appeared to originate from within the medial layer of the artery. The media was disrupted and the muscle cells oriented at right angles to main artery axis (Verhoeff-van Gieson; bar = 50 μ m).

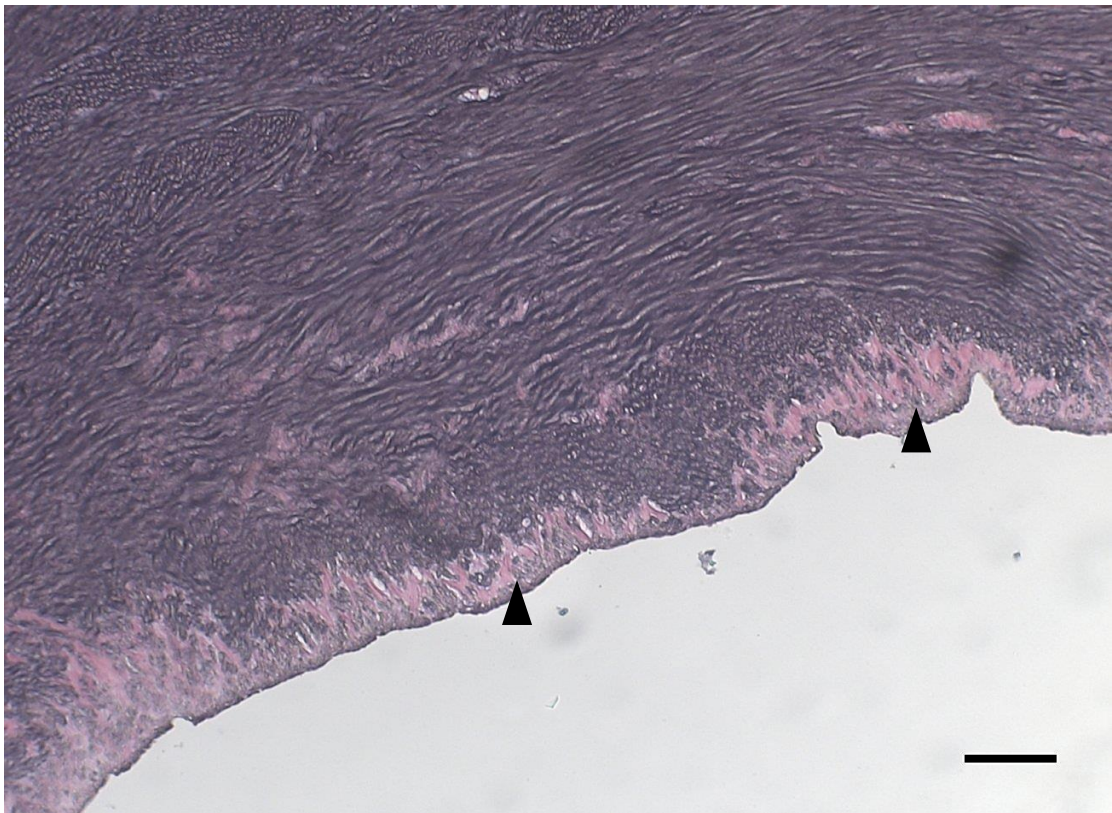


Figure 2.16 Intimal smooth muscle proliferations (arrowheads) within the Atlantic bluefin tuna coronary artery. In this artery the proliferation contained several fibrous elements (Verhoeff-van Gieson; bar = 50 μ m).

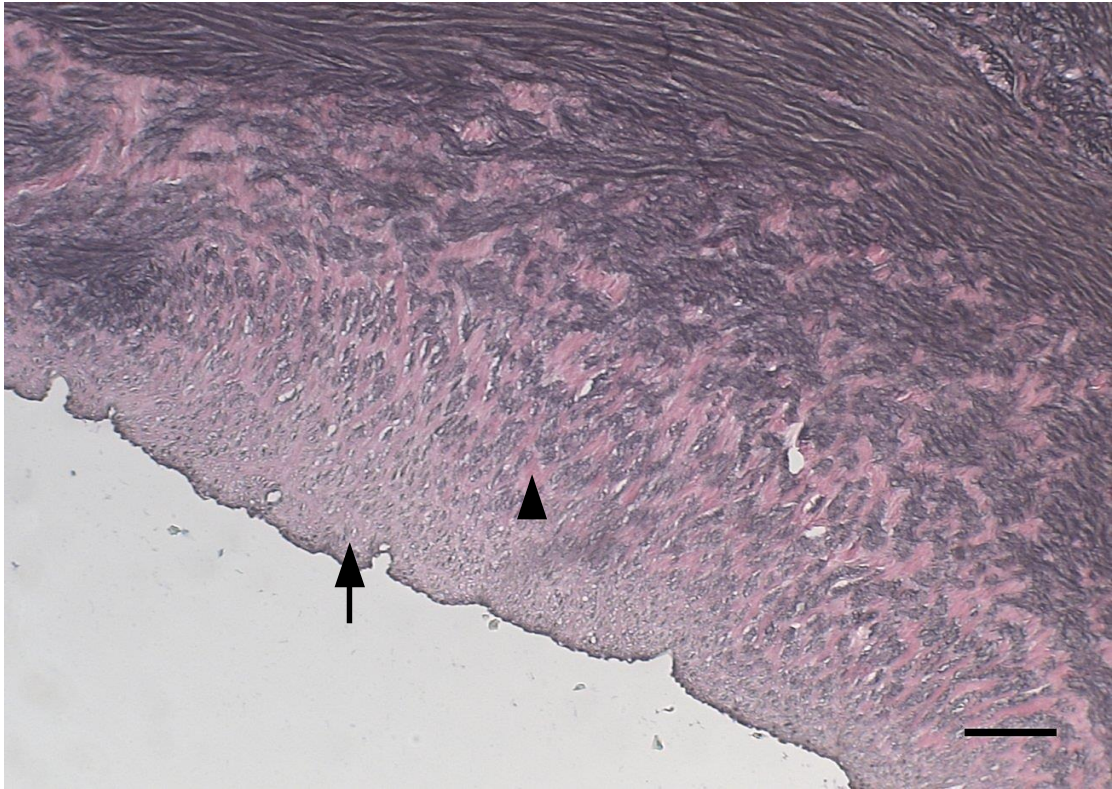


Figure 2.17 An arteriosclerotic lesion within the Atlantic bluefin tuna coronary artery. Generally, lesions had this appearance and consisted of large proportions of smooth muscle cells (arrow) oriented at right angles to the main artery axis, and lesser amounts of collagen (arrowhead) and fibrous components (Verhoeff-van Gieson; bar = 50 μ m).

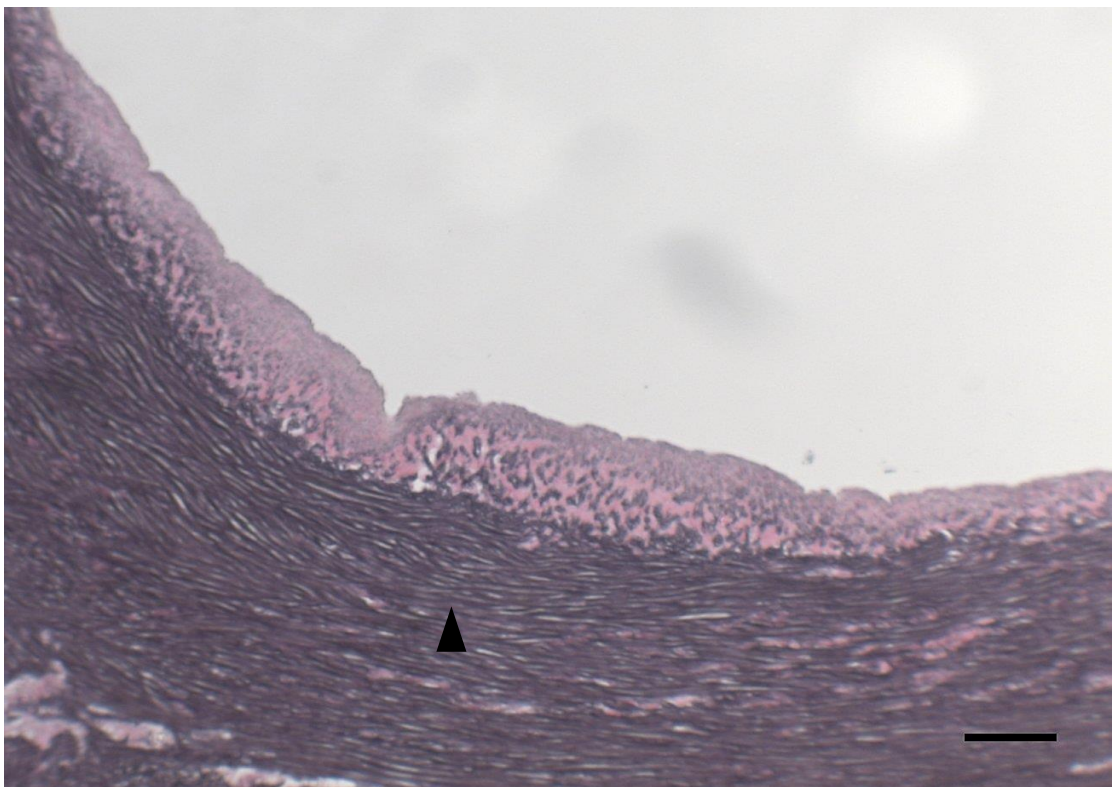


Figure 2.18 Arterial lesions from the coronary artery of an Atlantic bluefin tuna with an intact medial layer (arrowhead). Lesions with an intact medial layer were not commonly observed and lesion areas were often accompanied by a disrupted media (Verhoeff-van Gieson; bar = 100 μ m).

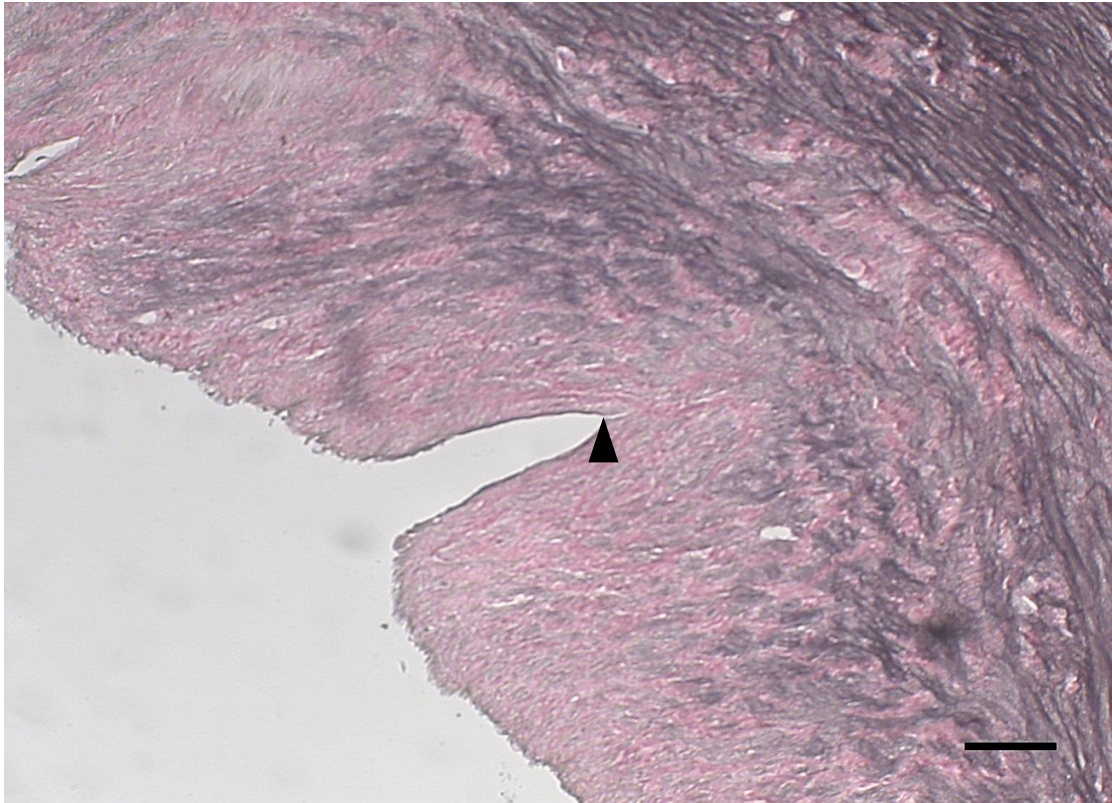


Figure 2.19 Fragmentation observed in lesions (arrowhead) from the Atlantic bluefin tuna coronary artery. A large amount of collagen can be observed within this particular lesion and the elastic lamina is continuous over the recess (Verhoeff-van Gieson; bar = 50 μ m).

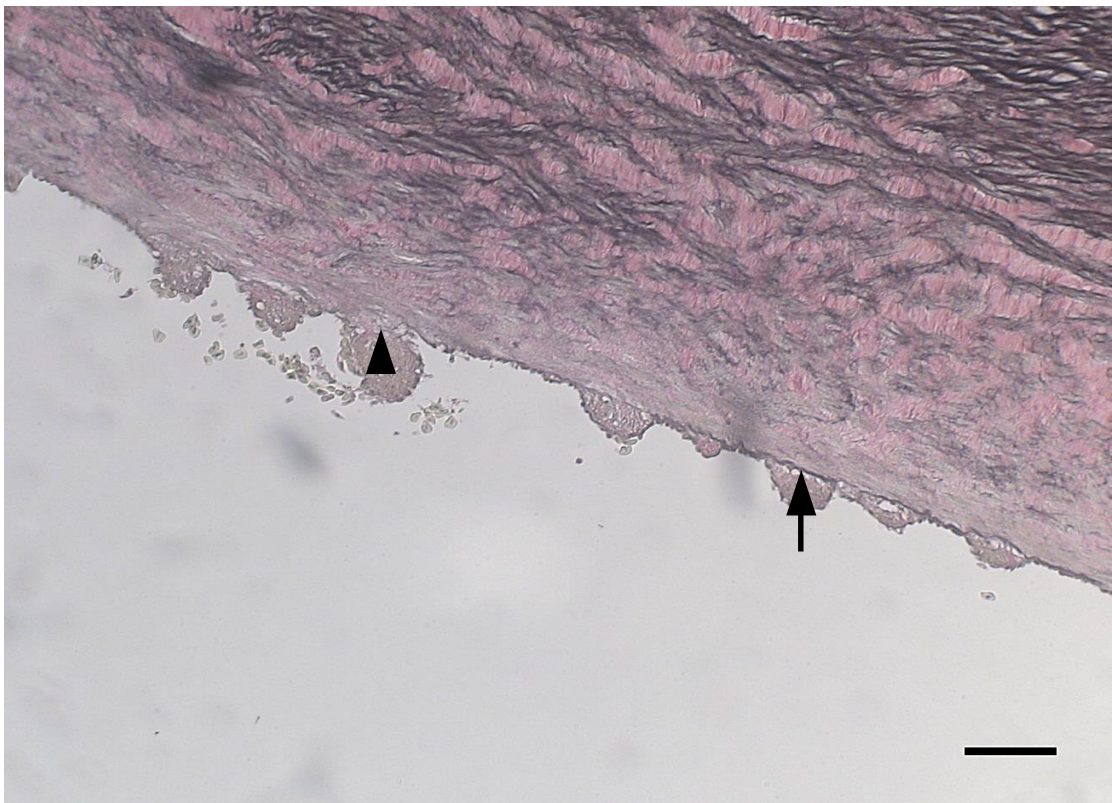


Figure 2.20 Section through the main coronary artery of an Atlantic bluefin tuna showing multiple focal proliferations along the endothelial layer. The elastic lamina is disrupted at locations immediately beneath the protrusions (arrowhead) and uninterrupted at others (arrow) (Verhoeff-van Gieson; bar = 50 μ m).

Sections through the major bifurcation showed an arterial structure composed almost entirely of smooth muscle and collagen (Figure 2.21). Lesions from the daughter branches were predominantly focal protrusions into the lumen (Figure 2.22). These were similar in appearance to cases of myointimal hyperplasia in salmonids, and consisted of proliferated smooth muscle and endothelial cells, and contained numerous vacuoles (Figures 2.23, 2.24). The IEL was usually present at locations but fragmented and confined to a point between the media and the hyperplastic protrusions.

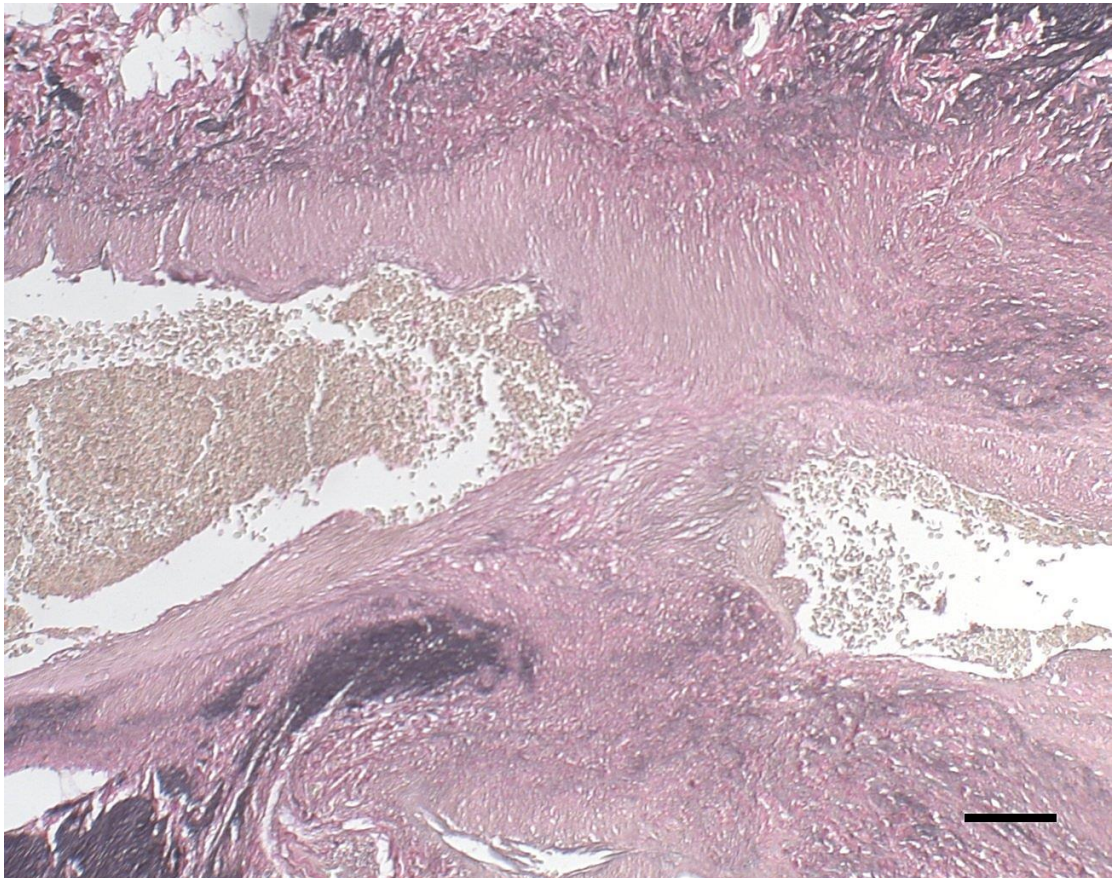


Figure 2.21 Section through the daughter branch after the major bifurcation of the coronary artery of an Atlantic bluefin tuna. Few structural elastic fibres were visible at this region and the structure mainly consisted of smooth muscle cells and collagen. Focal myointimal proliferations were most commonly observed in this area (Verhoeff-van Gieson; bar = 100 μ m).

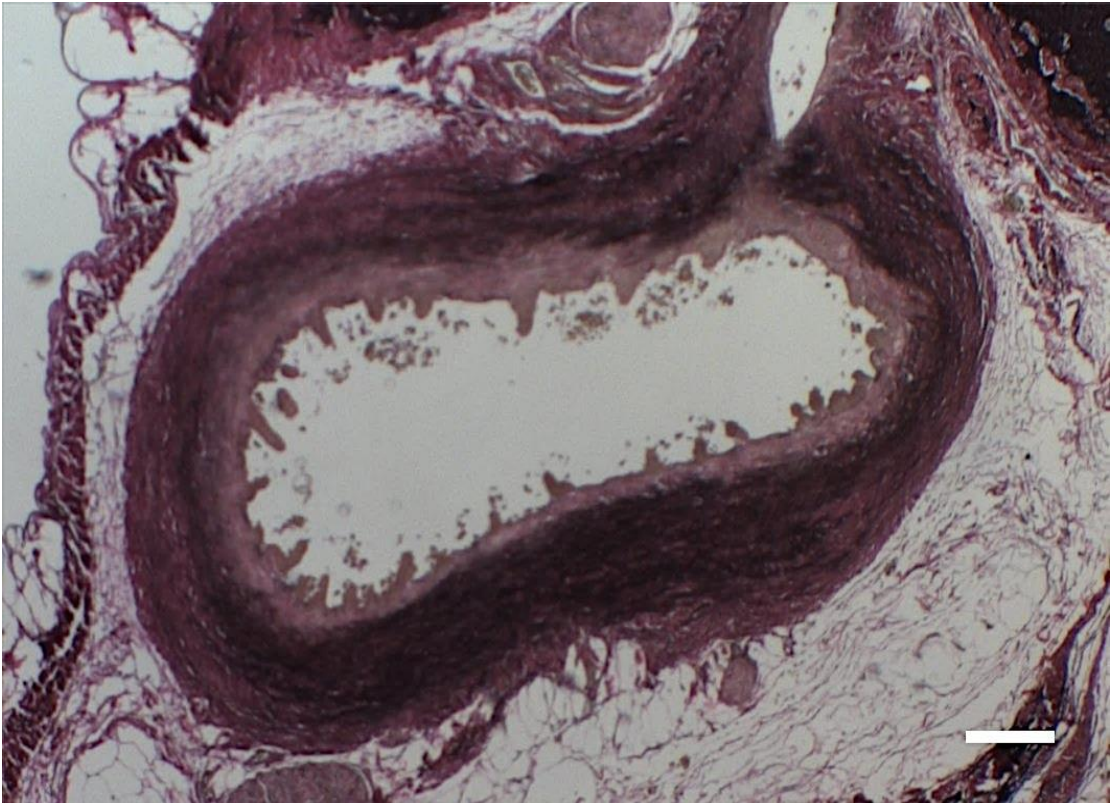


Figure 2.22 Section through daughter branch of the coronary artery of an Atlantic bluefin tuna specimen showing multiple focal myointimal proliferations along the entire luminal circumference (Verhoeff-van Gieson; bar = 200 μ m).

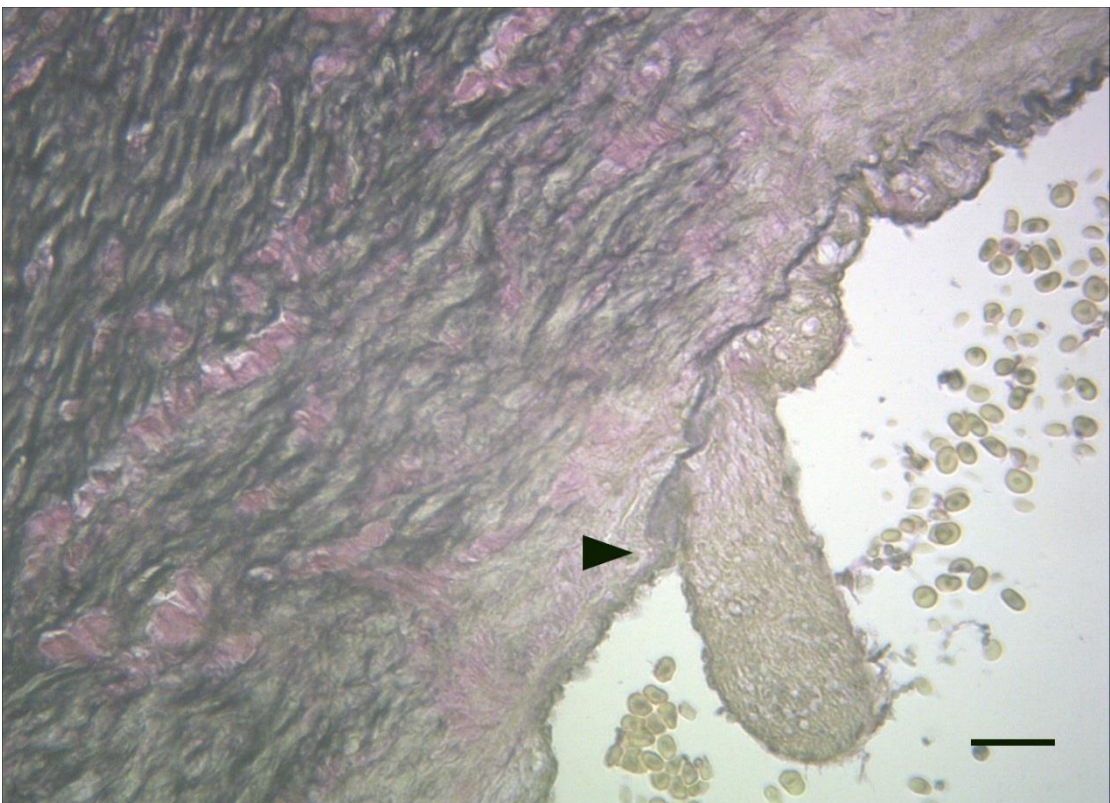


Figure 2.23 Focal myointimal hyperplasia in the first daughter branch of the main coronary artery of an Atlantic bluefin tuna specimen. The arrowhead demonstrates location where the inner elastic lamina is disrupted (Verhoeff-van Gieson; bar = 20 μ m).

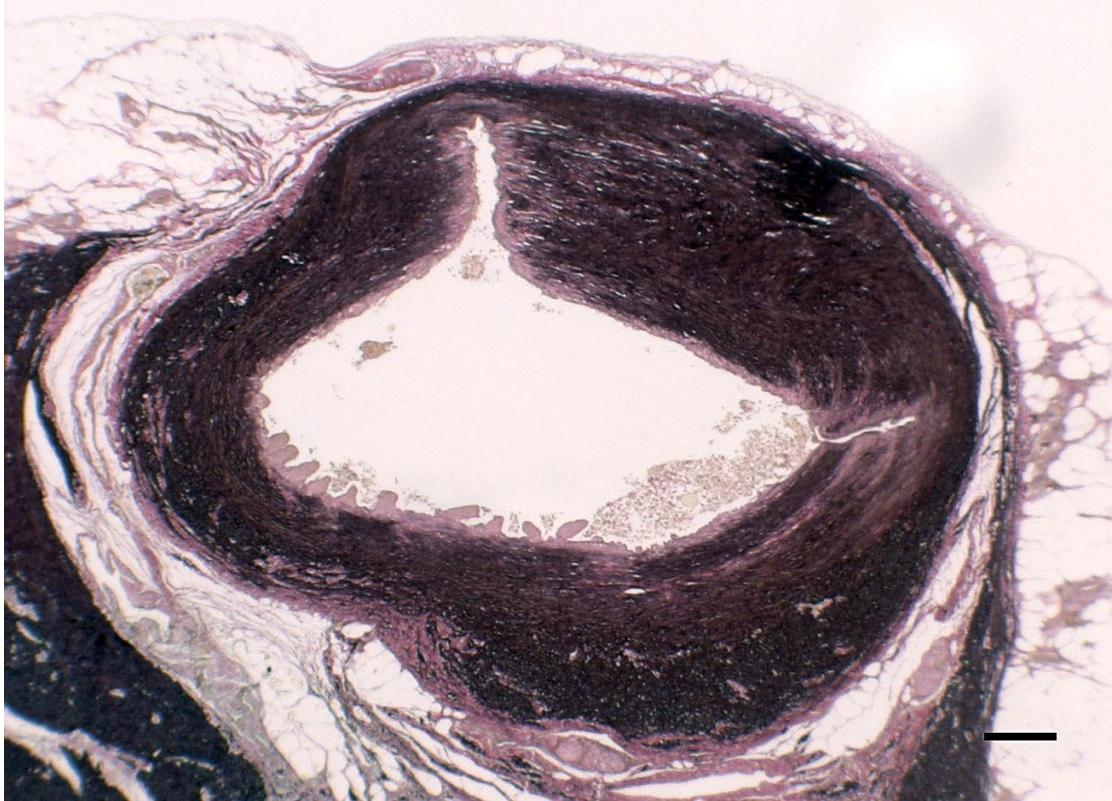


Figure 2.24 Section through the daughter branch of an Atlantic bluefin tuna coronary artery showing disrupted arterial structure with heavy fibrous deposition and multiple focal proliferations along the ventral region of the lumen (Verhoeff-van Gieson; bar = 200 μ m).

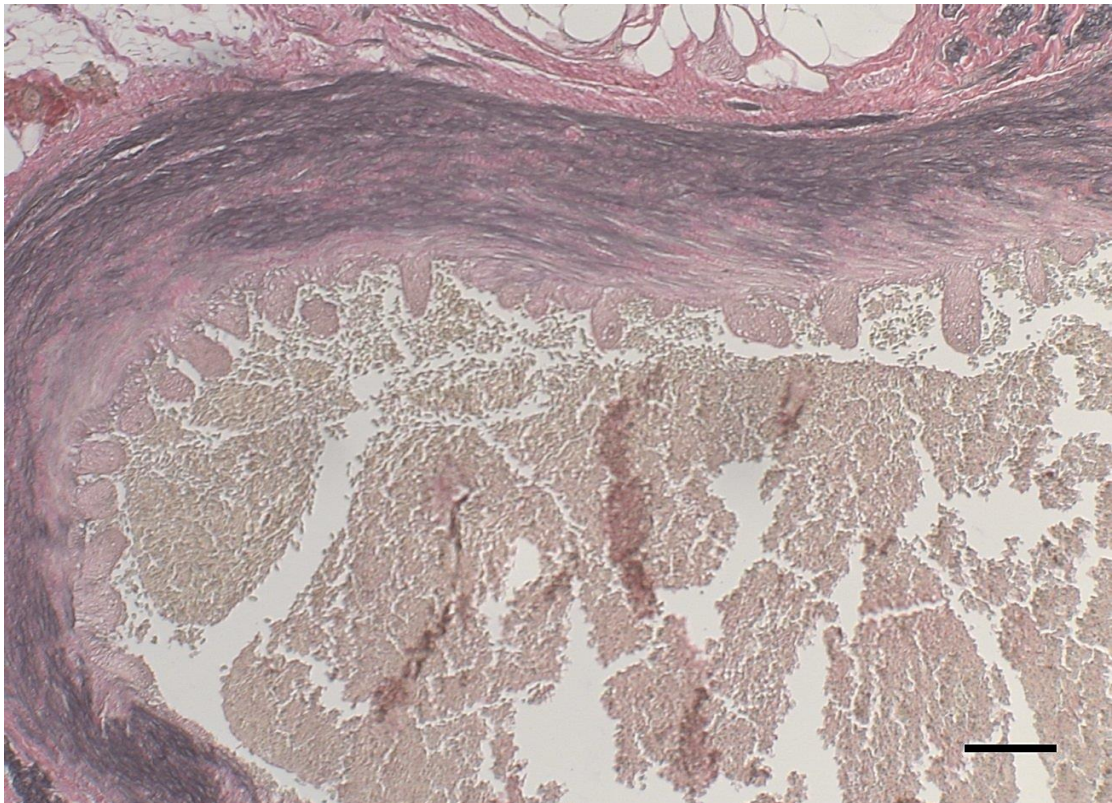


Figure 2.25 Section through the daughter branch of the main coronary artery of an Atlantic bluefin tuna. Arterial structure is non-uniform and there is no clear distinction between the media and intima. Multiple focal proliferations can be found around the lumen (Verhoeff-van Gieson; bar = 100 μ m).

Microscopy also revealed abnormalities within the artery network infiltrating the bulbus arteriosus (Figure 2.26). The arterioles were observed to have an adventitia composed of collagenous and elastic fibres, a relatively thick media incorporating smooth muscle and a small amount of dispersed elastic fibres, and a highly convoluted IEL. Abnormalities observed within these vessels were of two types. The first consisted of focal myointimal proliferations within the intima, and in these lesion types the underlying IEL was thinned and disrupted beneath the smooth muscle growths. The focal lesions were predominantly composed of endothelial and smooth muscle cells, but in cases were also found to include considerable amounts of collagen. Hyperplastic media were also observed and these demonstrated a disproportionately thick medial layer and a distorted lumen, the latter appearing to be a consequence of the unequal proliferation of the media along the vessel circumference. The smooth muscle fibres were arranged transversely to the main vessel axis as opposed to their circular arrangement in the normal arterial structure.

2.3.4. Lipid staining

Only a small number of coronary artery samples were processed for lipid staining due to the long pre-processing procedures to fix lipids within the tissue for routine histological processing. After staining for fats with oil red O, it was quickly evident that lipid inclusions are not a common feature of arteriosclerotic lesions in ABT. None of the arteries stained positive for lipid within the arterial structure, and Figure 2.27 shows the typical negative appearance of oil red O stained arteries.

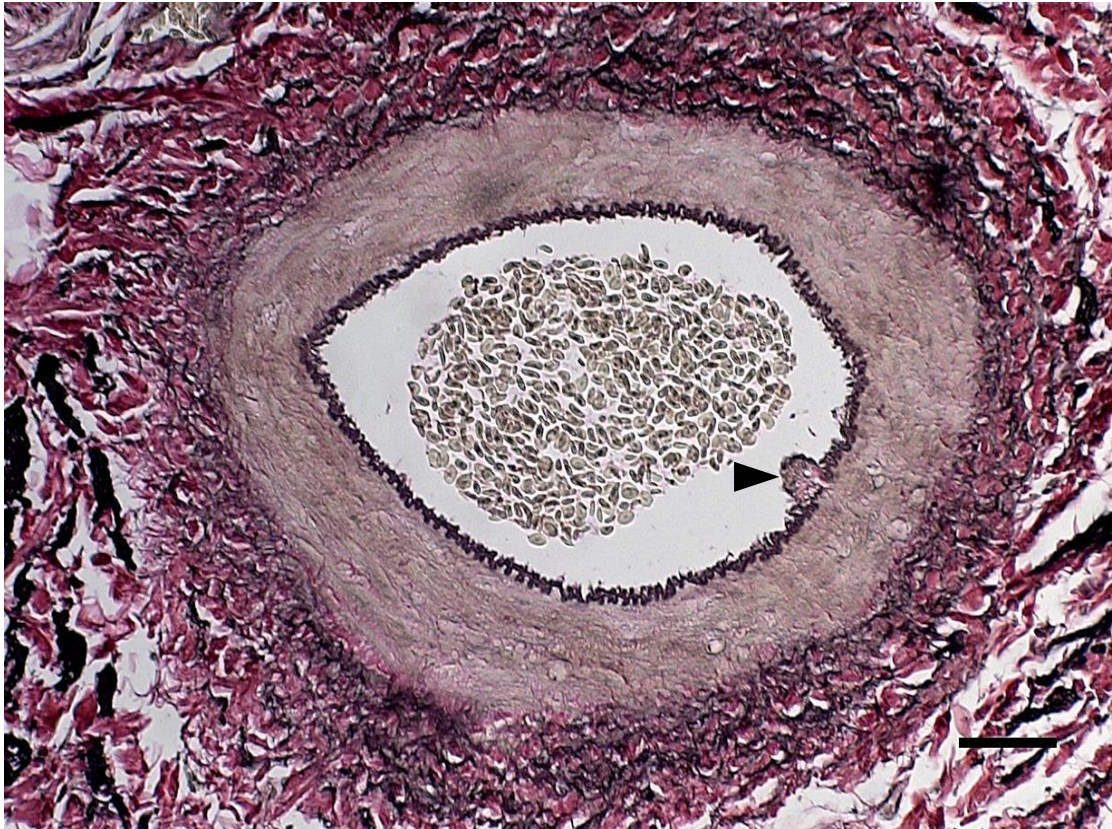


Figure 2.26 Focal myointimal proliferation in an arteriole supplying the bulbus arteriosus parenchyma of an Atlantic bluefin tuna (Verhoeff-van Gieson; bar = 50 μ m).

