Blood Metabolomes and Molecular Markers of Cellular Aging in Wild Fish

By

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A Thesis submitted to the Faculty of Graduate and Postdoctoral Studies

of the University of Manitoba

in partial fulfilment of the requirements for the degree of

Doctor in Philosophy

Department of Biological Sciences

University of Manitoba

Winnipeg

Abstract: Blood Metabolomes and Molecular Markers of Cellular Aging in Wild Fish

My work studies stress and aging physiology of wild fish using biochemical methods. The species used are two North-American temperate fish: *Sander vitreus*, walleye, and *Salvelinus namaycush,* lake trout, both species being upper-level predators of high ecological and economic importance. While using biochemical measurements is common in fish laboratory models, my thesis uses these techniques to gain insight into the biology of fishes in the wild with focus on possible environmental stress and the effect of biological aging. In my first research chapter, I determined by non-lethal sampling the whole blood metabolomic profile of *S. vitreus* from Winnipeg Lake to test for regional differences in metabolites across this population. Results show different blood metabolite patterns across the Southern versus the Northen basin of Lake Winnipeg. Northern *S. vitreus* exhibit higher concentrations of essential and branched-chain amino acids and metabolites of specific amino acid catabolism pathways, relative to Southern caught specimens. The pattern of amino acid metabolites in Northern S. vitreus may indicate heavier reliance on protein catabolism relative to the Southern fishes. Conversely, higher concentration of long-chain acylcarnitines in some Southern fish suggests higher reliance on β-oxidation for energy supply during spawning. My second research chapter investigated if patterns of changing metabolites with age in mammals may also be found in blood metabolites of Lake Winnipeg *S. vitreus* as they age in the wild. Results show amino acids, including essential, branched-chained and some non-essential amino acids, correlate positively with chronological age, while some medium and short-chained acyl-carnitines decrease with age. These results are similar to age-related patterns in mammals. The third research chapter investigated cellular senescence in wild-caught *S. namaycush,* using two common markers of aging associated with physiological decline, relative telomere length and lipofuscin accumulation. Results show no correlation of these aging markers with age in *S. namaycush,* in three tissues, heart, liver and red blood cells. These findings show that lipofuscin accumulation and relative telomere length data do not support increasing cellular senescence with age in this species and may represent negligible senescence in *S. namaycush.*

Acknowledgements

I extend my gratitude to my thesis committee for their patience and kindness during my defence process. Special thanks to Dr. J. R. Treberg for the trust and opportunity to work in his lab and for supporting my two major doctoral projects; I hope I did justice to them. Thank you to Dr. Huebner for providing teaching lab facilities and to Dr. E. Anjos for instructing me in histology procedures. I am deeply grateful to Maria Yusishena for assistance with histology and aging, and also for taking pictures of walleye spines and scales. For the contributions to Chapter 4, my thanks to Dr. Guzzo for his help and to Travis Durhack for assistance in collecting tissue samples. I also wish to thank Youseff Habibi for guidance in optimizing PCR procedures. I appreciate Dr. Gary Anderson for the use of his low-speed saw and other equipment for walleye spine processing, and Dr. Allison Loeppky for teaching me how to process spines for aging. Lastly, sincere thanks to Dr. Jen E. Jeffrey for guidance in R software, and to Dr. Matt Thorstensen for always offering help with statistics and R; I wish you the best in Toronto.

Dedication

I am a non-traditional Latina in Science. If you read it, cheers to you.

It takes a village, so I will like to dedicate my thesis document to my family: abroad, mom, dad, Tia Vera; and my family here: Ronni and all his sacrifices, and my daughter Perla, the one that understood, always, keep playing Music, and discovering Science, I hope my experiences may give you guidance and encouragement to never stop, or something to laugh about, you are so much smarter than me,

To all the immigrants out there, and my Spanish and Latino fellows, si se puede,

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1. Chapter 1: Introduction and background information on this thesis.

1.1 Contribution of Authors

Lilian Maria Wiens, Conceptualization, writing and editing; J.R. Treberg, editing.

This thesis on fish biology applies methods commonly employed in laboratory settings for application in the context of wild fish species. Lake Winnipeg will be the focus of the next two chapters' studies, both on walleye, *Sander vitreus.* The first research chapter uses metabolomics to measure metabolites in the blood of the fish to test for regional, or temporal, differences across geographical areas of Lake Winnipeg to link metabolomic data to organismal physiology; the third chapter uses metabolomics to investigate how age influences patterns found in the metabolites in the blood of walleye. The final research chapter studies lake trout, *Salvelinus namaycush,* a long-lived Salmonid, which was sampled from the Algonquin Lakes in Ontario. Continuing with the laboratory techniques, the final research chapter measures molecular markers associated with aging, specifically relative telomere length and advanced glycation products (lipofuscin) accumulation, to confirm if patterns of aging in more commonly studied models are similar to what is found in an aquatic ectotherm. The remainder of this chapter provides the general rationale and background for the three research chapters that follow.

1.2 Why Fish? Economic and Cultural Importance

Fishes are one of the most specious taxa of vertebrates and are vital to ecosystem health in many places (Holmlund & Hammer, 1999). However, fishes are under threat in many ecosystems, especially freshwater ones when limited connectivity constrains capacity to migrate away from poor conditions. Fish are the most important source of protein for humans worldwide (Holmlund & Hammer, 1999). Marine fish harvest (including by-catch) and aquaculture is on the level of over 100 million tons annually (Fisheries and Aquaculture Department, 2011). Many inland communities also depend on fisheries for economic and food necessities (Lynch et al., 2015). Furthermore, fishes provide many ecological services for the environment, often acting as principal members of aquatic food webs, regulating and influencing the stability, resilience and dynamics of food webs (Holmlund & Hammer, 1999). The importance of fish to ecosystems may occur at all levels of their life cycle, from the larval to adult stages (Holmlund & Hammer, 1999). Fish have been used as indicators of the health of ecosystems (Jiang et al., 2024). Some fish may also contribute to terrestrial food webs, for example, migratory salmon being preyed on by bears and other mammals (Houston et al., 1989). Furthermore, fish are not only important for subsistence and services to the environment; fishing is also important as a cultural practice for many communities worldwide. In the Amazon, artisanal and community traditional fishing is a major driver of economic subsistence and provides the major source of protein for some Amazonian aboriginal communities (Coimbra et al., 2017). Northern communities in Canada also have important cultural connections between human and fish populations; the health of fish populations and access of traditional communities to fisheries resources and sovereignty has a positive impact in aboriginal communities and is connected to wellbeing in the community (Blanchet et al., 2021). Further, aboriginal rights of fishing are embedded in the Canadian Law (Tough, 2000).

1.3 Comparing Laboratory-Reared and Wild Fish Studies

Experiments performed in laboratory settings with fish have advantages, such as controlled environments; laboratory work provides stability and reproducibility to experiments and therefore, clear and consistent results (Robinson et al., 2007; Sneddon et al., 2017). For example, studies performed in the laboratory with non-wild fish are crucial to understanding fish behaviour (Dunn et al., 2016a), and how temperature effects physiology and behavior (Schoenfuss et al., 2023) or larval development (Dunn et al., 2016b). However, the results of laboratory-based studies are not always easily extrapolated to the much more variable wild-context. Furthermore, it is known that laboratory reared fish can differ physiologically and behaviorally from their wild-counterparts (Smith & Fuiman, 2004). Wild-fish studies on the other hand, can bring a true ecological context to known fish physiology (Meka & McCormick, 2005), and behaviors like migration (Auer, 1996) and spawning (Martins et al., 2012). However, these studies on wild-fish tend to have highly variable results and little to no ability to manipulate animals in comparison to laboratory experiment settings.

1.4 Lake Winnipeg

Lake Winnipeg is situated in central North America, within the prairie region of Canada, and harbors water collected from rivers draining the Lake Winnipeg Basin, which extends nearly a million square kilometers (Environment and Climate Change Canada (ECCC) & Development, 2020). Lake Winnipeg is the tenth largest lake in the world (Wassenaar & Rao, 2012) and in the last few decades has undergone many environmental changes and challenges including eutrophication, warming, and invasive aquatic species introductions (Enders et al., 2021). Lake Winnipeg is divided into two large basins the Northern and Southern basins, connected by a region known as the Narrows, which is approximately 2 kilometers wide. Regional differences occur in water depth, temperature, and the specific river drainages that supply Lake Winnipeg across the Northern and Southern basins (Sheppard, 2013). The two basins of Lake Winnipeg also differ in their planktonic species (Patalas & Salki, 1992) and fish communities; for example, there tend to be higher abundance of sauger, Sander canadensis, in the Southern basin than in the North basin (Bozek et al., 2011; Sheppard, 2013). Furthermore, population shifts in major forage fishes, cisco (Coregonus artedi), emerald shiner (*Notropis atherinoides*), and Rainbow Smelt (Osmerus mordax), have been noted recently (Enders et al., 2021). Monitoring of catch-per-unit-effort (CPUE) found decreases in these pelagic prey fish species from 2005-2008 to 2015-2018 in Lake Winnipeg, coincident with the introduction of zebra mussels, *Dreissena polymorpha* (Enders et al. 2021).

Lake Winnipeg trophic dynamics differ between the Northern and Southern basins (Hobson et al., 2012). Isotopic data shows separated food web systems between both basins, with Northern predatory walleye having greater δ15N concentration in the muscle, suggesting a higher trophic position (within the carnivory diet) than Southern Lake Winnipeg walleye (Ofukany et al., 2014). Higher elevation of δ15N concentration may come as well from catabolism (O'Brien, 2015). Therefore, Lake Winnipeg is a system with possible intrinsic trophic and geographical differences, as well as environmental influences which may affect the physiology of the species that inhabit this lake system, amongst those species affected will be the walleye *Sander vitreus*, which is the focus of my first two research chapters.

1.5 Walleye of Lake Winnipeg

The walleye is the most abundant upper trophic level fish species in Lake Winnipeg and are part of a targeted fishery; therefore, this species is an ecologically and economically important fish and a prized species in Lake Winnipeg's fisheries in Manitoba (Conservation and Water Stewardship, 2017). Compared to walleye in other large systems, like Lake Ontario, the Lake Winnipeg population of walleye appears to have smaller gonads and disproportionately low gonad lipid content despite also having high lipid content in the rest of their body relative to other walleye populations (Moles et al., 2008). These differences in lipid partitioning for Lake Winnipeg walleye has been suggested to reflect increased mortality or possibly reproductive senescence (Johnston et al., 2012) but may also relate to a high degree of ‘skipped’ spawning as it was recently demonstrated less than 50% of tagged walleye did not return to the Red River spawning site the following year (Turner et al., 2025). There is debate about the health and status of the walleye and its fisheries in Lake Winnipeg (Palsson, 2021); according to The Province of Manitoba monitoring (Environment Canada 2011) the biomass of walleye in Lake Winnipeg has decreasing (Environment Canada 2011). Previous work by Sheppard (2013) showed faster growth and higher body condition (mass relative to body length) in the Northern basin walleye than in the South basin which was explained as being due to the Northern walleye feeding on Rainbow Smelt. However, the Smelt population has collapsed in the last few years, which may lead to possible negative consequences for the Northern walleye.