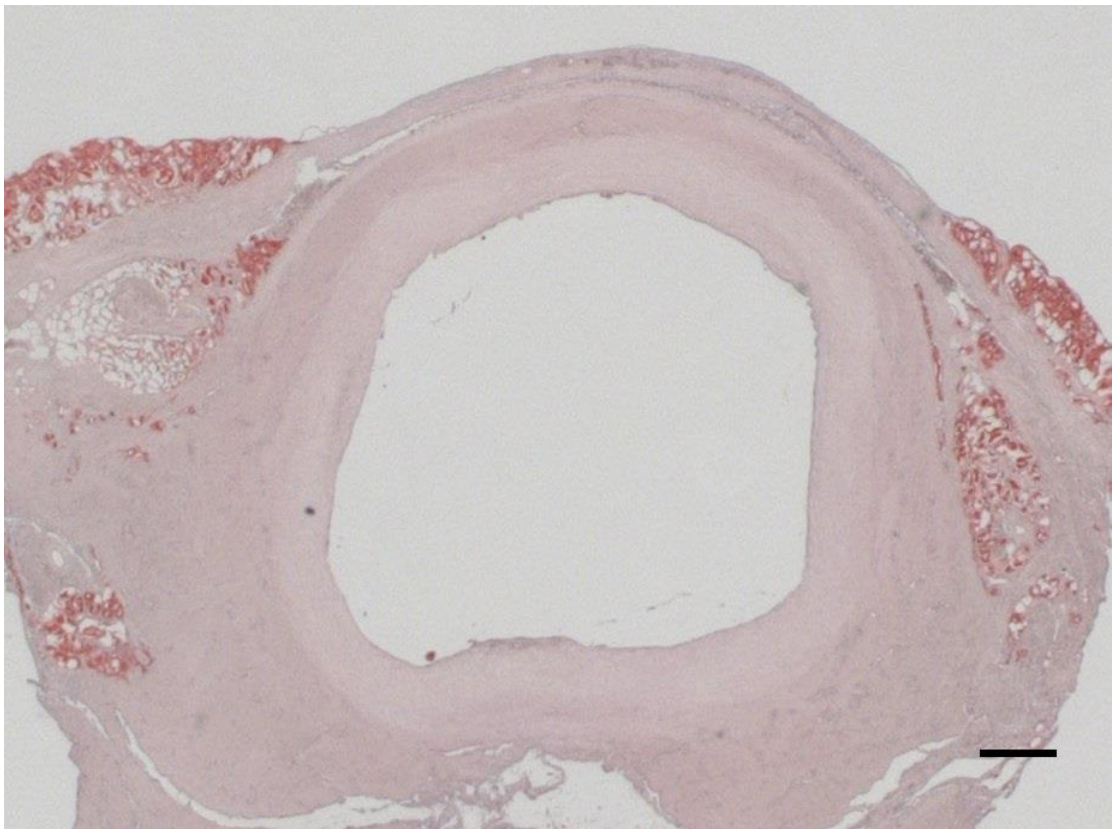


Figure 2.27 Atlantic bluefin tuna coronary artery section pre-processed for lipid fixation. Arteriosclerotic lesions appeared to be free of lipid inclusions (oil red O; bar = 300 μ m).

Figure 2.28 shows the only artery section where the lipid was detected close to the coronary artery structure. Although the intimal proliferations were free of lipid inclusions, a considerable number of lipid-impregnated vacuoles were detected at a location between the media and adventitia. Other artery sections, however, were not found to have such large amounts of lipid in the supporting tissue of the bulbus arteriosus, and this suggests that most likely the lipid bodies detected within the arterial structure are unrelated to the arteriosclerotic process itself, and this is further supported by the fact that no lipid was found within the proliferations themselves.

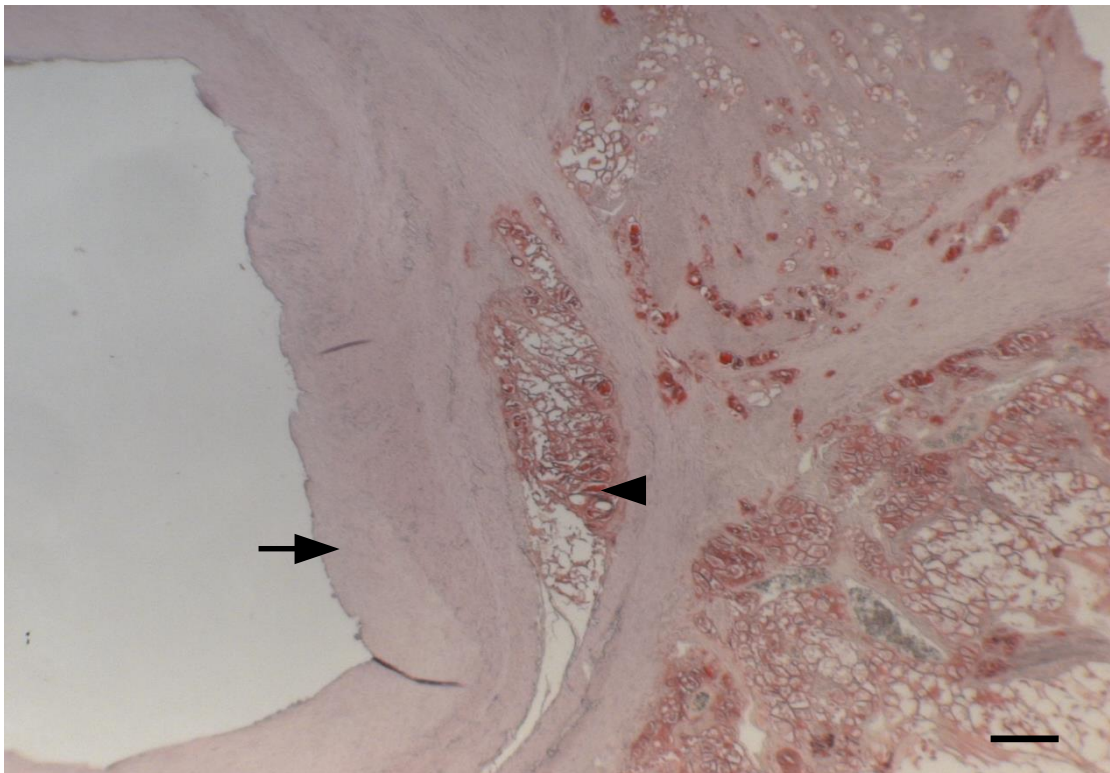


Figure 2.28 Micrograph of artery from a 200 kg farmed Atlantic bluefin tuna stained for lipids. Arrowhead indicates region where numerous lipid-containing vacuoles were detected while arrow shows arterial lesion which stained negative for lipids (oil red O; bar = 200 μ m).

The method used to fix lipids in formalin-fixed coronary arteries (Tracey & Walia, 2002) was validated by treating formalin-fixed liver tissue in the same manner as the artery specimens. Positive staining with oil red O of the control section indicated adequate lipid fixation within the fish tissue during pre-treatment.

2.3.5. Morphometric measurements

Lesion scoring for PLL and MLT was based on the measurement of nine coronary artery sections (*i.e.* three sections each from the proximal, medial and distal regions of the artery) per ABT specimen. Table 2.3 summarises the PLL and MLT values, which were significantly and positively correlated ($p < 0.01$), for the coronary arteries evaluated in this study. Generally, mean values for both PLL and MLT increased with increasing fish age as represented by weight and length values. The only exception to this trend was for coronary arteries collected from the Group 2 category where mean PLL and MLT values were higher than for specimens of a lower mean weight from Group 1. Scatter plots showing this trend are shown in Figures 2.29 and 2.30 for arteries collected from captive fish while those for ABT sourced from the wild are presented in Figure 2.31.

PLL figures ranged from 0.5 % in smaller fish to 58.5 % in larger specimens. Similarly, the lowest MLT value measured within the sample set was from small individuals collected from the wild and amounted to 8 μm , while a larger fish sourced from farm had the largest MLT at 1,018 μm .

Statistical results are shown in Tables 2.4 and 2.5. Lesion severity did not differ significantly between sexes ($p > 0.05$). Mean lesion scores were higher in females in Group 3 and Group 2 but these were higher in males than females in Group 1. Interestingly, in all groups there was a statistically significant trend ($p < 0.01$) for PLL to increase proximally to distally (*i.e.* increasing severity with increasing distance from the ventricle). As expected the same was observed for MLT ($p < 0.01$) with the exception of arteries collected from wild ABT (Group 3). Figures 2.32 and 2.33 show mean PLL and MLT by artery region respectively.

Table 2.3 Potential lumen loss and maximal lesion thickness of coronary arteries from Atlantic bluefin tuna specimens collected from different sources. Values are given as mean \pm standard deviation (SD) and values in parentheses represent upper and lower ranges (Group 1, farm 6 months; Group 2, farm 18 months; Group 3, wild).

Origin	Weight (kg)	Length (cm)	Potential lumen loss (%)	Maximum lesion thickness (μm)
Group 1, ≤ 120 kg ($n = 9$)	76 ± 23 (46-108)	153 ± 23 (123-187)	20.4 ± 10.6 (0.5-48.5)	149 ± 100 (35-695)
Proximal region			14.8 ± 10.5 (0.5-47.0)	90 ± 51 (35-232)
Medial region			22.2 ± 9.8 (6.5-41.3)	161 ± 90 (48-457)
Distal region			24.2 ± 9.3 (6.8-48.5)	195 ± 120 (46-695)
Group 1, ≥ 120 kg ($n = 16$)	270 ± 101 (131-476)	245 ± 28 (197-289)	24.2 ± 10.4 (2.9-58.5)	263 ± 180 (18-1018)
Proximal region			20.7 ± 10.3 (2.9-58.5)	171 ± 110 (18-467)
Medial region			24.8 ± 9.4 (9.2-49.2)	282 ± 180 (50-761)
Distal region			27.1 ± 10.6 (6.5-54.8)	336 ± 201 (35-1018)
Group 2, ≤ 120 kg ($n = 8$)	94 ± 17 (68-117)	166 ± 15 (141-183)	18.9 ± 6.5 (7.3-39.1)	119 ± 57 (29-273)
Proximal region			16.3 ± 4.8 (7.3-25.9)	107 ± 53 (38-287)
Medial region			20.2 ± 6.7 (7.9-36.2)	123 ± 60 (52-273)
Distal region			20.3 ± 7.2 (10.1-39.1)	126 ± 60 (29-250)
Group 2, ≥ 120 kg ($n = 8$)	182 ± 35 (126-241)	222 ± 18 (188-249)	22.9 ± 10.1 (4.3-46.5)	193 ± 132 (26-549)
Proximal region			17.5 ± 7.5 (4.3-35.7)	126 ± 74 (26-328)
Medial region			24.8 ± 11.1 (5.4-44.0)	225 ± 146 (83-488)
Distal region			26.5 ± 9.5 (9.9-46.5)	243 ± 138 (55-549)
Group 3 ($n = 12$)	35 ± 7 (23-49)	119 ± 7 (112-135)	10.3 ± 7.6 (0.8-42.4)	77 ± 52 (8-289)
Proximal region			7.4 ± 3.4 (0.8-13.9)	72 ± 41 (18-157)
Medial region			10.0 ± 7.0 (2.8-37.7)	76 ± 52 (8-215)
Distal region			13.4 ± 9.7 (2.8-42.4)	83 ± 62 (11-289)

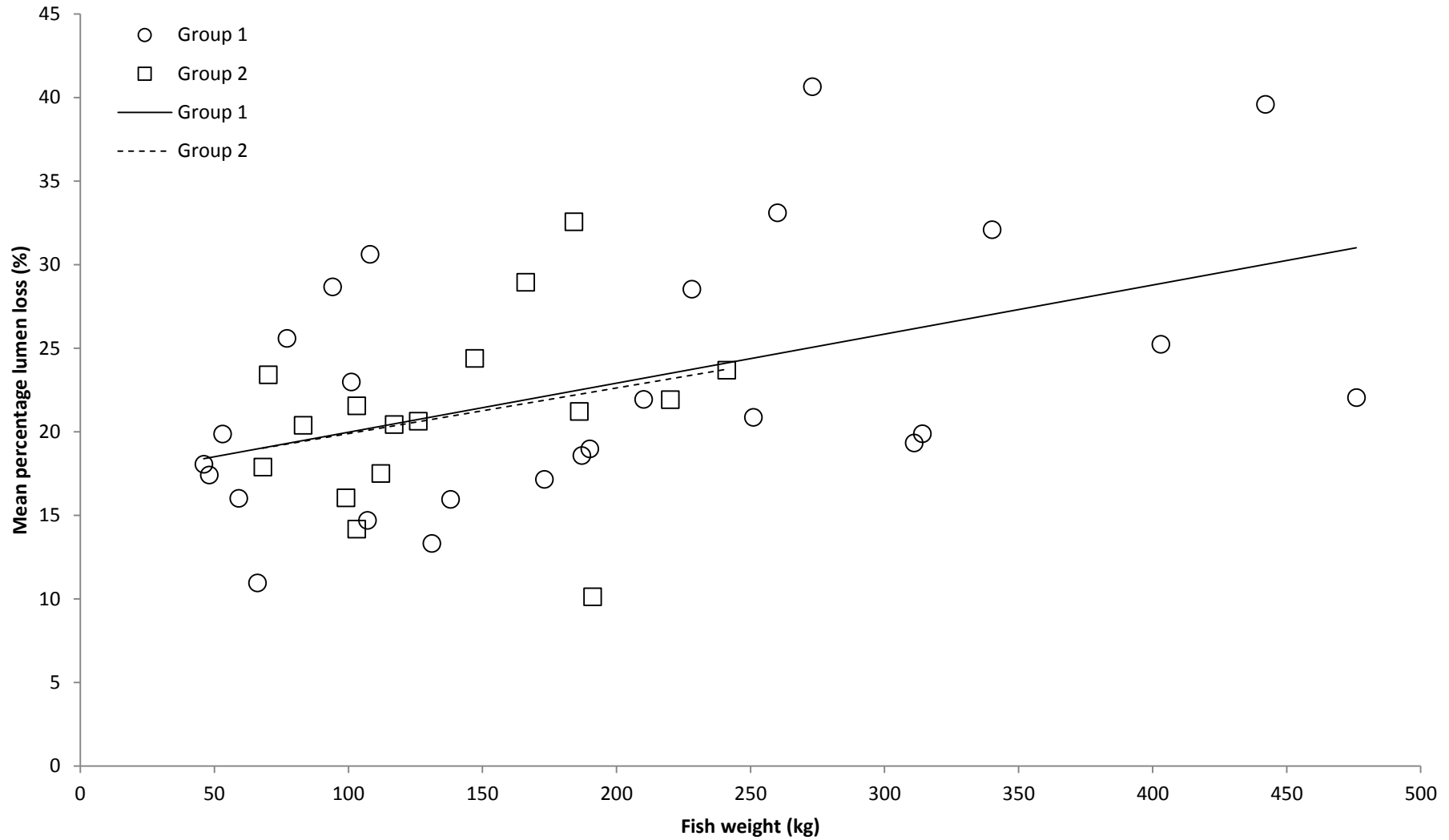


Figure 2.29 Mean percentage lumen loss as a function of weight for arteries collected from farmed Atlantic bluefin tuna (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$).

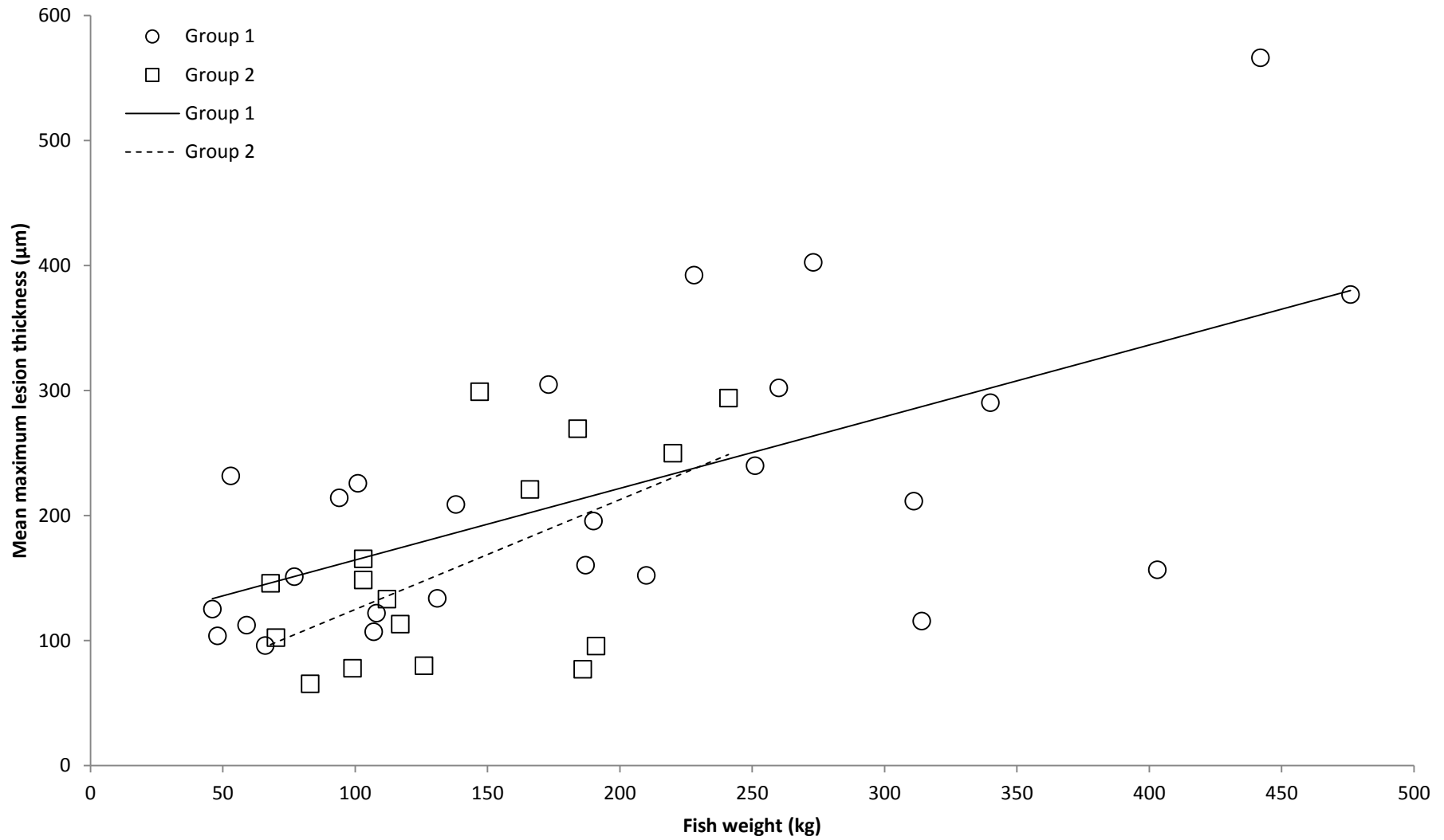


Figure 2.30 Mean maximal lesion thickness as a function of weight for arteries collected from farmed Atlantic bluefin tuna (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$).

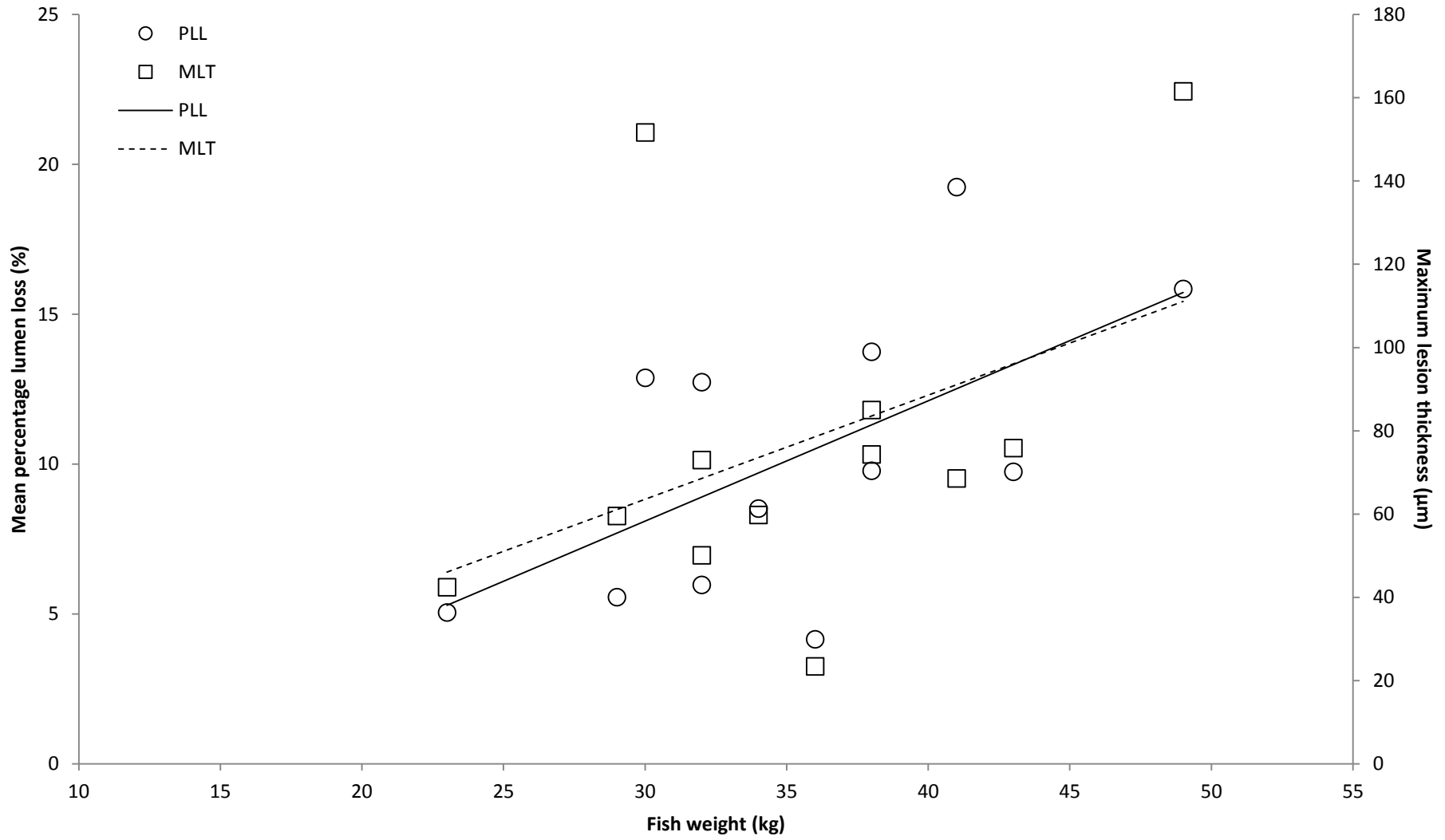


Figure 2.31 Mean percentage lumen loss and mean maximum lesion thickness as a function of weight for arteries from wild Atlantic bluefin tuna (Group 3, $n = 12$).

Within all groups there was a significant positive correlation ($p < 0.05$) between lesion severity indices and length and weight values. Pearson correlation coefficients were higher for weight rather than length values, indicating that weight has a stronger influence on lesion severity than length. Results from ANCOVA also showed weight to have a stronger influence on lesion severity than sex.

Table 2.4 Statistical p -values for the effects of various parameters on percentage lumen loss in the coronary arteries of Atlantic bluefin tuna. Values in parentheses denote Pearson correlation coefficients (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$; Group 3, wild, $n = 12$).

Parameter	Group		
	1	2	3
Sex	0.344	0.003	0.117
Artery region	<0.001	<0.001	0.006
Length	<0.001 (0.349)	0.014 (0.323)	0.001 (0.031)
Weight	<0.001 (0.368)	0.009 (0.385)	<0.001 (0.354)
ANCOVA: weight	<0.001	0.002	<0.001
ANCOVA: sex	0.462	0.198	0.235

Table 2.5 Statistical p -values for the effects of various parameters on maximum lesion thickness in the coronary arteries of Atlantic bluefin tuna. Values in parentheses denote Pearson correlation coefficients (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$; Group 3, wild, $n = 12$).

Parameter	Group		
	1	2	3
Sex	0.971	0.321	0.016
Artery region	<0.001	0.007	0.936
Length	<0.001 (0.414)	<0.001 (0.389)	0.024 (0.217)
Weight	<0.001 (0.407)	<0.001 (0.359)	0.004 (0.273)
ANCOVA: weight	<0.001	<0.001	0.008
ANCOVA: sex	0.714	0.156	0.034

Statistical analysis on pooled lesion indices found artery region, length and weight to have significant effects ($p < 0.01$) on both PLL and MLT values. ANCOVA results indicated weight and origin as the strongest predictors for PLL and MLT, while sex had the lesser influence. Results are summarised in Table 2.6.

Table 2.6 Statistical *p*-values for various parameters on pooled percentage lumen loss and maximal lesion thickness data from Atlantic bluefin tuna specimens from all origins. Values in parentheses denote Pearson correlation coefficients.

Parameter	Lesion severity index	
	Percentage lumen loss	Maximum lesion thickness
Sex	0.070	0.031
Artery region	0.000	<0.001
Length	<0.001 (0.504)	<0.001 (0.557)
Weight	<0.001 (0.466)	<0.001 (0.518)
ANCOVA: weight	<0.001	<0.001
ANCOVA: sex	0.110	0.035
ANCOVA: origin	<0.001	<0.001

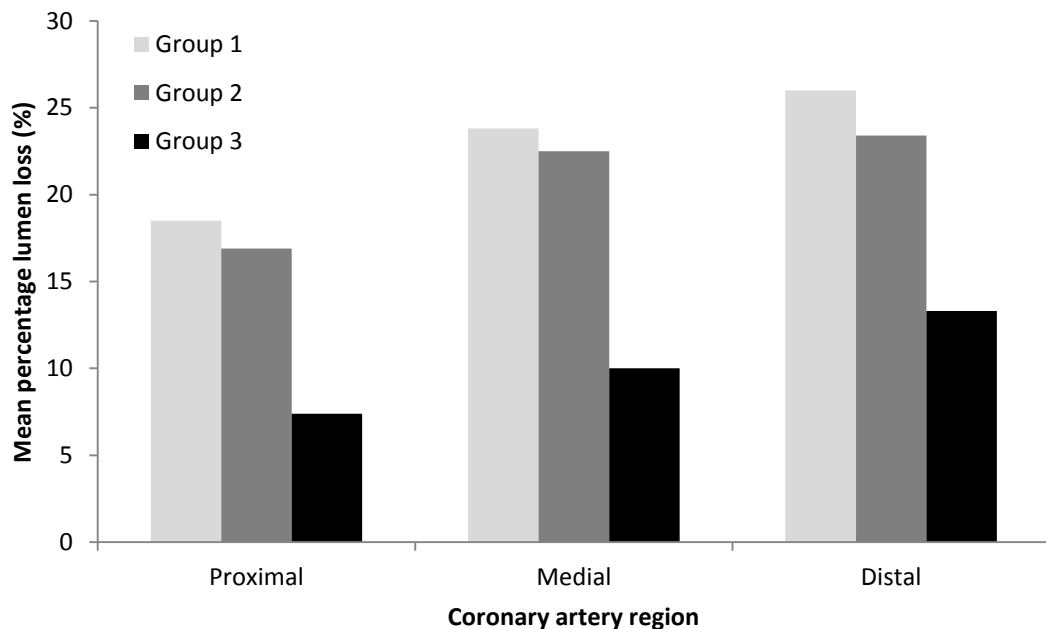


Figure 2.32 Mean percentage lumen loss in Atlantic bluefin tuna coronary arteries as a function of artery region and by specimen origin (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$; Group 3, wild, $n = 12$).

2.3.6. Effect of ventricle size on lesion severity

The pathology of lesions was identical to that described in **Section 2.3.3** above. All sections studied had at least one form of identifiable lesion so that prevalence was equivalent to 100%. Measurements were taken from artery sections from 57 ABT and descriptive data for the specimens are provided in Table 2.7.

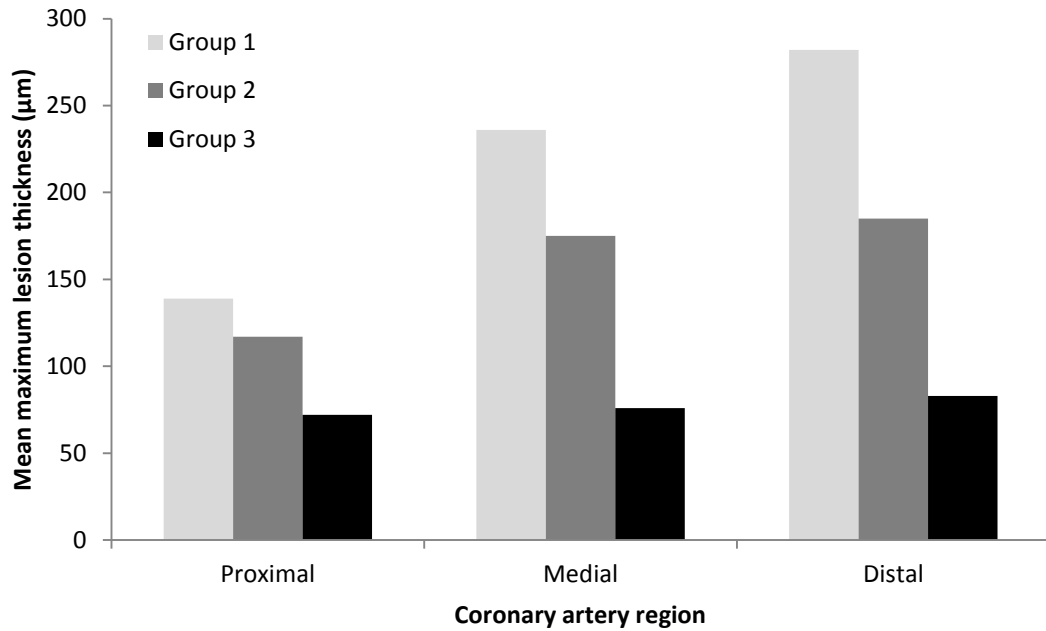


Figure 2.33 Mean maximal lesion thickness in Atlantic bluefin tuna coronary arteries as a function of artery region and by specimen origin (Group 1, farm 6 months, $n = 26$; Group 2, farm 18 months, $n = 16$; Group 3, wild, $n = 12$).

Table 2.7 Descriptive data for Atlantic bluefin tuna specimens. Values are given as the mean \pm standard deviation and values in parentheses represent upper and lower ranges ($n = 57$).

Parameter	Mean values
Specimen weight (kg)	190 \pm 121 (43-411)
Specimen length (cm)	205 \pm 56 (130-293)
Ventricle weight (g)	290 \pm 154 (110-655)
Percentage lumen loss (%)	22.9 \pm 7.4 (2.2-63.1)
Maximum lesion thickness (μm)	266 \pm 138 (39-1,280)

Ventricle weight was significantly ($p < 0.01$) and positively correlated with specimen weight (Pearson = 0.950), length (Pearson = 0.945), PLL (Pearson = 0.190) and MLT (Pearson = 0.342) values. Figure 2.34 plots ventricle weight as a function of fish weight, and plots of PLL and MLT as a function of ventricle weight are given in Figures 2.35 and 2.36 respectively.

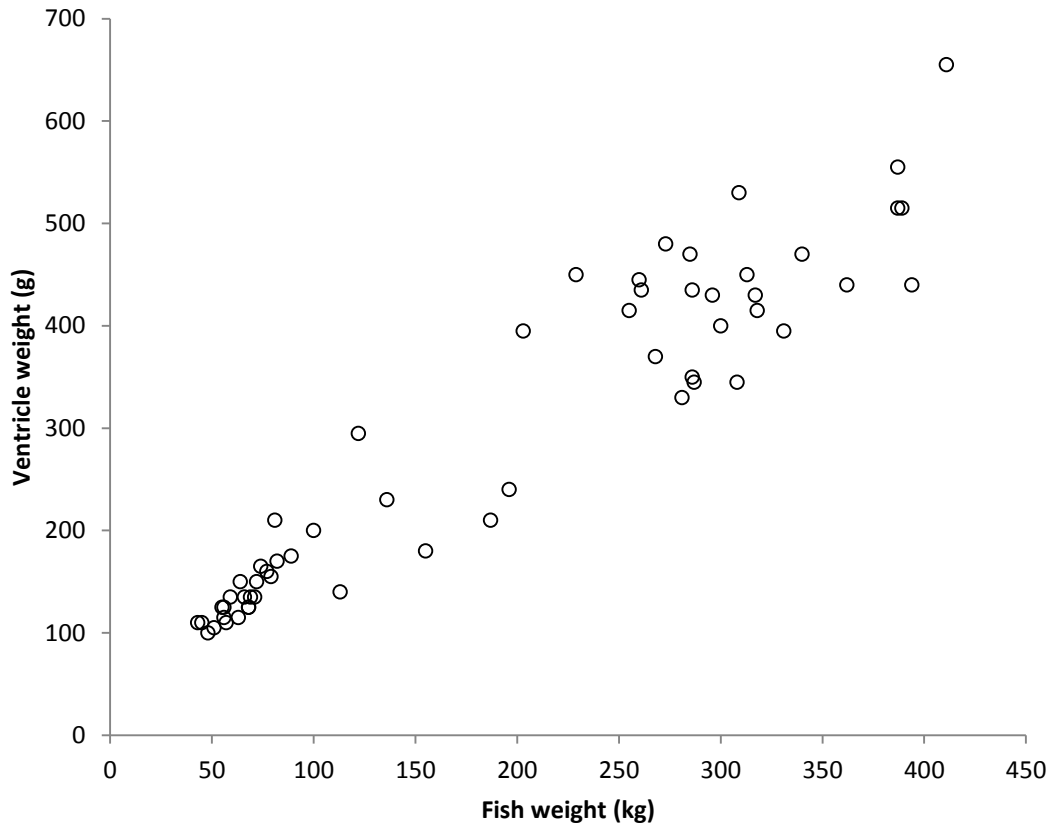


Figure 2.34 Ventricle weight as a function of specimen weight for Atlantic bluefin tuna (Pearson = 0.95; $n = 57$).