Diet and nutritional differences in walleye across basins of Lake Winnipeg may result in physiological variances (Olynyk et al., 2017) and if so, this may signify the presence of differential performance, reproductive output, body chemistry, metabolism, along with the general life history and survival across Lake Winnipeg walleye (Hobson et al., 2012; Ofukany et al., 2014). For example, the age at 50% maturity for both sexes has decreased over time for the Narrows and Northern basins while not changing significantly for the Southern basin walleye over the same period of 1981 to 2001 according to (Johnston et al. 2012). Study on the predicted length at age 7 was also lower in the Northern basin than the Southern walleye (Johnston et al., 2012). The rate of growth and diet constraints may be correlated, as the fish will only be able to grow if foraging success exceeds basic metabolic needs, including cellular maintenance and homeostasis costs, otherwise catabolism of body tissue is the only means of meeting energetic needs. Stable isotopes also suggest basin level differences in walleye foraging, although results are not definitive. While walleye in the North show a higher δ15N than the Southern basin fish, suggesting higher trophic level or a higher degree of nutrient recycling (indicative of food limitation), this appears due in part to basin-wide isotopic dynamics as forage fishes also have elevated δ15N in the North basin (Ofukany et al., 2014). However, consistent with basin-level trophic differences, the prey consumed by walleye in the North were larger than prey items found in walleye stomachs from the South Basin; therefore, northern Lake Winnipeg walleye may get more nutrition per prey item consumed on average (Sheppard et al., 2015). Furthermore, walleye display diversity in growth patterns spatially and temporally, with regional variations in the rate of growth between basins (Sheppard et al., 2018).

Adding to the debate over the current health state of the walleye in Lake Winnipeg (Turner et al., 2021), index gillnetting data (conducted by the Government of Manitoba between 2009 and 2018) has found lower length-at-age in young (2-3 year age class) walleye caught from the Northern basin of Lake Winnipeg (Johnston et al., 2012; Thorstensen et al., 2021). Decreasing size-at-age in a population may reflect pressure when fisheries target the heavier and larger individuals, nutritional constraints across this population, or a combination of both fishing and other environmental influences. Information that clarifies or extends the knowledge on walleye physiology in Lake Winnipeg will be of importance to better understand the general trends that are influencing walleye numbers and the current physiological health of this population in Lake Winnipeg.

1.6 Focus of Chapter 4: Lake Trout, Salvelinus Namaycush, the Longevous Salmonid

Lake trout, *Salvelinus namaycush,* is a temperate to Arctic member of the Salmonidae family and is the longest-living Salmonid that may live up to 50 years (Froese and Pauli 2018). Lake trout is an important species in North American (Johnson et al., 2012), where they spawn during the Fall in lakes and rivers of cold and deep waters of the Canadian Shield (Steward & Watkinson, 2007). Lake trout are apex predators in the waters they inhabit. As they grow, lake trout will not have significant natural predators outside of humans. While Lake Trout can reach a size of up to 46kg, making them the largest non-anadromous Salmonid in North America, they also mature slowly and take up to 12 years to reach maturity in larger lakes, therefore they can be affected by overfishing, as recovery to overfishing can be slow (Steward & Watkinson, 2007).

As a very long-lived Salmonid, lake trout is a suitable model to study senescence. Lake trout, which has the capacity to be an indeterminate grower, has been shown to have traits that suggest negligible senescence may occur in some ecosystems (Purchase et al. 2022). As an indeterminate grower, the growth potential of lake trout will be influenced by the quality of nutrition available (Shipley et al., 2023). Food web interactions, forage energy use and the size of prey will all influence the quality of diet that predators consume. Fish, with a few exceptions like pacus which become herbivores as adults (Pelster et al. 2015), go through a common diet shift during their life history. Larva are zooplankton and insect eaters, and as the fish grows and progresses to adult sizes it moves to consume larger prey (larger zooplankton, benthic invertebrates and fish); therefore; their capacity to shift diet will be under influence of the composition and availability of prey, which in this case will be an effect of the food-web structure (Pazzia et al., 2002). Upper trophic level predators in aquatic ecosystems with smaller and short food-webs tend not to reach the maximum size possible for the species, and tend not to have high-body condition, as these ecosystems often cannot support the higher total biomass that more complex food webs can (Shipley et al., 2023). This limitation of growth potential, or lack thereof, may be a key ecosystem trait related to lake trout size in lakes, and therefore, may influence traits related to the biology of these animals as they age. Smaller lakes may not be able to house and maintain lake trout that are large, an example is the work of (Purchase et al. 2022), where the environment limited growth potential of this species, while, in comparison, larger bodies of water, like Great Slave Lake, that are unexploited environments may house more resilient and larger lake trout (Kennedy, 1954).

1.7 Fish Physiology, Nutrition and Growth

As heterotrophs, consumption of food items is required for fish (Goeritz et al. 2014); however, nutrients may be indispensable (required for growth) or dispensable (not necessarily required) depending on species, developmental, dietary, and physiological state. Piscivorous fish, like other carnivorous vertebrates, are quite reliant on dietary protein intake for their energy needs (Hemre et al., 2002; Kaushik & Seiliez, 2010; Xu et al., 2017). Additionally, the amino acids that make up dietary protein are nutritionally crucial because in fishes many amino acids are indispensable. Fish can produce only a set of the amino acids needed for protein building and other metabolic needs; the rest of the needed amino acids have to be obtained from their diet. (Wolfe et al., 2022). Furthermore, as ectotherms, fish will be under the influence of the environment, with temperature considered the master factor for metabolic rate in ectotherms (Abele et al., 1998; Alabaster & Welcomme, 1962; Narum et al., 2013). When physicochemical effects push the physiology of the fish outside the range of adaptations for a particular species, this may cause cellular damage and physiological stress to the fish (Walker et al., 2020). Physiological stress together with activities that require large energy investments like migration, reproduction, and foraging can represent added constraints that may delay growth in the fish (Alfonso et al., 2021). Assessing the homeostasis and physiological health of aquatic animals in the wild is difficult, as the measure will vary across taxa and environmental adaptations (Jerez-Cepa & Ruiz-Jarabo, 2021). The way to measure stress is to determine the deviations from homeostasis in the organism (Sopinka et al., 2016) and in fishes this is often done by focusing on a metabolic or physiological stress marker (Kroon et al., 2017). To assess stress, most studies on fish focus on a few biological “markers”, i.e., enzymes, oxidative stress-derived molecules, glucocorticoids (Meka & McCormick, 2005; Verbeek et al., 2008). An example is cortisol, where chronic high levels in tissues show maladaptive physiological responses for the organism (Lee et al., 2015; Meka & McCormick, 2005). The growth and health of fish in the wild may also be measured by using morphometric values (Anumudu & Mojekwu, 2015), like condition factor (Holth et al., 2010; Seibel et al., 2021). However, there are disadvantages with these current methods of physiological assessment of stress (Dando 1969; Verbeek et al. 2008; Smit et al. 2009; Cooket al. 2011), as they do not show whole patterns of change within the organism. But, with the advent of “omics” studies, identification and assessment of a wider range of molecules from a single sample can be achieved and provide a more integrated measurement of the individual's physiology and metabolism at the time of sampling (Psychogios et al., 2011). A few examples of metabolomics studies in wild fish include: microplastic effects benthopelagic fish Serratus scriba (Zitouni et al., 2022a); oxidative stress induced by mercury effects (Cappello et al., 2016); environmental contamination and fish health (Long et al., 2020); and even work on sturgeon species (Zhou et al., 2023).

1.8 Metabolomics as a Method to Study Physiology

Metabolomics is the field of study that identifies and analyzes the whole or partial makeup of the molecular intermediates that are part of the metabolic pathways of the organism (Strimbu & Tavel, 2010). Metabolomic studies can either have a non-targeted approach, showing the complete range of the metabolites found in an assessment, which usually have no pre-existing knowledge of the previous metabolite data in the study. Alternatively, metabolomic studies can be based on a targeted approach, in which a specific group of molecules is searched and a specific hypothesis testing is underway (Schimpe-Ruthledge et al., 2016). Metabolites therefore will be any specific molecule that may be identified in these studies; they can be a marker of a disease or physiological state, reactant, the intermediate or final product in a metabolic pathway, or a marker of toxicological damage (Strimbu & Tavel, 2010). Metabolomic studies identify molecules in a tissue, organ, or the whole organism, then after sample and analysis these molecules can be grouped and patterns and changes in these molecules can pinpoint changes or disruptions in major metabolic pathways of organisms (Schimpe-Ruthledge et al., 2016).

Metabolomics can show a wide-ranging snapshot of the model's internal state based on the level of the metabolic intermediates measured and therefore can be a tool for a general understanding of physiology of the model sampled (Cox et al., 2017). While metabolomics is common in human research (Li et al., 2022), this type of research has expanded to other models, like birds (Zelentsova et al., 2022), fish (Samuelsson et al., 2011), and farm animals (Roques et al., 2020). As metabolomic data can give information on which metabolic pathways may be changing, impacts of factors like environmental constraints, toxins, and other factors that influence the general physiology and state of the organisms under study can be investigated.

Metabolomics approaches in fish research have looked at toxicological effects of effluents on rainbow trout, *Oncorrhyncus mykiss,* exposed to effluents across two years of sampling (Samuelsson et al. 2011a). Metabolomics work also has looked at the influence of cyanobacterial blooms on medaka fish (*Oryzias latipes)* liver health. Cyanobacterial blooms produced molecular markers of stress on liver tissue suggesting differential damage depending on sex, the strain of the bloom and modality of exposure of the fish (Le Manach et al. 2018a). Metabolomics profiles were also investigated in zebrafish, Danio rerio, to understand behavior changes in anxiety-induced experiments using the phenyl pyrazole pesticide finopril. The study found modified metabolic pathways in pesticide-treated whole larval fish in relation to non-treated controls, with higher fatty acid concentrations in anxiety-induced fish and lower concentrations of neurotransmitters glycine and glutamate, together with lower concentrations of branched-chain amino acids in anxiety-induced larva (Wang et al., 2016). In the New Zealand juvenile snapper, *Pagrus auratus*, a wild fish, metabolomic work showed separation in metabolite profiles across coastal nursery sites, which may indicate nursery quality differences in the coastal area for this species (Goode et al., 2020). Additional investigations have studied the impact of the pesticide isocarbophs on the metabolic profile of zebrafish (Jia et al., 2018), showing an increase in lactate concentration and amino acids alanine, and branched-chain amino acids together with decreasing creatine in whole fish samples exposed to pesticides versus controls, which may imply perturbation of energy metabolism in zebrafish exposed to isocarbophs. Comparison between laboratory hatchery Chinook Salmon versus wild fish, found salmon exposed to chemicals of emerging concern, found fishes exposed to chemicals had decreased amino acid concentration in their plasma, as well as, decrease in nucleotide metabolites, highlighting potential negative effects of contaminants in fish populations (Meador et al., 2020). Another toxicology study has investigated the correlation of exposure to microplastics in the environment to changes in liver metabolites in the painted comber, *Serranus scriba*, and found differences in 36 metabolites of fish exposed to microplastics (Zitouni et al. 2022a).