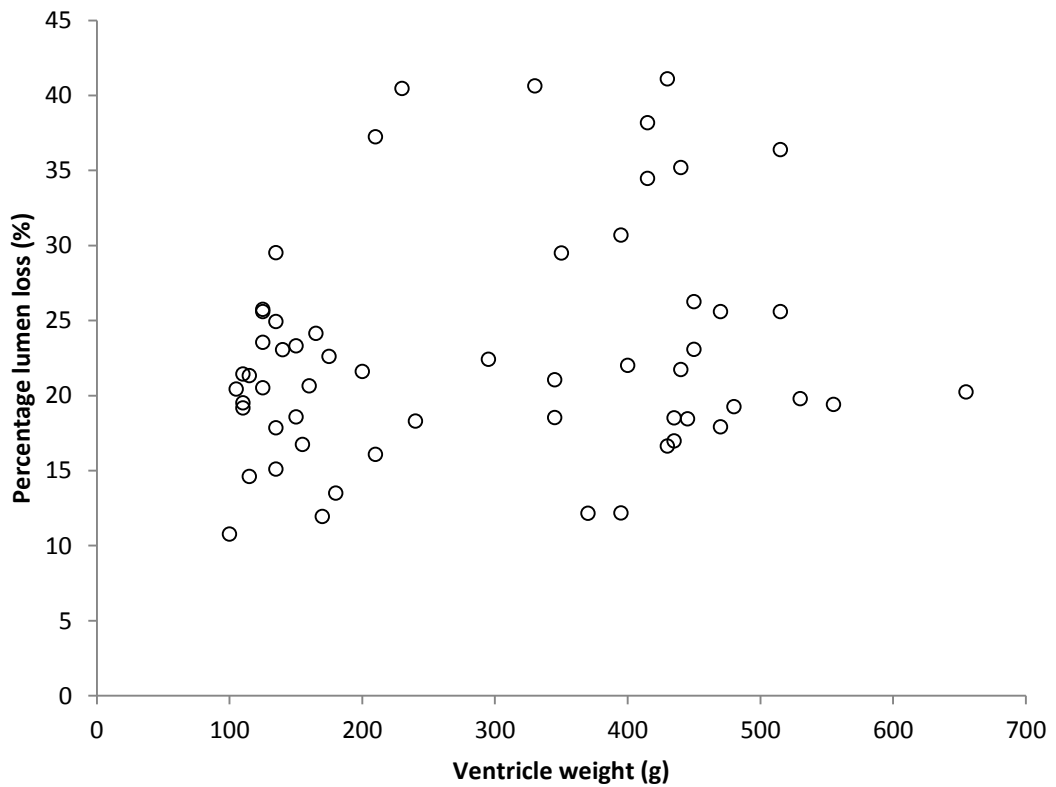


Figure 2.35 Percentage lumen loss as a function of ventricle weight for Atlantic bluefin tuna coronary arteries (Pearson = 0.19; $n = 57$).

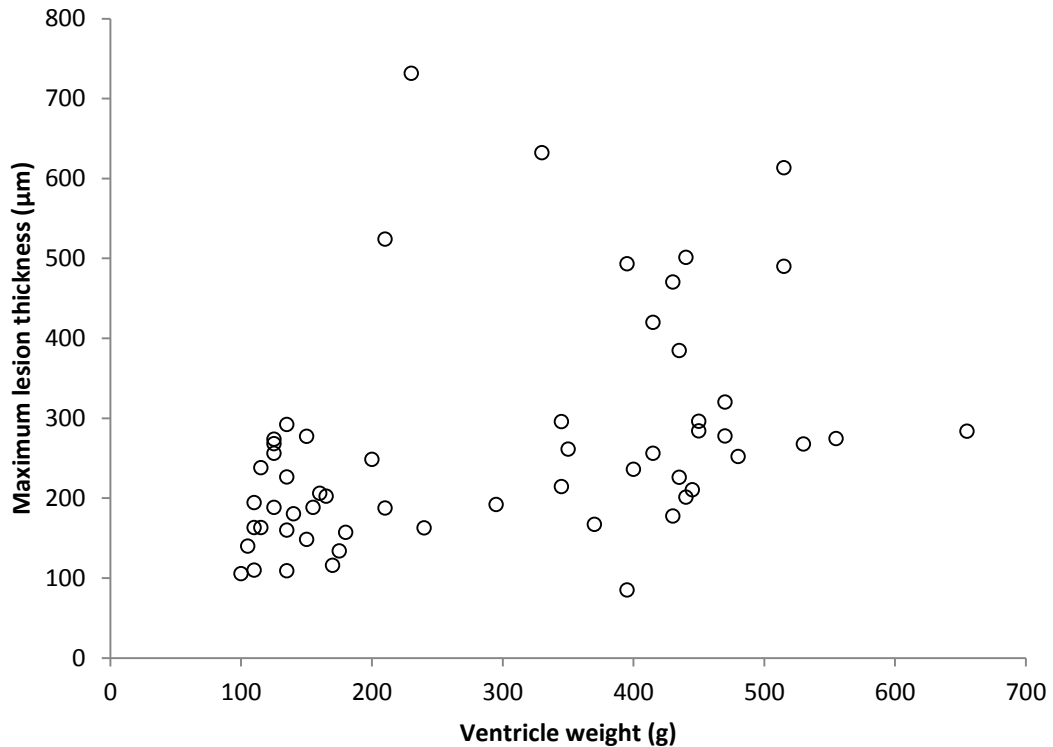


Figure 2.36 Maximum lesion thickness as a function of ventricle weight for Atlantic bluefin tuna coronary arteries (Pearson = 0.34; $n = 57$).

2.4. Discussion

Vastesaege *et al.* (1962) described the common occurrence of arteriosclerotic lesions in a restricted sample of wild ABT. Based on the results collected from a larger number of specimens, this study confirms these findings on the basis that all fish examined had varying degrees of arteriosclerotic lesions along their intimal boundary.

Locations around the arterial circumference that were free of lesions (Figure 2.7) had a distinctive appearance from the coronary arteries of other fish species. The intima consisted of a single layer of endothelial cells overlaid by a convoluted IEL, making this arterial layer similar in histological appearance to that of other teleosts. Remarkable differences, however, were observed in the medial layer, which in contrast to salmonid coronary arteries (Farrell, 2002), appeared to have a higher proportion of thick elastic

fibres. Skipjack and yellowfin tunas have higher blood pressures than ordinary teleosts (Brill & Bushnell, 2001). According to Farrell *et al.* (1992), skipjack tunas may also have an obligate dependence on coronary circulation for efficient cardiac function and have a well-vascularised myocardium (Tota, 1983). In consideration, this anatomical difference may be a physical adaptation to meet the extraordinary physiological requirements of the tuna cardiac system and larger amounts of elastic fibres within the artery, together with abundant collagen in the adventitia, should aid in maintaining artery form and function on a highly distensible bulbus arteriosus.

Although information on arteriosclerotic lesions in ABT is limited, the research was directed towards investigating the occurrence of such pathologies along the main coronary artery; severe lesions seem to be restricted to the part of the coronary artery lying on the bulbus arteriosus and ventral aorta in salmonids (Moore *et al.*, 1976a; García-Garrido *et al.*, 1993). Microscopically the lesions appeared diverse in form but morphologically similar to those encountered in other teleosts, although generally on a more substantial scale due to the larger dimensions of the ABT coronary artery.

Lesions varied from focal myointimal proliferations to larger proliferations extending over large segments of the intimal area and in the most severe cases proliferations covered the entire intimal border. Lesion morphology was non-uniform but mainly consisted of elastic fibres, smooth muscle cells, and in the more severe proliferations significant amounts of collagen. In their majority, the proliferative cells were oriented at right angles to the medial cells, from which they appear to originate, and this arrangement is similar to that encountered in salmonid lesions (Moore *et al.*, 1976c; McKenzie *et al.*, 1978). Most were observed to be highly vacuolated and to possess

basophilic nucleus-like structures, the latter being classified in other species as proliferating smooth muscle (Seierstad *et al.*, 2008). As described for arteriosclerotic pathologies from salmonids (Farrell *et al.*, 1986; Kubasch & Rourke, 1990; García-Garrido *et al.*, 1993), the IEL was found to be disrupted in lesion areas but in more severe pathologies it was generally absent. Although reduplication of the IEL has been documented in other species (Seierstad *et al.*, 2005b; Durán *et al.*, 2010), it was not encountered in ABT coronary arteries in this study.

In the proximal part of the coronary artery, the lesions were focal myointimal proliferations, and consisted exclusively of smooth muscle. Further away from the ventricle, however, these gave way to lesions of increasing width indicating that these may merge to form larger and more diffuse luminal protrusions. The latter bear a striking resemblance to mild atherosclerotic lesions in human aortas (Stouffer, 2005).

Even though lesions predominantly took the form of luminal protrusions originating from the media, the latter appeared to possess decreasing structural integrity in more acute lesions, and this was more apparent in the larger fish. Similar to lesions described in salmon (Van Citters & Watson, 1968; McKenzie *et al.*, 1978), the media was thinned and its usually dense array of elastic fibres was dispersed and infiltrated with smooth muscle and collagenous components, the latter presumably assisting in the maintenance and strengthening of the arterial structure (Safar, 2007). This loss in structural integrity was also observed to increase with increasing fish size, with the media having increasing proportions of collagen. Interestingly, this is also the case in humans where collagen is known to replace fatigued elastin with age, thereby increasing arterial stiffness (Izzo Jr. & Mitchell, 2007).

Frozen sections are the preferred material for examination of tissues for the presence of lipids, however facilities for these procedures were not available at the point of collection and so the tissues were pre-treated to fix lipid for routine histological processing (Tracey & Walia, 2002). Using this method, control tissues stained positive for lipids so that it was presumed that lipid fixation was adequate for the purposes of this study. No lipid deposits were detected in any of the arteriosclerotic lesions in the coronary arteries and this conflicts with earlier reports of lipid deposits in ABT (Vastesaegeer *et al.*, 1962). Only one artery was found to have lipid deposits within the bulbus arteriosus tissue but the inclusions were outside the arterial structure so that it differs from atheroma-type pathologies in that the latter originate from the intima (Safar, 2007).

Traditionally, arteriosclerotic lesions in salmonids and other teleosts are graded based on a five-level system developed by (Moore *et al.*, 1976a, b, c), based on the number of cells constituting an intimal proliferation. As expected, the lesions encountered within the ABT coronary artery are much larger in size (due to the larger size of the artery when compared with that from smaller fish species) so that using a system of grading lesions based on cell size might be inadequate in accurately detailing arteriosclerosis in the species. Although this method is still valid for grading arteriosclerotic lesions, it tends to give slightly different results to computerised measurements as performed here (Seierstad *et al.*, 2005a). The procedure used here estimates two parameters – the PLL and MLT – which when considered together may reflect better the state of the arteriosclerotic artery. The first gives an estimate of the actual luminal area lost due to proliferative lesions while the latter reflects the degree of protrusion into the lumen and

may be considered as an indirect measure of lesion size. These two parameters were strongly correlated across the entire sample set so that increasing lumen loss was linked to an increase in lesion size.

Initially changes associated with spawning were thought to bring about these pathologies in salmonids, but later it was acknowledged that lesions progress with age and may only be secondarily influenced by other events (Saunders & Farrell, 1988; Saunders *et al.*, 1992; Farrell, 2002; Seierstad *et al.*, 2008). The results obtained confirm this is also the case for ABT since in all sample groups lesion severity – in terms of both PLL and MLT – increased with specimen size indicating that lesions were accumulating in the artery over time.

Davie and Thorarensen (1996) found no differences in lesion severity between sexes and the same was observed in the current study with arteries from the ABT. There was a non-significant trend for lesion scores to be higher in females in wild fish (Group 3) and fish held in cages for 18 months (Group 2). The opposite was true for fish farmed for a period of 6 months (Group 1). This may be related to the fact that at weights in excess of approximately 250 kg the proportion of males to females increases so that the larger individuals – where lesion severity is expected to be high – are more likely to be males (personal observation). This trend, where male capture frequency is higher at larger weights, has also been described in wild capture fisheries (Mather *et al.*, 1995).

Although the aetiopathogenic processes leading to the arteriosclerotic lesions in salmonids are multifactorial and sometimes speculative, it is widely accepted that these lesions originate mainly from tissue responses following vascular injury (Farrell, 2002) and from mechanical stresses on the artery wall caused by the distension of the

underlying bulbus arteriosus during blood ejection from the ventricle (Saunders *et al.*, 1992). This so-called 'response-to-injury' theory is also recognised to be one of the main initiating factors of atherosclerosis in humans (Ross, 1999). Lesions were more pronounced moving away from the ventricle and their occurrence was also observed to be more common on the luminal surface away from the bulbus arteriosus. Provided that this pattern in severity was found across all of the coronary arteries inspected, it is plausible to presume that mechanical stresses play an important role in arteriosclerotic lesions in ABT and that their influence on the arterial structure increases with distance away from the ventricle attachment. Lesion progression in fish may also be aided by haemodynamic factors (Maneche *et al.*, 1972; Muñoz-Chápuli *et al.*, 1991; Farrell, 2002; Seierstad *et al.*, 2008) and their interaction with the arterial wall. Several studies have identified hypertension (Kannel, 1996) and several haemodynamic factors such as low shear stress and high flow oscillation (Glagov, 1972; Stary *et al.*, 1992; Stouffer, 2005) as significant risk factors in human atherosclerosis since it facilitates endothelial damage (Berliner *et al.*, 1995). Several tuna species have been documented to have a higher blood pressure than other teleosts (Brill & Bushnell, 1991) so that they may be predisposed to arterial lesions, especially during periods of intense activity and their consequential effects on the coronary artery.

Statistical treatment indicated that fish origin, whether from the farm or wild, influenced the severity of arteriosclerotic lesions in ABT. Although the statistical analysis used treats the parameters independently from each other, the outcome may have potentially been biased by poor sample weight representation across the different categories. The mean specimen weight of ABT collected from the wild was low when

compared to individuals sourced from farm, since no large wild ABT were available for recovery of the coronary artery at the time of study. It was also difficult to assess for differences in lesion severity between fish held in cages for different periods of time since fish sizes were different across the two farmed fish categories. Even though efforts were made so that each category had an adequate representation in terms of size and sex, sampling ABT specimens is opportunistic and relies upon the availability of these during commercial harvest. Although fish from the farm and ocean have different lifestyles, the major differences are expected to be in diets, swimming speed and its associated demands on the cardiac system, and growth rates. Vastesaege *et al.* (1962) linked lesions in ABT to their diets but findings in salmonids suggest that arteriosclerotic lesions in these species are not associated with dietary FA composition (Saunders *et al.*, 1992; Seierstad *et al.*, 2008) and at most, dietary intake plays a secondary role in lesion progression (Farrell *et al.*, 1986). Swimming speeds are also likely not to differ to a large degree between wild and farmed specimens since tuna species are obligate ram ventilators and need to maintain a constant adequate swimming speed for gaseous exchange through their gills (Brown & Muir, 1970). Although not considered as an initiating factor, faster growth places higher demand on physiological systems leading to a more stressful lifestyle and its ensuing effects. Growth rates are expected to differ between wild and farmed fish due to the higher food availability under captive conditions and this may stimulate lesion development through vascular injury or accelerate lesion progression (Farrell, 2002).

Partial correlation analysis demonstrated a significant correlation between ventricular mass and PLL and MLT values, indicating increasing severity with increasing mass.

Similar findings have been reported in rainbow trout (Davie & Thorarensen, 1996) and Atlantic salmon (Seierstad *et al.*, 2005b; Seierstad *et al.*, 2008). Although correlation between ventricle weights and lesion severity was significant, it was relatively dispersed for some of the specimens so that some lower ventricle weights showed higher severities than larger ventricles. The hearts were collected from specimens with differing weights and ventricle mass varied correspondingly. The correlation between these two parameters, however, was strong and linear with few outliers indicating that ventricle weights increase uniformly with increasing fish age or weight.

Although the correlation between ventricle weight and PLL and MLT values was significant, the correlation was in itself weak. This goes to suggest that ventricle mass is not the only determinant in lesion severity in ABT and other factors might be involved. It is important to note also that due to the variability in sample size the ventricle weights also fluctuated over a wide range so that it is not possible to draw clear conclusions on the effect of ventricle weight on lesion severity.

2.5. Conclusion

The high value of the fish together with difficulties in conducting controlled experiments, make ABT a difficult subject to investigate. Although it was not possible to identify a single parameter as having the most influence on lesion severity, it was clear that coronary artery lesions in ABT are widespread and structural degeneration continues throughout the life of the fish. Restrictions in force in the ABT fishery make experimental design difficult and for example the sample set did not include any immature fish and only smaller mature specimens could be sourced from the wild. This necessitates careful interpretation when testing for parameter effects on such lesions.

Several factors are likely to be involved in the development of arteriosclerotic lesions in the ABT especially considering that the species is long-lived and is also highly migratory so that it is exposed to different environments through the course of its life. The understanding of these warrants further investigation but the scope for this may be limited unless controlled experiments may be performed.

CHAPTER 3

DESCRIPTION OF HAEMATOLOGICAL AND NON-SPECIFIC HUMORAL IMMUNE PARAMETERS FROM FARMED ATLANTIC BLUEFIN TUNA

3.1. Introduction

The ABT has been fattened commercially for over a decade but owing to several practical limitations, including their commercial value, little is known about their physiology when held in captivity. Different fish have different lifestyles and this is reflected in variations in their physiology. Tunas are high-energy demand teleosts (Korsmeyer & Dewar, 2001) and so their biological characteristics are expected to be different from those of less active teleosts.

Blood is one of the major components allowing an organism to function physiologically. To date there have been few studies examining the haematology or plasma biochemistry of ABT held under commercial culture conditions. Haematological data is widely available for other teleosts, showing a wide variation in blood parameters between species (Ellis, 1977; Hine *et al.*, 1987), so much so that specific investigations are required to establish functional reference ranges for a particular species.

Blood is an easily accessible component of the fluid system of vertebrates and it is routinely assessed to detect changes in the physiological status of an organism (Houston, 1997). Peripheral blood analysis is used to evaluate the health of several organisms (De Pedro *et al.*, 2005) but it is necessary to establish normal species-specific ranges for this routine analysis.

Fish are in close contact with their aquatic environment and respond physiologically to any changes within it (Casillas & Smith, 1977). Haematological and plasma biochemical indices are influenced by several exogenous factors and are therefore useful for monitoring animal health (Adham *et al.*, 2002; Barcellos *et al.*, 2003; Borges *et al.*, 2004), nutritional status (Spannhof *et al.*, 1979), disease (Řehulka, 2002) and general management stress (Svobodová *et al.*, 2006). For example, changes in erythrocyte numbers may reveal the presence of infection (Řehulka, 2002) and fluctuation in leucocyte proportions may be an indicator of disease (Nussey *et al.*, 1995a; Harikrishnan *et al.*, 2003; Silveira-Coffigny *et al.*, 2004) or environmental toxicants (Nussey *et al.*, 1995a).

The immune system of teleosts consists of both innate and adaptive components (Du Pasquier, 2001). The adaptive response is specific, has a lag phase and is based on the secretion of antibody (Ab) molecules produced by B-cells and the action of T-cells (Manning & Nakanishi, 1997). In contrast the innate immune response comprises several non-specific elements that act immediately against foreign bodies and serves to prevent infection, or delay pathogen establishment until an adequate targeted response can be developed (Alexander & Ingram, 1992). Innate immunity is an essential

component of pathogen defence in fish since their specific immune responses are constrained due to their poikilothermic nature and limited Ab repertoire (Whyte, 2007).

The more prominent non-specific defence components found in the teleost immune system include natural Ab, lysozyme, haemagglutinins (lectins) and complement (Yano, 1996). To date these non-specific immune parameters have only been investigated in tuna species other than ABT.

Ab have an important role in teleost immunity and are involved in the resistance of the fish to bacteria and other pathogens (Uchida *et al.*, 2000). They are expressed on the surfaces of B lymphocytes (Fillatreau *et al.*, 2013) and can be found in the serum and mucus of fish. Immunoglobulin M (IgM) is the predominant immunoglobulin (Ig) class found in teleost serum (Boes, 2001) and is associated with adaptive immunity (Whyte, 2007; Ehrenstein & Notley, 2010) but they are also involved in complement fixation (Magnadóttir *et al.*, 1997; Boshra *et al.*, 2004), agglutination (Arnold *et al.*, 2006) and moderating cellular cytotoxicity (Magnadóttir *et al.*, 1997; Stafford *et al.*, 2006).

Lysozyme is a bacteriolytic enzyme which hydrolyses linkages between molecular constituents of the peptidoglycan layer in bacterial cell walls and is commonly found in teleost mucus, serum and tissues (Jollès & Jollès, 1984; Ellis, 1999). Complement consists of several serum proteins and has two modes of activity. The alternative pathway is Ab independent and is activated, among other things, by lipopolysaccharide on bacterial surfaces while the classical pathway is triggered through the presence of antigen-Ab complexes. Complement activation results in the synthesis or release of important molecules involved in inflammatory responses which possess opsonising and bactericidal properties (Yano, 1996). Lectins are responsible for haemagglutination and

the aggregates they form promote phagocytosis and the clearance of pathogens. They have been reported in many teleost species (Ellis, 1999).

The main objective of this study was to investigate haematological and plasma biochemical parameters in farmed ABT. Available data is scarce and new findings may contribute to the establishment of standard reference values that may aid in welfare assessment of the animals. Additionally, the research aimed to test for the presence of several innate immune components in captive ABT. Both haematological and immunological features are known to vary with fish age (Magnadóttir *et al.*, 1999b). Since ABT are a species with a large size/age range, this study aimed to analyse for the effect of size on the measured haematological, biochemical and immune parameters.

3.2. Materials and Methods

3.2.1. Study population

ABT were captured in the Mediterranean (FAO Statistical Area 14 [see Figure 2.1]; 35°10'N, 014°46'E) in June 2012 by purse seine vessels, transferred to sea cages and towed to farm. They were held in offshore marine cages, off southern Malta (35°49'N, 014°35'E) for a period of 6 months and maintained on a trash fish diet – a combination of mackerel, sardine and herring of varying origin and fat composition. The fish were fasted for one day prior to harvest, and then killed by a shot to the head as per standard industry procedures and transferred to a landing boat. Forty-five ABT specimens were rinsed with running water and blood was withdrawn from the lateral blood vessel beneath the pectoral recess (Figure 3.1) using a syringe fitted with an 18G needle and transferred to 6 mL haematology (EDTA), plasma (lithium heparin) or serum (no

additive) blood collection tubes (Becton Dickson, Oxford, UK). Plasma was separated from heparinised blood by centrifugation at 400 $\times g$ for 5 min, transferred to sterile cryovials and stored at -70°C until use. For serum collection, the blood was allowed to clot at ambient temperature for 30 min, stored overnight at 4°C for clot retraction, centrifuged at 400 $\times g$ for 10 min and then stored at -70°C until use. Whole blood for haemoglobin (Hb) determination was stored at -70°C without separation. Time between slaughter and blood sample retrieval was 15 min.



Figure 3.1 Blood collection from the lateral blood vessel located beneath the pectoral recess of an Atlantic bluefin tuna.

Average sea temperature during the farming period was 25°C while water temperature at time of sample collection was 17°C . Oxygen levels within the cages were always at or near saturation and there were no mortalities attributed to disease during the entire period the fish were held in cages. All specimens appeared clinically healthy and visual inspection of the internal organs did not show any apparent signs of infection or other disorders.

3.2.2. Measurement of haematological and biochemical parameters

Erythrocyte (EC) and leucocyte (LC) counts were determined from whole blood samples diluted 1:200 in Natt-Herrick solution (66 mM NaCl, 18 mM Na₂SO₄, 11 mM Na₂HPO₄, 2mM KH₂PO₄, 0.01 % methyl violet, 0.75 % formaldehyde). Counts were made using an improved Neubauer chamber (Fisher Scientific, Loughborough, UK).

Differential LC counts were made from whole blood smears fixed in 100 % methanol and stained with a Rapi-Diff staining kit (Raymond Lamb, Thermo Scientific; 10 sec Solution A, 20 sec Solution B, 15 sec Solution C, 20 sec dH₂O). Two hundred white blood cells were counted under oil immersion at × 1,000.

Packed cell volume (*syn.* haematocrit; Hct) was calculated following centrifugation at 10,000 ×g for 5 min in a microhaematocrit centrifuge. This procedure was performed within 30 min from sample withdrawal to prevent the influence of external factors on Hct readings.

Haemoglobin (Hb) levels in whole blood were determined with a cyanmethaemoglobin assay using Drabkin's Reagent (DR) containing 0.05 % Brij 35 solution (30 % w/v; Sigma, Dorset, UK). A Hb standard curve (0-30 g dL⁻¹) was prepared using haemoglobin (from bovine blood, Sigma H2500). Standards and whole blood samples were diluted 1:250 in DR, mixed and allowed to stand for 15 min at room temperature (22°C). Aliquots of 100 µL of the diluted solutions were then added in triplicate to a 96-well microplate (Sterilin, Newport, UK) and the absorbance at 540 nm was measured in a microplate reader (Synergy HT, BioTek, Potton, UK). Negative control was DR without any additions and

Hb levels in the unknowns were calculated from the absorbance of the standard concentration curve.

Mean cell volume (MCV), mean corpuscular Hb (MCH) and mean corpuscular Hb concentration (MCHC) were determined with the following equations:

$$\text{MCV (fL)} = \frac{10 \times \text{Hct (\%)}}{\text{EC (}10^6 \mu\text{L}^{-1}\text{)}}$$

$$\text{MCH (pg)} = \frac{\text{Hb (g dL}^{-1}\text{)}}{\text{EC (}10^6 \mu\text{L}^{-1}\text{)}}$$

$$\text{MCHC (g dL}^{-1}\text{)} = \frac{\text{Hb (g dL}^{-1}\text{)}}{\text{Hct (\%)}}$$

Frozen plasma samples were submitted to a diagnostic laboratory (Easter Bush Veterinary Centre, Edinburgh, UK) for biochemical analysis on an automated analyser (iLab650, Instrumentation Laboratory, Bedford MA, USA).

3.2.3. Measurement of immunological parameters

3.2.3.1. Purification of IgM from serum

IgM from ABT serum was purified on an ÄKTAprime chromatography system (GE Life Sciences, Little Chalfont, UK) equipped with a HiTrap IgM Purification HP column (GE Life Sciences) according to the manufacturer's instructions. The column was prepared for purification by treating with filtered binding buffer (0.02 M PBS [5 mM NaH₂PO₄·2H₂O, 14 mM Na₂HPO₄·2H₂O, 150 mM NaCl, pH 7.2], 0.8 M (NH₄)₂SO₄). Serum samples were diluted 1:1 in binding buffer and eluted through the IgM purification

column. The purified IgM was collected from appropriate fractions after elution with 0.02 M PBS pH 7.5.

The eluted fractions were washed three times with sterile 0.02 M PBS by centrifugation at 4,000 $\times g$ in 3 kDa centrifugal filter units (Amicon Ultra-25, Millipore, Watford, UK) and then concentrated in the same buffer until the desired volume was achieved. The protein concentration of the purified IgM sample was determined using a protein assay kit (BCA protein assay kit, Pierce Biotechnology, Rockford IL, USA) using bovine serum albumin as standard and stored at -70°C until use.

3.2.3.2. Electrophoresis and western blotting

Affinity-purified ABT IgM was examined by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting procedures. The IgM was added to an equal volume of 2 \times SDS-PAGE sample buffer (125 mM Tris-HCl, 20 % glycerol, 0.4 % w/v SDS, 200 mM dithiothreitol, 0.01 % bromophenol blue, pH 6.8), heated for 3 min at 95°C, cooled on ice and centrifuged at 4,000 $\times g$ for 3 min.

The sample was separated with an Amersham ECL gel system (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. Electrophoresis of the purified serum was performed on a 10 % precast gel (Amersham ECL Gel System, GE Healthcare). The precast gel was removed from packaging, rinsed with dH₂O and pre-run as per the manufacturer's instructions in 1 \times SDS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS, pH 8.3) at 160 V for 12 min. Ten microlitres each of pre-stained broad range protein markers (Spectra Broad Range Protein Ladder, Thermo

Scientific) and IgM sample (5 µg protein) were loaded onto the gel and proteins subjected to 160 V for 60 min.

The electrophoresed proteins were transferred to a nitrocellulose membrane (Amersham Hybond ECL, GE Life Sciences) at 60 V for 45 min in transblot buffer (191 mM glycine, 25 mM Tris, 13.3 % methanol, pH 8.3). The membrane was blocked with 3 % BSA in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5) for 1 h at 22°C and washed three times for 5 min each with TTBS (20 mM Tris, 0.5 M NaCl, 0.05 % Tween 20, pH 7.5). The membranes were then incubated for 1 h with either an anti-ABT IgM (AquaMab-F19, Aquatic Diagnostics Ltd., Stirling, UK) monoclonal Ab (mAb; IgG1 isotype, recognising the IgM heavy-chain heavy chain; reconstituted in 0.02 M PBS) diluted 1:20 in TBS, or an anti-ABT IgM polyclonal Ab from mouse (Aquatic Vaccine Unit, Institute of Aquaculture, University of Stirling, Stirling, UK) diluted 1:250 in TBS. The blots were washed three times with TTBS incubated for 1 h at 22°C with a 1:200 dilution of anti-mouse IgG biotin in TBS (Sigma) and re-washed as above. Amplification for the membrane treated with the mAb was for 1 h at 22°C with horseradish peroxidase streptavidin (Vector Laboratories, Peterborough, UK; 1:200) and washed with TTBS incubating for 15 min on the last wash. Signal amplification was not performed for the membrane treated with the polyclonal Ab. The membranes were developed with a 4CN membrane peroxidase substrate kit (KPL Inc., Gaithersburg MD, USA), dried and scanned.

3.2.3.3. Measurement of serum total IgM

Serum was assayed for IgM using a direct enzyme-linked immunosorbent assay (ELISA). An IgM standard curve ($5-2 \times 10^{-3}$ µg mL⁻¹) was prepared in carbonate-bicarbonate

coating buffer pH 9.6 (Sigma) from affinity-purified ABT IgM. Serum samples were diluted 1:6,400 in the coating buffer and serially diluted to 1:25,600 in the same buffer. Lyophilised anti-ABT IgM mAb (Aquatic Diagnostics Ltd.) was resuspended as described above and diluted 1:33 in 0.02 M PBS according to the manufacturer's instructions.

The wells of a 96-well ELISA plates (Immulon, Thermo Scientific) were coated in duplicate with 100 μ L of each standard curve concentration and with an equal volume of each of the serum dilutions in triplicate. The plate was incubated overnight at 4°C. Non-fixed antigen was removed by washing five times with low salt wash buffer (LSWB; 20 mM Tris, 380 mM NaCl, 0.05 % Tween 20, pH 7.3), non-specific binding sites were blocked with 250 μ l well⁻¹ 3 % skimmed milk in LSBW for 2 h at 22°C and then drained off. The plate was incubated for 1 h at 22°C with 100 μ L well⁻¹ anti-ABT IgM mAb diluted 1:33 in PBS, then washed five times with high salt wash buffer (HSWB; 20 mM Tris, 500 mM NaCl, 0.05 % Tween 20, pH 7.3). The samples were incubated for 1 h at 22°C with 100 μ l well⁻¹ of goat anti-mouse IgG-HRP (Sigma; 1:2,000), washed five times with HSWB and treated with 100 μ L chromogen (3,3',5',5-tetramethylbenzidine; TMB; Sigma) in substrate buffer (15 mL TMB substrate buffer, 150 μ L H₂O₂, 5 μ L TMB) for 10 min at 22°C. The reaction was stopped with the addition of 50 μ L of 2 M H₂SO₄ and the plate read at 450 nm. Negative controls (blanks) consisted of wells without serum, primary and secondary Ab. Blank-adjusted readings were used to construct an IgM standard curve. The standard curve was used to obtain IgM levels in the serum samples.

3.2.3.4. Measurement of serum lysozyme activity

Serum lysozyme activity was measured using a turbidimetric assay. Frozen sera were brought to 22°C and 10 μ L were added in triplicate to microplate wells. A 0.2 mg mL⁻¹

Micrococcus lysodeikticus (Sigma) suspension was prepared in 0.04 M PBS (10 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 28 mM $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 300 mM NaCl, pH 7.2). Aliquots of 190 μL of the bacterial suspension were added to each sample well and the reduction in absorbance at 540 nm was measured at 5 min intervals with continuous shaking at 25°C on a microplate reader. One unit (U) of lysozyme activity was defined as the amount of sample causing a reduction in absorbance of 0.001 min^{-1} . Negative control consisted of 200 μL bacterial suspension and results were blanked against PBS absorbance. Lysozyme activity was assayed at pH 5.8 and 6.2 to determine optimal conditions for enzyme activity.

3.2.3.5. Measurement of serum complement activity

Complement activity of serum was measured using complement-mediated haemolysis assay using methods adapted from Marsden *et al.* (1996) and described in Watts *et al.* (2002). Defibrinated sheep red blood cells (SRBC) in Alsever's solution (Oxoid, Basingstoke, UK) were washed three times in 0.02 M PBS pH 7.2 by centrifugation at $450 \times g$, resuspended to 3 % v/v in Hank's balanced salt solution (HBSS; Thermo Scientific) containing calcium (Ca) and magnesium (Mg) but without phenol red, and concentration adjusted for optimal absorbance at 450 nm. The classical complement pathway requires the presence of both Ca and Mg ions for its activation (Marsden *et al.*, 1996) but the alternative pathway is only dependent on the latter (Watts *et al.*, 2002). For measurement of the alternative pathway, 10 mM EGTA (Sigma) were added to HBSS to chelate free Ca and inhibit the classical pathway.

One hundred microlitres of SRBC suspension were added to 96-well round-bottom microplate (Sterilin) wells. Serum samples were diluted in assay buffer to give final

serum concentrations of 2.5, 5, 10 and 20 % and an equal volume of each dilution added to triplicate wells. Plate preparation was performed at 4°C to preserve complement activity (Yano, 1992) prior to incubation, which was for 1 h at 25°C to reflect the internal temperature of endothermic tuna species (Graham & Dickson, 2001). Following incubation the reaction was stopped by centrifuging the plate at 300 ×g for 10 min at 4°C, and 100 µL aliquots of well contents were transferred to a fresh 96-well microplate and absorbance measured at 450 nm in a plate reader. Background and 100 % haemolysis readings were obtained by adding equal amounts of the SRBC suspension to assay buffer and dH₂O respectively.

One unit (U) of activity was defined as the quantity of serum sufficient to generate 50 % haemolysis of a 1.5 % SRBC suspension at 25°C. Serum dilution curves against mean haemolysis were plotted individually per sample and CH₅₀ and ACH₅₀ U mL⁻¹ serum were calculated from this data (Yano, 1992).

3.2.3.6. Measurement of serum haemagglutinating activity

ABT serum was tested for haemagglutinating activity in a round-bottom microplate. Defibrinated SRBCs were washed three times with 10 volumes of TBS buffer (50 mM Tris-HCl, 150 mM NaCl, pH 7.2) by centrifugation and resuspended to 2.5 % (v/v) in the same buffer. Serum samples were inactivated by heating at 56°C for 20 min, double serial dilutions were made in TBS buffer and 25 µL were added to an equal volume of 2.5 % SRBC suspension. The microplate was incubated for 1 h at 25°C to reflect ABT internal temperature (Graham & Dickson, 2001) and the reciprocal of the highest dilution showing visible agglutination expressed as the titre value.

3.2.3.7. Bacterial inhibition assays

Antibacterial activity in ABT serum was tested with an inhibitory zone assay. Bacterial fish pathogens were sourced from the Institute of Aquaculture (University of Stirling) bacterial culture collection. *Vibrio anguillarum* (NCIMB 6), *V. vulnificus* (ATCC 27562 biovar I) and *Photobacterium damsela* subsp. *piscicida* (SSV1 Greece) stored at -80°C were revived and cultured in tryptic soy broth (Oxoid) supplemented with 1 % NaCl for 20 h at 22°C. Tryptic soy agar plates (Oxoid) containing 1 % NaCl were inoculated with the bacterial culture and the bacteria distributed evenly with a sterile spreader. Blank antimicrobial susceptibility discs (Oxoid) were impregnated with untreated or complement-inactivated serum (heated at 56°C for 1 h) and aseptically transferred to the agar plates. Positive and negative controls were 30 µg oxytetracycline antibiotic disc (Oxoid) and a blank filter disc with sterile PBS respectively. Growth inhibition was observed after a 24 h incubation period at 24°C.