Some limitations of the study of fish that are sampled in the wild, and handled, before sampling, is the acute stress response that may appear shortly after (Twardek et al., 2018). This response may cloud results of experiments that study markers of stress or other molecules that may respond short term, like lactic acid (Dando, 1969) and glucose (Acerete et al., 2004). Therefore, it is of importance in field work studies which measure stress to choose metabolites which will not be likely influenced by the acute fast stress response. Choosing molecules with known pathways, and specific reactions that are not influenced in great extent by the first acute stress response is needed for metabolic studies that include sampling in the wild. Examples of molecules which may be good markers of specific pathways includes amino acids and some lipids (Pan et al., 2023). Amino acids, have known pathways, are signaling molecules that reflect nutrient status in the animal (Kaushik & Seiliez, 2010). As well some amino acids like the branched chained category rise during insulin resistance (Guillet et al., 2004). Amino acids also, participate in important pathways within the cell, like gluconeogenesis (Aikawa et al., 1973), ketogenesis (Baquet et al., 1991), and the one-carbon metabolism, important for gene expression within the cell (Eudy et al., 2020). Another type of molecule, which can represent a good candidate for metabolomic studies and is of known metabolism, pathway and turnover rates may specify mitochondrial function, are the acylcarnitines, Acylcarnitines are molecules that derive from fatty acids and the catabolism of some amino acids bonded to a carnitine molecule. This reaction is reversible, it happens on the outer mitochondrial membrane as well as within the matrix by carnitine-CoA acyltransferase enzymes (Xiang et al., 2025). Therefore, their flux may be a good representation of energy shifts within tissues (Jarrell et al., 2020), they are accessible in the bloodstream for sampling (Muilwijk et al., 2018), and the chemical composition of the carbon acyl chains, specifies specific metabolic pathways according to how many carbons the fatty acid chain possesses (De Pasquale et al., 2020; Stryer, 1981).

1.9 Senescence and the Aging Process

Senescence and aging are in some literature used interchangeably. Senescence has been defined in several ways, but in general refers to any changes that alter the physiology of the organism which leads to increase in mortality, with increasing age. Aging, therefore will be the natural life history and development with time of the organism, while senescence refers to the negative changes that occur during aging (Finch, 1990). In the scientific literature there are many theories of senescence: the free radical theory (Harman, 1956), the mitochondrial theory (Harman, 1972) and the mutation genome theory (Szilard, 1958) and the antagonistic pleiotropy hypothesis of aging (or senescence), being among the most relevant. According to the current literature, the process of senescence can be influenced by many factors. The intrinsic factors that are known to have effects on the rate of senescence related processes (Fig. 1.1) include oxidative stress (Harman, 1956), DNA methylation (Jung & Pfeifer, 2015), telomere shortening (Shammas, 2011) and genetic mutations (Verheijen et al., 2018), among others (Dimri et al., 1995). Senescence is also influence by extrinsic factors including diet (Finch, 1990), temperature for ectotherms (Anttila et al., 2013) and adverse environmental conditions like toxins and radiation (Neary, 1961). Organisms show three types of senescence: rapid. nematodes, annual plants, some insects, semelparous fishes like Pacific salmons, gradual, humans and most placental mammals are a good example, and negligible, trees, sea anemones, and possibly some fish and reptiles (Finch, 1990). As well, senescence can happen at many levels of biological organization. For example, senescence may be recognizable at the population level through shifts in life history traits; at the individual organism level through changes in patterns seen at the tissue level (Nakano et al., 1989) down to the cell and molecular levels (Kotarska et al., 2017).

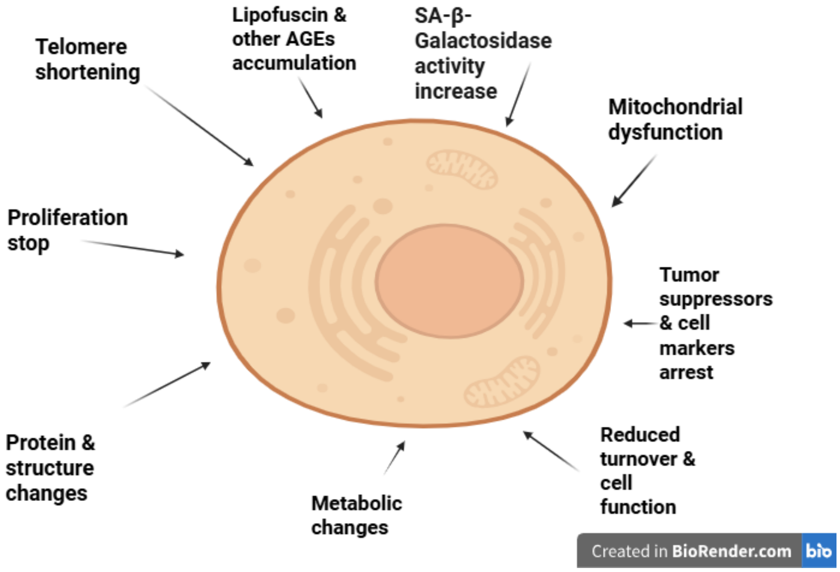

Figure. 1.1. *Diagram of the main characteristics of a senescent cell: Telomere shortening, advanced glycation products, AGEs, accumulation, SA-β-galactosidase activity increase, mitochondrial dysfunction, protein changes, proliferation stop, tumour suppressors and cell marker arrest, reduced turnover and cell function. (Dimri et al., 1995; Jacobson et al., 2010; Sims et al., 2010; Stoll et al. 2011; Burton and V. Krizhanovsky, 2014; Ott et al., 2014) Created in https://BioRender.com*

The antagonistic pleiotropy theory of aging was first proposed by Medawar (1952), and expanded on by Williams (1957), in which the idea that earlier traits which are advantageous to reproduction and early life for the organisms may become deleterious later in life. This effect comes from the lack of selection against deleterious traits later in life of the organism, thereby increasing the possibility of death and disease in old age. What is notable is the chance for selection for longevity, in cases where the organism has increased reproductive output as it ages, which is possible for some indeterminate growing species. Furthermore, as reviewed elsewhere (Williams et al., 2006) the deleterious traits which may increase with age can interact with environmental factors. Therefore, a question to be addressed in senescence is whether physiological senescence increases susceptibility to environmental hazards.

1.9.1 Senescence in Fishes

Fish are the most diverse vertebrate taxa, their life history has all the spectrum from short lived to long-lived species, like the Greenland shark (Somniosus microcephalus), considered the oldest vertebrate to date (Nielsen et al., 2016). Further study of senescence on non-model organisms may give a better and broader perception of the senescence process as a whole, especially in fishes, as human and other mammal species include only a portion of the diverse evolution of vertebrates. Most studies in senescence in fish have included small, semelparous (reproduce only once in their lifespan) and/or short-lived species, like zebrafish (Yu et al., 2006), a species that is of determinate growth. However, some of the species of ectotherm vertebrates have indeterminant growth (Hariharan et al., 2016) and many of them like some fish and as well as some reptilians (Blanco & Sherman, 2005; Rose et al., 2019), show no apparent decrease in reproductive capacity with age (Nielsen et al., 2016). Furthermore, a few invertebrate animals are considered to be immortal and lack senescence all together (hydras) (Barve et al. 2021), emphasizing the necessity of further study of non-model organisms to broaden the understanding of the underlying mechanisms that influence senescence.

1.10 Summary of the Following Chapters

In Chapter 2, walleye are sampled from a range of sites, including the spawning Red River followed by northward sampling of non-spawning fish ending with Dauphin River from the Northern basin of Lake Winnipeg, Manitoba. Chapter 2 is a targeted metabolomics study of spatiotemporal variation of whole blood metabolites. Past use of transcriptomics in gill biopsies from walleye in Lake Winnipeg found Northern walleye have higher expression of chaperones, mammalian target of rapamicyn (mTOR) signaling transcripts, and other stress molecules compared to walleye from the Southern basin (Jeffrey et al., 2022). The main hypothesis of Chapter 2 is that the metabolome will differ as a function of capture location, with differences across sites from different basins predicted to be the strongest pattern due to the basin differences described in Chapter 1. T

Chapter 3 tests if chronological age has an effect on the metabolic concentrations of major molecules sampled on a wild fish. Fishes unlike mammals and birds, are indeterminate growers (Sebens, 1987) so reproductive fitness may increase with age in some fishes. Different selective pressures may act on determinate growers versus indeterminant growing species (Froehlich et al., 2013). The third chapter uses the walleye metabolomics from Chapter 2, combined with measured chronological age of the fishes to testing for change in the blood metabolome with age in Lake Winnipeg Walleye. The hypothesis is that, molecules like amino acids, some lipids and biogenic amines will be metabolized at a slower rate than in younger, faster growing, individuals, predicting that these metabolites will tend to increase with age in walleye. This chapter also tested in a common marker of cellular senescence, relative telomere length, is a function of chronological age in a wild fish.

Finally, Chapter 4 expands on previous work (Purchase et al., 2022) showing possible lack of senescence in lake trout, which inhabits two smaller lakes in the Experimental Lakes Area, in Ontario. Expanding on this past study, and the study of walleye aging in Chapter 2, Chapter 4 primarily compares relative telomere length and lipofuscin accumulation in in four population of lake trout. A key differentiation from other work on aging in wild lake trout (Purchase et al., 2022), Chapter 4 studies two populations with indeterminant growth and two with determinant growth to evaluate if growth potential influences patterns seen with these common markers of cellular senescence and age of the fish. Notably, the results in this chapter showed no significant increase in markers of cellular senescence with age in any of the lake trout populations, suggesting the possibility that negligible senescence is widespread in this species.

1.11 References

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2. Chapter 2: Whole blood metabolomic profiles from Lake Winnipeg walleye implicate regional differences in oxidative fuel use

2.1 Contribution of Authors

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2.2 Introduction

Climate change is one of the principal threats to watersheds, rivers, and lake environments (Allen & Ingram, 2002; Gál et al., 2019; Reist et al., 2006; Wang et al., 2021). Increasing average temperatures are the most widely known effect of climate change (Van Vliet et al., 2011). However, rising annual mean temperatures can also increase variation in seasonal patterns and unpredictable weather events that affect ecosystem health (Partanen et al., 2017). Environmental instability is expected to exacerbate the effect of other challenges that already affect aquatic freshwater species, such as anthropogenic influences, invasive species, contamination, and nutrient efflux entering waters. Due to these accumulating environmental challenges (Van Vliet et al., 2016), aquatic organisms may not be able to thrive and survive (Sauchyn et al., 2020). Inland lakes and other freshwater environments may be particularly affected by environmental and anthropogenic changes happening currently across continental masses (LeMoine et al., 2020; Marsh et al., 2021). The ability to monitor the health of species sampled from their natural habitat will be valuable for conservation consideration as the strain of climate change takes effect, especially for those species under additional pressures such as targeted harvesting such as fisheries.

A system where assessments of the physiological condition of its inhabitants may be particularly valuable is Lake Winnipeg, the tenth largest lake in the world (Environment and Climate Change Canada (ECCC) and Manitoba Agriculture and Resource Development (MARD), 2020; Wassenaar & Rao, 2012). Lake Winnipeg is divided into the Northern and Southern basins, which are connected by a narrow channel (Fig. 2.1). These basins possess different ecological conditions. For example, summer algal blooms are prevalent in the Southern basin, aligning with increased anthropogenic nutrient input, which also has higher summer average temperatures (ECCC and MARD 2020). Warmer temperatures combined with algal blooms lead to oxygen deprivation in the water (Gardner et al., 2017) and may also have secondary influences through toxins produced in these blooms that are then released to the environment (Mielewczyk et al., 2023). Food web structures vary (Ofukany et al., 2014), with nutrient and river inputs differing across basins in Lake Winnipeg (Sheppard, 2013). Stable isotope ratios of carbon (13C/12C) and nitrogen (15N/14N) in tissues of fishes across the lake support patterns of differing nutrient supply between basins (Hobson et al., 2012; Ofukany et al., 2014). In the last few decades Lake Winnipeg has been challenged by multiple factors (Mooij et al., 2007) including emerging effects of climate change (Shrestha et al., 2012) and general human disturbances (Enders et al., 2021). Additional fishery pressures influence the Lake Winnipeg ecosystem, with the walleye (*Sander vitreus*) being the predominantly targeted species in both basins (Manitoba Government 2018). Recent analysis of inter-basin movement and survivorship of walleye supports reducing fishery pressure on Lake Winnipeg walleye (Turner et al., 2021). Given that walleye is an upper trophic level predatory fish in Lake Winnipeg (Ofukany et al., 2014), the ability to assess the nutritional status of this species may be a particularly valuable means of monitoring overall ecosystem health.

The population size of walleye in Lake Winnipeg has been declining over time, with evidence implicating that the current fisheries harvest may be a contributing factor for almost a decade (Conservation and Water Stewardship, 2017; Manitoba Government, 2018). In Lake Winnipeg, the rate of growth of walleye varies by basin, with the Southern basin fish showing two different morphologies, a dwarf and a larger-sized subpopulation (Moles et al., 2010).

Furthermore, the mean age of walleye has declined in the Northern basin since the 1980s through to the early 2000s (Johnston et al., 2012). The Northern basin walleye has been found to have lower growth rates compared to Southern walleye since the collapse of an important prey species, the Rainbow Smelt, *Osmerus mordax*, in 2013 (Thorstensen et al., 2020). Importantly, as reported elsewhere, forage fish density is higher in the Southern basin than in the North and this is paralleled by higher density of walleye, per unit lake surface area, in the South (Moles et al., 2010; Sheppard et al., 2015; Turner et al., 2021); however, the forage fish to walleye ratio is larger in the North than the Southern basin, indicating walleye in the North may have access to abundant food but at the potential cost of increased activity to compensate for lower encounter rates with lower prey density. The differences in growth rate between basins may reflect basin-specific physiological variance within Lake Winnipeg walleye. Such physiological differences may extend to differential performance, reproductive output, metabolism, and general survival in Northern versus Southern basin groups (Hobson et al., 2012; Ofukany et al., 2014). Recent transcriptomic comparisons of gill biopsies from Lake Winnipeg walleye support basin-specific differences in physiological challenges (Jeffrey et al. 2023).

Measuring physiological state of organisms can be a valuable tool for monitoring the health and nutritional status of wild animals; however, accurate assessment tools are limited. For example, monitoring of growth and body condition is robust but slow and cumbersome to collect data because of the need to repeatedly sample large numbers of animals. Biochemical and molecular tools may provide more rapid assessments but come with their own challenges due to the lack of reference values, true control conditions, and the variation introduced by population-specific differences in responses across environmental gradients (Jerez-Cepa & Ruiz-Jarabo, 2021). For example, local adaptation in separated populations can have different cortisol responses to environmental stress (King et al. 2016). Markers of stress in the blood of fishes, often including major metabolites (glucose and lactate) as well as regulatory molecules like glucocorticoids (Sopinka et al., 2016, Kroon et al., 2017), can be valuable but are also limited in the specific nature of the information they provide and require sampling within minutes post-capture (Lawrence et al., 2018).

There have been recent advances showing that mRNA responses in gill biopsies from Walleye can show evidence of hypoxia (Jeffrey et al., 2020) indicating the potential of non-traditional approaches to evaluate poor environmental conditions for animals sampled from the wild. This rationale was expanded to a transcriptomics approach to investigate regional differences in Lake Winnipeg walleye using non-lethal gill biopsies (Jeffrey et al., 2023). The comparative transcriptomics indicated elevation of transcripts associated with tissue remodeling and cell turnover, preferential expression of glycolytic and hypoxia-related genes in relation to mitochondrial proteins involved with oxidative phosphorylation, and overall suggested increase cellular stress, or at least increased cell turnover, in walleye sampled from the Dauphin River area in comparison with walleye from the South basin (Jeffrey et al., 2023). Important to the current study, the differences in cell mRNA signatures in walleye gills appear more dependent on the South to North sampling and less influenced by whether the walleye were spawning (Red River) or post-spawn (sampled from Mattheson). Recently, it was also shown that blood levels of three essential amino acids and their metabolic derivatives, including catabolic intermediates and post-translational modifications of amino acids that occur while they are within proteins, supported the contention that protein turnover was higher in walleye from the Dauphin River area than for walleye from the Southern basin (Thorstensen et al., 2021). Unfortunately, for the amino acid catabolism metabolites, results were inconclusive, indicating a more comprehensive assessment of metabolites may be needed to gain insight into the nutritional status of wild walleye based on blood-borne metabolites.

Compared to traditional stress markers in fishes, metabolomics, where large numbers of metabolites involved in different metabolic pathways are measured simultaneously, may provide a more comprehensive assessment of an animal's physiological condition (Shokry et al., 2017; Waś et al., 2025; Yang et al., 2025). Metabolomics have been applied successfully across several tests of the effects of environmental disturbance on fishes (Samuelsson et al. 2011, Jia et al. 2018, Le Manach et al. 2018, Zitouni et al. 2022,Goode et al. 2020), and the current study aims to evaluate if metabolomics can provide information on the nutritional status of Lake Winnipeg walleye. To do so, we take advantage of sampling walleye starting with the annual spawn of walleye, which occurs in spring at ice-off in Lake Winnipeg, and following ice-off to sample post-spawn walleye from the South to the North. Lake Winnipeg walleye are known for having disproportionately high lipid in their muscle, compared to walleye from other large lake systems, while having comparable growth rates in mature fishes (Moles et al., 2010). This unusual metabolic phenotype is consistent with the speculation that the preferential lipid accumulation reflects a metabolic strategy that favors energy storage for walleye in Lake Winnipeg, with a trade-off of a lower annual energetic commitment to reproduction. It was recently shown that at over the years of 2017 to 2018, approximately 50% of walleye caught during spawning in the Red River in 2017 skipped spawning in 2018 (Turner et al., 2025), which is unusual for walleye in large lake ecosystems (Henderson et al., 1996; Henderson & Nepszy, 1994) but suggests they are under nutritional limitation for reproductive output. An unusual metabolic phenotype, combined with regional variation in growth, feeding dynamics and environmental conditions plus transcriptomic evidence of regional differences in gill metabolism and tissue remodeling make Lake Winnipeg an excellent system to test regional differences in metabolomic responses.

To investigate this, and to discern the metabolites present in the same group of fish and study approach applied to evaluate wild Lake Winnipeg walleye physiology using gill transcriptomics (Jeffrey et al., 2023), we investigated the whole blood metabolome of walleye sampled from 4 sites in Lake Winnipeg (Fig 2.1): the Red River during spawning, Sandy Bar post-spawn and within the Southern basin, Mattheson (also post-spawn) which is in the Narrows region and is transitional from the Southern and Norther basin, along with Dauphin River walleye as representative animals caught post-spawn from the North basin.

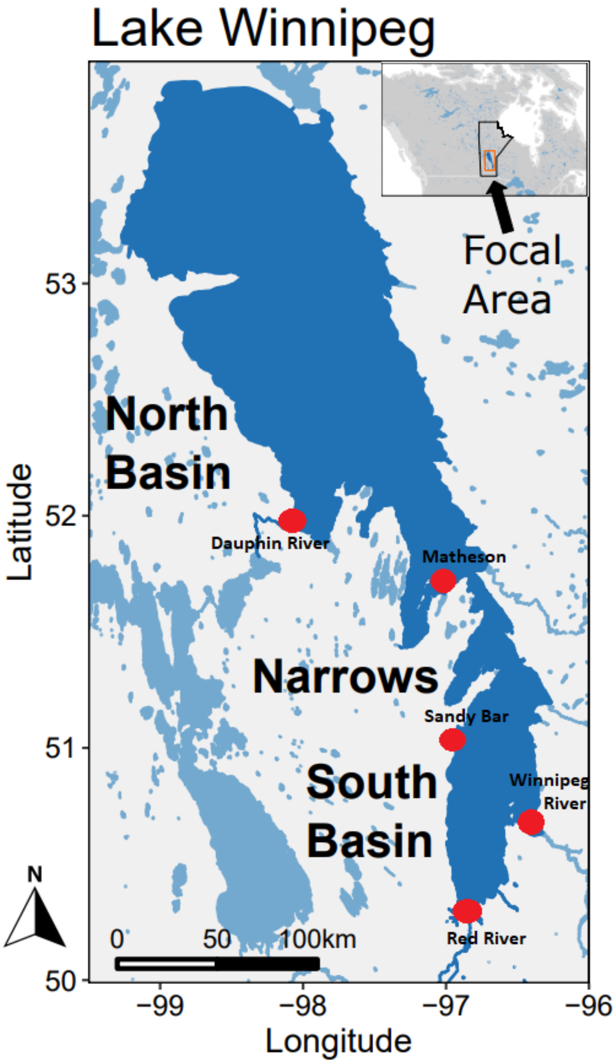

Figure 2.1. *Map showing the sampling sites for walleye, Sander vitreus, in Lake Winnipeg., Dauphin River signifies the Northern basin site; Matheson represents the walleye of the Narrows canal; and Sandy Bar and Red River represent the Southern basin walleye. Map data were downloaded from: rworldmap-package (South, 2023), raster v3.6-20 (Hijmans, 2023), rnaturalearth v0.3.2 (Massicotte et al., 2023), and lake and river data from the Statistics Canada (2011) census project (Statistics Canada, 2011).*