3.2.4. Statistical analysis

3.2.4.1. Haematological and immune parameters

For statistical purposes and to identify significant parameter difference between fish of different weights, specimens were classified into two weight classes – Group 1 and Group 2 – consisting of fish of weight above and below 100 kg respectively.

Data were tested for normality with a one-sample Kolmogorov-Smirnov test and logarithmically (\log_{10}) transformed when it deviated from normality. Pearson correlation analysis was used to compare haematological, biochemical and immunological parameters within groups. Student's *t*-test was used to analyse

differences between lysozyme activity at different pH, and differences between classical and alternative pathway activities. One-way analysis of variance (ANOVA) was used to test for significant differences in parameter readings between weight classes. Statistical analysis was performed with SPSS Statistics 20 (IBM Corp.) and significance level was set at 0.05.

3.2.4.2. Principal component analysis

Principal Component Analysis (PCA) was used to identify structuring within the datasets. PCA was individually performed on plasma biochemistry, haematological and immune measurements. All variables were \log_{10} transformed to correct for increasing variance with increasing mean size of the measured variables and then those with coefficient of variation (CV) values greater than an arbitrarily set level of 20 % were removed prior to PCA analysis. PCA was performed with Statistica 10 (StatSoft Inc., Tulsa OK, USA).

3.3. Results

The ABT specimens showed significant weight ranges between individual fish. For purposes of comparing the haematological, biochemical and immunological parameters across size groups, the fish were classified into two separate classes based on their mass.

Table 3.1 shows descriptive data for the relevant groups.

Table 3.1 Descriptive statistics for Atlantic bluefin tuna specimens used for haematological, plasma biochemical and immunological measurements. Values represent mean \pm standard deviation and figures in brackets show upper and lower ranges (Group 1, >100 kg; Group 2, \leq 100 kg).

	Haematological and immunological measurements		Plasma biochemical measurements		
	Weight (kg)	Length (cm)	Weight (kg)	Length (cm)	
Group 1 (n = 30)	286 \pm 42 (217-361)	245 \pm 11 (222-263)	Group 1 (n = 15)	281 \pm 40 (221-361)	242 \pm 12 (222-263)
Group 2 (n = 15)	81 \pm 12 (61-100)	145 \pm 8 (130-157)	Group 2 (n = 15)	81 \pm 12 (61-100)	145 \pm 8 (130-157)

3.3.1. Haematological parameters of Atlantic bluefin tuna

Light microscopy showed ECs to have an elliptical profile with a similarly shaped, strongly basophilic and central nucleus (Figure 3.2). The different LC types from ABT are shown in Figure 3.3. The LCs with the highest abundance were the lymphocytes which were small in size and round, with a large and densely staining basophilic nucleus which almost occupied the entire cell volume. Thrombocytes were observed to be elongate, spindle-shaped and less frequently oval in form and their basophilic nucleus always occupied more than half the cell volume. Granulocytes were round with a central to an off-central nucleus with a neutrophilic or eosinophilic granular cytoplasm. No basophils were observed from any of the peripheral blood smears in this study. Monocytes were the least abundant of the LCs observed. They were of an irregular form, possessed a large and strongly basophilic nucleus occupying more than 60 % of the cell, and with an occasionally vacuolar cytoplasm.

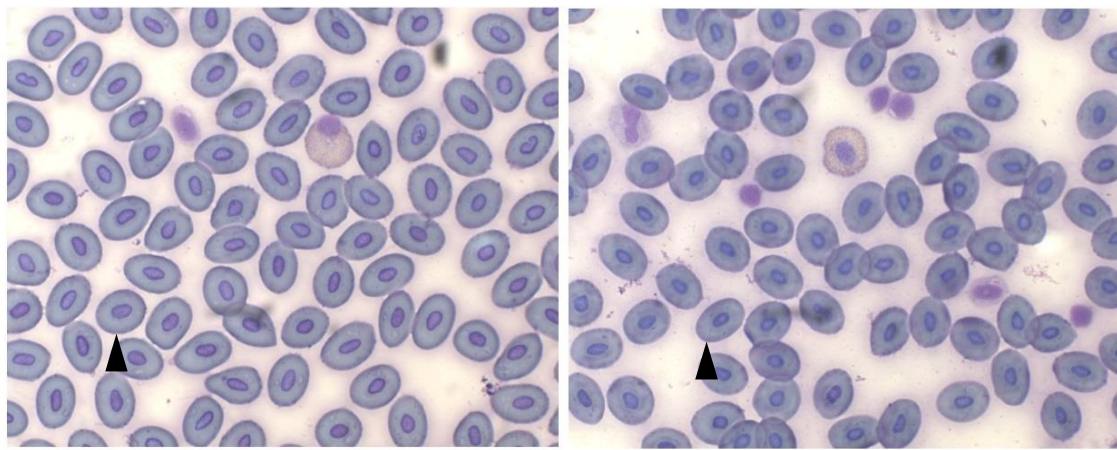


Figure 3.2 Representative Rapi-Diff stained peripheral blood smears from Atlantic bluefin tuna showing the elliptical morphology of the erythrocytes (arrowheads) and different leucocyte types (bar = 10 μ m).

The haematological parameters of ABT measured in this study are listed in Table 3.2.

Mean EC and LC counts, HCT and Hb were higher in Group 2 indicating that these

haematological characteristics vary with fish size. The variation was statistically significant for EC ($p < 0.05$) and Hb ($p < 0.05$). EC counts were significantly and inversely correlated with weight ($p < 0.01$) as were Hb levels ($p < 0.01$).

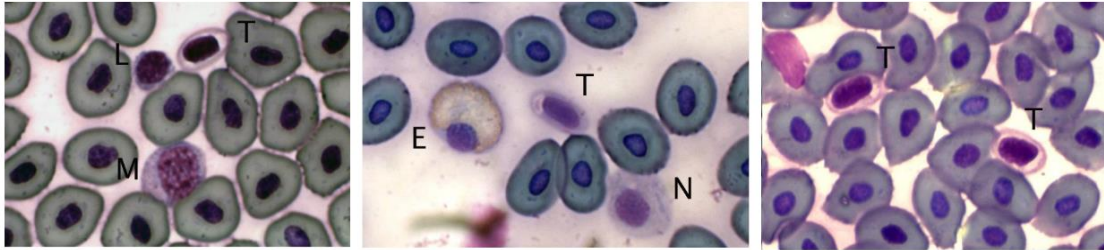


Figure 3.3 Representative Rapi-Diff stained peripheral blood smear from captive Atlantic bluefin tuna showing different leucocyte types and their morphology (L, lymphocyte; T, thrombocyte; M, monocyte; E, eosinophilic granulocyte; N, neutrophilic granulocyte; bar = 10 μ m).

Table 3.2 Haematological parameters and differential counts from Atlantic bluefin tuna. Values represent mean \pm standard deviation (SD) and upper and lower ranges (Group 1, >100 kg, $n = 30$; Group 2, ≤ 100 kg, $n = 15$).

Parameter	Group 1		Group 2	
	Mean \pm SD	Range	Mean \pm SD	Range
EC ($\times 10^{12} \text{ L}^{-1}$)* ^a	2.53 \pm 0.12	2.28-2.81	2.61 \pm 0.08	2.48-2.73
LC ($\times 10^9 \text{ L}^{-1}$)	199.8 \pm 13.8	172-227	206.9 \pm 16.3	180-226
HCT (%)	46.30 \pm 2.68	41.2-51.6	46.91 \pm 3.30	41.18-53.57
Hb (g dL ⁻¹)* ^a	17.77 \pm 0.80	16.30-19.64	19.81 \pm 0.35	19.25-20.27
MCV (fL)	183.51 \pm 11.73	153.10-206.60	179.54 \pm 11.71	162.80-201.40
MCH (pg)*	70.48 \pm 45.29	61.29-80.91	75.87 \pm 3.25	70.51-81.10
MCHC (g dL ⁻¹)*	38.54 \pm 3.22	33.10-46.40	42.40 \pm 3.07	37.00-49.20
Lymphocyte (%)	57.05 \pm 6.96	45.77-67.82	57.57 \pm 5.83	47.03-66.18
Thrombocyte (%)	30.56 \pm 9.39	14.80-49.25	28.78 \pm 8.56	15.08-47.03
Monocyte (%)	2.10 \pm 1.41	0.00-4.08	2.12 \pm 1.12	0.97-4.02
Neutrophil (%)	4.07 \pm 1.79	1.00-7.14	3.78 \pm 1.60	1.93-6.09
Eosinophil (%)	6.20 \pm 3.52	0.98-11.94	7.75 \pm 3.84	1.94-12.14

* $p < 0.05$; a, parameters showing significant Pearson correlation ($p < 0.05$); EC, erythrocyte; LC, leucocyte; HCT, haematocrit; Hb, haemoglobin; MCV, mean cell volume; MCH, mean corpuscular haemoglobin; MCHC, mean cell haemoglobin concentration.

In contrast, there was no clear pattern between the two size groups as regards differential LC counts. Variation between the group means was minor and no significant differences were observed ($p > 0.05$).

The PCA model explained 70 % of the variation in the haematological variables (Table 3.3) and the principal factors (F1-F2) displayed clustering of haematological parameters (Figure 3.4) based on the size of the fish.

Table 3.3 Univariate statistics for major haematological variables of 45 farmed Atlantic bluefin tuna specimens. The component loadings and the percentage of the variance explained by each variable ($n = 6$) for the first two principal factors (F1-F2) are shown and component loading values above ± 0.70 are shown in bold.

Variable	Mean \pm SD	CV (%)	F1	F2
Weight (kg)	213 \pm 104	0.12	-0.967	-0.120
Length (cm)	209 \pm 49	0.05	-0.961	-0.153
EC ($\times 10^{12}$ L ⁻¹)	2.56 \pm 0.12	0.02	0.561	-0.485
LC ($\times 10^9$ L ⁻¹)	2.02 \pm 0.15	0.03	0.283	-0.393
HCT (%)	46.51 \pm 2.85	0.03	0.195	-0.818
Hb (g dL ⁻¹)	18.45 \pm 1.17	0.03	0.854	0.327
Variance proportion (%)			50.4	20.0
Cumulative (%)			50.4	70.4

SD, standard deviation; CV, coefficient of variation; EC, erythrocyte; LC, leucocyte; HCT, haematocrit; Hb, haemoglobin.

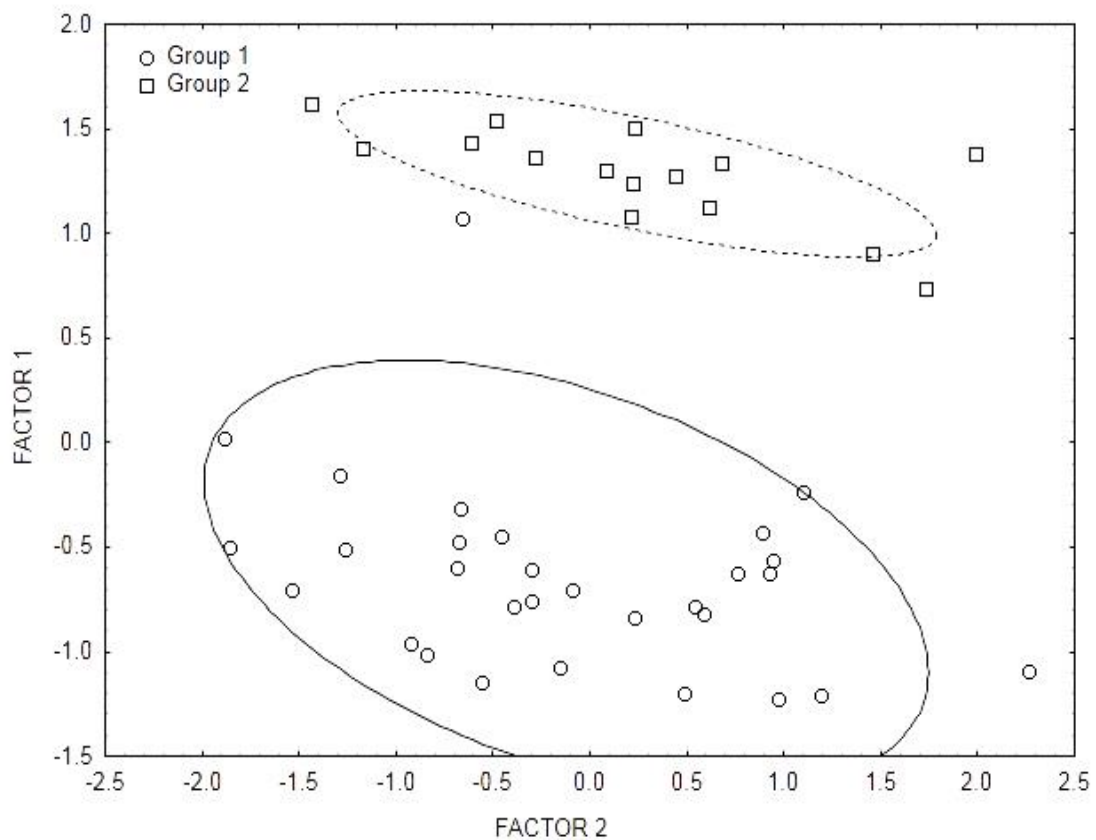


Figure 3.4 Plot of F1 and F2 generated by PCA on the major haematological indices of farmed Atlantic bluefin tuna specimens. There is clear separation in the haematological variables of different size classes along F1. Ellipses represent 95 % confidence interval of the mean (Group 1, >100 kg, $n = 30$; Group 2, ≤ 100 kg, $n = 15$).

3.3.2. Plasma biochemical parameters of Atlantic bluefin tuna

The biochemical parameters measured from ABT plasma are presented in Table 3.4. The mean biochemical values for alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase, total bilirubin, direct bilirubin and inorganic phosphate were higher in Group 1 while others parameters were higher in blood plasma samples of the smaller specimens from Group 2. Some of the biochemical parameters, notably alanine aminotransferase, alkaline phosphatase, aspartate aminotransferase and total bilirubin, varied over a broad range between individual specimens. Statistical analysis showed that some parameters were significantly different ($p < 0.05$) between the two groups. Pearson correlations between several weight and the biochemical parameters were significant ($p < 0.05$).

The principal factors generated by PCA accounted for 78.9 % of the variance within the sample set (Table 3.5). Plots of F1 and F2 showed distinct clustering between fish of different weight groups (Figure 3.5).

Table 3.4 Mean plasma biochemical values measured for Atlantic bluefin tuna specimens with an automated analyser. Values represent mean \pm standard deviation (SD) and upper and lower ranges (Group 1, >100 kg, $n = 15$; Group 2, ≤ 100 kg, $n = 15$).

Parameter	All Groups		Group 1		Group 2	
	Mean \pm SD	Range	Mean \pm SD	Range	Mean \pm SD	Range
Albumin (g L ⁻¹)* ^a	24.49 \pm 2.34	19.80-27.90	22.91 \pm 2.09	19.80-27.50	26.07 \pm 1.29	24.20-27.90
ALT (U L ⁻¹)* ^b	2.86 \pm 1.52	1.00-7.00	3.73 \pm 1.71	2.00-7.00	2.00 \pm 0.53	1.00-3.00
ALP (U L ⁻¹)	14.67 \pm 15.06	1.00-57.00	19.80 \pm 17.45	1.00-57.00	9.53 \pm 10.43	1.00-39.00
AST (U L ⁻¹)* ^b	61.60 \pm 59.26	18.00-278.00	84.30 \pm 47.67	24.00-185.00	32.20 \pm 8.96	18.00-54.00
Tot. bilirubin (μ mol L ⁻¹) ^b	1.84 \pm 1.38	0.10-6.40	2.53 \pm 1.15	1.00-6.40	1.16 \pm 1.29	0.10-5.40
D. bilirubin (μ mol L ⁻¹)*	0.50 \pm 0.20	0.20-1.00	0.55 \pm 0.20	0.20-1.00	0.46 \pm 0.19	0.20-0.80
Calcium (mmol L ⁻¹)* ^a	4.94 \pm 0.70	3.68-6.22	4.39 \pm 0.45	3.68-5.15	5.49 \pm 0.38	4.87-6.22
Chloride (mmol L ⁻¹)* ^a	176.43 \pm 9.50	150.00-199.00	172.40 \pm 10.11	150.00-191.00	180.47 \pm 7.07	171.00-199.00
TCHO (mmol L ⁻¹)* ^a	6.69 \pm 1.25	4.70-9.40	6.03 \pm 1.19	4.70-9.40	7.36 \pm 0.94	6.30-9.20
Creatinine (μ mol L ⁻¹)* ^a	40.93 \pm 5.43	29.00-50.00	38.27 \pm 6.10	29.00-50.00	43.60 \pm 2.92	39.00-50.00
Globulin (g L ⁻¹)* ^a	43.84 \pm 5.23	30.90-50.20	41.12 \pm 6.00	30.90-50.20	46.57 \pm 2.18	41.90-50.00
In. phosphate (mmol L ⁻¹)	2.90 \pm 0.43	2.21-3.96	3.04 \pm 0.49	2.33-3.96	2.76 \pm 0.33	2.21-3.42
Potassium (mmol L ⁻¹)	5.73 \pm 1.17	4.00-8.70	6.10 \pm 1.33	4.40-8.70	5.36 \pm 0.89	4.00-6.60
Total protein (g L ⁻¹)* ^a	68.32 \pm 7.22	50.60-77.60	64.02 \pm 7.71	50.60-76.90	72.63 \pm 3.00	66.20-77.60
Sodium (mmol L ⁻¹)* ^a	217.73 \pm 13.30	193.00-242.00	207.67 \pm 10.22	193.00-225.00	227.80 \pm 6.70	217.00-242.00
Triglyceride (mmol L ⁻¹)* ^a	6.07 \pm 1.00	4.36-7.77	5.64 \pm 0.91	4.36-6.99	6.50 \pm 0.93	5.18-7.77
BUN (mmol L ⁻¹)	0.86 \pm 0.22	0.40-1.30	0.82 \pm 0.22	0.40-1.20	0.89 \pm 0.22	0.50-1.30
Uric acid (mmol L ⁻¹)* ^a	90.00 \pm 31.17	40.00-154.00	69.87 \pm 24.77	40.00-123.00	110.13 \pm 23.01	76.00-154.00
Glucose (mmol L ⁻¹)	9.62 \pm 2.48	6.6-13.20	9.76 \pm 2.08	6.60-13.20	10.00 \pm 1.38	8.30-13.40

* $p < 0.05$; a, significant ($p < 0.05$) negative Pearson correlation with specimen weight; b, significant ($p < 0.05$) positive Pearson correlation with specimen weight; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; Tot. bilirubin, total bilirubin; D. bilirubin, direct bilirubin; TCHO, total cholesterol; In. phosphate, inorganic phosphate; BUN, blood urea nitrogen.

Table 3.5 Univariate statistics for 20 blood chemistry variables measured on 28 specimens of farmed Atlantic bluefin tuna. Figures shown in bold with coefficient of variation values greater than 20 % were rejected prior to analysis with a PCA model. The component loadings and the percentage of variance explained by each variable ($n = 15$) for the first principal factors (F1-F3) are shown. Component loading values above ± 0.70 are shown in bold.

Variable	Mean \pm SD	CV (%)	F1	F2	F3
Weight (kg)	170 \pm 103	0.13	0.848	-0.185	-0.249
Length (cm)	189 \pm 50	0.05	0.850	-0.211	-0.259
Albumin (g L ⁻¹)	24.53 \pm 2.23	0.03	-0.902	0.050	0.050
ALT (U L ⁻¹)	2.86 \pm 1.52	0.52	-	-	-
ALP (U L ⁻¹)	14.11 \pm 15.81	1.28	-	-	-
AST (U L ⁻¹)	59.50 \pm 58.39	0.18	0.704	-0.414	0.096
D. bilirubin (μ mol L ⁻¹)	0.49 \pm 0.20	-0.53	-	-	-
Tot. bilirubin (μ mol L ⁻¹)	1.80 \pm 1.43	4.05	-	-	-
Calcium (mmol L ⁻¹)	4.95 \pm 0.71	0.09	-0.887	0.058	0.229
TCHO (mmol L ⁻¹)	6.67 \pm 1.12	0.09	-0.852	-0.156	0.232
Creatinine (μ mol L ⁻¹)	41.00 \pm 5.45	0.04	-0.659	-0.043	-0.504
Globulin (g L ⁻¹)	43.67 \pm 5.30	0.04	-0.857	-0.274	-0.124
In. phosphate (mmol L ⁻¹)	2.89 \pm 0.44	0.14	0.263	-0.765	0.220
Potassium (mmol L ⁻¹)	5.72 \pm 1.21	0.12	0.199	-0.864	0.223
Total protein (g L ⁻¹)	68.19 \pm 7.25	0.03	-0.901	-0.197	-0.079
Sodium (mmol L ⁻¹)	219.21 \pm 12.49	0.01	-0.844	0.109	0.209
Triglyceride (mmol L ⁻¹)	6.09 \pm 1.03	0.10	-0.677	-0.487	0.175
BUN (mmol L ⁻¹)	0.84 \pm 0.22	-1.35	-	-	-
Uric acid (mmol L ⁻¹)* ^a	92.29 \pm 30.63	0.08	-0.879	-0.122	-0.253
Glucose (mmol L ⁻¹)	9.84 \pm 1.64	0.07	-0.395	-0.415	-0.679
Variance proportion (%)			56.4	14.4	8.1
Cumulative (%)			56.4	70.8	78.9

SD, standard deviation; CV, coefficient of variation; ALT, alanine aminotransferase; ALP, alkaline phosphatase; AST, aspartate aminotransferase; Tot. bilirubin, total bilirubin; D. bilirubin, direct bilirubin; TCHO, total cholesterol; In. phosphate, inorganic phosphate; BUN, blood urea nitrogen.

3.3.3. Western blotting

Purified ABT IgM was separated by electrophoresis, transferred to a nitrocellulose membrane and incubated with the mAb against the ABT IgM heavy chain. The reaction between the mAb and polyclonal Ab and the IgM heavy chain were observed at a molecular weight (MW) of approximately 74 kDa (Figure 3.6).

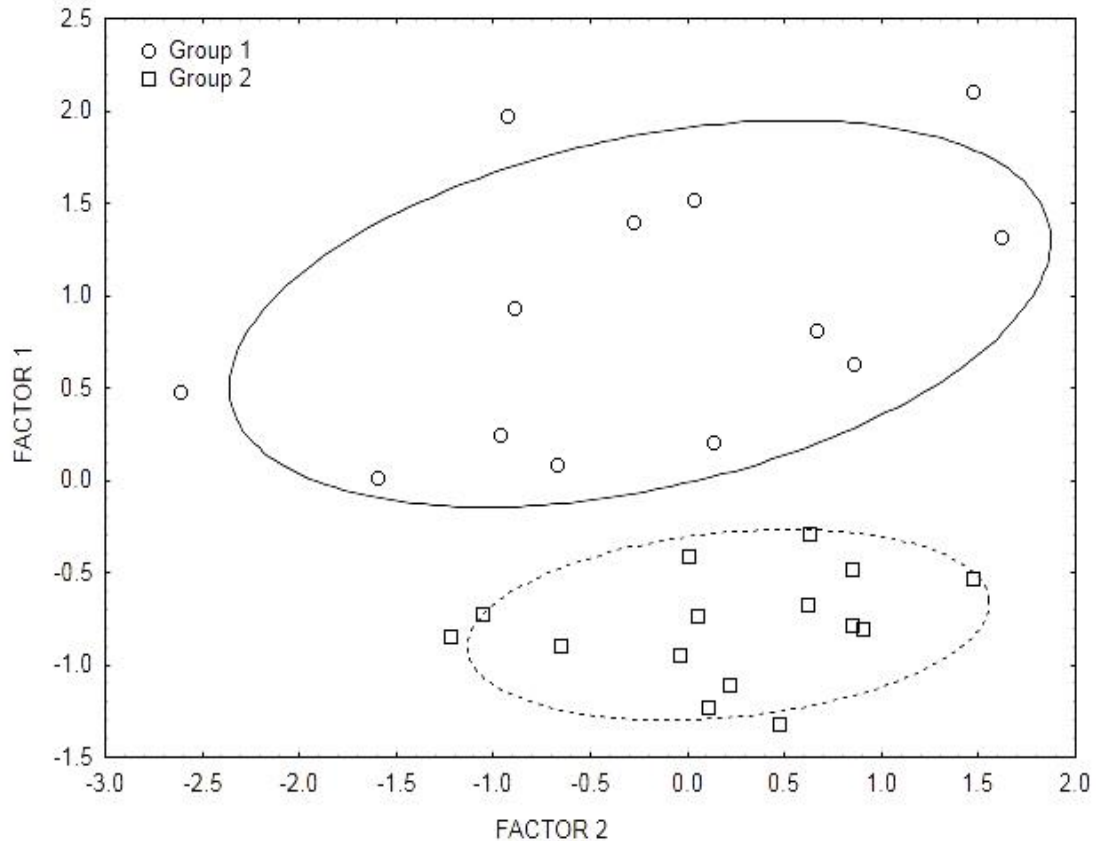


Figure 3.5 Plot of F1 and F2 generated by PCA on the plasma biochemical measurements from 28 farmed Atlantic bluefin tuna specimens. There is clear separation between different size classes along F1. Ellipses represent 95 % confidence interval of the mean (Group 1, >100 kg, $n = 14$; Group 2, ≤ 100 kg, $n = 14$).

3.3.4. Total IgM levels, and lysozyme and complement activities in Atlantic bluefin tuna serum

Results for immunological measurements on ABT serum are presented in Table 3.6. Total IgM levels in serum from ABT were quantified using an ELISA. IgM levels were significantly higher ($p < 0.01$) in sera collected from smaller individuals (Group 2). Lysozyme activity was marginally higher ($p > 0.05$) at pH 6.2 than at pH 5.8 while both classical and alternative complement activities were higher in Group 1 fish but not statistically different ($p > 0.05$).

Results from PCA on the immunological measurements described above are shown in Table 3.7. Plots of the principal components showed clear separation between Group 1

and Group 2 fish (Figure 3.7) suggesting that the measured immune system indices vary with fish size or age.

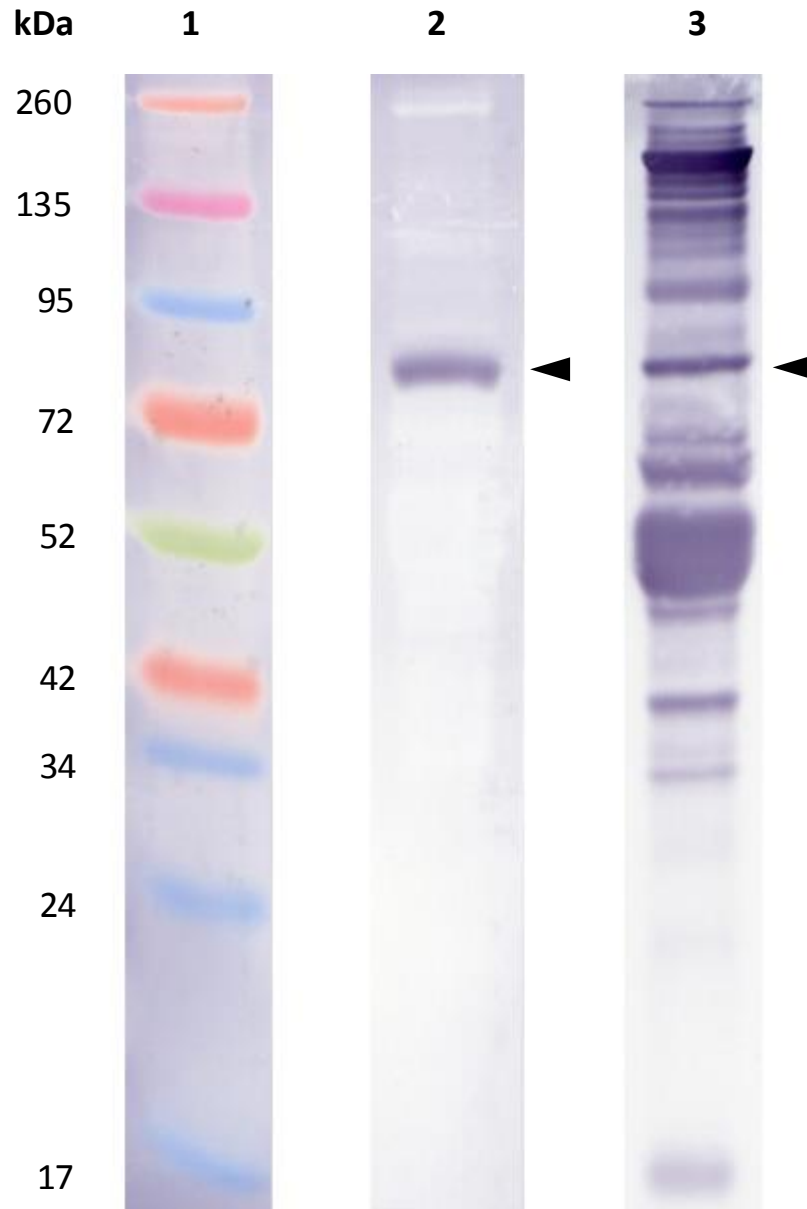


Figure 3.6 Immunoblot analysis of reactivity of monoclonal (mAb) and polyclonal antibody (Ab) against purified Atlantic bluefin tuna (ABT) IgM. Lane 1, molecular weight protein marker; Lane 2, immunoblot with anti-ABT IgM mAb; Lane 3, immunoblot with polyclonal Ab. Arrowheads show reactivity of the antibodies with purified IgM at a MW of approximately 74 kDa.

Table 3.6 Measured values for several immune parameters from farmed Atlantic bluefin tuna serum. Values represent mean \pm standard deviation (SD) and upper and lower ranges (Group 1, weight >100 kg, $n = 30$; Group 2, ≤ 100 kg, $n = 15$).

Parameter	Group 1		Group 2	
	Mean \pm SD	Range	Mean \pm SD	Range
Total IgM (mg mL ⁻¹)*	8.05 \pm 0.56	7.12-9.45	9.67 \pm 0.74	8.19-11.99
Lys pH 5.8 (U mL ⁻¹)	1,866 \pm 97	1,705-2,058	1,882 \pm 118	1,715-2,054
Lys pH 6.2 (U mL ⁻¹)	1,920 \pm 142	1,654-2,110	1,894 \pm 141	1,702-2,105
CH ₅₀ (U mL ⁻¹)	91 \pm 7	79-101	88 \pm 7	75-98
ACH ₅₀ (U mL ⁻¹)	52 \pm 3	48-59	50 \pm 4	46-58

* $p < 0.01$; Lys, lysozyme activity; CH₅₀, classical complement activity; ACH₅₀, alternative complement activity.

3.3.5. Haemagglutinating activity in Atlantic bluefin tuna serum

All serum samples tested showed strong haemagglutinating activity and titre values ranged from 16,384 to 32,768. There were no visible differences between the haemagglutinating capacities of serum from different size classes.

3.3.6. Bacterial inhibition in Atlantic bluefin tuna serum

Bacterial inhibition assays did not reveal any activity against bacterial growth in the serum samples tested. Positive controls (30 μ g oxytetracycline) inhibited the growth of all species tested.

Table 3.7 Univariate statistics for immunological parameters of 45 farmed Atlantic bluefin tuna specimens. The component loadings and the percentage of the variance explained by each variable ($n = 6$) for the first two principal factors (F1-F2) are shown and component loading values above ± 0.70 are shown in bold.

Variable	Mean \pm SD	CV (%)	F1	F2
Weight (kg)	213 \pm 104	0.12	0.962	0.061
Length (cm)	209 \pm 49	0.05	0.968	0.079
Lys (pH 6.2; U mL ⁻¹)	1911 \pm 139	0.03	-0.074	0.769
CH ₅₀ (U mL ⁻¹)	90.6 \pm 6.8	0.03	0.337	0.492
ACH ₅₀ (U mL ⁻¹)	51.6 \pm 3.7	0.03	0.472	-0.512
Total IgM (mg mL ⁻¹)	8.59 \pm 1.0	0.05	-0.891	0.003
Variance proportion (%)			50.0	18.4
Cumulative (%)			50.0	68.4

SD, standard deviation; CV, coefficient of variation; Lys, lysozyme; CH₅₀, complement activity; ACH₅₀, alternative complement activity.

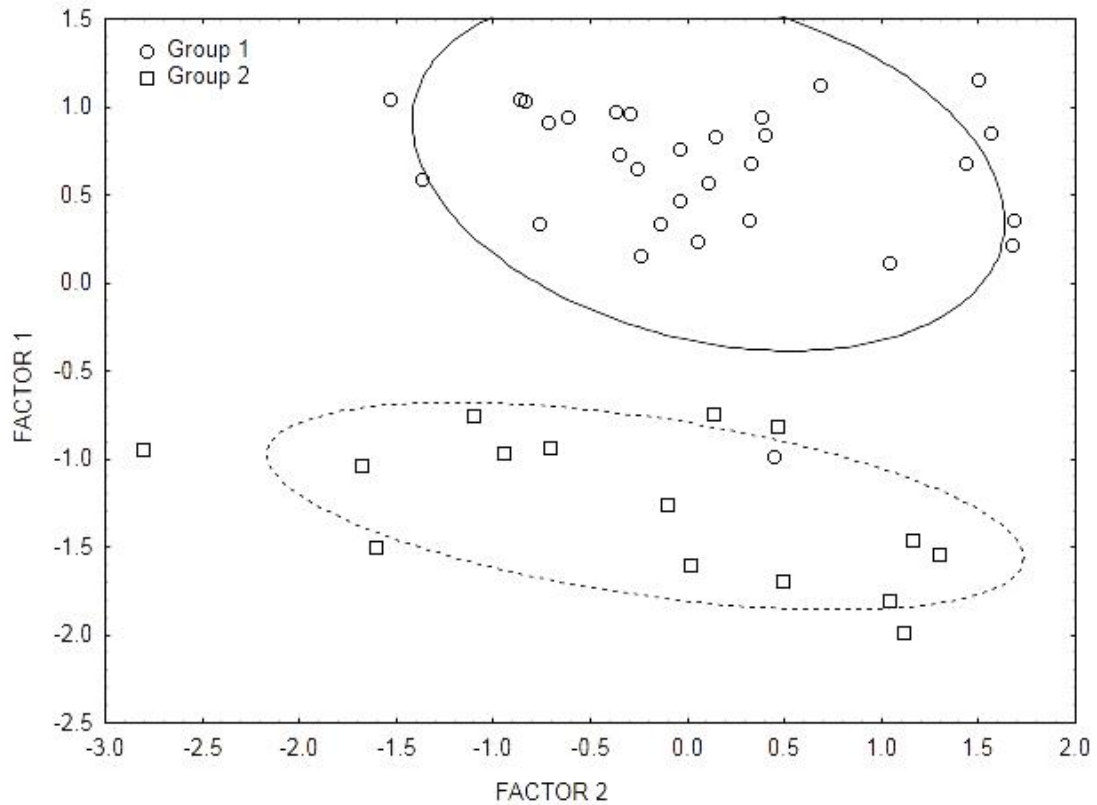


Figure 3.7 Plot of F1 and F2 generated by PCA on immunological measurements from the serum of 45 farmed Atlantic bluefin tuna specimens. There is separation between different size classes along F1. Ellipses represent 95 % confidence interval of the mean (Group 1, >100 kg, $n = 30$; Group 2, ≤ 100 kg, $n = 15$).

3.4. Discussion

Due to their sensitivity towards changes in physiological conditions (Dalmo *et al.*, 1997; Castillo *et al.*, 1998) reference values for haematological and biochemical indices may be applied in the evaluation of an organism's physiological and health status. Data on haematological parameters for farmed ABT, however, have never been investigated and limited data is available for plasma biochemical values.

Light microscope observations of stained peripheral blood smears have shown that both erythrocytes and leucocytes are morphologically similar to those described in other teleosts and those reported from other tunas including the SBT (Rough *et al.*, 2005), and yellowfin and skipjack tunas (Alexander *et al.*, 1980).

Comparison of the data obtained here to those published previously for tunas reveals similarities to certain species but differences from others. EC counts are similar to those reported for wild ABT (Becker *et al.*, 1958; Gutierrez, 1967, 1970). The ranges described here are also comparable to those from the SBT (Rough *et al.*, 2005) but lower than those reported for yellowfin and skipjack tuna (Alexander *et al.*, 1980).

Similar to results from other thunnids (Gutierrez, 1967, 1970; Alexander *et al.*, 1980; Rough *et al.*, 2005) high mean Hb levels (18.45 g dL⁻¹) were measured in farmed ABT in this study. Values for Hct and Hb agree with those of Rough *et al.* (2005) for wild caught SBT but are lower (Gutierrez, 1967, 1970; Alexander *et al.*, 1980) or higher (Brill *et al.*, 1998; Lowe *et al.*, 1998, 2000; Brill & Bushnell, 2001) than reported in other tuna species. Published reports of haematological values in tunas to date have mostly relied on the capture of wild specimens and it is well known that tunas demonstrate high activity when captured using traditional line methods. In contrast, the data reported here originates from specimens that have been killed by a shot to the head so that death is immediate and sample retrieval is quick. Stress has been reported to increase the Hct value in tuna species (Wells *et al.*, 1986; Brill & Bushnell, 1991; Bushnell & Brill, 1991) and Gallagher *et al.* (2001) have described the splenic release of erythrocytes and the consequent increase in Hct values in chinook salmon following increased activity. Taking this into account the Hct values reported here may better reflect the actual *in vivo* values but a firm conclusion will be presumptive since this may also be due to inter-species differences, measurement error or the actual physiological status of the specimens.

High EC counts, Hb and Hct values are characteristics of active fish (Cao & Wang, 2010; Satheeshkumar *et al.*, 2012). MCV (a measure of EC volume), MCH (a measure of the amount of Hb per EC) and MCHC (a measure of Hb concentration in a standard volume of EC) were calculated using standard equations to identify variability between the haematological properties of ABT and other teleosts. These indices have their basis on EC counts, Hb and Hct values and the latter are normally lower in non-thunnid species (Sandnes *et al.*, 1988; Sadler *et al.*, 2000; De Pedro *et al.*, 2005; Shigdar *et al.*, 2007; Cao & Wang, 2010; Hrubec & Smith, 2010). MCV lies towards the median of the accepted ranges while MCH and MCHC fall outside the higher ranges normally reported from other teleosts (Hrubec & Smith, 2010). This denotes that mean EC size is comparable to other fish species but the amount of Hb contained within a cell and a given volume respectively is much higher, reflecting a necessity for the blood to have a high oxygen-carrying capacity. In fact there exists an inverse relationship between EC size and the aerobic swimming ability of fish (Lay & Baldwin, 1999) and these haematological features allow tunas to support their high metabolic rates (Brill & Jones, 1994) which have been documented extensively (Korsmeyer *et al.*, 1996).

LC counts in farmed ABT may be considered as relatively high but still fall within ranges described from other fish species (Hrubec & Smith, 2010). In decreasing order of frequency, the LCs detected were lymphocytes, thrombocytes, eosinophils, neutrophils and monocytes and this follows patterns seen in other tuna species (Becker *et al.*, 1958; Alexander *et al.*, 1980; Rough *et al.*, 2005). Lymphocytes are one of the central components of defensive responses and have roles in Ab production and regulating the amount of circulating macrophages in the blood in outbreaks of infection (Rough *et al.*,

2005; Roberts & Ellis, 2012) so that their high presence may aid in the development of an immune response under such circumstances. Thrombocytes are important in the blood clotting process and their presence in tuna blood should promote rapid clotting when required (Casillas & Smith, 1977; Roberts & Ellis, 2012). The relative percentages of monocytes, eosinophils and neutrophils were low compared to the major two cell types as has been reported in other *Thunnus* spp. (Saunders, 1966; Alexander *et al.*, 1980; Hine *et al.*, 1987). All of these cell types have phagocytic roles (Ainsworth, 1992; Roberts & Ellis, 2012) and neutrophils are thought to mediate acute inflammatory responses (Nagamura & Wakabayashi, 1983; Suzuki, 1986; Suzuki & Hibiya, 1988).

The haematological indices were subjected to PCA and this statistical treatment resulted in the separation of fish according to their weight (Figures 3.4, 3.5, 3.7). Additionally, one-way ANOVA has shown significant differences in EC numbers, Hb, MCH and MCHC between the two size groups. Both in the wild and in captivity, smaller tunas are observed to be more active and swim at a quicker pace than larger individuals (personal observations). Higher activity in fish is marked by high Hb levels and a lower MCV (Lay & Baldwin, 1999) and this may be the reason behind the higher Hb levels measured in the smaller weight class. Although no statistical differences were found in MCV between the two groups, the index calculated for the smaller fish was marginally smaller than that for the other weight class.

Blood is essential for homeostasis and proper physiological function so that it is expected to reflect the general status of an animal. Consequently, conditions that impact or require change in an organism's physiology are likely to result in changes in several haematological variables. The influence of these factors on the haematology of

several teleost species has been detailed in several reports. For example disease outbreaks and general husbandry stress have been reported to result in changes of EC levels, Hct and Hb levels (Harbell *et al.*, 1979; Waagbo *et al.*, 1988; Pagés *et al.*, 1995; Řehulka, 2002; De Pedro *et al.*, 2005; Skov *et al.*, 2011) and modify circulating LC numbers (Ellis, 1981; Roubal, 1986; Møyner *et al.*, 1993; Tierney *et al.*, 2004; Martins *et al.*, 2009). Pollutants also cause similar changes (Witters, 1986; Nussey *et al.*, 1995a, b).

Apart from the effects above there are also changes brought about naturally without the influence of external stressors. Changes in haematological variables have been linked to season (Sandnes *et al.*, 1988; De Pedro *et al.*, 2005; Rough *et al.*, 2005; Pascoli *et al.*, 2011), environmental temperature (Langston *et al.*, 2002; Magill & Sayer, 2004), age (Svetina *et al.*, 2002), nutritional status (Řehulka & Adamec, 2004) and photoperiod (Leonardi & Klempau, 2003). De Pedro *et al.* (2005) also reported diel rhythms in tench, *Tinca tinca* (L.).

Plasma biochemical values have a similar use to haematological parameters in fish health diagnosis and management (De Pedro *et al.*, 2005; Cao & Wang, 2010), if suitable biochemical ranges are available. Data on the serum biochemical values of ABT is restricted and there is an element of uncertainty in comparisons with other teleosts considering that such values are affected by sampling and measurement variables (Korcock *et al.*, 1988; Barham & Schoonbee, 1990), and environmental factors (Lochmiller *et al.*, 1989; LeaMaster *et al.*, 1990; Bayir *et al.*, 2007; Campbell, 2012).

Most of the serum biochemical values reported here are higher than those described for farmed ABT of a similar weight range from the Adriatic Sea (Popovic *et al.*, 2008) but the particular causes for this are difficult to identify. In contrast to this, the electrolyte

levels measured in this study compare well with those described by Popovic *et al.* (2008) and with those reported from smaller (mean weight = 59 kg) captive ABT in Turkey (Percin *et al.*, 2010). The serum electrolyte values are also similar to those from other fish species (Liu *et al.*, 2007) and this inter-species conservation demonstrates their importance in osmotic, ionic and acid-base regulation in teleosts. In fact serum electrolyte levels are the best understood among biochemical parameters in fish (Hrubec *et al.*, 1996) and may be used to investigate gill function or damage to the branchial epithelium, and are also indicators of stress (Campbell, 2012). Gills are also the site of urea excretion so that electrolyte levels in serum may reflect improper function (Campbell, 2012) or elevated protein breakdown in outbreaks of disease (Řehulka, 2002).

Cholesterol levels were found to be towards the lower range of those generally reported in teleosts (Sandnes *et al.*, 1988; Lee *et al.*, 2003; Liu *et al.*, 2007; Cao & Wang, 2010). The long-term effects of these levels on the health status of fish is unknown (Campbell, 2012) although they have been shown to decrease in clinically diseased non-thunnid species (Řehulka, 2002), may be important indicators towards the nutritional status of the fish (De Pedro *et al.*, 2005) and do not fluctuate greatly with age (Svetina *et al.*, 2002). Total cholesterol and triglyceride levels in farmed ABT have been reported to be higher than those in wild specimens so that it might be affected by nutritional intake (Jelavic *et al.*, 2012). Cholesterol levels measured from farmed ABT in this study are similar to comparable sized specimens in Turkey (Percin *et al.*, 2010) but higher than those reported for farmed ABT from Croatia (Popovic *et al.*, 2008).

Bilirubin, which originates from the breakdown of Hb in the liver, and ammonia, alanine and aspartate aminotransferases are linked to liver function but there is little information on expected ranges in blood from teleosts (Vangen & Hemre, 2003; Zhang *et al.*, 2010a; Campbell, 2012). Therefore, reliable ranges for bilirubin may act as indicators of proper liver function and are useful in assessment of physiological status.

Albumin and globulin form part of the total serum protein and the latter has been shown to decline in diseased salmonids (Mulcahy, 1971) and carp (Harikrishnan *et al.*, 2003) and a similar pattern was observed in fish with restricted nutritional intake (Peres *et al.*, 2013). It has also been linked to feed intake in tunas where higher concentrations were found in wild specimens (Jelavic *et al.*, 2012). As for glucose levels, serum protein concentration reflects the nutritional state of the fish (Vangen & Hemre, 2003) and may also be useful in assessing fish welfare (Coourdacier *et al.*, 2011).

Blood glucose concentration is associated with fish activity and more active species possess higher blood glucose concentrations (Cao & Wang, 2010). The levels measured in this study are relatively high when compared to available data (Popovic *et al.*, 2008; Jelavic *et al.*, 2012). It is difficult to identify a reason for this finding considering that glucose levels are affected by environmental stressors (Svoboda *et al.*, 2001; Lermen *et al.*, 2004), age (Hrubec *et al.*, 2001; Coz-Rakovac *et al.*, 2005) and nutrition (De Pedro *et al.*, 2005) but these high values may mirror the high activity shown by the species.

There was pronounced inter-specimen variation in the plasma biochemical values of the enzymes alanine and aspartate aminotransferases, and alkaline phosphatase. It is known that plasma biochemical ranges vary considerably in teleosts and it is difficult to identify a cause for this since such variation is brought about by several endogenous and

exogenous factors (Campbell, 2012). Amongst the three enzymes, alanine and aspartate aminotransferases had the lowest and highest concentrations respectively, and this order of concentration follows that observed in other species (Ferguson, 2006).

Statistical analysis of the data has demonstrated several weight-based differences in the biochemical values measured. Glucose levels were found to be marginally, but not significantly, higher in fish of smaller weight and this is in agreement with the results of other research on farmed ABT (Jelavic *et al.*, 2012). Differences were relatively pronounced when considering that PCA conducted on biochemical values showed clear separation between fish from different groups suggesting that tunas of different weights and age have differing physiological requirements, and in consequence differing biochemical indices.

Several precautions were taken to enable meaningful comparison of haematological and biochemical parameters between fish of different weights. All the specimens were kept together from the time of capture until they were harvested so that culture conditions were identical for all fish used in this study. Furthermore, all samples were collected on the same day and within the shortest timeframe possible so that readings would represent a single time point and therefore not be subject to the effect of external factors or other stressors. As in all other work with tuna species, it is difficult to carry out controlled experiments and sample collection is generally opportunistic. Nonetheless, the results obtained here compare well with those reported from other tuna species and add information to the limited data on useful haematological and plasma biochemical ranges in tuna species and may contribute to the establishment of normal reference parameters in ABT.

Immunoglobulin M (IgM) is the predominant Ab in the serum of teleosts (Bengtén *et al.*, 2000). Purified IgM isolated from ABT serum was screened with an anti-ABT IgM mAb by Western blot analysis. The mAb showed reaction with the heavy chain of the IgM molecule, showing a single band at a MW of approximately 74 kDa. Expectedly, this is the same MW reported for the heavy chain of SBT (Watts *et al.*, 2001b) and that of other teleosts including Atlantic salmon (Havarstein *et al.*, 1988; Magnadóttir, 1998).

ELISA results have shown that IgM levels in ABT serum varied between 8 and 9.5 mg mL⁻¹ serum in the specimens used in this study. Watts *et al.* (2002) reported lower levels ranging from 0.5 to 5 mg mL⁻¹ in sera from SBT. Data for other species were also relatively lower than those measured for ABT in this study (Magnadóttir, 1998; Nielsen, 1999; Lange *et al.*, 2001) but higher levels have been measured in cod, *Gadus morhua* L. (see Magnadóttir, 1998).

Given the limited availability of data on IgM levels in tuna species, it is difficult to draw conclusions from the results obtained here, considering that IgM concentrations are affected by several factors. Results from studies on other teleosts have shown IgM to fluctuate with age (Matsubara *et al.*, 1985; Klesius, 1990; Sanchez *et al.*, 1993; Estévez *et al.*, 1995; Magnadóttir *et al.*, 1999b), environmental conditions (Klesius, 1990; Magnadóttir *et al.*, 1999a) and disease status (Magnadóttir *et al.*, 1995). In SBT higher levels have been measured in serum from the same cohort when sampled in winter when sea temperature was lower (Watts *et al.*, 2001a).

Husbandry limitations make investigations into the effect of such exogenous factors on IgM levels difficult since no suitable methods are presently available to maintain ABT under controlled conditions. Higher IgM levels were measured from smaller fish as

compared to larger specimens and this differs from several studies showing increasing IgM levels with increasing fish age. It is not possible to isolate a single cause for this considering that the fish were being kept under uncontrolled conditions, but since the specimens were sampled at a single time point to reduce the effects of exogenous modulators, these variances may reflect differences between the immune status or parameters of fish of different age. It is also possible that captivity affects smaller individuals to a larger extent so that their immune response is more pronounced.

Investigations into the innate immune parameters of ABT in this study have confirmed that serum possesses complement, lysozyme and haemagglutinating activities as described in other teleosts (Alexander & Ingram, 1992). These immune components have been reported already from SBT (Watts *et al.*, 2002) and complement activity has been demonstrated in yellowfin (Giclas *et al.*, 1981). Activities of all three parameters were relatively high although it is difficult to compare these against published results due to the use of different assay procedures.

Using identical methods to those described in Watts *et al.* (2002), the activities of both the alternative and classical complement pathways were detected at levels similar to those found in SBT in the same study. The classical pathway, which showed the higher activity of the two, is activated by the antigen-Ab complexes (Yano, 1996) but may also be activated by acute phase proteins such as C-reactive protein, viruses and bacteria (Holland & Lambris, 2002). In contrast, the alternative pathway is directly triggered through the presence of foreign bodies such as the lipopolysaccharides of Gram-negative bacteria (Yano, 1996). Teleost complement has been shown to be bactericidal (Sakai, 1992), virucidal, opsonic and possess detoxifying properties (Yano, 1996) so that

it is an important part of the innate immune response. Complement activation generally results in a depletion in the levels of complement proteins in the blood and synthesis of the individual components happens at a slow rate so that their activities may be useful as indicators of the health status of an organism (Yano, 1992).

Similar to findings in the SBT (Watts *et al.*, 2002), lysozyme activity was elevated but there were no significant differences between the activities of lysozyme at different pH. Lysozyme has been detected in several teleosts (Alexander & Ingram, 1992) and among its more important functions is its anti-bacterial and cell lysis activities (Yano, 1996). A high titre value causing visible haemagglutination against SRBC was observed for the serum samples. Haemagglutination is mediated by lectins, proteins originating from outside the immune system, and these have been shown to inhibit the growth of virulent bacteria although their function is largely unknown (Yano, 1996).

The humoral immune parameters measured in this study are affected by several exogenous factors including size of the fish, culture conditions, environmental temperature, and nutritional and disease status of the fish (Alexander & Ingram, 1992; Montero *et al.*, 1998; Magnadóttir *et al.*, 1999a, b; Montero *et al.*, 1999; Nikoskelainen *et al.*, 2004; Dominguez *et al.*, 2005). Although not strictly endothermic, tunas are capable of maintaining an elevated body temperature with respect to their environment (Graham & Dickson, 2001) so that the influence of environmental temperature on their physiology is, to a certain degree, controlled. This has been substantiated by Watts *et al.* (2002) who measured higher immune activities in the sera of SBT acclimated to lower sea temperatures. There are no reports of disease outbreaks or mass mortalities in adult

captive ABT and this may be due to the quicker response of the immune system made possible by a constantly elevated body temperature.

PCA of the immunological parameters measured here resulted in separations identical to those obtained for haematological and plasma biochemical parameters. Except for IgM levels, none of the other parameters were found to differ significantly between Group 1 and Group 2 fish, but PCA gave two separate clusters. As for the above, the degree of clustering between the different groups suggests that important innate immune parameters in the serum vary with fish size or age.

Serum in bacterial inhibition assays did not demonstrate antibacterial properties against the fish pathogenic bacteria tested in this study. This may signify that either the serum did not possess any bacteriolytic activity against the specific bacterial strains used or that additional components, necessary for the promotion of antibacterial activity, were absent or not present at sufficient concentrations.

3.5. Conclusion

This study is the first report of several haematological indices in farmed ABT. Blood from ABT has been shown to have special features that enable it to function in support of the high metabolic demands of the species. The activities of lysozyme, complement and haemagglutination were measured and found to be considerably high, although comparisons with published reports are difficult to perform. Little can be inferred from the plasma biochemical values measured but these add to limited data on these parameters in these species. Haematological parameters may find use in the

assessment of fish welfare, and the data reported in this study should contribute in this regard.

Owing to restrictions on experimental design and specimen numbers, few conclusions can be made from the results. Notwithstanding, statistical analysis has shown that there are clear differences in the haematology of captive ABT based on their size but further investigations in this field are required to determine the reasons leading to such separation between size classes.

CHAPTER 4

NON-SPECIFIC HUMORAL IMMUNE COMPONENTS IN ATLANTIC BLUEFIN TUNA EPIDERMAL MUCUS

4.1. Introduction

The body surface of teleosts is covered by epidermal mucus, a biochemically diverse secretion that acts as a distinctive physical and chemical barrier between the organism and the aquatic environment which it inhabits. Amongst other functions, it has important roles in maintaining fish health and is involved in ionic and osmotic regulation, excretion and disease resistance (Shephard, 1994). Mucus is mainly produced by the epidermal goblet cells, which are sparingly distributed along the fish epidermis (Pickering, 1974; Laurent, 1984), but is supplemented with secretions from other subepithelial cells or glands (Shephard, 1994; Thornton & Sheehan, 2004).

The main structural components of mucus are high molecular weight glycoproteins (\approx 106 kDa) referred to as mucins (Pickering, 1974; Fletcher *et al.*, 1976; Tabak, 1995). They are similar in structure to mammalian mucins (Alexander & Ingram, 1992) and are capable of inter-molecular interactions so that they maintain a three-dimensional structure in water (Shephard, 1994).

Teleosts are ectothermic and their adaptive immune response is slow and restricted by the effects of external factors on their metabolism (Bly *et al.*, 1988), so they rely on non-specific immune defences for initial protection against potential pathogens (Alexander & Ingram, 1992). Fish are also more susceptible to bacterial infection since their skin is non-keratinised and offers less resistance to microbial entry (Suzuki, 1985). The epidermal mucus of fish plays a significant role in this regard. Firstly, it prevents foreign objects from coming into direct contact with the epidermal layer maintaining the structural integrity of the latter (Shephard, 1994) and it is impermeable to most bacterial pathogens (Cone, 2009). In addition, constant mucus production ensures that pathogens are immobilised and shed prior to their contact with the epithelial surfaces (Pickering, 1974).

Apart from mechanical protection, fish skin mucus also confers humoral protection and is a key component of the innate immune defence of fish (Ellis, 2001). Several elements of the innate immune system are known to exist in the mucus of teleost fish, and these include lysozyme, immunoglobulins (Ig), complement, antimicrobial proteins, several enzymes and lectins (Hjelmeland *et al.*, 1983; Alexander & Ingram, 1992; Bergsson *et al.*, 2005; Uribe *et al.*, 2011).

Natural antibodies in fish exist mainly as IgM isotypes (Lobb & Clem, 1981; St. Louis-Cormier *et al.*, 1984; Salinas *et al.*, 2011) and are found in serum and mucosal secretions of the skin and gut (Ellis, 2001). They are relatively non-specific and bind with pathogens even without prior exposure (Baumgarth *et al.*, 2005), and are involved in complement activation and pathogen neutralisation among other functions. They have been reported in the skin mucus of several teleost species including carp (Rombout *et al.*,

1993), Atlantic salmon (Hatten *et al.*, 2001), channel catfish, *Ictalurus punctatus* (Rafinesque) (see Lobb, 1987), and olive flounder, *Paralichthys olivaceus* (Temminck et Schlegel) (see Palaksha *et al.*, 2008), and their presence in epidermal skin secretions must provide some form of protection against invading pathogens.

Another type of Ig, termed IgT/IgZ, has been discovered in skin mucus of rainbow trout (Hansen *et al.*, 2005), and zebrafish, *Danio rerio* (Hamilton) (see Danilova *et al.*, 2005), respectively. IgT has also been reported to be the major Ig involved in mucosal immunity in rainbow trout (Zhang *et al.*, 2010b; Xu *et al.*, 2013).

Lysozyme (*syn.* muramidase) is a bacteriolytic enzyme found widely within living systems including fish (Jollès & Jollès, 1984; Alexander & Ingram, 1992). Its lytic action lies in its ability to hydrolyse linkages in the cell walls of bacteria and its presence in cutaneous mucus provides a degree of protection against harmful agents. Other enzymes described in teleost mucus and known to be involved in fishes' natural resistance to infection include proteases and cathepsins (Ingram, 1980; Alexander & Ingram, 1992; Ellis, 2001; Fast *et al.*, 2002) and these have been reported from the epidermal mucus of several different fish species (Alexander & Ingram, 1992; Aranishi & Mano, 2000b; Subramanian *et al.*, 2007; Palaksha *et al.*, 2008; Nigam *et al.*, 2012). These enzymes cleave protein structures within bacteria, thus reducing bacterial progression, and in turn limiting the opportunity for the bacteria to infect their host (Ingram, 1980). They may also act indirectly and are known to activate or enhance synthesis of complement, Ig and antimicrobial peptides, and modify mucus consistency to increase its shedding rate (Aranishi *et al.*, 1998; Cho *et al.*, 2002a; Morrissey, 2013). Alkaline phosphatase is another innate immune component identified in teleost mucus,

and its levels have been shown to increase in Atlantic salmon mucus during infection with the salmon louse, so levels of the enzyme may act as a potential stress indicator (Ross *et al.*, 2000). They are also considered to provide some form of protection during initial stages of wound healing (Iger & Abraham, 1990).

The complement system is another non-specific response elicited by the vertebrate innate immune system, and has also been reported from the skin mucus of rainbow trout (Harrell *et al.*, 1976). It is activated by several external agents such as Gram-negative bacteria, and its activation stimulates the production of peptides necessary for inflammatory responses (Yano, 1996).

Agglutinins (lectins) are carbohydrate-binding proteins of non-immune origin able to agglutinate cells, glycoconjugates and molecules (Denis *et al.*, 2003). They have been detected in several fish species, but their function in epidermal mucus is not fully understood although it has been suggested that their action may aid in the prevention of epidermal colonisation by bacterial pathogens (Ingram, 1980).

At present there is no information on the mucosal immunity of the ABT. Furthermore, to date there are no reports of disease outbreaks in farmed ABT. The main objective of this research was to examine the mucus from this tuna species for the presence of non-specific immune factors, quantify their activity and analyse their antibacterial properties. Additional information on enzyme levels may be useful in determining the health of individuals and may aid in the determination of the physiological status of the fish under captive conditions.

4.2. Materials and Methods

4.2.1. Collection and processing of Atlantic bluefin tuna skin mucus

Twelve ABT specimens ranging in weight from 220 to 380 kg, held in offshore cages in Malta (35°49'N, 014°35'E), were killed by a shot to the head and transferred to a landing boat. The fish were rinsed with running seawater and left untouched for a few minutes to allow the secretion of fresh epidermal mucus. Mucus was collected with a sterile spatula moved in a head to tail direction from the dorsal surface of the fish, transferred aseptically to cryovials and stored at -70°C until used. During collection adequate care was taken to prevent damage to the epidermal surface in order to prevent contamination with blood or other subepidermal secretions. Additionally, no mucus was collected from the ventral surface of the fish to prevent contamination of the specimens with intestinal flora. Sea temperature at time of collection was 17°C and gross external and internal inspection of the specimens did not reveal any clinical signs of disease.

The crude mucus was purified in the laboratory following methods adapted from Palaksha *et al.* (2008). The mucus samples were thawed, pooled and homogenised (Ultra-Turrax, IKA, Staufen, Germany) with four volumes of Tris-buffered saline (TBS; 50 mM Tris-HCl, 150 mM NaCl, pH 8.0). The homogenate was centrifuged at 4,000 ×g for 25 min at 4°C. The supernatant was collected, transferred to freeze-drying tubes and lyophilised overnight. The lyophilised powder was reconstituted in TBS buffer and centrifuged at 4,000 ×g for 30 min at 4°C to remove insoluble mucus components. It was passed through a 0.45 µm syringe filter (Minisart, Sartorius, Surrey, UK), transferred to 3 kDa centrifugal filter units (Amicon Ultra-25, Millipore) and concentrated according to the manufacturer's instructions at 4°C. The mucus extract (ME) collected was aliquoted

and stored at -70°C until use. All the procedures above were carried under controlled temperature conditions to minimise protein degradation and preserve enzyme activities.

The protein concentration of the extracts was determined spectrophotometrically with a protein assay kit (BCA protein assay kit, Pierce Biotechnology) using bovine serum albumin (BSA) as standard and based on the colorimetric detection and quantification of total protein using bicinchoninic acid (Smith *et al.*, 1985). Briefly, this involved the preparation of a linear standard curve from the BSA standard provided with concentrations 0-2,000 µg mL⁻¹. Twenty five microlitres of each standard or ME were placed into triplicate wells of a microtitre plate (Sterilin). Two hundred microlitres of the assay kit working reagent were added to each well, the plate was shaken for 30 sec and then incubated at 37°C for 30 min. The plate was then cooled to room temperature (RT; 22°C) and read at 562 nm in a microplate reader (Synergy HT, BioTek). Readings (with blanks subtracted) were used to calculate the protein concentration of the ME.

4.2.2. Measurement of immunoglobulin in the mucus extract

4.2.2.1. Total Ig

Total Ig in the ME was measured by Ig precipitation using methods described by Siwicki *et al.* (1994). The Ig was precipitated out of the ME by mixing 100 µL with an equal volume of polyethylene glycol in a microplate and incubating for 2 hrs at 22°C with constant agitation. The precipitate was separated by centrifugation at 3,000 ×g for 15 min. The protein concentration of the supernatant was measured as described in **Section 4.2.1** and the amount of Ig precipitated out was calculated by subtracting the remaining protein concentration from that of the ME.

4.2.2.2. Total IgM

The ME was assayed for IgM using an ELISA. An IgM standard curve was prepared from purified ABT IgM isolated from serum (see **Section 3.2.3.1**). ME was adjusted to an initial protein concentration of 100 $\mu\text{g mL}^{-1}$ in coating buffer and 2-fold serial dilutions made in the same buffer (*i.e.* assay protein concentrations at 10, 5 and 2.5 $\mu\text{g well}^{-1}$). ELISA procedures were as described in **Section 3.2.3.3**. Negative controls (blanks) consisted of wells without added ME, primary or secondary Ab. Blank-adjusted readings were used to construct an IgM standard curve. The standard curve was used to obtain IgM levels in the ME.

4.2.3. Electrophoresis and western blotting

The protein composition of the ME was examined by SDS-PAGE and analysed for IgM with western blotting.

4.2.3.1. SDS-PAGE

ABT ME was subjected to SDS-PAGE under denaturing conditions. The TBS buffer in the ME was replaced with DNase/RNase-free water using 3 kDa centrifugal filter devices (Amicon Centricon, Millipore). Ten microlitres of the desalted ME were added to an equal volume of 2 \times SDS-PAGE sample buffer (125 mM Tris-HCl, 20 % glycerol, 0.4 % w/v SDS, 200 mM dithiothreitol, 0.01 % bromophenol blue, pH 6.8), heated for 3 min at 95°C, cooled on ice and centrifuged at 4,000 $\times g$ for 3 min.

A 12 % SDS gel was prepared for protein separation. The separating gel buffer for the SDS gels was prepared by mixing 5 mL separating buffer (1.5 M Tris, 0.4 % SDS), 7 mL dH₂O and 8 mL 30 % acrylamide/bis-acrylamide solution (Severn Biotech, Kidderminster,

UK), and degassed for 15 min *in vacuo*. Fifteen microlitres tetramethylethylenediamine (TEMED; Fisher Scientific) and 70 μ L of 10 % ammonium persulphate (Sigma) were added to the buffer prior to pouring into 10 \times 8 cm gel casters (Mighty Small, GE Life Sciences). Two hundred microlitres of iso-butanol (Fisher Scientific) were layered on top, the gel was allowed to polymerise at 22°C for 1 h and then washed with dH₂O prior to the addition of a 4 % stacking gel. This was prepared by mixing 2.5 mL stacking gel buffer (0.5 M Tris, 0.4 % SDS, pH 6.8), 6.1 mL dH₂O and 1.34 mL 30 % acrylamide/bis-acrylamide solution and degassing for 15 min. Ten microlitres TEMED and 50 μ L 10 % ammonium persulphate were added to the solution prior to pouring onto the substrate gels. After gel polymerisation was complete the gels were transferred to an electrophoresis apparatus.

Ten microlitres each of pre-stained broad range protein markers (Spectra Broad Range Protein Ladder, Thermo Scientific) and sample (10 μ g protein) were loaded onto the gel, and the proteins subjected to 60 V for the first 15 min followed by 160 V until the dye front reached the bottom of the gel.

The gels were stained overnight with constant agitation in 0.25 % (w/v) Coomassie Blue R-250 (Fisher Scientific) in ethanol/acetic acid/dH₂O (50:10:40) and destained with ethanol/acetic acid/dH₂O (40:10:50) until the desired band resolution was achieved. For increased staining sensitivity the gels were treated with a silver stain kit (ProteoSilver silver stain kit, Sigma) according to the manufacturer's instructions. In brief, the polyacrylamide gel was fixed overnight with constant agitation in ethanol/acetic acid/dH₂O (50:10:40). The gel was washed in 30 % ethanol for 10 min followed by a 10 min wash in ultrapure H₂O. The gel was incubated in a 1 % sensitising solution for 10

min, washed twice for 10 min in H₂O and equilibrated with a 1 % silver solution for 10 min. The gel was briefly washed in H₂O and developed (developing solution: 5 mL Developer 1, 100 µL Developer 2, 99 mL H₂O) for 4 min. Once band staining was adequate, development was stopped by the addition of 5 mL stop solution, incubated for 5 min and then washed in H₂O. Relative mobility (R_f) values were calculated to estimate band MW.

4.2.3.2. Western blotting

Additional electrophoresis procedures were performed on the ME for immunoblotting. Sample preparation prior to loading onto the gel was as described in **Section 4.2.3.1**.

The proteins were separated with an Amersham ECL gel system (GE Healthcare) according to the manufacturer's instructions. Electrophoresis of the ME was performed on a 10 % precast polyacrylamide gel (GE Healthcare). Electrophoresis and western blotting procedures were as described in **Section 3.2.3.2**.

4.2.4. Evaluation of enzymes and their activity in the mucus extract

4.2.4.1. API-ZYM kit assay

The ME was examined for the presence of various enzymes using an API-ZYM kit (Bio-Merieux, Basingstoke, UK) as per the manufacturer's instructions with modifications (Palaksha *et al.*, 2008). In summary, the ME was diluted to a protein concentration of 1 mg mL⁻¹ and 100 µL of this was added to each cupule. The strip was incubated for 6 h at 25°C in a humidified API strip container. Following incubation, one drop each of ZYM-A and ZYM-B reagents were added to each cupule and the strip was incubated for 5 min

at 22°C. The strip was briefly placed under intense light and the results were recorded according to colour change and intensity according to the manufacturer's instructions.

4.2.4.2. Protease activity: azocasein hydrolysis assay

Protease activity in the ME was measured by an azocasein hydrolysis assay as outlined in Ross *et al.* (2000). The ME was mixed with an equal volume of 100 mM ammonium bicarbonate buffer containing 0.7 % azocasein (Sigma) in a microplate and incubated for 19 h at 30°C with constant agitation. The reaction was stopped by the addition of trichloroacetic acid to a final acid concentration of 4.4 % (Fisher Scientific), cooled on ice, and centrifuged at 5,000 ×g for 5 min. One hundred microlitres of the supernatant was transferred to microplate wells containing an equivalent amount of 0.5 M NaOH and the absorbance measured at 450 nm in a microplate reader. Positive and negative controls were trypsin (Sigma) and assay buffer respectively. The protease activity was quantified as the increase in absorbance.

4.2.4.3. Protease activity: zymography assays

Protease activity in the ME was examined by gelatin and β -casein substrate zymography with methods according to Ross *et al.* (2000) with modifications. A 10 % SDS gel was prepared from a 10 % separating buffer consisting of 5 mL buffer (1.5 M Tris, 0.4 % SDS), 8.35 mL dH₂O and 6.65 mL 30 % acrylamide/bis-acrylamide solution. Other procedures were as described in **Section 4.2.3.1** but 0.1 % gelatin (from porcine skin, Sigma G8150) or 0.15 % β -casein (from bovine milk, Sigma C6905) was added to the separating solution prior to pouring into gel casters.

For zymography, desalted ME was diluted with an equivalent volume of 2× non-reducing SDS sample buffer (125 mM Tris-HCl, 20 % glycerol, 0.4 % w/v SDS, 0.01 % bromophenol blue, pH 6.8). The sample was mixed thoroughly but not heated to preserve enzyme activity. The gel was loaded with 10 µL of pre-stained broad range protein markers (Thermo Scientific) and 15 µL sample (15 µg protein). The gel was also loaded with 2 µg trypsin (Sigma) in sample buffer as positive control and a sample buffer blank as a negative control. The gel was run at 60 V for the first 15 min and at 200 V until the dye front reached the bottom of the gel in 1× SDS running buffer. System temperature throughout the procedure was maintained at 4°C to preserve enzyme activity.

Following electrophoresis, the gels were washed three times for 5 min at 4°C with 50 mM Tris-HCl, pH 7.5 containing 2.5 % Triton X-100 (Sigma). After washing the substrate gels were incubated at 30°C for 19 h in the same buffer containing 5 mM MgCl₂ and 62.5 mM CaCl₂, with frequent shaking. The gels were stained in 0.1 % Amido Black (Sigma) in methanol/acetic acid/dH₂O (45:10:45) and destained with methanol/acetic acid/dH₂O (50:2:48) until adequate contrast was achieved.