2.3 Methods

2.3.1 Animal sampling

This study was possible because of a multiyear project tracking fish movement by acoustic telemetry in the Lake Winnipeg basin (see also, Munaweera et al., 2021; Thorstensen et al., 2020; Turner et al., 2021). Animals were captured by electrofishing beginning in the South and moving Northward due to annual ice-off conditions. We determined the whole blood metabolites of the walleye sampled from Lake Winnipeg May to June, pre and post-spawning in 2017 and 2018, across four sampling sites: Red River (May 2, 2017; May 7, 2018), Sandy Bar (May 10–16, 2017; May 17–23, 2018), Matheson (May 18–19, 2017; May 28–29, 2018), and Dauphin River (May 29–June 1, 2017; May 30–June 5, 2018; Fig. 2.1). As explained elsewhere (Turner et al., 2025), the Red River walleye were assumed to be in the spawning period based on gametes being released during handling, whereas walleye caught from other sites did not display this response and were considered to be post-spawning fish. Our sample included 58 walleyes in 2017 (Red River 17, Sandy Bar 19, Matheson 5, Dauphin River 17) and 60 in 2018 (Red River 15, Sandy Bar 15, Matheson 15, Dauphin River 15). Sample collection is described in more detail elsewhere (Thorstensen et al. 2020). Briefly, after capture, fish were held in aerated live wells (limited to approximately < 30 min) before surgical implantation of telemetry transponders. During surgery to implant acoustic telemetry devices, the surgeon attempted to sex the individual walleye; however, this was not always possible or definitive and was not prioritized for animal welfare reasons. Additionally, the target fish size in 2017 was limited to large individuals due to the size of the telemetry devices, making that year's data unreliable to analyze for sex differences because of the sexual dimorphism of walleye (females are larger).

Access to smaller acoustic transponders in 2018 allowed for a wider range of fish selection for surgery and, consequently more males were included; however, due to the issues in definitive identification and unbalanced sampling in 2017, sex data are reported for completeness but were not included in the statistical analysis. A small (≤0.1 mL of blood per 100 g of fish mass) whole blood sample was taken during the surgical procedure by caudal puncture using a heparinized syringe and frozen as whole blood in the field with a liquid nitrogen dry-shipper and stored at -75°C or colder until analyzed.

2.3.2 Blood analysis: LC-MS/MS

Metabolites in the samples were measured by either direct injection mass spectrometry following reverse-phase liquid chromatography (LC-MS/MS) or by nuclear magnetic resonance (NMR) at The Metabolomics Innovation Centre (TMIC). A TMIC Prime Assay LC-MS/MS Method with a targeted quantitative metabolomics approach to analyze the samples using a combination of direct injection mass spectrometry with a reverse-phase LC-MS/MS custom assay. The assay was done in combination with an ABI 4000 Q-Trap (Applied Biosystems/MDS Sciex) mass spectrometer. Isotope-labelled internal standards and other internal standards are used for metabolite quantification. The method combines the derivatization and extraction of analytes, and the selective mass-spectrometric detection using multiple reaction monitoring (MRM) pairs. Mass spectrometric analysis was performed on an API4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, CA). The samples were delivered to the mass spectrometer by a liquid chromatography method followed by a direct injection (DI) method. Data analysis was done using Analyst 1.6.2.

2.3.3 Blood analysis: NMR

Following deproteinization, involving ultra-filtration as described in (Psychogios et al., 2011), 250 µL of the sample was transferred to a 3mm SampleJet NMR tube for spectral analysis. The 1H-NMR spectra were collected on a 700 MHz Avance III (Bruker) spectrometer with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe (Saude et al., 2006). 1H-NMR spectra were done at 25°C using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr). All 1H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 8.1 (Chenomx Inc., Edmonton, AB).

Both mass spectrometry and NMR procedures were performed at The Metabolomics Innovation Centre. No attempt to optimize for matrix effects on metabolite extraction was done for either LC-MS/MS or NMR, however, several metabolites were detected by both methods which were compared (2.2.4.1) and consistently show high agreement indicating matrix and extraction effects were not confounding factors for at least those metabolites quantified by both approaches.

2.3.4 Statistical Analysis

Statistical analyses were performed with the software R version 4.2.1 (The R. Core Team 2024).

2.3.4.1 Metabolites used for Multivariate Statistics and the Averaging Procedure for Metabolites Detected by both NMR and LC-MS/MS

There were some inconsistencies between the metabolites detected and the year; however, 157 metabolites were detected in both years of sampling. A small number of metabolites were detected in one year, but not the other (22 metabolites were detected in 2017 but not 2018 and 32 were detected in 2018 but not 2017). Metabolites not detected in both years were excluded from the multivariate analysis.

Several metabolites were detected by both NMR and LC-MS/MS, in which case, a linear regression between the values was performed to compare values from both analytical approaches. If both NMR and LC-MS/MS were reporting comparable results, a slope of 1 is expected, and we set a threshold of r2 > 0.9 for acceptable precision between analytical methods and within 10% of expected (i.e. slope within the range of 0.9-1.1) as the threshold of accuracy. Likewise, a zero intercept is predicted when both methods give comparable results. We set this threshold for the predicted intercept to be equal to zero, plus or minus 10% of the mean value of the metabolite, averaging the NMR and LC-MS/MS results for each walleye. All metabolites that fit these criteria were included as a single value in the multivariate statistics by averaging the NMR and LC-MS/MS results for each walleye. The metabolites that diverged from this pattern were carnitine (slope of 0.81) and glucose (1.18) in 2017; the optical visualization of their graph showed a high correlation of their data, however.

2.3.4.2 Linear Models for Fork Length versus Mass, by Site and Condition Factor Calculation

The relationship between the length (measured here as fork length (FL)) and wet-mass (W) of fish indicates their 'body condition', which, if variable between sites, would suggest differing energy balance status between groups of sampled walleyes. We fitted a linear model (estimated using ordinary least squares, OLS) relating FL (in mm), W (in kg). The mass to length relationship was linearized by converting values to the natural log (ln). We initially the formula (ln (W) ~ ln (FL) by Site*Year), which tests if there are any interactions between Site and Year in the model. If no interaction was found, then the formula: ln (W) ~ ln (FL) * Site was used;

however, in cases where there is not strong evidence for a significant interaction with year ($p < 0.05$), data from both years were pooled for each site. Marginal means were calculated using the means function in R to evaluate contrasts across the qualitative factors (site and year) used in the model, and the trends' function was used to compare the slopes of ln W and ln FL, which measures the mass gain per unit length gain, analogous to condition factor.

2.3.4.3 Principal Component Analysis (PCA)

To initially decipher trends for metabolites and give direction for which metabolites to further investigate in Lake Winnipeg walleye, exploratory principal components analysis (PCA) was performed using the FactoMineR software package (Le et al., 2008), with a variance-covariance matrix and standardization (mean zero and one unit of standard deviation) provided by the package. The data were from 118 walleye, 58 individuals from 2017 and 60 individuals from 2018 and included a total of 157 metabolites. Only metabolites that were measured in both years were included in the analysis, and those detected by both NMR and LC-MS/MS were averaged (based on details in 1.2.4.1). The function 'dimdesc' on the package FactoMineR was used to determine the metabolites with the highest correlation with the first two Principal Components. To derive a list of the most influential metabolites, results were sorted by highest-to-lowest correlation for Dimensions 1 and 2 in the PCA and the top 10% of all measured metabolites were further examined by ANOVA to evaluate the influence of sampling year and site.

2.3.4.4 ANOVA of specific metabolites

A 2-way ANOVA was used to compare metabolites with the highest loading on the PCA. Initially, each metabolite was assessed assuming a possible interaction between site and year:

metabolite ~ Site * Year; however, in cases where interaction was not significant, a simpler additive model was then used: metabolite ~ Site + Year. In all cases, data were confirmed to fit the assumptions of ANOVA although for several metabolites (indicated in results) data required being Ln-transformed to meet the assumption of equal variance. In all cases $p < 0.05$ was considered significant.

2.4 Results

2.4.1 Metabolite Identification and Common Metabolite Correlation

Using mass spectrometry, a total of 113 metabolites were identified in 2017 and 122 metabolite molecules were identified in the year 2018. Amongst both methods NMR and mass spectrometry, some common metabolites were captured each year (2018, n=22, and 2018, n=15). The subset of metabolites that were measured using both detection methods were considered as the same molecule. Accordingly, the common metabolites identified by NMR and mass spectrometry, were averaged. Correlation tests confirmed that metabolites measured by both NMR and mass spectrometry were significantly correlated (Pearson's correlation test, p value of ≤0.05). After this the combined list of molecules found identified for both years by both methods combining common metabolites, and non-common metabolites as well, were a total of 157 metabolites.

2.4.2 Mass Versus Fork-Length, Site and Years Linear Model

We fitted a linear model (estimated using OLS) to compare wet mass (M) with fork length (FL) relationships across sites and years of data collection (statistical model using the equation: $\ln(W) \sim \ln(FL) * Site * Year$). The overall patterns in fish size were similar across years and sites (Fig. 2.2); however, was a significant interaction with Year because of a difference in the relationship between 2017 and 2018 for walleye caught at the Matheson site (t=-2.778, p=0.0065). Pair-wise comparisons, using the emmeans and emtrends features, confirmed no significant effect of Year on the M and FL relationship within a site of capture ($p > 0.05$); however, there was a trend towards difference between years for Matheson ($t = –2.94$, $p = 0.076$). Scrutinizing these results for Matheson, relative to the other walleye size data (Fig. 2.2) indicates fish from Matheson in 2017 includes a small number of individuals over a very narrow range of sizes, leaving this significant contrast likely prone to a Type 1 (false positive). Moreover, the interpolated intercept for the 2017 Matheson fish is extremely distant from both the 2018 Matheson walleye, as well as the walleye from all other sites (Fig 2.2). Since the 2018 Matheson walleye intercept is in general agreement with all other data, it was determined the more conservative interpretation is that the difference across years in Matheson was a false positive and thus it is prudent to ignore the statistical difference at Matheson, leading to combining fish size data from both 2017 and 2018 for all further comparisons between sites of capture.

We next test the relationships across sites, now with 2017 and 2018 data pooled (statistical model equation: log(W) ~ log(FL) * Site). The model explains a statistically significant and substantial proportion of variance ($R^2 = 0.96$, $F(7, 110) = 430.39$, $p < .001$, adjusted $R^2 = 0.96$). With both years of data combined, significant pairwise differences are found between sites: Dauphin River < Red River (estimate = –0.0884, $p = 0.0292$) and Matheson < Red River (estimate = –0.1222, $p = 0.0031$), indicating that Dauphin River fish and those from Matheson were smaller than those from Red River. No other significant differences were found across the sites. Comparing the nature of the W to FL slope (emtrends), which estimates an increase in wet mass per unit increase in length analogous to estimates of condition factor, found significant differences are present between: Dauphin River < Red River (estimate = –0.7185, p = 0.0005). Marginal slope differences (0.05 < p < 0.10) are shown between: Dauphin River < Sandy Bar (estimate = –0.4223, p = 0.0803), indicating the Dauphin River fish shown less increase in mass per increase in length than their Southern counterparts.

2.4.3 Principal Components Analysis on the Walleye Blood

The analysis performed on the whole blood and 157 total individual metabolites found across 2017 and 2018 for Lake Winnipeg walleye showed high contributions in the first two dimensions for amino acids (proline, leucine, isoleucine, lysine, phenylalanine, histidine, tryptophan), some of their breakdown metabolites like trans-hydroxyproline, biogenic amines, some sphingolipids, acylcarnitines (long-chained and short-chained), and phospholipids.

Thirty-five percent of the variance was explained by the first two dimensions, with 23.1% being explained by PC1 and 12.1% by PC2 (Fig. 2.3, 2.4 and 2.5). The eigenvalues for the PCA analysis are 36 for axis 1 and 19 for axis, the data for the first two dimensions of the PCA show two major clusters of metabolite types (Fig. 2.4): The Northern site, Dauphin River, presents higher concentrations of amino acid metabolites, and the Southern localities of Red River, Winnipeg River, Matheson, and Sandy Bar show a higher concentration of particular acylcarnitine molecules. A 2-way ANOVA was used to evaluate patterns across sites and years for those metabolites with the highest correlations related to the PC scores (loadings), with most metabolites indicating significant differences between sites and years, with several having a significant interaction between site and year (Table 2.1).

2.4.4 Linear Model of Metabolites Results

The metabolites with significant interaction terms needed to be compared by a post-hoc test (Tukey in this case); however, when all 30 molecules that show high correlation (contribution of >0.65) on the two first dimensions of the PCA were assessed in this manner, which is more conservative than the 2-way ANOVA results, all metabolites fell into three different types of responses: 1) metabolites that only consistently show year-to-year differences (with a few inconsistent site differences), methionine, C16:2, Arginine, lysine, Taurine, C18:2, Malate, C14:2OH, C9. Xanthine, C5:1, Phosphocholine, C14:2, ornithine, C16:2OH, hypoxanthine, IMP and guanidoacetate; 2) metabolites that show consistent differences between sites over both years (at least where levels in Dauphin differ from at least one other site, typically Red River), valine, isoleucine, trans-OH-proline, asparagine, tryptophan, proline, C5, and C3; and 3) metabolites those that show no consistent patterns in relation to either site or year, leucine, phenylalanine, C5MDC, glutamate, 18-1SM and C6.

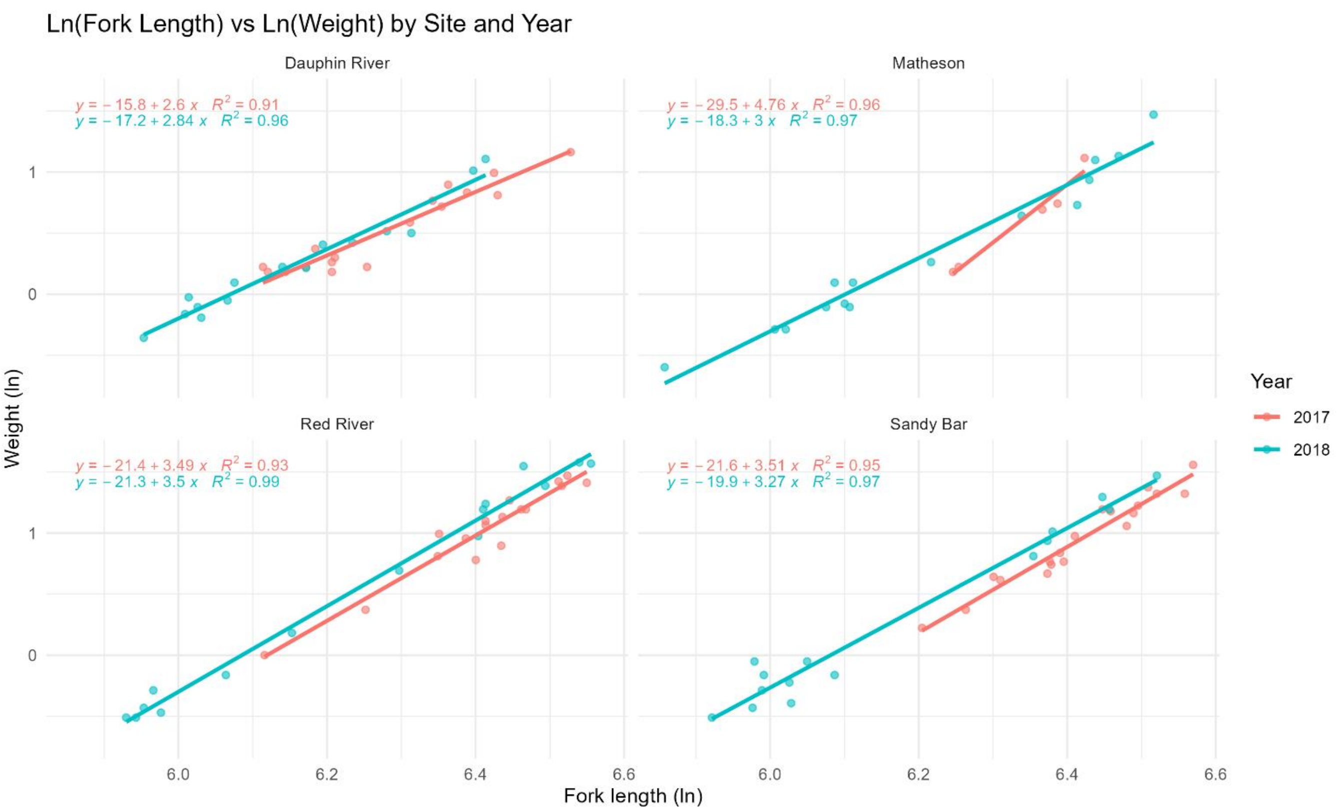

Figure 2.2 *Natural log of fork length and the natural log mass in grams for walleye from Lake Winnipeg. Fork length in mm, wet-mass(W), in g. The log-linear regression lines are shown for each site and year. All lines are statistically significant for a log of fork length, for all sites. Comparisons found that overall, Dauphin River and Matheson fish are smaller (lower Mass) than the Red River walleye, while the slope (indicative of condition factor) is shallower for the Dauphin River walleye than the Red River fish (p = 0.0005), with Sandy Bay walleye also trending (p = 0.083) towards a steeper slope than the Dauphin River Walleye. See section 2.3.2 for further details. Year breakdown of average and standard deviation, SD, summaries: 2017: Red River n=17, FL 613.12 ±61.24, and W 2.96 ±0.94; Sandy Bar n=19, FL 615.26 ±60.41, and W 2.74 ±0.95; Matheson n=5, FL 565.60 ±45.15, and W 1.92 ±0.76; Dauphin River n=17, FL 537.47 ±67.52, and W 1.79 ±0.63. Year breakdown of average and standard deviation, SD, summaries: 2018: Red River n=15, FL 525.93 ±126.04, and W 2.34 ±1.71; Sandy Bar n=15, FL 490.13 ±110.27, and W 2.34 ±1.71; Matheson n=15, FL 508.8 ±104.25, and W 1.68 ±1.12; Dauphin River n=15, FL 475.73 ±72.04, and W 1.39 ±0.68.*

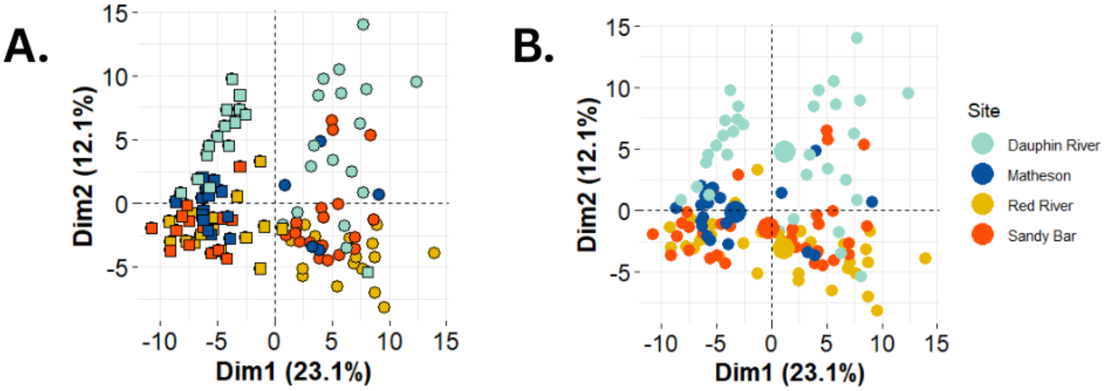

Figure 2.3. *Principal component analysis for the whole blood metabolome of Lake Winnipeg walleye. The walleye were sampled in 4 locations across Lake Winnipeg (Fig. 2.1). A. Biplot showing the separation by year for all the individuals n=118, circles represent individuals from 2017, and squares represent those from 2018. B. Biplot eigenvector averages, according to site of capture, for the walleye. The % contributions to the first two dimensions (Dim) are shown with the axis title.*