4.2.4.4. Lysozyme activity

Lysozyme activity in the ME was measured using a turbidimetric assay (Ross *et al.*, 2000). Freeze-dried ME was reconstituted in 40 mM PBS, vortexed and centrifuged at 4,000 ×g for 5 min at 4°C to remove insoluble material. Twenty five microlitres of the reconstituted ME (0.45 mg mL⁻¹ protein) were added to triplicate wells with 175 µL of lysozyme assay substrate, containing 0.75 mg mL⁻¹ *Micrococcus lysodeikticus* (Sigma) suspended in 0.1 M PBS, in a 96-well flat-bottomed microplate. The reduction in absorbance at 450 nm was measured continuously every 5 min at 25°C in a microplate

reader. One unit (U) of activity was defined as the amount of enzyme that catalysed a reduction in absorbance of 0.001 min^{-1} at 450 nm and the initial rate of reaction was used to calculate lysozyme activity in the ME.

4.2.4.5. Alkaline phosphatase activity

Alkaline phosphatase activity in the ME was quantified by incubating 100 μL of ME with an equal amount of 4 mM *p*-nitrophenyl phosphate (Sigma) in 100 mM ammonium bicarbonate buffer with 1 mM MgCl_2 , pH 7.8 at 30°C as described by Ross *et al.* (2000). The increase in optical density (OD) at 405 nm was measured continuously for 3 h in a microplate reader. Alkaline phosphatase activity was calculated from the initial rate of reaction and one unit (U) of activity was defined as the amount of enzyme required for the release of 1 μmol *p*-nitrophenol product in 1 min. The extinction coefficient of *p*-nitrophenol under assay conditions was determined experimentally.

4.2.4.6. Esterase activity

Esterase activity in the ME was determined according to the method of Ross *et al.* (2000). One hundred microlitres of ME was incubated with 100 μL of 0.4 mM *p*-nitrophenyl myristate substrate (Sigma) in 100 mM ammonium bicarbonate buffer containing 0.5 % Triton X-100, pH 7.8 at 30°C. Absorbance readings were measured continuously for 3 h at 405 nm in a microplate reader. The extinction coefficient of *p*-nitrophenol under assay conditions was determined experimentally.

4.2.4.7. Cathepsin B activity

Cathepsin B activity in the ME was measured as described in Easy and Ross (2010). Five microlitres of ME was added to 50 μL of assay buffer (0.1 M NaH_2PO_4 , 0.08 % w/v Brij

35, 1 mM EDTA, pH 6.0), 20 μ L of 1 mM dithiothreitol and 60 μ L of ultrapure water in a 96-well microplate. The plate was incubated for 5 min at 30°C, and 50 μ L of 25 μ M carbobenzoxy-L-phenylalanyl-L-arginyl-4-methylcoumaryl-7-amide (Sigma) was added after incubation. The fluorescence of 7-amino-4-methylcoumarin (AMC) was measured continuously for 30 min at 30°C in a microplate reader. Excitation and emission wavelengths were 380 and 405 nm respectively. The extinction coefficient of AMC under assay conditions was determined experimentally and the initial rate of reaction was used to calculate enzyme activity. One unit (U) of activity was defined as the amount of enzyme required to release 1 μ mol of AMC in 1 min.

4.2.5. Alternative complement activity of the mucus extract

The ME was assayed for alternative complement by measurement of spontaneous haemolytic activity with methods adapted from Bagni *et al.* (2005). Defibrinated SRBC in Alsever's solution (Oxoid) were washed three times with 10 volumes of gelatin veronal buffer, prepared by dissolving a barbitone complement fixation tablet (Oxoid) and 0.1 g gelatin (Sigma) in 100 mL dH₂O, resuspended to 5 % (v/v) in the same buffer and adjusted for optimal absorbance. Twenty five microlitres of the 5 % SRBC suspension were added to serial dilutions of ME in 150 μ L gelatin veronal buffer in a microplate and incubated at 25°C for 100 min. One hundred percent lysis was obtained by adding an equal volume of 5 % SRBC suspension to 150 μ L dH₂O. Following incubation the plate was centrifuged at 1,500 \times g for 2 min and 50 μ L of supernatant was transferred to a clean microplate. Alternative complement activity was estimated by measuring the OD of the supernatant at 540 nm in a microplate reader.

4.2.6. Haemagglutinating activity in the mucus extract

The ME was tested for haemagglutinating activity in a round-bottom microplate (Sterilin) as outlined in **Section 3.2.3.6** but without heat treatment of the ME and with modifications from Palaksha *et al.* (2008). Fifty microlitres of ME was serially diluted with an equal amount of TBS and mixed with 25 μL of 2.5 % SRBC suspension. The microplate was incubated for 1 h at 24°C and the inverse of the highest dilution showing visible agglutination was expressed as the titre value.

4.2.7. Antibacterial activity of the mucus extract

Antibacterial activity in the ME was tested by a well diffusion assay in agar plates. The bacterial fish pathogens used were as described in **Section 3.2.3.7**. Tryptic soy agar plates (Oxoid) containing 1 % NaCl were inoculated with the bacterial culture and spread evenly with a sterile spreader. Twenty microlitres of ME, TBS buffer and dH_2O were added to separate 4 mm wells made with a sterile pipette. Growth inhibition was observed after a 24 h incubation period at 24°C.

4.3. Results

4.3.1. Characteristics of the mucus extract

The mean protein concentration of the ME as determined from the protein assay was 1,600 $\mu\text{g mL}^{-1}$, while that for the desalted extract prepared for electrophoretic procedures was 2,000 $\mu\text{g mL}^{-1}$. The extract was colourless.

4.3.2. Immunoglobulin in the mucus extract

The ME was separately assayed for total Ig and IgM using a chemical precipitation and a direct ELISA method respectively.

4.3.2.1. Total Ig

Mucus was assayed for total Ig by precipitation with polyethylene glycol. A protein assay was used to determine the protein concentration prior to and after precipitation and the difference between the two measurements was taken as the total Ig value in the ME. The absorbance of the ME at 562 nm prior to incubation with polyethylene glycol was measured as 1.227 ± 0.002 and decreased to 1.153 ± 0.002 after precipitation ($n = 3$). The protein assay standard curve ($y = 0.0011x + 0.0634$; $R^2 = 0.99126$) was used to calculate protein concentrations ($\mu\text{g mL}^{-1}$). Total Ig for the ME amounted to $67.9 \pm 3.73 \mu\text{g mL}^{-1}$.

4.3.2.2. Total IgM

Total IgM in the ME was quantified with an ELISA using an anti-ABT mAb. The IgM standard curve ($y = 0.3626x + 0.1366$; $R^2 = 0.977$) from the assay is shown in Figure 4.1. Blank-adjusted readings were used to calculate mean total IgM concentration in the ME and calculated values are given below (Table 4.1). Blank-adjusted control absorbance was 0.041 ± 0.001 . Absorbance readings for the 1:400 dilution approached the detection limit of the ELISA assay but the total IgM concentration derived from the equation is similar to those obtained for lower dilutions indicating that IgM in the ME was still adequately detected at such concentrations.

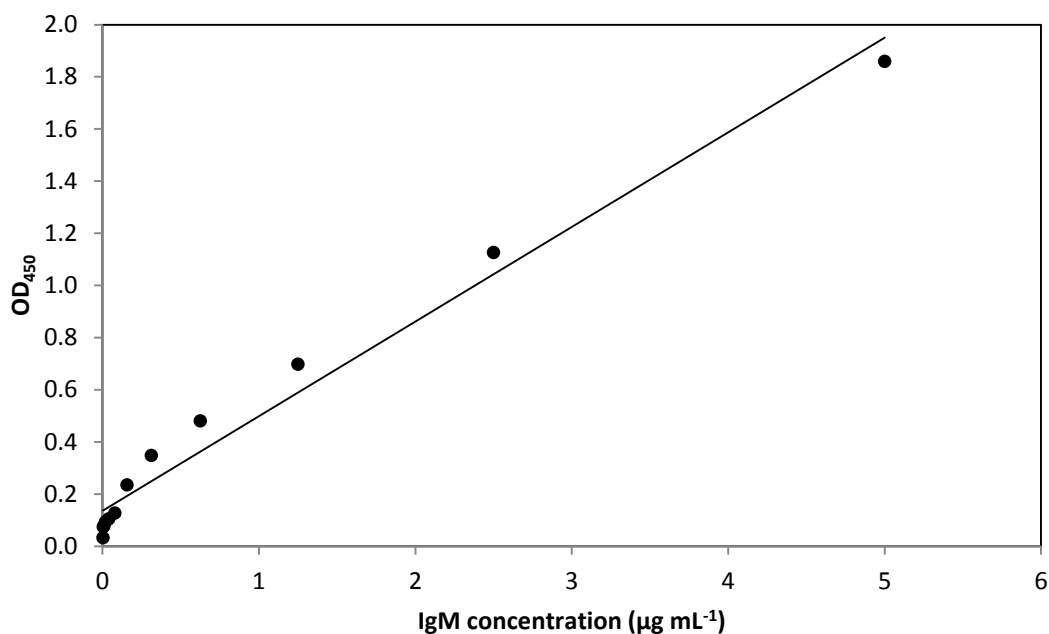


Figure 4.1 ELISA standard concentration curve obtained for Atlantic bluefin tuna IgM purified from serum. The equation ($y = 0.3626x + 0.1366$; $R^2 = 0.977$) was used to calculate IgM values in the mucus extract.

Table 4.1 Total IgM concentrations in the Atlantic bluefin tuna ME ($n = 3$). OD₄₅₀ values represent blank-adjusted absorbance values and are expressed as mean \pm standard deviation.

Protein ($\mu\text{g well}^{-1}$)	OD ₄₅₀	Total IgM ($\mu\text{g mL}^{-1}$)
10.0	0.314 ± 0.003	56.39 ± 0.81
5.0	0.215 ± 0.002	56.89 ± 0.94
2.5	0.158 ± 0.001	56.04 ± 1.38

4.3.3. Electrophoresis and western blotting

SDS-PAGE and western blot results for the ME are shown in Figure 4.2. Staining revealed a total of 10 bands with MW of 90, 70, 50, 42, 32, 30, 28, 26, 22 and 17 kDa. Highest band intensity was at the 42 kDa region and the remaining bands had a similar appearance. A relatively strong background was observed for both the Coomassie Blue and silver-stained gels.

Immunoblotting with the anti-ABT IgM mAb showed a very faint reaction at approximately 78-72 kDa on the membrane but negative results were obtained for the membrane treated with the anti-ABT polyclonal Ab (Figure 4.2).

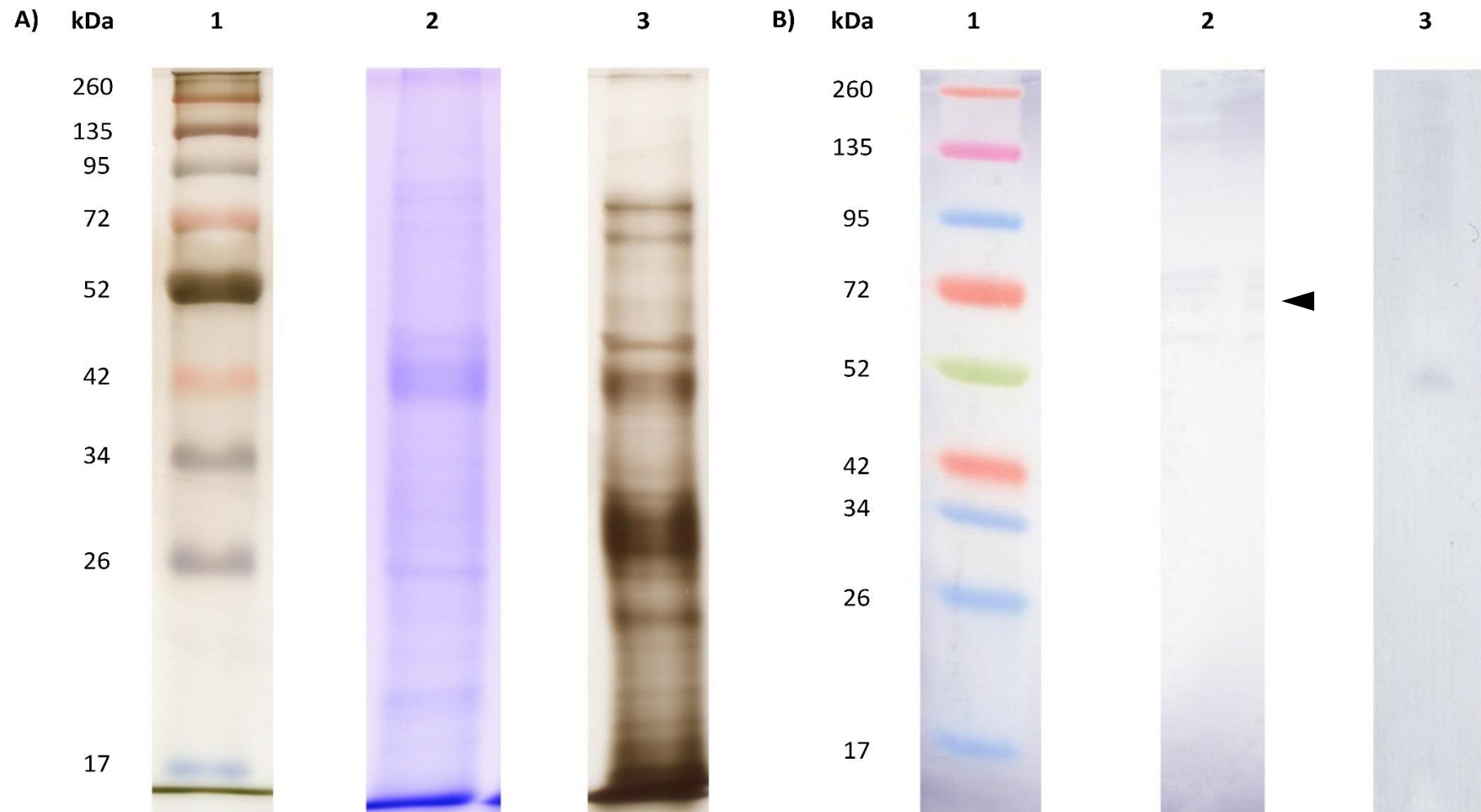


Figure 4.2 A) SDS-PAGE protein profile of the mucus extract (ME) prepared from Atlantic bluefin tuna (ABT) specimens. Lane 1, prestained protein marker; lane 2, protein profile stained with Coomassie Blue R-250; lane 3, protein profile stained with silver; B) western blots of the ME of ABT separated on a 10 % precast gel. Lane 1, prestained protein marker; lane 2, immunoblot assay incubated with anti-ABT IgM mAb; lane 3, immunoblot assay incubated with anti-ABT polyclonal antibody. Arrowhead shows reaction of the mAb on the membrane at a molecular weight of approximately 72 kDa.

4.3.4. Evaluation of enzymes in the mucus extract

The ME was examined for enzymes using an API-ZYM kit. Reaction intensity was recorded through comparison with a colour chart provided by the manufacturer. After addition of the developing reagents, the control cupule did not show any colour changes. The API-ZYM kit tests for 19 enzymes and of these a total of 11 enzymes were shown to be present in the ME at varying concentrations. A strong reaction (≥ 3) was observed for alkaline phosphatase, leucine arylamidase, acid phosphatase, naphthol-AS-BI-phosphohydrolase, β -galactosidase and N-acetyl- β -glucosamidase. Weaker reactions were recorded for esterase, valine arylamidase, trypsin, β -glucuronidase and α -fucosidase. The ME tested negative for all other enzymes (Figure 4.3).

4.3.5. Protease activity in the mucus extract

Results from the API-ZYM assay indicated the presence of proteases in the ME so it was assessed further for the activity of protease-type enzymes.

4.3.5.1. Azocasein hydrolysis assay

The azocasein hydrolysis assay confirmed the presence of protease-like activity in the ME. Mean protease activity in the ME was 28.95 ± 0.58 U mg^{-1} protein ($n = 3$). Activities in the positive and negative control were as expected.

Cupule	Enzyme	Result
1	Control	----
2	Alkaline phosphatase	4+
3	Esterase (C 4)	+
4	Esterase Lipase (C 8)	-
5	Lipase (C 14)	-
6	Leucine arylamidase	3+
7	Valine arylamidase	+
8	Cystine arylamidase	-
9	Trypsin	1+
10	α -chymotrypsin	-
11	Acid phosphatase	4+
12	Naphthol-AS-BI-phosphohydrolase	4+
13	α -galactosidase	-
14	β -galactosidase	3+
15	β -glucuronidase	2+
16	α -glucosidase	-
17	β -glucosidase	-
18	N-acetyl- β -glucosamidase	3+
19	α -mannosidase	-
20	α -fucosidase	1+

Figure 4.3 API-ZYM assay for the evaluation of enzymes in the mucus extract (ME). Values on the right represent the intensity of reaction and give an indication to the amount of the respective enzyme in the ME.

4.3.5.2. Zymography

Zymograms were used to visualise protease activity against both gelatin and β -casein substrates and ME, electrophoresed on gels, showed activity against both substrates (Figure 4.4). The gelatin substrate gel showed two distinct zones of clearing; the first was at 260-90 kDa and a second at around 26 kDa and below, indicating that gelatine was digested by protease-type enzymes in the ME. Zones of clearing were evident at 260-150 kDa, 34-30 kDa and at 22 kDa for the β -casein zymogram. Both zymograms showed a clear band in the 22-26 kDa region (trypsin molecular weight = 23.8k Da) for the positive control. Distinct bands did not appear in the zymograms and substrate degradation was visible over a broad MW range.

4.3.5.3. Cathepsin B activity

Cathepsin B activity was detected within the ME and quantified as 98.322 ± 9.148 U mg^{-1} protein ($n = 3$).

4.3.6. Lysozyme, alkaline phosphatase and esterase activity in the mucus extract

The ME was analysed for the activities of lysozyme, alkaline phosphatase and esterase activity. The activities of these different enzymes in ABT ME are summarised in Table 4.2 ($n = 3$). Results for positive and negative controls were as expected for all assays.

Table 4.2 Summary of enzyme activities measured in the mucus extract of Atlantic bluefin tuna. Results are expressed as mean \pm standard deviation ($n = 3$).

Enzyme	Activity (U mg^{-1} protein)
Alkaline phosphatase	0.511 ± 0.002
Cathepsin B	98.322 ± 9.148
Esterase	0.075 ± 0.002
Lysozyme	198.62 ± 4.80
Protease	28.947 ± 0.578

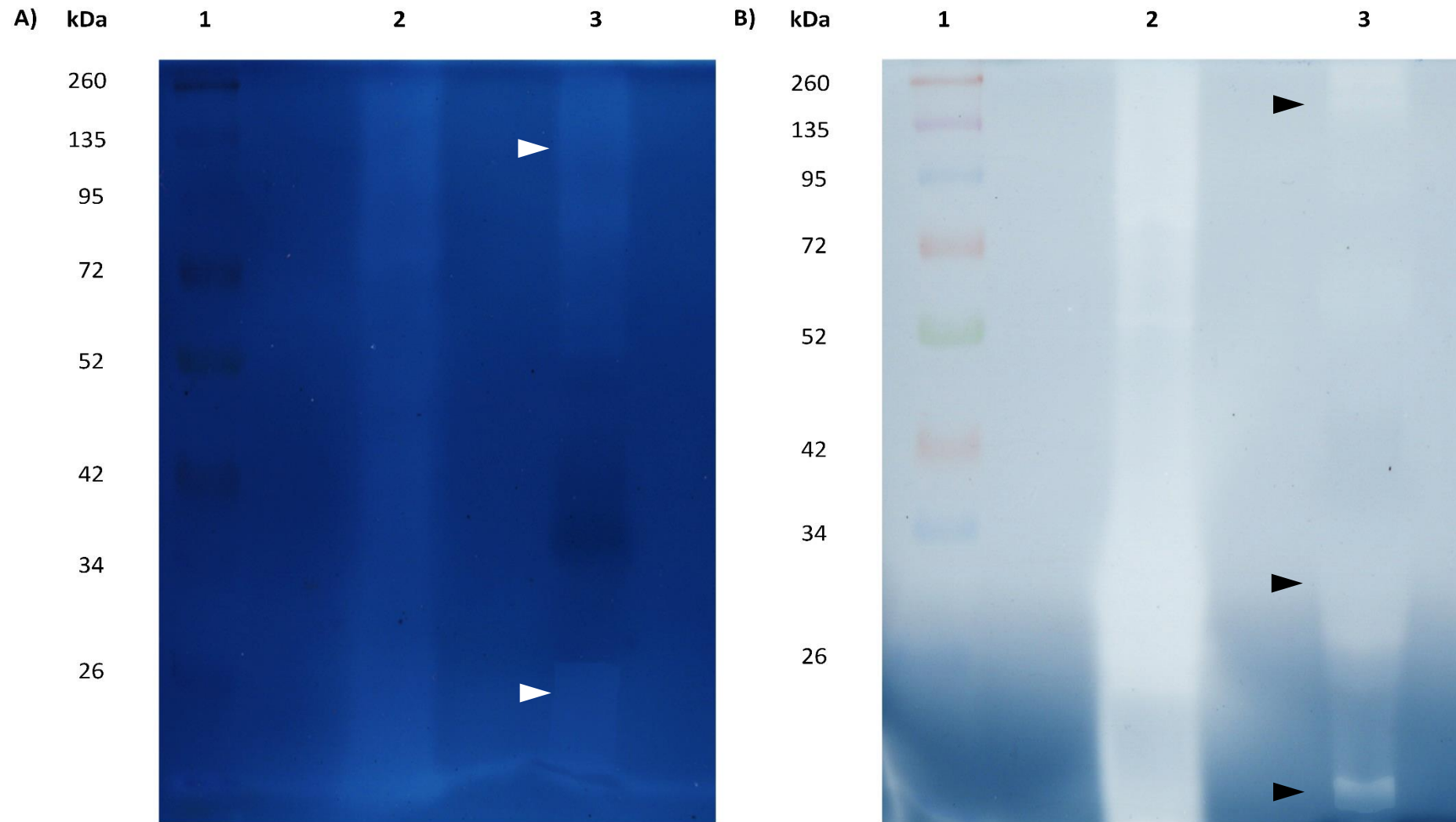


Figure 4.4 A) Gelatin zymogram of the mucus extract (ME) showing substrate degradation at various regions (arrowheads). Lane 1, prestained protein marker; lane 2, trypsin control (2 µg); lane 3, ME; B) β-casein zymogram of ME showing substrate degradation at various regions (arrowheads). Lane 1, prestained protein marker; lane 2, trypsin control (2 µg); lane 3, ME.

4.3.7. Alternative complement activity in the mucus extract

ABT ME was analysed for alternative complement activity with a spontaneous haemolytic activity assay. No haemolysis was observed indicating that no complement activity could be detected in the ME using this particular assay system.

4.3.8. Haemagglutinating activity of the mucus extract

The ME showed visible haemagglutination activity at a titre value of 8 (Figure 4.5).

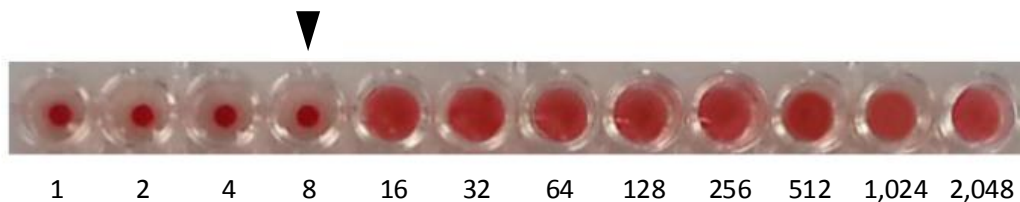


Figure 4.5 Mucus extract haemagglutination assay showing visible agglutination at a titre factor of 1:8 (arrowhead). Values below the plate represent assay dilution factors.

4.3.9. Bacterial inhibition in the mucus extract

Bacterial inhibition of ME was tested using an agar well diffusion assay. The extract was tested against *Vibrio anguillarum* (NCIMB 6), *V. vulnificus* (ATCC 27562 biovar 1) and *Photobacterium damsela* subsp. *piscicida* (SSV1 Greece). No visible inhibition of bacterial growth was observed with any of the bacterial species used in the well diffusion assay.

4.4. Discussion

Fish live in a marine environment containing several different classes of pathogens, and epidermal mucus represents an important biological interface between the fish and its environment. In several teleost species, fish epidermal mucus has been shown to exhibit non-specific response characteristics of the innate immune system (Ingram, 1980;

Alexander, 1985; Alexander & Ingram, 1992), including Ig, agglutinins and enzymes. The function of external mucus as a first barrier of protection, and to a degree its differentiation from internal immune systems, has been demonstrated by Rombout *et al.* (1993) who found molecular differences between the serum and mucus Ig in carp. IgM type natural antibodies in fish cutaneous secretions presumably confer some form of protection against invading pathogens (Sinyakov *et al.*, 2002; Baumgarth *et al.*, 2005; Salinas *et al.*, 2011) and have been detected in the mucus of numerous teleost species (Palaksha *et al.*, 2008; Salinas *et al.*, 2011; Esteban, 2012).

The presence of Ig in the mucus of ABT was analysed using three different experimental procedures in this study; precipitation with polyethylene glycol and ELISA for total Ig and IgM respectively, and immunoblots. Results between the former two assays differed slightly in that total Ig was estimated to be present at slightly higher levels than IgM, but both indicate that Ig are present in the epidermal mucus of this tuna species. Both methods gave relatively low values indicating that Ig levels in the mucus are low, and this is consistent with findings for other teleosts (Lobb & Clem, 1981; Rombout *et al.*, 1986; Lobb, 1987; Itami *et al.*, 1988; Al-Harbi & Austin, 1993; Hatten *et al.*, 2001; Palaksha *et al.*, 2008). At such low levels, however, it is always difficult to accurately estimate the levels of Ig in mucus especially considering that it was processed post-collection, and the results serve more to confirm the presence of Ig rather than to estimate their exact concentration. The measured difference between total Ig and IgM may reflect the presence of an Ig isotype similar to IgT/IgZ found in rainbow trout or zebrafish skin mucus respectively, but more specific investigations in this field are required to confirm this, since such differences may be due to assay limitations.

Natural Ab are present within an organism in the absence of external antigens and are predominantly of the IgM isotype (Baumgarth *et al.*, 2005). Additionally they are known to be capable of binding bacterial pathogens actively; teleost fish are capable of generating IgM-type natural antibodies against exogenous antigens (Whyte, 2007). Therefore the presence of natural Ab in epidermal secretions should aid in protection of the organism against infection, or at least delay the onset of infection until an adaptive immune response is established.

Although the presence of the IgM molecule could be detected by ELISA it was not seen in the immunoblot using a polyclonal Ab against ABT IgM. In contrast to this, very faint bands in the region of the IgM heavy were obtained with an anti-ABT IgM mAb, although it was unclear if this was due to a specific reaction with IgM-type molecules present in the mucus or background staining. Polyclonal Ab, due to their lower specificity and recognition of multiple antigen epitopes, would be expected to show a stronger reaction than mAb, which recognise a single epitope. Consequently it is difficult to explain the results obtained here. Rombout *et al.* (1993) reported slight structural differences between mucosal and serum Ig, because a mAb developed by them against mucosal Ig did not react with Ig isolated from serum; they attributed this to molecular differences in the Ig heavy chain. In contrast, other teleost species have been reported to have identical IgM forms in serum and mucus (Lobb, 1987; Shin *et al.*, 2007). The same anti-ABT IgM used in ELISA detected the presence of an IgM-like component. Considering this, it is probable that the negative or slight reactions observed in the immunoblot assays in this study are more likely to be due to the low concentrations of the natural Ab in the mucus rather than to any structural differences within the Ig molecule itself.

Similarly, Jung *et al.* (2012) observed positive immunoblot reactions when olive flounder mucosal IgM levels were high but negative reactions when Ab levels were low.

The protein profile of the ME was examined using gel electrophoresis. All protein bands detected by denaturing SDS-PAGE had a MW below 100 kDa. The MW of the proteins detected were similar to those described for olive flounder (Palaksha *et al.*, 2008), but more investigations are required to draw conclusions regarding similarities between proteins. ABT mucus has a relatively thick consistency and this is probably due to the presence of a large amount of mucins. This may have given rise to large amounts of breakdown products following reduction and denaturation of the sample in sample buffer prior to electrophoresis and may account for the relatively strong background obtained in protein electrophoresis. The latter, however, may also be due to a degree of sample breakdown prior to storage during sample retrieval, or during laboratory processing.

The presence and possible immunological function of lysozyme, alkaline phosphatase and proteases in fish skin mucus of species other than tunas has been reported (*e.g.* Ingram, 1980; Ellis, 1981; Alexander & Ingram, 1992; Shephard, 1994; Ellis, 2001). An API-ZYM bacterial enzyme kit was adapted for testing the presence of these and other enzymes in ABT epidermal mucus. The test strip was set to an incubation temperature of 25°C to represent their body temperature (Graham & Dickson, 2001) and the average seawater temperature the fish experience during their time in the cages. The strip tested positive for the presence of several enzymes with both alkaline and acid phosphatases showing high concentrations. Positive readings were also obtained for the protease-type enzymes leucine arylamidase, cystine arylamidase and trypsin. The

presence of several other enzymes was also detected but there are no reports on their physiological significance in fish mucus. Cutaneous mucus is in constant contact with a marine environment containing several bacterial species that may become trapped within the mucus itself. Considering this, it is difficult to determine whether the enzymes detected originate from the mucosal secretions or those of the bacterial flora sustained within (Palaksha *et al.*, 2008).

The activities of some of the enzymes detected by API-ZYM were analysed in more detail. Proteases have been histochemically localised in mucus-secreting cells (Braun *et al.*, 1990), are known to have protective function and are an important component of the innate immune response (Ingram, 1980). Their presence in the ABT epidermal mucus was investigated using an azocasein assay and substrate gel electrophoresis. Azocasein hydrolysis detected a relatively potent protease activity ($28.9 \text{ U mg}^{-1} \text{ protein}$) in the ME and this corroborates the presence of leucine arylamidase and trypsin detected with the API-ZYM strip. The activity of proteases on protein substrates in zymography further substantiate these findings and are a clear indicator that proteases were not only present in the cutaneous ME, but were also active. Diffuse substrate digestion zones were observed in the zymograms and this is similar to that obtained for fish mucus post-processed in the laboratory using similar procedures (Palaksha *et al.*, 2008). Electrophoresis on a 12 % SDS substrate gel did not appear to allow for efficient separation of reduced non-denatured protein so that final zymography assays were performed on a 10 % SDS substrate gel. Considering this, it could be that diffuse bands may have been obtained due to the restricted mobility of the non-denatured proteins within the gel so that digested bands were obtained over a diffuse area rather than at

defined MW. Some separation of the clearing zones was still evident and protease activity at a MW of approximately 100 kDa has been reported from the skin mucus of other fish species (Subramanian *et al.*, 2007). Low MW protease bands have been found in extracts prepared from the mucus of rainbow trout (Hjelmeland *et al.*, 1983), Atlantic salmon (Ross *et al.*, 2000) and others (Subramanian *et al.*, 2007). Significant cathepsin B activity was detected in the ABT ME. Proteases are known to have bacteriolytic activity against Gram-negative bacteria; a trypsin-like protease from rainbow trout mucus has been shown to inhibit the growth of *V. anguillarum* (Hjelmeland *et al.*, 1983). Cathepsin B is a cysteine protease which can degrade proteoglycans in bacterial cell walls (Nguyen *et al.*, 1990) and has demonstrated growth inhibiting activity against *Edwardsiella tarda*, *Flavobacterium columnare* and *V. anguillarum* (Aranishi, 1999; Aranishi & Mano, 2000a). They are also known to stimulate or enhance the production of several components of the innate immune system including complement and Ig in mammals and antibacterial peptides in teleosts (Yoshikawa *et al.*, 2001; Cho *et al.*, 2002a, b; Morrissey, 2013). In humans, cathepsins are involved in antigen processing (Zavašnik-Bergant & Turk, 2006), so that they are also important during the generation of adaptive immune responses. Proteases may also prevent pathogen invasion or attachment indirectly by modifying mucus consistency so that it is shed at a faster rate (Aranishi *et al.*, 1998), in consequence reducing opportunity for infection or epidermal attachment.

The presence and activity of other enzymes were confirmed and quantified by the API-ZYM kit and substrate assays respectively. The specific activity of lysozyme in the extract was very high (198.6 U mg⁻¹ protein) but although present, alkaline phosphatase and esterase enzymes showed low activities. Results from the API-ZYM assay suggested a

high activity, but the corresponding microplate assay using a *p*-nitrophenyl phosphate substrate showed that this, in fact, was low. Similar discrepancies were found between API-ZYM and the substrate assay used in the work of Palaksha *et al.* (2008). Differences between the two methods may be due to the different substrates and assay conditions used – 2-naphthyl phosphate and *p*-nitrophenyl phosphate respectively. Alternatively it may be that the *p*-nitrophenyl phosphate is not sensitive enough, especially considering that Ross *et al.* (2000) reported low enzyme activities in salmon infected with the salmon louse, *Lepeophtheirus salmonis*, and no or low activity was detected in other work on salmon epidermal mucus (Fast *et al.*, 2002; Easy & Ross, 2010).

Lysozyme is an enzyme ubiquitous within organisms. It is known to demonstrate strong antibacterial behaviour capable of damaging the peptidoglycan layer of Gram- positive bacteria, and to act indirectly on Gram-negative bacteria, after action by the complement system of proteins, on the outer cell wall (Yano, 1996). Alkaline phosphatase levels have been shown to increase in salmon epidermal mucus following infection with *L. salmonis* (Ross *et al.*, 2000) and have been demonstrated in the rodlet cells of superficially wounded carp (Iger & Abraham, 1990) so that it is believed that they confer some form of protection during epithelial repair. Consequently they may act as indicators of stress (Iger & Abraham, 1997; Subramanian *et al.*, 2007). The role of esterase enzymes in skin epidermal secretions is presently not understood but in higher vertebrates functions as an inhibitor in the classical complement pathway (Tamura *et al.*, 1976).

Different studies have used different procedures to quantify enzyme activities so that it is impractical to make comparison between the specific enzyme activities reported here

and other research. Inter-species comparison also has limited significance considering that enzyme levels have been found to fluctuate according to environmental conditions (Jung *et al.*, 2012), stress and other conditions (Balfry & Iwama, 2004). However when tested in conjunction with other enzymes, lysozyme and proteases have consistently been found to have high specific activities in comparison to other enzymes (Ross *et al.*, 2000; Subramanian *et al.*, 2007; Palaksha *et al.*, 2008; Jung *et al.*, 2012; Nigam *et al.*, 2012). Subramanian *et al.* (2007) also found lysozyme levels in fish epidermal mucus to be higher in marine than freshwater species while the opposite was true for proteases. Enzyme activities were also found to be higher in bottom-dwelling species than those occupying cleaner habitats by Nigam *et al.* (2012), perhaps indicating that more sedentary lifestyles require a higher degree of protection against pathogens.

Complement has an important role in innate immune responses (Alexander & Ingram, 1992) and their mediation is essential for several inflammatory responses (Yano, 1996). No haemolysis of SRBC was detected in the ME in this study, so it could be that alternative complement activity is absent in ABT mucus. Components of the complement system have been found in the mucus of salmonid fish (Harrell *et al.*, 1976), but similarly the skin mucus of olive flounder did not demonstrate complement activity (Palaksha *et al.*, 2008).