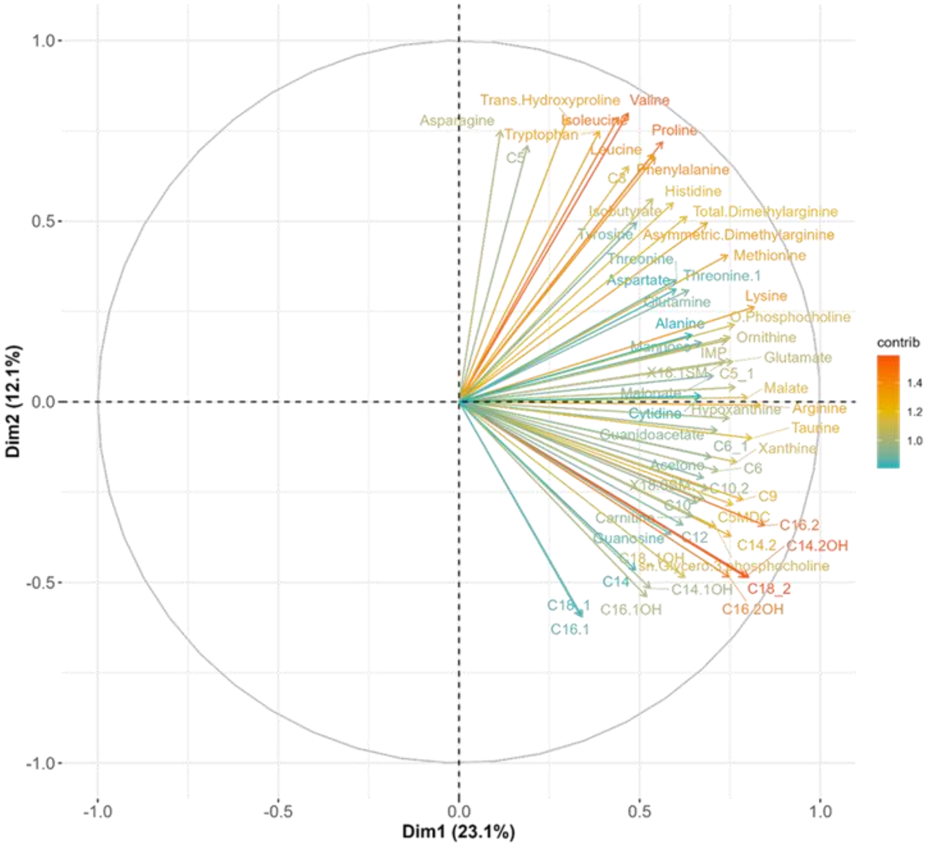

Figure 2.4. *Principal component analysis biplot for the whole blood metabolome of the walleye sampled over 2017 and 2018. The walleye were sampled in 4 locations across Lake Winnipeg (Fig. 2.1). PCA biplot for the metabolite contributions of the first 60 metabolites with selected eigenvector variances >1.0. The lines represent arrows identifying the individual metabolite away from their vector within the graph, and the % variance explained in each dimension (Dim) by the PCA is shown in brackets.*

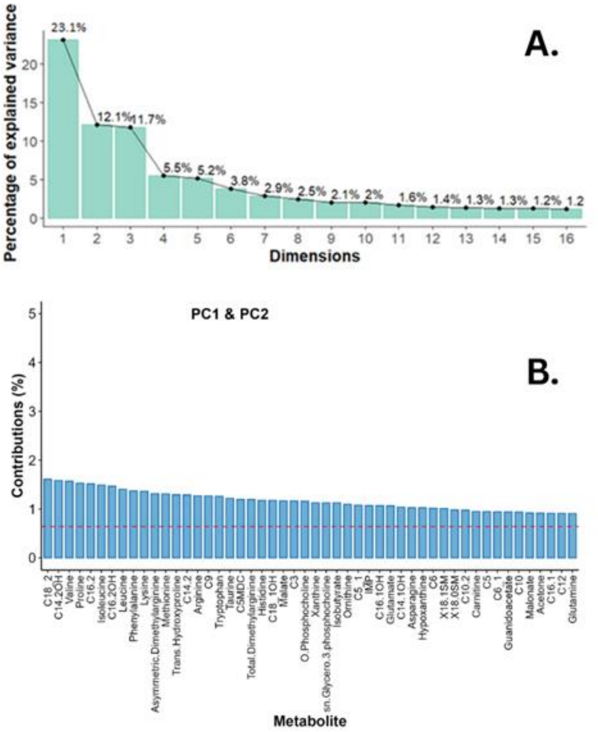

Figure 2.5. *PCA Screeplot of the whole blood metabolites found to be influential to the first two dimensions in the walleye sampled in 2017 and 2018. The graph includes the individual walleye fish, n=118 (n=58, 2017; n=60, 2018) from Lake Winnipeg. A. The plot shows the ranking of the dimensions of the first 16 axes on the PCA. B. The plot shows the highest 50 metabolites shared by axes 1 and 2 in the PCA.*

***Table 2.1**. Summary of the ANOVA results for the metabolites with greatest influence on the PCA analyses (correlation of > 0.65). Metabolite classes and PCA correlation provided with metabolites separated by the greatest influence on Dimension 1 or 2. Empty cells for the interaction term (Site: Year) indicate p > 0.05, and non-significant relationships are in bold.*

Metabolite information			Test Statistics			p-values		
Name	Class	PCA Correlation	Site	Year	Site: Year	Site	Year	Site: Year
Dimension 1								
C16:2	AC	0.846	8.536	201.52		<0.001	<0.001	
Arginine	AA	0.835	8.01	256.884		<0.001	<0.001	
Lysine	AA	0.817	7.777	251.918	5.515	<0.001	<0.001	0.001
Taurine	AA	0.810	4.059	246.193		0.009	<0.001	
C18:2	AC	0.801	12.126	109.51		<0.001	<0.001	
C14:2OH	AC	0.793	9.855	107.891		<0.001	<0.001	
C9	AC	0.786	1.965	90.271		0.123	<0.001	
Xanthine	Py+Pur	0.769	10.298	245.078	6.042	<0.001	<0.001	0.001
C5:1	AC	0.764	3.037	125.205		0.032	<0.001	
O-Phosphocholine	Cholines	0.762	4.991	148.078		0.003	<0.001	
C5MDC	AC	0.758	3.362	79.753		0.021	<0.001	
Glutamate	AA	0.757	8.764	91.079		<0.001	<0.001	
C14:2	AC	0.753	5.78	92.094		0.001	<0.001	
Ornithine	AA	0.751	4.054	168.223	4.669	0.009	<0.001	0.004
C16:2OH	AC	0.748	5.856	70.891		0.001	<0.001	
Hypoxanthine	Py+Pur	0.747	6.633	173.381		<0.001	<0.001	
Methionine	AA	0.743	13.792	95.428	3.572	<0.001	<0.001	0.016
IMP	Py+Pur	0.742	6.164	212.349		0.001	<0.001	

18:1SM	SL	0.738	3.76	63.816		0.013	<0.001	
C6	AC	0.718	0.797	65.739		0.498	<0.001	
Guanidoacetate	AA	0.714	6.378	154.956		<0.001	<0.001	
Dimension 2								
Valine	AA	0.799	24.954	21.064		<0.001	<0.001	
Isoleucine	AA	0.788	18.41	14.417		<0.001	<0.001	
Trans-Hydroxyproline	AA	0.786	32.317	8.562		<0.001	0.004	
Asparagine	AA	0.751	24.257	0.291		<0.001	0.591	
Tryptophan	AA	0.749	24.081	3.917		<0.001	0.05	
Proline	AA	0.719	18.013	34.567		<0.001	<0.001	
C5	AC	0.709	22.04	0.115	4.473	<0.001	0.735	0.005
Leucine	AA	0.686	11.542	32.736	4.552	<0.001	<0.001	0.005
Phenylalanine	AA	0.672	14.35	23.023	2.705	<0.001	<0.001	0.049
C3	AC	0.652	16.862	16.64	3.944	<0.001	<0.001	0.01

Note, for metabolites with a significant ($p<0.05$) interaction term, the degrees of freedom for Site, Year, Interaction, and Residuals were 3, 1, 3 and 110, respectively. In cases with no significant ($p >0.05$) interaction, the degrees of freedom for Site, Year and Residuals were 3, 1 and 113, respectively. Metabolite classes are abbreviated as: AA, amino acids and their derivatives; AC, acylcarnitine esters (note number of carbons are indicated by first number, with unsaturated bonds following the colon, OH indicating a hydroxylated fatty acyl chain; Py + Pur, pyrimidines, purines and their derivatives; Cholines, indicate choline and metabolites of choline; SL, sphingolipids.

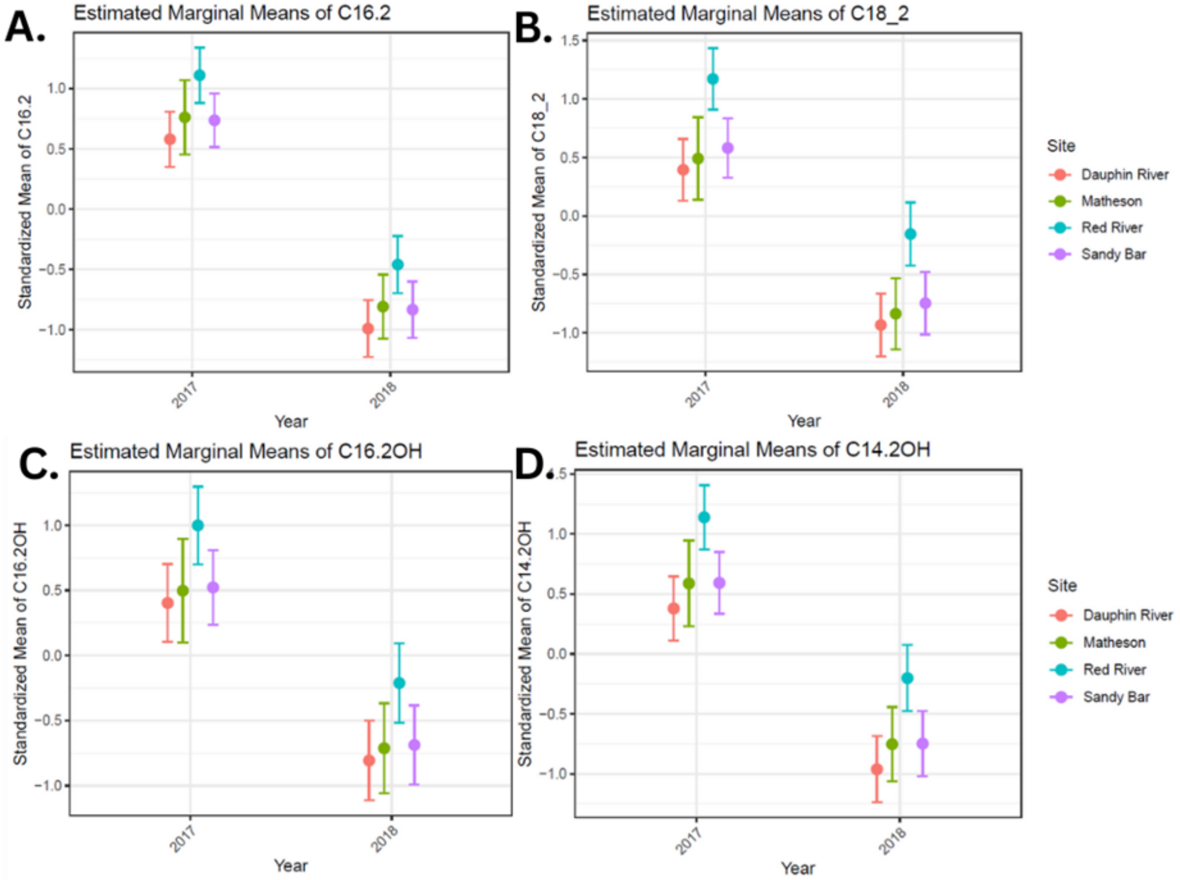

Figure 2.6. *Marginal means comparison of representative long-chained (carbon chain of ≥ 14) acylcarnitine metabolites in the blood of Walleye caught from Lake Winnipeg in 2017 and 2018. A. Hexadecadienoylcarnitine (C16:2) B. Octadecadienoylcarnitine (C18:2). C. 3-Hydroxyhexadecadienoylcarnitine (C16:2OH). D. 3-Hydroxytetradecadienoylcarnitine (C14:2OH). The dot represents the estimated marginal mean and the bars are the 95% confidence interval. Data were standardized to a mean of zero before analysis. 2017 data, n = 17, 19, 5 and 17 for Red River, Sandy Bar, Matheson and Dauphin River, respectively. For 2018 data, n = 15 for all sites. Sites are expressed in the graph alphabetically, not shown as they were sampled in the study.*