Fish mucus contains natural agglutinins or lectins (Ingram, 1980), they are non-specific (Alexander & Ingram, 1992) and may provide a degree of inhibition against bacterial growth (Yano, 1996). Haemagglutination of SRBC was visually observed in the ME and this is similar to findings in the mucus of other teleosts (Suzuki, 1985; Itami *et al.*, 1993; Palaksha *et al.*, 2008; Jung *et al.*, 2012). Although their exact function in fish epidermal

mucus is not understood, they have been reported to stimulate phagocytic activity (Ottinger *et al.*, 1999) and assist in wound healing (Al-Hassan *et al.*, 1983). In mammals, they are known to be involved in the activation of the complement pathway (Matsushita *et al.*, 2004).

Although antibacterial activity against both fish and terrestrial pathogens has been shown to be present in extracts from the skin mucus of several teleosts (Austin & McIntosh, 1988; Hellio *et al.*, 2002; Palaksha *et al.*, 2008; Subramanian *et al.*, 2008; Ruangsri *et al.*, 2010; Balasubramanian *et al.*, 2012), no such activity was evident in radial diffusion assays with the ME from ABT in this study. This contrasts with results obtained for the different enzymes, but it is possible that the volumes of ME used in the assays were too low to inhibit the growth of the bacterial pathogens used in this study, or that the ME had no activity against these particular bacterial strains. Furthermore, the lack of bacteriolytic activity observed may be due to other essential components being absent, or present at insufficient concentrations to inhibit the pathogens.

Tunas are fast-swimming, obligate ram ventilators and their physiology requires them to sustain their swimming speed for efficient gas exchange (Korsmeyer & Dewar, 2001). The skin mucus from ABT has been shown to have several non-specific humoral immune components, which would be expected to work synergistically to defend the organism against pathogenic attachments or invasions. Being epipelagic species they inhabit open water and their high swimming speed should make pathogenic invasion or attachment more difficult so that they may rely less on such a cutaneous defence system. It has been shown that primitive and sedentary fish have higher enzyme activities and this presumably compensates for the lack of an adaptive immune response (Subramanian *et*

al., 2007) and an increased chance of pathogen attachment respectively. Although tunas are considered amongst the most evolved teleosts they still possess components of the innate immune system within their epidermal mucus suggesting that epidermal secretions must have an important role in defending the organism from pathogen infection or attachment. Being lower vertebrates, adaptive immune responses in fish are less sophisticated and diverse than in higher vertebrates (Du Pasquier, 2001; Tort *et al.*, 2003). Although not strictly endotherms, tunas are able to sustain heat generated by muscle activity and maintain elevated body temperatures (Graham & Dickson, 2001) so that in the event of infection they may mount an immune response in a shorter time than ectothermic fish (Watts *et al.*, 2001a). The innate immune components of the mucus act without delay (Ellis, 2001) so that their action may serve either to prevent infection or at least delay pathogen invasion until a proper specific immune response can be generated. Coupling the possibility of a swift immune response with the immune components in epidermal mucus, tunas may offer high resistance to disease and in fact there are no reports of disease in mature captive tunas.

4.5. Conclusion

This is the first report on the innate immune parameters found in ABT epidermal mucus. Among other non-specific immune components, the study has assessed the presence of IgM, lysozyme, protease and alkaline phosphatase. Further studies are required to better understand the functions and importance of these to the species, and how they may reflect on the welfare of the animals. Although visual inspection of the specimens confirmed that they were macroscopically healthy prior to sample retrieval, it was not possible to ascertain that the fish were entirely free of disease so that more

investigations are required to relate these parameters to the health status of the fish. This task is made more complex considering that these immune parameters are known to show seasonal variation in other fish species (Jung *et al.*, 2012) and also due to the limitations encountered when working with the ABT.

CHAPTER 5

STUDIES ON FATTY ACID COMPOSITION AND LONG-CHAIN PUFA BIOSYNTHESIS GENE EXPRESSION IN FARMED ATLANTIC BLUEFIN TUNA

5.1. Introduction

Lipids are the preferential energy source for teleosts (Sargent *et al.*, 2002), with the energy being released from them through oxidative metabolism pathways. Lipids act as the main metabolic energy source throughout the life of the fish (Tocher *et al.*, 1985), with FA such as palmitic acid (16:0), oleic acid (18:1 n -9), eicosanoic acid (20:1 n -9) and cetoleic acid (22:1 n -11) being their major energy source (Sargent *et al.*, 2002). Fish muscle contains a high proportion of mono- (MUFA) and polyunsaturated (PUFA) FA and the more prominent PUFA are arachidonic acid (ARA; 20:4 n -6) and its metabolic precursor linoleic acid (18:2 n -6) from the n-6 series FAs and eicosapentaenoic acid (EPA; 20:5 n -3), docosahexaenoic acid (DHA; 22:6 n -3) together with their metabolic precursor linolenic acid (18:3 n -3) from the n-3 series FAs (Sargent *et al.*, 2002). The FA composition of highly migratory fish, including tunas, contrasts with those of other teleosts in having higher amounts of DHA, and this is thought to be linked to a form of

selective FA catabolism during active migration (Medina *et al.*, 1995; Murase & Saito, 1996; Saito *et al.*, 1997, 1999, 2005; Osako *et al.*, 2006).

Tuna species are highly migratory and their muscular arrangement displays specialisations for high-performance swimming and long-distance cruising (Westneat & Wainwright, 2001). As in other teleosts, aerobic red muscle is employed for sustained swimming over long distances, while anaerobic white muscle is active when faster speeds are required (Altringham & Shadwick, 2001). In ABT, the red muscle constitutes only a small proportion of the total body mass and has a high concentration of myoglobin and mitochondria, the former imparting it with its characteristic dark red colouration (Korsmeyer *et al.*, 1996). In teleosts, lipids are the energy source in red muscle and energy is released through FA oxidation. To this effect, tuna red muscle demonstrates high aerobic and metabolic performance to sustain their fast swimming speeds (Korsmeyer & Dewar, 2001). The white muscle of tuna occupies a larger proportion of the tuna body weight, but is distinct from that of other teleosts in that it has a relatively high aerobic capacity and intracellular lipid content (Korsmeyer *et al.*, 1996; Goñi & Arrizabalaga, 2010).

The FA composition of fish muscle is influenced by various factors, including the composition of their diet (Hearn *et al.*, 1987; Farndale *et al.*, 1999), and changes in nutritional state affect their muscle lipid content (Ackman, 1989; Sprague *et al.*, 2012). At present, the tuna farming industry relies on capture from wild-capture fisheries, and fish are maintained on a trash fish diet consisting mainly of sardine, herring and mackerel of varying nutritional composition. Research on other species, including tunas, has shown that dietary FA intake influences the FA composition of the end product

(Saito *et al.*, 1996; Farndale *et al.*, 1999; Hemre & Sandnes, 1999; Sargent *et al.*, 2002; Skog *et al.*, 2003).

The ABT is renowned for its meat quality, particularly with regard to texture and taste. Lipid content has an important influence on these, and in this regard several studies on the lipid components of several tuna species have been undertaken (Saito *et al.*, 1997, 1999; Nakamura *et al.*, 2005, 2006; Roy *et al.*, 2010), but information on the lipid composition of ABT is still limited. Fish lipid levels vary with the size and age of the fish, and studies on the PBT have shown that younger fish possess lower lipid levels in their dorsal muscle (Nakamura *et al.*, 2007). It has also been demonstrated that there is variation in the lipid composition of the different regions of tuna dorsal muscle (Roy *et al.*, 2010).

Fish, like higher animals, have the ability to biosynthesise C₂₂ long-chain PUFA (LC-PUFA) from C₁₈ shorter chain PUFA (Sargent *et al.*, 2002). This involves desaturation and elongation of the FA chain, mediated by fatty acyl desaturase (*fad*) and elongase (*elovl*) enzymes, respectively (Morais *et al.*, 2011). Teleosts have the ability to synthesise several saturated FA (SFA) *de novo* (Sargent *et al.*, 2002), but lack certain desaturases that are essential for the *de novo* synthesis of PUFA (Tinoco, 1982). The conversion of linolenic acid to EPA involves desaturation at the $\Delta 6$ position followed by a chain elongation and a second $\Delta 5$ desaturation (Sprecher, 1981; Sargent *et al.*, 2002). Although the conversion of EPA into DHA is not entirely understood, it involves an additional $\Delta 6$ desaturation and 2-carbon chain elongation step so that it involves a C₂₄ intermediate (Sprecher *et al.*, 1995; Sprecher, 2000).

Freshwater teleosts are capable of synthesising DHA from linolenic acid (Buzzi *et al.*, 1997; Bell *et al.*, 2001), but marine species show limited capabilities in this biosynthesis due to apparent deficiencies in the conversion pathway (Buzzi *et al.*, 1997; Ghioni *et al.*, 1999; Tocher & Ghioni, 1999; Sargent *et al.*, 2002). *Fad* genes have been described from several teleost species and with the exception of $\Delta 5fad$ from Atlantic salmon and a bifunctional $\Delta 5/\Delta 6fad$ from zebrafish (Hastings *et al.*, 2004), these are of the $\Delta 6$ type ($\Delta 6fad$) (Zheng *et al.*, 2004a, 2005, 2009; Tocher *et al.*, 2006; González-Rovira *et al.*, 2009). Activity of *fad* and *elovl* has been described in ABT larvae (Morais *et al.*, 2011) and a highly conserved *fad* has been identified in the SBT (Gregory *et al.*, 2010).

The objectives of this study were to examine the FA profiles of farmed ABT, the effects of size on lipid levels and composition, and the relationship between diet and ABT FA composition. Furthermore, there is no information on how the absence of a controlled diet affects the FA composition of the fish when farmed over longer periods than the standard 6 to 8 months. For this purpose, flesh samples from specimens farmed for different durations were collected for comparative analysis of their FA profiles. Finally, there are no reports on the expression of $\Delta 6fad$ and *elovl5* genes in farmed ABT, and this study attempted to quantify the expression of these two genes from several organs of the fish to acquire a better understanding of the FA metabolism of this species in captivity.

5.2. Materials and Methods

5.2.1. Study population and sample collection

Samples were collected from two populations of ABT held in cages for different durations. They were captured in the Mediterranean in June 2011 (FAO Statistical Area 10 [see Figure 2.1]; 39°04'N, 014°41'E; Groups 1 and 2) and June 2010 (FAO Statistical Area 21 [see Figure 2.1]; 33° 31'N, 013° 14'E; Group 3) and using purse seine methods, transferred to sea cages and towed to farm. They were held in offshore marine cages, off southern Malta (35°49'N, 014°35'E), for a period of 6 or 18 months (depending on the sample population) and maintained on a trash fish diet. The fish were fasted for one day prior to harvest. Specimens were killed by a shot to the head as per industry standards, and transferred to a processing vessel.

Flesh samples for analysis of FA composition were collected from the caudal end of the fish ($n = 17$) so that the market value of the dorsal and ventral muscle product was retained (Figure 5.1). To analyse for differences in lipid composition between the different regions of the dorsal muscle, a single cross-sectional sample through cephalic dorsal ordinary muscle (Ce-OM) was collected from a fish weighing 50 kg that was held in cages for 6 months. Analysis was performed on muscle tissue collected from three separate body regions termed Ce-OM 1 to 3 (Figure 5.2). The samples were frozen and stored at -60°C until analysed in the lab.

Tissue samples from the kidney, liver, spleen and brain were collected for quantification of the expression of *Δ6fad* and *elov15* genes. The tissues were excised, transferred into RNAlater (Sigma) and stored at 4°C for 24 h, after which the RNAlater was decanted and the organ specimens were stored at -20°C until analysed.

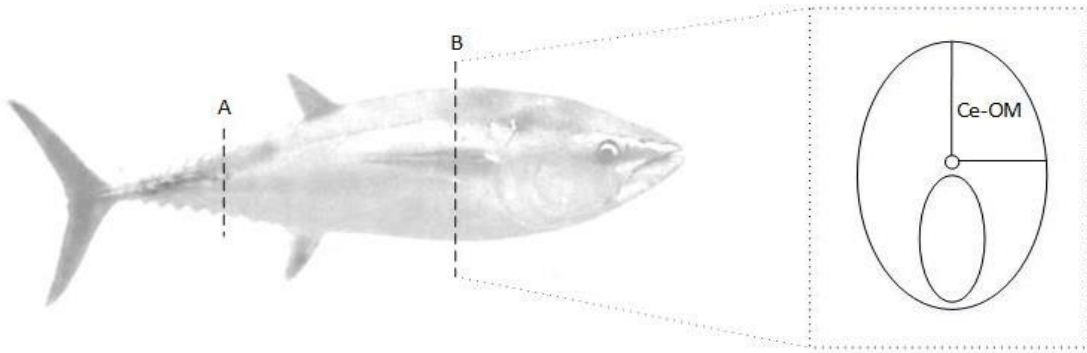


Figure 5.1 Sampling collection points for: A, caudal muscle and B, dorsal ordinary muscle.

The ABT specimens in this study were maintained on a trash fish diet consisting of Atlantic herring, *Clupea harengus* L., Pacific herring, *Clupea pallasii* Valenciennes, European pilchard, *Sardina pilchardus* (Walbaum), and Atlantic chub mackerel, *Scomber colias* Gmelin, during the farming period. Representative samples ($n = 5$) of the diet were collected and homogenised whole (including gonads and internal organs), mixed thoroughly and stored at -60°C until analysis.

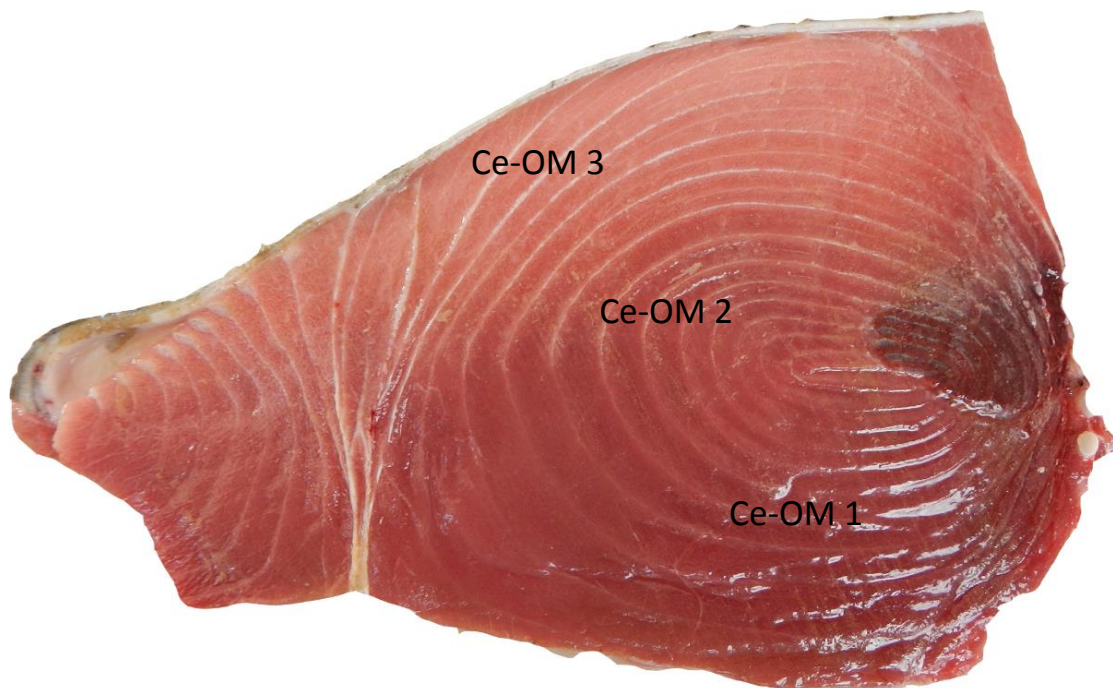


Figure 5.2 Cross-sectional cut through cephalic dorsal ordinary muscle (Ce-OM) of farmed Atlantic bluefin tuna showing locations at which dorsal ordinary muscle was analysed for fatty acid composition.

Fish weight ranged from 52-350 kg and 76-116 kg for fish farmed for 6 and 18 months respectively. All specimens appeared clinically healthy and visual inspection of the organs did not show any apparent signs of infection or other disorders.

5.2.2. Measurement of total lipid content

Total lipid (TL) from the caudal and dorsal ordinary muscle, and from the homogenised trash fish samples was measured gravimetrically according to methods from Folch *et al.* (1957). One gram of tissue sample was placed in 20 mL of an ice-cold 2:1 (v/v) chloroform-methanol mixture containing 0.01 % butylated hydroxytoluene (Sigma) as antioxidant and homogenised with a tissue disperser (Ultra-Turrax, IKA). Five millilitres of 0.88 % KCl were added, the solution mixed thoroughly and allowed to stand for 1 h on ice to allow for the separation of the organic phases, containing the extracted lipid, from the aqueous phase. TL content was determined gravimetrically after evaporating the solvent with oxygen-free nitrogen gas and desiccating under a vacuum overnight.

5.2.3. Analysis of fatty acids

FA analysis was performed according to Christie (1993). FA methyl esters (FAME) were prepared through acid-catalysed trans-esterification at 50°C for 16-18 h using heptadecanoic acid (Sigma) as a standard at 10 % of TL mass. FAMEs were separated and quantified with gas-liquid chromatography equipment (Vega 8160, Carlo Erba, Wigan, UK) fitted with a 30 m × 0.32 mm ID capillary column (CP-Wax 52CB, Agilent, Wokingham, UK) and on-column injection at 50°C. Carrier gas was hydrogen and the temperature was 50-150°C at 40°C min⁻¹ and to 230°C at 2°C min⁻¹. Identification of

unknowns and confirmation of FA identity was performed with gas chromatography-mass spectrometry.

5.2.4. RNA isolation and cDNA synthesis

RNA was isolated from sampled brain ($n = 1$), kidney ($n = 5$), spleen ($n = 5$) and liver ($n = 5$) using a TRIzol RNA purification kit (Applied Biosystems, Paisley, UK) according to the manufacturer's instructions. The tissues were partially defrosted and homogenised in TRIzol (Sigma; 1 mL reagent 100 mg⁻¹ tissue) and incubated for 5 min at RT (22°C). Then 200 µL chloroform (Sigma) mL⁻¹ TRIzol were added to the homogenate, mixed thoroughly and incubated at 22°C for 3 min. The samples were centrifuged at 12,000 ×g for 15 min at 4°C, 600 µL of the upper phase transferred to an RNase-free container and 600 µL of ethanol were added and the reaction mixture vortexed. Seven hundred microlitres of the sample was transferred to a spin cartridge and centrifuged at 12,000 ×g for 15 sec at 22°C and the flow-through discarded. Seven hundred microlitres of Wash Buffer I were added to the spin cartridge and the mixture centrifuged and processed as above. This was followed by the addition of 500 µL of Wash Buffer II with identical processing as for Wash Buffer I. The spin cartridge was then centrifuged at 12,000 ×g for 1 min at 22°C to attach the RNA to the membrane, and the cartridge inserted into a recovery tube. Purified RNA was collected after three sequential elutions with 100 µL RNase-free water followed by centrifugation for 2 min at 12,000 ×g at 22°C. RNA quality and quantity was assessed with ethidium bromide agarose gel electrophoresis and spectrophotometry (ND-1000, NanoDrop, Wilmington DE, USA). Extracted RNA samples were stored at -80°C until analysed.

RNA was reverse transcribed into cDNA with a cDNA reverse transcription kit (Applied Biosystems) as per the manufacturer's instructions. Briefly, an appropriate volume of 2× reverse transcriptase (20 % reverse transcription buffer, 8 % 25× dNTPs, 20 % 10× random primers, 10 % MultiScribe reverse transcriptase [50 U μl⁻¹], 42 % nuclease-free H₂O) was prepared and stored on ice until use. A cDNA archive reaction plate was prepared at RT by the addition of 50 μL of 2× master mix and an equal volume of RNA sample (1-2 μg) into wells of a 96-well microplate (Sterilin). The microplate was centrifuged briefly to eliminate air bubbles, and the cDNA was obtained after thermal cycling at 25°C and 37°C for 10 and 120 min respectively.

5.2.5. Quantitative polymerase chain reaction

The expression of *Δ6fad* fatty acyl desaturase (GenBank: HQ214238) and *elov15* elongase (GenBank: HQ214237), two genes involved in the synthesis of LC-PUFA, was quantified with real-time quantitative polymerase chain reaction (qPCR) together with two reference genes, elongation factor-1α (*elf-1α*; FM995222) and β-actin (GU046791). Primers used in this procedure are listed in Table 5.1. The amplification efficiency for primer pairs was evaluated with serial dilutions of cDNA from pooled tissue samples. Amplifications were performed in a final volume of 20 μL containing 2 or 5 μL cDNA diluted 1:20 for reference genes and tissue samples respectively, 0.5 μM of each primer and 10 μL absolute TM qPCR SYBR Green mix (Thermo Scientific). qPCR initial activation was at 95°C for 15 min and then 30-40 cycles: 15 sec at 95°C, 15 sec at annealing T_m and 30 sec at 72°C. A 75-90°C melt curve was performed after amplification to confirm single products in each reaction (Morais *et al.*, 2011). Negative controls consisted of amplifications without cDNA and these were performed in conjunction with a non-

template control (NTC). The presence of primer-dimers in the NTC was analysed with agarose gel electrophoresis. Results were calculated from the cDNA standard curve and assumed as expression values normalised by the averages of the two reference genes.

Table 5.1 Sequence, annealing temperature (T_m) and fragment size produced by the primer pairs used in quantitative PCR (qPCR).

Transcript	Primer	Primer sequence	Fragment	T _m
Δ6fad	Tt-qD6DES-F	CCGTGCACTGTGTGAGAAAC	152 bp	60°C
	Tt-qD6DES-R	CAGTGTAAGCGATAAAATCAGCTG		
elov15	Tt-qElo5-F	CCACGCTAGCATGCTGAATA	236 bp	60°C
	Tt-qElo5-R	ATGGCCATATGACTGCACAC		
elf-1α	Tt-qEF1-F	CCCCTGGACACAGAGACTTC	119 bp	60°C
	Tt-qEF1-R	GCCGTTCTTGGAGATACCAG		
β-actin	Tt-qBACT-F	ACCCAACACAGTGCCCATCTA	155 bp	61°C
	Tt-qBACT-R	TCACGCACGATTTCCCTCT		

Adapted from Morais *et al.* (2011); bp, base pairs.

5.2.6. Statistical analysis

Statistical analysis was performed on the results obtained for the FA analysis. For comparison purposes, the specimens were classified into three categories according to the time spent in captivity and their weight. Group 1 fish consisted of fish held for 6 months and weighing less than 100 kg, Group 2 of fish held captive for 6 months and weight exceeding 100 kg and Group 3 represented fish kept in cages for 18 months. Differences in FA levels between Groups were statistically analysed using one-way ANOVA and a Tukey *post-hoc* test for multiple comparisons between parameters where relevant. Pearson correlation was used to detect correlations between FA levels and specimen weight in pooled data from Group 1 and Group 2 specimens. One-way ANOVA was used to test for differences in FA between different regions of the dorsal ordinary muscle. Significance levels were set at 0.05 and analysis was performed with SPSS Statistics 20 (IBM Corp.).

5.3. Results

5.3.1. Fatty acid composition of different groups

A similar FA profile was observed for all farmed ABT muscle tissues analysed. On average, the major constituents of muscle lipid were SFA at around 29 % of TL, approximately 41 % MUFA, predominantly in the form of oleic acid (18:1*n*-9) and 25 % PUFA, principally as EPA and DHA. The DHA/EPA was close to 1 across all groups.

For purposes of comparing lipid profiles between fish of different size and time in captivity, the fish were classified into three groups according to these parameters (see **Section 5.2.6**). Significant differences between groups were limited to the SFA docosanoic acid (22:0), which was higher in the larger fish from Group 2, the MUFA 24:1*n*-9 (nervonic acid), which was lower in Group 2, and total *n*-6 PUFAs which increased significantly with farming duration. There was a non-significant increase in TL and PUFA levels with increasing farming duration, while EPA/DHA was constant between specimen groups. The EPA/ARA ratio decreased significantly with time spent in captivity. Table 5.2 lists the FA profile of analysed ABT caudal muscle samples and Figure 5.3 shows the major lipid classes as a function of specimen groups.

Table 5.2 Fatty acid composition of Atlantic bluefin tuna tail cuts. Values represent mean \pm standard deviation (SD) (Group 1, 6 months captive, weight <100 kg, $n = 5$; Group 2, 6 months captive, weight >100 kg, $n = 7$; Group 3, 18 months captive, $n = 5$).

Parameter	Group 1	Group 2	Group 3
Body weight (kg)	67 \pm 13	281 \pm 60	96 \pm 17
Total lipid	13.8 \pm 2.6	14.2 \pm 2.3	14.8 \pm 4.2
14:0	5.0 \pm 0.4	5.4 \pm 0.2	4.9 \pm 0.5
15:0	0.4 \pm 0.1	0.5 \pm 0.1	0.4 \pm 0.1
16:0	18.8 \pm 1.4	18.8 \pm 1.1	18.2 \pm 0.6
18:0	4.4 \pm 0.3	4.5 \pm 0.4	4.4 \pm 0.2
20:0	0.3 \pm 0.0	0.3 \pm 0.1	0.3 \pm 0.0
22:0*	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^b	0.1 \pm 0.0 ^a
Total SFA	28.9 \pm 2.0	29.6 \pm 1.8	28.4 \pm 0.8
16:1 n -7	7.7 \pm 0.4	7.8 \pm 0.2	7.7 \pm 0.5
18:1 n -9	17.4 \pm 0.7	16.0 \pm 1.5	16.9 \pm 0.6
18:1 n -7	3.6 \pm 0.1	3.4 \pm 0.2	3.6 \pm 0.1
20:1 n -9	5.9 \pm 1.2	5.7 \pm 0.4	4.8 \pm 0.9
20:1 n -7	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0
22:1 n -11	6.4 \pm 0.9	6.4 \pm 0.5	5.5 \pm 1.1
22:1 n -9	0.6 \pm 0.1	0.5 \pm 0.0	0.5 \pm 0.1
24:1 n -9*	0.7 \pm 0.0 ^a	0.7 \pm 0.1 ^b	0.7 \pm 0.0 ^a
Total MUFA	42.6 \pm 2.3	40.8 \pm 2.3	39.9 \pm 2.0
18:2 n -6	1.2 \pm 0.1	1.2 \pm 0.1	1.3 \pm 0.1
18:3 n -6	0.2 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0
20:2 n -6	0.2 \pm 0.1	0.2 \pm 0.1	0.2 \pm 0.0
20:3 n -6	0.1 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.1
20:4 n -6	0.9 \pm 0.1	1.0 \pm 0.1	1.1 \pm 0.1
22:4 n -6	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.1
22:5 n -6	0.2 \pm 0.0	0.2 \pm 0.1	0.3 \pm 0.0
Total n-6 PUFA*	2.9 \pm 0.3^a	2.9 \pm 0.3^a	3.3 \pm 0.1^b
18:3 n -3	0.8 \pm 0.0	0.8 \pm 0.1	0.8 \pm 0.0
18:4 n -3	1.7 \pm 0.1	1.8 \pm 0.2	1.8 \pm 0.1
20:3 n -3	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0
20:4 n -3	0.5 \pm 0.0	0.6 \pm 0.1	0.6 \pm 0.0
20:5 n -3	9.5 \pm 1.3	10.1 \pm 1.5	10.5 \pm 1.2
22:5 n -3	1.5 \pm 0.2	1.6 \pm 0.3	1.8 \pm 0.2
22:6 n -3	9.9 \pm 1.2	9.7 \pm 1.5	11.2 \pm 0.4
Total n-3 PUFA	24.0 \pm 2.7	24.8 \pm 3.4	26.8 \pm 1.7
Total PUFA¹	28.5 \pm 3.1	29.6 \pm 3.8	31.7 \pm 2.1
n -3/ n -6	8.4 \pm 0.2	8.4 \pm 0.7	8.2 \pm 8.2
DHA/EPA	1.1 \pm 0.1	0.9 \pm 0.1	1.1 \pm 0.2
EPA/ARA*	10.5 \pm 0.6 ^a	10.9 \pm 0.6 ^a	9.7 \pm 0.3 ^b

SD values of 0.0 denote values < 0.05; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; ¹Total PUFA includes 16:2, 16:3 and 16:4; *denotes significant differences between groups ($p < 0.05$); ^{a,b} represent significantly differing parameters.

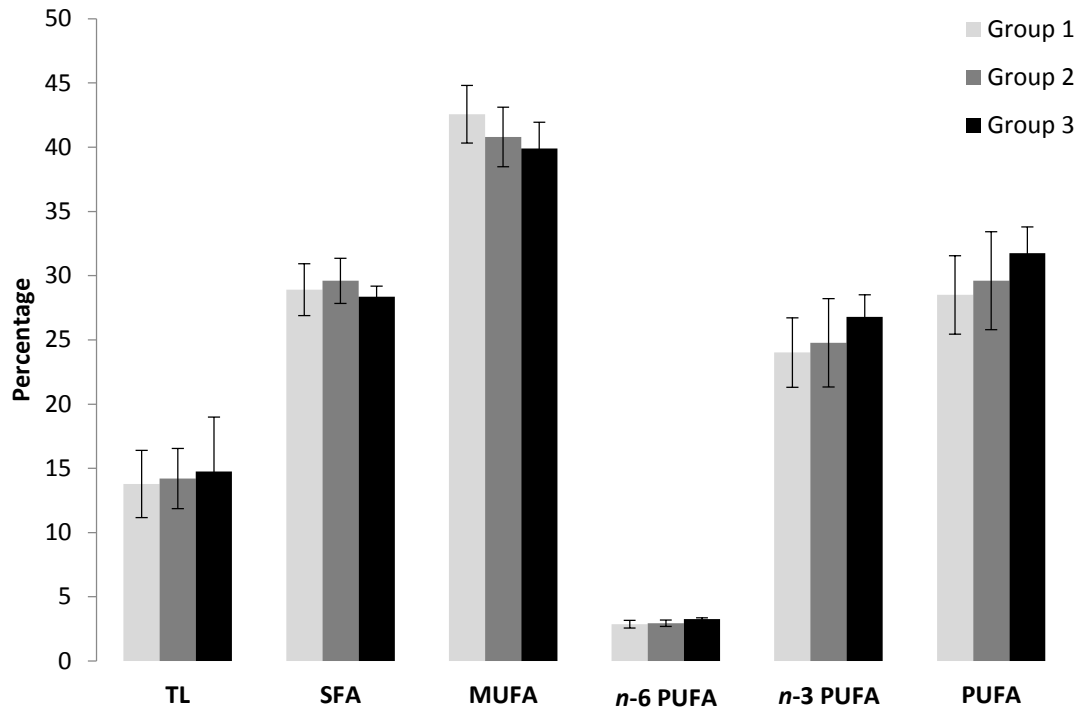


Figure 5.3 Major fatty acid groups (as a percentage of the total lipid composition) of the experimental sub-groups of farmed Atlantic bluefin tuna. Error bars represent standard deviation of the mean (Group 1, 6 months captive, weight <100 kg, $n = 5$; Group 2, 6 months captive, weight >100 kg, $n = 7$; Group 3, 18 months captive, $n = 5$).

5.3.2. Effect of fish size on fatty acid composition

Pooled data from Group 1 and Group 2 fish were subjected to a Pearson correlation analysis to examine for variation in FA composition with fish size (Table 5.3). Only 20:3 n -6 (dihomo- γ -linolenic acid) had a positive and significant correlation with fish size, while 18:1 n -9 (oleic acid) and 18:1 n -7 (vaccenic acid), both MUFA, were significantly and negatively correlated with fish weight. DHA/EPA decreased with increasing fish size ($p < 0.05$).

Table 5.3 Pearson coefficients for fatty acid measurements correlated with specimen weight.

Parameter	Pearson coefficient	<i>p</i>	Parameter	Pearson coefficient	<i>p</i>
Total lipid	0.113	0.72	18:3 <i>n</i> -6	0.347	0.27
14:0	0.513	0.09	20:2 <i>n</i> -6	(0.147)	0.65
15:0	0.040	0.90	20:3 <i>n</i> -6*	0.664	0.02
16:0	(0.060)	0.85	20:4 <i>n</i> -6	0.379	0.22
18:0	0.144	0.66	22:4 <i>n</i> -6	0.338	0.28
20:0	(0.105)	0.75	22:5 <i>n</i> -6	(0.049)	0.88
22:0	0.541	0.07	Total <i>n</i> -6 PUFA	0.277	0.38
Total SFA	0.127	0.70	18:3 <i>n</i> -3	(0.110)	0.73
16:1 <i>n</i> -7	0.339	0.28	18:4 <i>n</i> -3	0.381	0.22
18:1 <i>n</i> -9*	(0.724)	0.01	20:3 <i>n</i> -3	0.285	0.37
18:1 <i>n</i> -7*	(0.638)	0.03	20:4 <i>n</i> -3	0.461	0.13
20:1 <i>n</i> -9	(0.167)	0.60	20:5 <i>n</i> -3	0.397	0.20
20:1 <i>n</i> -7	0.328	0.30	22:5 <i>n</i> -3	0.425	0.17
22:1 <i>n</i> -11	(0.108)	0.74	22:6 <i>n</i> -3	(0.005)	0.99
22:1 <i>n</i> -9	0.328	0.32	Total <i>n</i> -3 PUFA	0.254	0.42
24:1 <i>n</i> -9	(0.486)	0.11	Total PUFA ¹	0.305	0.33
Total MUFA	(0.526)	0.08	<i>n</i> -3/ <i>n</i> -6	0.058	0.86
18:2 <i>n</i> -6	(0.242)	0.45	DHA/EPA	(0.685)	0.01

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ¹Total PUFA includes 16:2, 16:3 and 16:4; *denotes significant correlation ($p < 0.05$); figures in parentheses denote negative correlations.

5.3.3. Fatty acid distribution in farmed Atlantic bluefin tuna cephalic dorsal ordinary muscle

To examine differences in the dorsal ordinary muscle of farmed ABT, three different locations from one specimen were sampled and analysed for FA composition. Results for the major FA components are given in Figure 5.4 and percentage composition of TL for various FAs are listed in Table 5.4. The levels of TL differed significantly between the various dorsal muscle regions sampled; the TL was 5.0 % in Ce-OM1 taken close to the spinal column, but increased to 28.2 % in the outer areas of the dorsal muscle. SFA showed a decreasing trend between Ce-OM 1 and Ce-OM 3. Significant differences between regions of muscle were also measured for MUFA, with values of these being higher in samples from the Ce-OM 1 region. Of the individual monoene components, only oleic acid (18:1*n*-3) differed significantly between the different areas of muscle

sampled. The percentage of MUFA was seen to decrease from Ce-OM 1 to Ce-OM 3, while PUFA showed an opposing trend. The DHA/EPA ratios were similar in Ce-OM 1 and 2, but differed significantly from that in Ce-OM 3.

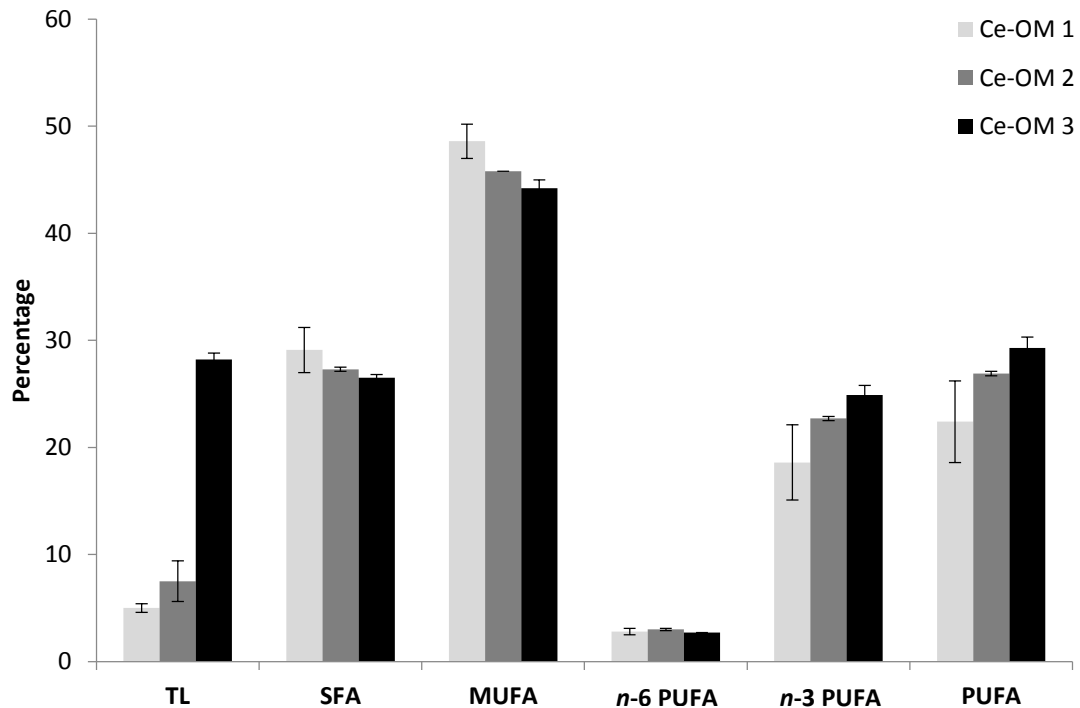


Figure 5.4 Major fatty acid groups (as a percentage of the total lipid composition) from different regions of the cephalic dorsal ordinary muscle of a farmed Atlantic bluefin tuna. Ce-OM 1-3 represent different regions of the dorsal muscle ($n = 1$).

5.3.4. Diet fatty acid composition

Table 5.5 shows the FA composition of the trash fish diet supplied to the fish during their time in captivity. FA levels varied between the different species. TL varied between 8 and 12 %. SFA levels were highest in chub mackerel. Total MUFA were higher in the herring species, while maximum PUFA levels were recorded in the pilchard. DHA/EPA ratios ranged from approximately 0.8 in Atlantic herring to 1.6 in the mackerel.

Table 5.4 Fatty acid composition of different regions of the cephalic dorsal ordinary muscle of a farmed Atlantic bluefin tuna specimen. Results are expressed as mean \pm standard deviation (SD). Ce-OM 1-3 represent different regions of the dorsal muscle ($n = 1$).

Parameter	Ce-OM 1	Ce-OM 2	Ce-OM 3
Total lipid*	5.0 \pm 0.4^a	7.5 \pm 1.9^b	28.2 \pm 0.6^{ab}
14:0	4.7 \pm 0.3	5.1 \pm 0.3	5.3 \pm 0.2
15:0	0.5 \pm 0.1	0.4 \pm 0.0	0.4 \pm 0.0
16:0	18.5 \pm 1.3	17.2 \pm 0.1	16.7 \pm 0.4
18:0*	5.0 \pm 0.4 ^a	4.3 \pm 0.1 ^b	3.7 \pm 0.1 ^{ab}
20:0	0.3 \pm 0.0	0.3 \pm 0.0	0.2 \pm 0.0
22:0*	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^b	0.1 \pm 0.0 ^b
Total SFA	29.1 \pm 2.1	27.3 \pm 0.2	26.5 \pm 0.3
16:1 n -7	6.7 \pm 0.2	6.8 \pm 0.1	7.2 \pm 0.1
18:1 n -9*	19.4 \pm 0.8 ^a	17.1 \pm 0.4 ^a	14.8 \pm 0.4 ^b
18:1 n -7	3.6 \pm 0.4	3.3 \pm 0.1	3.1 \pm 0.1
20:1 n -9	7.8 \pm 0.3	7.6 \pm 0.1	7.8 \pm 0.1
20:1 n -7	0.3 \pm 0.0	0.3 \pm 0.0	0.3 \pm 0.0
22:1 n -11	9.5 \pm 0.5	9.2 \pm 0.2	9.5 \pm 0.3
22:1 n -9	0.7 \pm 0.0	0.7 \pm 0.0	0.7 \pm 0.0
24:1 n -9	0.5 \pm 0.5	0.8 \pm 0.0	0.7 \pm 0.0
Total MUFA*	48.6 \pm 1.6^a	45.8 \pm 0.0^{ab}	44.2 \pm 0.8^b
18:2 n -6	1.3 \pm 0.1	1.4 \pm 0.0	1.2 \pm 0.0
18:3 n -6	0.1 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0
20:2 n -6	0.2 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0
20:3 n -6*	0.1 \pm 0.0 ^a	0.1 \pm 0.0 ^{ab}	0.1 \pm 0.0 ^b
20:4 n -6	0.8 \pm 0.1	0.8 \pm 0.0	0.8 \pm 0.0
22:4 n -6	1.5 \pm 0.2	0.1 \pm 0.0	0.1 \pm 0.0
22:5 n -6	0.1 \pm 0.0	0.2 \pm 0.0	0.2 \pm 0.0
Total n-6 PUFA	2.8 \pm 0.3	3.0 \pm 0.1	2.7 \pm 0.0
18:3 n -3	0.8 \pm 0.1	0.9 \pm 0.0	0.9 \pm 0.0
18:4 n -3	1.5 \pm 0.2	1.9 \pm 0.1	2.1 \pm 0.1
20:3 n -3	0.1 \pm 0.0	0.1 \pm 0.0	0.1 \pm 0.0
20:4 n -3	0.4 \pm 0.1	0.5 \pm 0.0	0.5 \pm 0.0
20:5 n -3	6.4 \pm 1.2	8.1 \pm 0.0	9.7 \pm 0.2
22:5 n -3	1.0 \pm 0.2	1.2 \pm 0.0	1.4 \pm 0.0
22:6 n -3	8.5 \pm 1.8	10.1 \pm 0.1	10.2 \pm 0.4
Total n-3 PUFA	18.6 \pm 3.5	22.7 \pm 0.2	24.9 \pm 0.9
Total PUFA¹	22.4 \pm 3.8	26.9 \pm 0.2	29.3 \pm 1.0
n -3/ n -6*	6.7 \pm 0.6 ^a	7.7 \pm 0.1 ^{ab}	9.1 \pm 0.2 ^b
DHA/EPA*	1.3 \pm 0.6 ^a	1.3 \pm 0.4 ^a	1.1 \pm 0.3 ^b

SD values of 0.0 denote values < 0.05 ; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ¹Total PUFA includes 16:2, 16:3 and 16:4; *denotes significant differences between the various muscle regions ($p < 0.05$); ^{a,b} represent significantly differing parameters.

Table 5.5 Mean fatty acid compositions of the different components of the trash fish diet supplied to the tuna during the farming period ($n = 5$).

Parameter	Atlantic herring	Pacific herring	European pilchard	Chub mackerel
Total lipid	8.64	8.33	11.95	9.70
14:0	5.35	8.48	10.46	8.49
15:0	0.43	0.47	0.50	1.41
16:0	23.79	15.16	20.50	30.54
18:0	3.30	1.48	3.50	7.09
20:0	0.23	0.29	0.34	0.99
22:0	0.19	0.00	0.13	0.41
Total SFA	33.29	25.89	35.43	48.94
16:1 $n-7$	8.82	4.51	10.58	7.52
18:1 $n-9$	30.16	6.98	7.17	12.38
18:1 $n-7$	4.64	1.78	3.45	3.65
20:1 $n-9$	1.07	14.88	1.42	2.21
20:1 $n-7$	0.20	0.35	0.15	0.37
22:1 $n-11$	1.66	24.95	0.47	1.99
22:1 $n-9$	0.23	0.18	0.29	0.41
24:1 $n-9$	0.33	1.12	0.56	0.94
Total MUFA	47.10	54.76	24.08	29.48
18:2 $n-6$	1.36	1.45	1.19	1.97
18:3 $n-6$	0.14	0.11	0.35	0.16
20:2 $n-6$	0.24	0.23	0.12	0.41
20:3 $n-6$	0.11	0.09	0.25	0.16
20:4 $n-6$	1.14	0.21	1.25	0.88
22:4 $n-6$	0.07	0.10	0.18	0.27
22:5 $n-6$	0.07	0.08	0.13	0.29
Total $n-6$ PUFA	3.14	2.26	3.46	4.14
18:3 $n-3$	0.63	1.07	0.92	1.17
18:4 $n-3$	1.46	2.68	3.32	1.30
20:3 $n-3$	0.07	0.11	0.00	0.15
20:4 $n-3$	0.22	0.41	0.83	0.35
20:5 $n-3$	7.13	5.13	18.37	4.86
22:5 $n-3$	0.30	0.55	1.77	0.92
22:6 $n-3$	5.66	5.88	6.27	8.04
Total $n-3$ PUFA	15.47	15.82	31.47	16.78
Total PUFA¹	19.61	19.35	40.49	21.58
$n-3/n-6$	4.93	6.99	9.10	4.05
DHA/EPA	0.79	1.15	0.34	1.65
EPA/ARA	6.27	23.97	14.71	5.56

SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ARA, arachidonic acid; ¹Total PUFA includes 16:2, 16:3 and 16:4.

5.3.5. Expression of $\Delta 6$ fatty acyl desaturase ($\Delta 6fad$) and fatty acyl elongase ($elov15$) in ABT organs

Quantitative expression of $\Delta 6fad$ and $elov15$ is shown in Figure 5.5. For both genes, expression was highest in the brain followed by the liver. No expression of $\Delta 6fad$ and $elov15$ was detected in the spleen, while only $elov15$ expression was detected in the kidney.

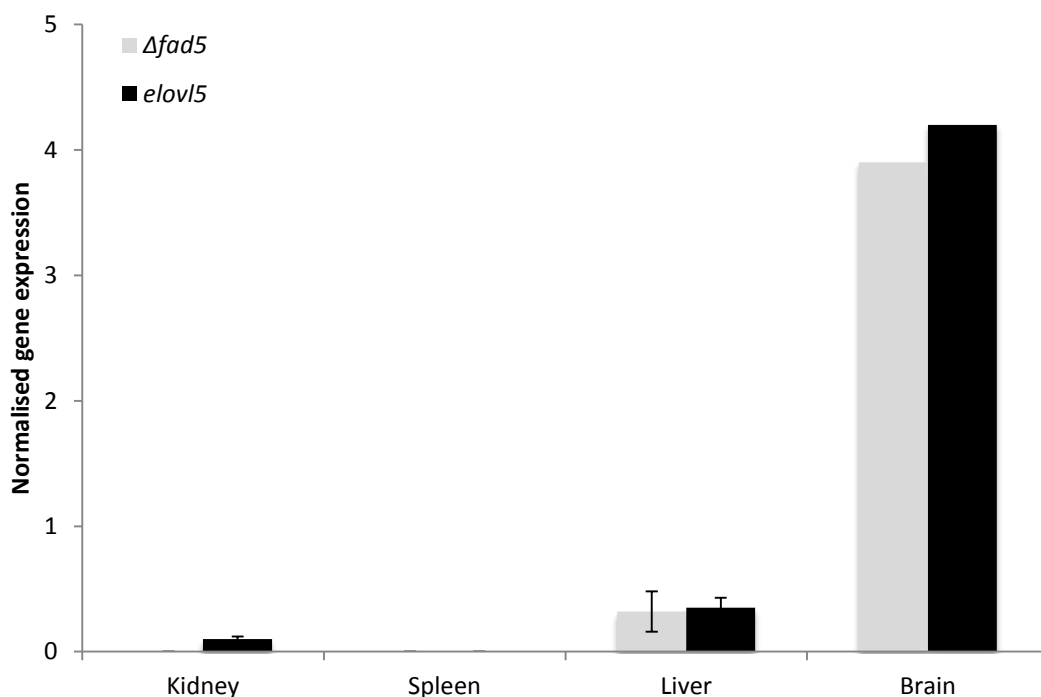


Figure 5.5 Normalised gene expression ratios for $\Delta 6fad$ and $elov15$ in different organs of farmed Atlantic bluefin tuna. Bars represent mean \pm standard deviation (kidney, spleen, liver, $n = 5$; brain $n = 1$).

5.4. Discussion

Data on the lipid composition of ABT is scarce due to the the costs involved in sourcing flesh samples for analysis. The main objective of this study was to examine the FA composition of farmed ABT muscle tissue. Mean total lipid in the caudal muscles of specimens in this study was in the region of 14 % while SFA, MUFA and PUFA were 29, 40 and 30 % respectively. Several FA, including the SFA palmitic acid (16:0) and the monoenic FA, 18:1 n -9 (oleic acid), 20:1 n -9 and 22:1 n -11, are known to be important for

energy generation in fish (Sargent *et al.*, 2002). In addition palmitic and oleic acids, together with EPA and DHA are important constituents of the cell membrane bilayer (Sargent *et al.*, 2002). It is expected, therefore, that these would be found in higher concentrations than other FA. In fact, of the entire FA composition, palmitic and oleic acids each accounted for 18 %, levels of both EPA and DHA amounted to 10 %, while 20:1*n*-9 and 22:2*n*-11 each amounted to approximately 6 % of TL.

Comparison of the FA composition obtained for farmed ABT in this study with that reported from Croatian specimens (Topic Popovic *et al.*, 2012) revealed more similarities than differences and all the major FA components (palmitic and stearic acid, DHA and EPA) were found at approximately similar levels. Also in migrating wild ABT, levels of SFA, MUFA and PUFA (Sprague *et al.*, 2012) are similar to those found in this study. This indicates some form of endogenous control over FA metabolism.

This is further corroborated by the lack of difference found in the FA composition of caudal muscle between fish held in cages for different durations. During the farming period, the fish are restricted to a controlled trash fish diet but this did not result in any major differences between the different groups of fish, regardless of the amount of time they had been maintained in captivity. In addition there were wide variations in SFA, MUFA and PUFA within the herring, sardine and mackerel supplied to the fish, but these variations were not reflected in muscle FA profiles. This further supports the hypothesis that some form of selective FA catabolism may be present in the ABT. Similar results were obtained in studies on PBT, where the lipid composition of a trash fish diet was not linked to the FA composition of tuna flesh (Nakamura *et al.*, 2007).

Selective use of FA for energy generation is widespread in several fish species (Sargent *et al.*, 2002) and migratory fish selectively retain high DHA levels within their tissues and organs (Medina *et al.*, 1995; Ishihara & Saito, 1996; Murase & Saito, 1996; Saito & Ishihara, 1996; Saito *et al.*, 1996; Mourente *et al.*, 2002; Osako *et al.*, 2006) so that DHA/EPA ratios are high in comparison with other teleost species (Mourente & Tocher, 2009). In fact, relatively high DHA/EPA levels have been reported from ABT from both wild and farm origins in other studies (Salvo *et al.*, 1998; Mourente *et al.*, 2002; Sprague *et al.*, 2012; Topic Popovic *et al.*, 2012). Several authors have suggested that the high DHA/EPA ratio in highly migratory species is due to the retention of DHA during long-distance migrations (Ishihara & Saito, 1996; Saito *et al.*, 1996, 1999; Mourente & Tocher, 2009). This is reasonable, especially considering that fish have restricted capabilities to biosynthesise LC-PUFA and *n*-3 PUFA are not metabolised as efficiently as SFA and MUFA for energy generation (Tocher, 2003). In addition, DHA may have crucial roles in gonadal development in female ABT (Mourente *et al.*, 2002) and is an essential dietary requirement in tuna species (Glencross *et al.*, 2002). DHA/EPA reported from wild ABT after migration and prior to their entry into the Mediterranean for spawning (Mourente *et al.*, 2002; Sprague *et al.*, 2012) are about two-fold higher than those reported from farmed ABT specimens in this study and others (Topic Popovic *et al.*, 2012). This difference arises from the lower EPA levels found in the wild fish muscle following their annual migration into the Mediterranean (Sprague *et al.*, 2012) rather than differences in DHA levels. Differences, therefore, exist in the nutritional status between wild and captive fish and since ABT still undergo sexual maturation in cages (personal observation) this should be due to the constant nutrient availability and restricted energy use in captivity.

There were no statistically significant differences in lipid levels between the different experimental sub-groups, however there was an expected trend in the results. Among the different groups, lipid levels were lowest in the smaller specimens and this is probably because energy is directed towards somatic growth rather than lipid being retained as an energy reserve. A similar trend was observed in farmed PBT specimens (Nakamura *et al.*, 2007). Lipid levels were highest in fish held captive for the longer duration, although the TL difference was minimal between the groups. There are some variations between the TL quantities reported here and those found in other studies. Previous studies on farmed ABT have reported lipid levels of 9 % (Topic Popovic *et al.*, 2012), 16 % (Yerlikaya *et al.*, 2009) and 20 % (Giménez-Casalduero & Sánchez-Jerez, 2006). Studies on wild fish have found muscle TL of 13 % (Mourente *et al.*, 2002) and 8 % (Sprague *et al.*, 2012). TL levels in tuna are known to vary depending on the region of muscle sampled (Nakamura *et al.*, 2007), and since most of these studies do not specify the region from where muscle was collected, a direct comparison of results cannot be made. It is, however, natural to expect TL levels to be influenced by several other factors including origin (Roy *et al.*, 2010), prey lipid levels (Saito *et al.*, 1996, 1997) and reproductive cycle stage (Mourente *et al.*, 2002).

In contrast to regional variation in TL levels in tuna ordinary muscle, FA compositions have been reported to be relatively similar in flesh samples collected from different locations of the ordinary muscle of PBT (Roy *et al.*, 2010). FA analysis of the different regions of the dorsal muscle of farmed ABT (Ce-OM 1-3) were analysed in this study and there were several variations in the major lipid classes between these. Ce-OM 1 corresponds to the dorsal *akami* region, while Ce-OM 2 and 3 come from the *chuo-toro*

region of ABT dorsal muscle (Figure 5.2). Interestingly, tuna dorsal muscle becomes paler with increasing distance from the spinal column, and this is easily observed and clearly evident in Figure 5.2. In fact, TL levels were significantly different between the three regions and increased from 5 to 28 % in Ce-OM 1 and 3 respectively. As for the variation in the major FA classes between the different regions, SFA and MUFA decreased between the inner and outer regions but PUFA increased. Although different FA compositions in dorsal muscle also occur in other fish species (Katikou *et al.*, 2001), the differences in the dorsal muscle of ABT in this study are more pronounced. The physiological basis of this phenomenon has not been investigated to date, but it may be linked to energy generation requirements of the different muscle groups in the fish. Interestingly this distinction in ABT dorsal and ventral muscle is more pronounced at lower sea temperatures so that increased lipid levels beneath the skin may be important in restricting heat loss to the environment and aid in homeothermic regulation.

ABT muscle samples were collected close to the caudal end of the fish in order to limit value depreciation of the marketable end product. Instead of relying on traditional tail cuts, dorsal muscle specimens were collected from a region known to be similar in appearance to the rostral end of the dorsal muscle. This was done in order to achieve a better representation of the entire tuna muscle. Only a single cross-sectional slice was collected from the anterior dorsal muscle, and this again is due to marketability reasons. Sample numbers were also limited, but despite this, variation within the separate groups of fish was relatively small so that it may be presumed that the results give an appropriate representation of the FA profile of the captive tuna population.

There was no opportunity to collect wild ABT specimens for muscle FA analysis. Considering the restricted energy use and constant feed availability it is expected that farmed specimens will possess higher TL values in comparison to their wild counterparts (Hirano *et al.*, 1980; Aoki *et al.*, 1991; Alasalvar *et al.*, 2002; Grigorakis *et al.*, 2002). At least, this is the case for the PBT, where research has shown that lipid is found at higher levels in farmed as opposed to wild specimens (Katada *et al.*, 1960; Nakamura *et al.*, 2006; Tsukamasa *et al.*, 2007).

The expressions of *Δ6fad* and *elov15* were quantified with qPCR to analyse variation in their expression within different organs. Both genes have been previously described in tuna species including ABT (Morais *et al.*, 2011), while Gregory *et al.* (2010) isolated a fatty acyl elongase from the liver of SBT. Brain tissue displayed the strongest expression for both *Δ6fad* and *elov15* and such pronounced expression of these elongase and desaturase genes within the teleost brain has been described in other species including Atlantic cod, cobia, *Rachycentron canadum* L., and Asian sea bass, *Lates calcarifer* (Bloch) (see Zheng *et al.*, 2004a, 2009; Tocher *et al.*, 2006; Mohd-Yusof *et al.*, 2010). Liver had lower expression ratios for both genes while only *elov15* was detected in the kidney, an expression pattern similar to that reported from the Atlantic cod with the exception that the liver of the latter displayed the presence of both *Δ6fad* and *elov15* (Tocher *et al.*, 2006). *Δ6fad* is involved in desaturation of C₂₄ intermediates in the conversion of EPA to DHA but its exact role in LC-PUFA biosynthesis has been the subject of conjecture (Tocher, 2010). Their significant expression in the teleost brain has given rise to the hypothesis that the role of these enzymes in marine fish is that of being essential to the maintenance of adequate DHA levels in the brain and neural tissues

(Zheng *et al.*, 2004a, 2009; Tocher *et al.*, 2006). In fact, $\Delta 6fad$ and *elov15* levels have been shown to increase during ABT larval development (Morais *et al.*, 2011). The important roles these play in the maintenance of the nervous system has been demonstrated by Monroig *et al.* (2010) in Atlantic salmon who reported that there are three types of $\Delta 6fad$ genes and considerable expression of all $\Delta 6fad$ genes occurs within the brain. No expression of either transcripts was detected in the spleen for farmed ABT and reasons for this are unclear.

Nutritional factors are also known to influence the expression of both $\Delta 6fad$ and *elov15* (Seilliez *et al.*, 2001; Zheng *et al.*, 2004b; González-Rovira *et al.*, 2009; Monroig *et al.*, 2010) but the experimental design of this study did not allow for such investigations.

5.5. Conclusion

This study investigated lipids in farmed ABT and relatively similar FA profiles were detected in all the fish studied. Although TL increased slightly with farming duration, FA profiles remained similar indicating some form of endogenous control or selective FA use within ABT. The FA composition of the trash fish diet supplied did not result in any differences in specimen lipids, similarly indicating that FA metabolism in ABT may be selective towards FA substrates. It has also been shown that lipid levels, and to a much lesser degree FA compositions, vary by region in the dorsal muscle of the fish with TL being higher closer to the skin. Finally, the expression of two genes, $\Delta 6fad$ and *elov15*, involved in FA metabolism was examined in organs of ABT. High expression ratios were measured in the brain suggesting important roles for these within the nervous system of the ABT.

CHAPTER 6

GENERAL DISCUSSION

The ABT has been farmed on an industrial scale in the Mediterranean for over a decade, but owing to several limitations encountered in working with the species, including but not limited to their value and size (see **Section 1.6**) there is a paucity of information relating to the health and welfare of captive ABT.

The health and welfare of captive fish is a subject which has recently gained international attention and importance. It is of increased significance in the case of farmed fish sourced from wild-capture fisheries in that their domestication is accompanied by changes which may affect their lifestyle and wellbeing. The objectives of this work were to attempt to investigate the effect of captivity on several aspects of the health and welfare of wild-caught ABT.

6.1. Coronary artery lesions in wild and captive Atlantic bluefin tuna

One study has reported on the occurrence of arteriosclerotic lesions in wild ABT, but there has been no follow up work to investigate this further. The study outlined in Chapter 2 investigated the effects of captivity, if any, on coronary arterial lesions in ABT.

It analysed the occurrence of arteriosclerotic lesions in captive and wild ABT, and used computer software to measure the severity of these lesions. The coronary arterial lesions described in this investigation are similar in morphology to those described previously for wild ABT (Vastesaege *et al.*, 1962), but lack the lipid inclusions described in the latter. Lesions in the coronary artery in salmonids are a progressive condition so that severity generally increases with fish age (Farrell, 2002) and results from this study indicate that a similar trend exists in the case of ABT since lesion severity increased with fish size. At the time of study, the only accessible source of wild ABT specimens were small specimens caught in the Adriatic Sea, and therefore it was not possible to compare coronary arterial lesions between wild and captive large specimens. Although results obtained here do not indicate that captivity adversely affects ABT in this regard, such direct comparisons would have given a clearer indication on the effects of farming on arteriosclerosis in ABT.

The larger specimens in this study had only been held in cages for 6 months. It may be possible that the effects of captivity on arterial lesions in ABT become more evident with an extended farming duration. Further work should attempt to address this in order to provide a more thorough report on the effects of farming on arteriosclerotic lesions in the ABT.

Observations of severe lesions in the wild specimens, despite their relatively smaller size, confirms that the formation of coronary arterial lesions in ABT commences prior to their capture for farming purposes. This study has attempted to represent a large proportion of the size/age ranges encountered in ABT farming, but could not source fish under the minimum catch limit, which presently stands at 30 kg. The wild specimens

sourced in this study were relatively small in size (mean weight = 35 kg) when compared to those obtained from the farm, and all were found to have some form of identifiable lesion within their coronary arteries. Additional research into the occurrence and prevalence of coronary artery lesions throughout the life-cycle of the ABT will be useful in providing further information on arteriosclerosis in the species, including its origins and possibly its aetiology.

The origins of arteriosclerotic lesions in salmonids are unknown but it is thought that vascular injury arising from mechanical stress is the primary initiating factor (Saunders & Farrell, 1988; Farrell, 2002). In this study, only the main coronary artery lying on the bulbus arteriosus was examined. Incidentally, several examples of such proliferations were found in the arterial vessel network within the bulbus arteriosus parenchyma, indicating that these lesions are not restricted only to the artery investigated here. Further work should attempt to analyse the vessels supplying the ventricle for lesions of this type, since these are also subject to mechanical stresses during contraction and relaxation cycles.

It has been postulated that fast growth, by way of increased demand on the physiological systems of the fish, promotes arterial lesions (Saunders *et al.*, 1992; Farrell, 2002). ABT growth is exceptionally high, and investigation of the consequences of this on lesion severity, and to a lesser degree prevalence, will be of interest in regard to the welfare of the fish under commercial farming conditions.

To date no research into the origins of arteriosclerosis in fish has been performed but studies have found that cholesterol (Farrell & Munt, 1983) and sex hormones (House *et al.*, 1979) may promote lesions in the coronary artery of salmonids. In practical terms,

investigations of the effects of such factors on arteriosclerosis in ABT will be interesting but may prove complicated to perform considering the constraints which would be encountered in such experimental designs. For example, wild-captured mature fish feed exclusively on whole fish so that research on the effects of diet on arteriosclerosis, requiring the delivery of dietary substances through the feed are, at the moment, not possible.

Due to time constraints during sampling, it was not possible to collect blood samples from specimens that had their hearts removed so that correlation of plasma cholesterol, which is known to promote lesions in mammalian arteries, and lesion severity could not be performed. Future work in the field should attempt to investigate this in order to give an indication on the effect of cholesterol levels on the severity of arteriosclerotic lesions in ABT.

6.2. Haematological parameters and immune components of farmed Atlantic bluefin tuna

Haematological and plasma biochemical indices are useful in the assessment and monitoring of animal health but require the availability of species-specific information, since these parameters vary considerably between fish species. There is only little information on haematological and plasma biochemical parameters for ABT and the second part of the thesis examined several parameters from the blood of captive ABT. It described basic haematological parameters, plasma biochemical values and screened for non-specific immune parameters in farmed ABT.

It is known that haematological variables show seasonal variation in Atlantic salmon (Sandnes *et al.*, 1988). It would be expected that the same applies for ABT and further

research into temporal or seasonal variation is essential to obtain reliable haematological indices for the species. Considering this the results obtained in this work should be developed further in this way to obtain more reliable indices which may be used year-round.

In addition, further research in the field should investigate the relation between haematological or plasma biochemical indices and the physiological or nutritional status of the animals. The results obtained from such types of studies can act as a reference in the welfare assessment of captive ABT, given that post-harvest blood collection is non-invasive and therefore does not affect the market value of ABT specimens.

Presently there is no information on how haematological and plasma biochemical indices and immune parameters change once ABT are made captive. Results from research on the SBT have shown that some haematological and immune parameters change during the transition of the fish from the wild to captivity (Kirchhoff *et al.*, 2011b). Similar variation would also be expected to occur in the ABT and investigations into this field will be of interest in that they may provide a better understanding of the physiological changes the fish undergo during this transition.

Statistical analysis revealed a clear separation in the haematological and biochemical indices of different size classes of fish. Similar separation was observed in the immune parameters measured, and this is mostly due to the higher IgM levels measured in the blood of smaller fish. The fish were being held in sea cages so that there were several uncontrolled environmental variables in the experimental setup and so it is difficult to ascertain whether this separation is due to intrinsic or extrinsic factors. ABT differ from the majority of teleosts in that they show a large size variation, are long-lived and may

live up to 40 years (ICCAT, 2014), so that it is possible that physiological requirements change throughout the life of the fish and this is the reason behind the variations observed here. It may also be, however, that the observed differences are because animals of different size or age are affected by captivity to differing degrees and the variations observed here should be investigated further.

There is very low disease prevalence in commercial ABT farming and although disease has not presented major problems to the industry, the immune responses of tunas to disease should be an interesting field of research especially considering that their elevated body temperatures may enable them to mount quicker immune responses. Such responses to parasitic infection have been demonstrated in the SBT (Aiken *et al.*, 2008; Kirchhoff *et al.*, 2012) and immunostimulants have been successfully delivered to the same species through their feed in small-scale experiments (Kirchhoff *et al.*, 2011a), and so similar studies may be designed for the ABT. To date such studies in the ABT have not been performed. The reasons for this are several and include the difficulties encountered in handling large fish and the cost of individual specimens. This, coupled with the apparent resistance of the species, may provide little justification for such a considerable research investment.

6.3. Non-specific immune components in Atlantic bluefin tuna epidermal mucus

Preliminary investigations have shown that disease prevalence in farmed ABT is very low and fish epidermal mucus is an important interface between the organism and its environment. Chapter 4 examined the properties of ABT epidermal mucus as a first barrier against pathogen entry and infection. An epidermal ME, prepared in the lab from

pooled mucus samples from farmed ABT, was examined for the presence of several non-specific humoral immune components. Treatment of western blots of the ME with polyclonal and monoclonal antibodies showed no reaction and faint bands between 78-72 kDa respectively, so it could not be confirmed if the latter was due to the presence of an IgM-type moiety or non-specific staining, but the presence of IgM in the ME was also detected in an ELISA. Precipitation of total Ig with polyethylene glycol detected a higher concentration of Ig than the ELISA so it may be that the ME contained another Ig isotype. This has been reported from other teleost species (Danilova *et al.*, 2005; Hansen *et al.*, 2005), and further investigation is necessary to confirm if this is also the case for ABT.

The ME was tested for the presence of enzymes with an API-ZYM strip, and the activities of certain enzymes detected in the former quantified in separate assays. Zones of clearing in zymograms and activity in azocasein hydrolysis assays confirmed the presence of proteases in the ME. Lysozyme, alkaline phosphatase and esterase activities were confirmed in separate assays. The ME also possessed haemagglutinating activity but did not inhibit bacterial growth in radial diffusion assays.

It has been shown that these immune components vary with season in the olive flounder (Jung *et al.*, 2012). The mucus in the present study was collected at a single time point from several specimens and pooled and seasonal variation could not be studied. Mucus enzyme levels have also been shown to change in disease outbreaks in the Atlantic salmon (Ross *et al.*, 2000) and due to changes in environmental parameters in the turbot, *Scophthalmus maximus* (L.) (see Huang *et al.*, 2011).

This is the first study describing the non-specific immune parameters of epidermal mucus of ABT. Further experimentation in this area should attempt to address the seasonality of the individual components described here, as well as changes in their levels brought about by general husbandry stress and infection. The ME used in this study did not appear to show bacteriolytic activity although, it was found to possess proteases and lysozyme. The antimicrobial activity of crude mucus may show different results, since the laboratory processing performed on the crude mucus in this study may have inactivated or removed some of its active components or more probably, diluted their concentration to insufficient levels for the inhibition of bacterial growth.

This research, especially in relation to IgM and other non-specific immune components, will contribute to knowledge on the importance and function of these components in the skin mucus of ABT.

6.4. Fatty acid composition of farmed Atlantic bluefin tuna and long-chain PUFA gene expression

In captivity ABT are fed on whole fish, and there is no information on how the absence of a non-formulated diet affects the species. The final part of this project examined the FA profile of ABT specimens farmed for different durations, and how prolonged farming affects the FA composition of the fish. The expression of genes involved in LC-PUFA biosynthesis in the kidney, spleen, liver and brain of captive ABT was also assessed.

Only slight differences were found in the FA profiles of fish held in cages for different periods of time. In addition, the final FA composition of the ABT was not affected significantly by the FA profile of the trash fish diet. These two findings reveal the presence of a degree of control over the metabolic substrates used by the ABT for

energy generation. This hypothesis is further supported by the findings of similar FA profiles in farmed ABT specimens from Croatia.

In addition, the FA composition of the dorsal muscle of the fish differed by location and TL increased at regions closer to the skin. At lower sea temperatures, the distinction between outer and inner layers of the dorsal and ventral muscle can be visually observed. Research investigating the TL and FA profile of different regions of the cephalic muscle during different times of year may explain this phenomenon better and give an indication to its relation, if any, with heat conservation in the species.

The expression of genes involved in LC-PUFA biosynthesis differed by organs, implying that the latter have differing roles in PUFA biosynthesis. Availability of organs in this study was somewhat restricted, however, and a more exhaustive examination of the expression in other organs should be done to gain a better understanding of the relation of these to the physiology of the ABT. It would also be of interest to analyse for differences in gene expression between wild and farmed ABT.

At present, tunas are being sourced from wild-capture fisheries so that the use of artificial feed is not possible, and this limits nutritional studies in the species. Notwithstanding, it would be useful to investigate in detail the effects of varying diet FA compositions on fish since this would aid commercial operations to better tailor their end product to different markets.

Comparison of the FA composition of farmed ABT with wild ABT would be useful but also has its limitations in that FA composition would be expected to vary with several

conditions, especially considering the significant energy investment that ABT put into gonadal development and maturation.

6.5. General conclusion

This study has investigated and provided novel information on several aspects of the health and welfare of ABT held under commercial conditions. It has shown that husbandry, at least for the durations investigated here, does not have significant impacts on the ABT as regards coronary arterial lesions and nutritional status. More detailed investigations into general health and welfare are required to confirm this.

Experimentation with wild-captured ABT encounters several difficulties, and this has resulted in a general lack of information on the species. New information on the closely related PBT is becoming available from juveniles sourced from hatcheries in Japan. Similarly, it is expected that experimentation with ABT will be facilitated once progress is made in the hatchery production of the species, but several obstacles remain to be overcome for this to attain commercial viability. Due to this, at present only basic experimentation is possible with ABT and detailed studies into various aspects of the species are difficult to achieve since their growth only progresses in sea cages, where control over husbandry and environmental conditions is always difficult to achieve.

As such there will always be a degree of limitation in the manipulations which can be performed with large ABT specimens so that understanding of their biology, whether captive or in the wild, may remain enigmatic and the subject of scientific speculation until suitable methods for their husbandry in containment are developed.

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