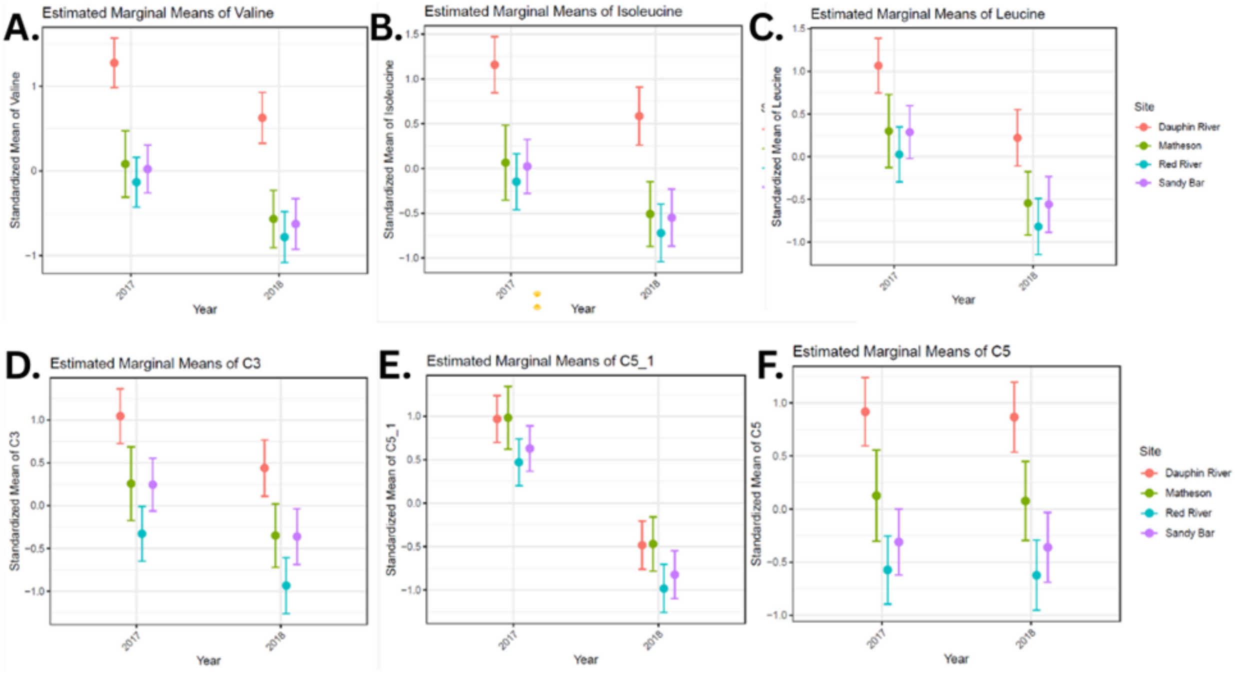

Figure 2.7. *Marginal means comparison of Branched chain amino acids and acylcarnitine metabolites associated with their catabolism in the blood of Walleye caught from Lake Winnipeg in 2017 and 2018. A. Valine. B. Isoleucine. C. Leucine. D. Propionyl carnitine (C3). E. Tigylcarnitine (C5:1). F. Isovaleryl carnitine (C5). The dot represents the estimated marginal mean and the bars are the 95% confidence interval. Data were standardized to a mean of zero before analysis. 2017 data, n = 17, 19, 5 and 17 for Red River, Sandy Bar, Matheson and Dauphin River respectively. For 2018 data, n = 15 for all sites. Sites are expressed in the graph alphabetically, not shown as they were sampled in the study.*

2.5 Discussion

This study used metabolomics to investigate Lake Winnipeg walleye for possible geographical divergences in metabolite patterns, as past literature found differences in mass, growth, amino acid and protein breakdown metabolites, along with differential patterns of gene expression consistent with metabolic disruptions across geographical sampling sites between the Northern and Southern basins (Jeffrey et al., 2023; Thorstensen et al., 2020). In the current study, we found that Dauphin River walleye was, on average, smaller and had a lower wet mass relative to a specific body length (Fig. 2.2 and section 2.2.1) than the walleye in the Red River area, with a trend towards lower mass per unit length relative to the Southern basin Sandy Bay site as well. The metabolites identified across walleye blood highlighted how differences in growth may be consistent with different metabolite profiles of walleye captured from different locations within the lake. Furthermore, while many differences found for specific metabolites were due to annual variation within a site (Table 2.2), for many metabolites this pattern was consistent across both 2017 and 2018. Therefore, while caution should be used in the interpretation of our results, whole blood metabolomics of wild-caught walleye did show the capacity to illustrate potential metabolic differences, on a year-to-year basis, during our sampling period over Lake Winnipeg. That several long-chain acylcarnitines were high in the spawning Red River walleye (Table 2.2, Fig. 2.6), in both years, even compared to the other Southern walleye that were post-spawn illustrates the capacity for metabolomics to demonstrate consistent differences occur in Lake Winnipeg walleye metabolites depending on whether they are spawning or not. Also noteworthy, several essential amino acids were elevated in the Dauphin fish, including most branched chain amino acids and several acylcarnitine metabolites of their catabolism (Fig. 2.7). The elevated essential amino acids in the blood of the most northern sampled walleye supports previous suggestions (Thorstensen et al., 2020; Jeffrey et al., 2023) that the walleye sampled from this region are under a different metabolic state than the post-spawning walleye sampled from the more Southern waters of Lake Winnipeg.

2.5.1 Study considerations and limitations

Before expanding on the findings themselves, particular limitations of this study warrant consideration. Because of logistical constraints during field sampling, it was not possible to separate a plasma or serum fraction of the blood samples. Some metabolites show different levels in the whole blood versus plasma (Kronenberg et al., 1998; Ogata, 1985; Ogata & Arai, 1985), which means we cannot identify a molecule's origin as intracellular or extracellular; therefore, differences between regions reported in this work may be of intracellular and extracellular origin. Likewise, we could not standardize or normalize for the duration fish were held post-electrofishing. Not surprisingly, metabolites that show consistent patterns across years or sites are from classes with lower turnover than other metabolites with well-known and rapid changes in blood, such as lactate (Lawrence et al., 2018), likely being confounded by the sampling protocol and thus being identified but not of great influence on the PCA analysis. Additionally, although we can be reasonably confident Red River fish were spawning, while those from other sites were not, we cannot definitively ascribe metabolite differences as spatial and not temporally influenced because post-spawn fish were sampled about 1-2 weeks apart. Similarly, while several metabolites varied in a consistent spatial pattern in both years of sampling, many metabolites mostly varied only by year (Table 2.2). We cannot exclude possible ‘year-to-year’ effects due to analytical inconsistencies but given that the same approach to analytical standards and metabolite quantification was used in both years’ sample analysis, it is plausible that metabolite differences across years are true biological differences. In support of the assertion of year-to-year comparability of the data, the M to FL relationship was not different between years, except Matheson in 2017 (Fig. 2.2), which as already explained (2.3.2) may have been a consequence of the small sample size that year in this site. The consistency of fish size and mass at a particular FL across years shows walleye sampled from Lake Winnipeg having repeatable biological traits and fish from each site would be in similar a similar state of long-term energy balance across sites in both years of sampling.

2.5.2 Metabolite Divergence Between Sites in Lake Winnipeg Walleye

Acylcarnitines carried in the blood are thought to reflect changes in the pool of acylcarnitines that are within the mitochondrial matrix (Sewell and Böhles 1995; Violante et al. 2013). We focus on the acylcarnitine differences due to the overlapping carnitine ester metabolites and subsequent oxidation as Coenzyme-A (CoA) derivatives in the mitochondria (Fig. 2.8). The Southern walleye from the Red River had higher whole blood long-chained acylcarnitine concentrations, which may indicate reliance on stored lipid reserves and elevated β-oxidation of long-chain fatty acids for energy demands during the early spring spawning season. (Mozsár et al., 2019). However, the Northern walleye, in our study, the Dauphin River fish, had higher concentrations of amino acids and intermediates of these specific amino acid breakdown pathways, specifically, we focused on the valine, leucine, and isoleucine breakdown pathways (Fig. 2.7). However, most molecules above are part of the essential amino acids’ breakdown or recycling, and short-chain carnitine esters. These molecules are in higher concentration in the

Northern cohort of the walleye sampled, the Dauphin River site, which may represent the reallocation of amino acids from protein within the tissues (Dunstan et al., 2019). The presence of higher concentrations of intermediates in the amino acid catabolism pathway may be evidence of protein catabolism in the Northern cohort of the Lake Winnipeg walleye (Rodrigues et al., 2023).

There is evidence that the Northern basin Lake Winnipeg walleye may be growing more slowly compared to the Southern counterparts (Thorstensen et al, 2020). This may be due to possible nutrition differences across basins, or reflect the differing arial forage fish density between basins (see discussion in Turner et al., 2025), suggesting these Northern fish may be more constrained in size and growth, in comparison with their Southern counterparts (Thorstensen et al. 2020; Turner et al. 2021). Other work on the same Lake Winnipeg walleye found a pattern of transcriptomic (mRNA) changes in Northern versus the Southern sites (Jeffrey et al., 2023). They found that walleye from the Northern basin exhibited higher gill mRNA levels of genes related to stress, gill remodeling, fatty acid synthesis, protein catabolism, mTOR pathway regulation, and molecular chaperones compared to walleye from the Southern basin. Paralleling our results, spawning Red River fish were more similar to Southern caught post-spawn fish than Northern post-spawn fish were to the Southern post-spawn Walleye transcriptomes (Jeffrey et al., 2023). These Differences in mRNA transcript abundance support the presence of long-term physiological stress and regional differences in physiology occurring in the walleye of Lake Winnipeg. While both transcriptomic and metabolomic approaches support a gradient in physiological states in Lake Winnipeg walleye, it is important to note that these molecular signatures integrate on different time scales, with several metabolites potentially responding faster than cellular changes in mRNA levels (Maan et al., 2023; Machuca et al., 2021).

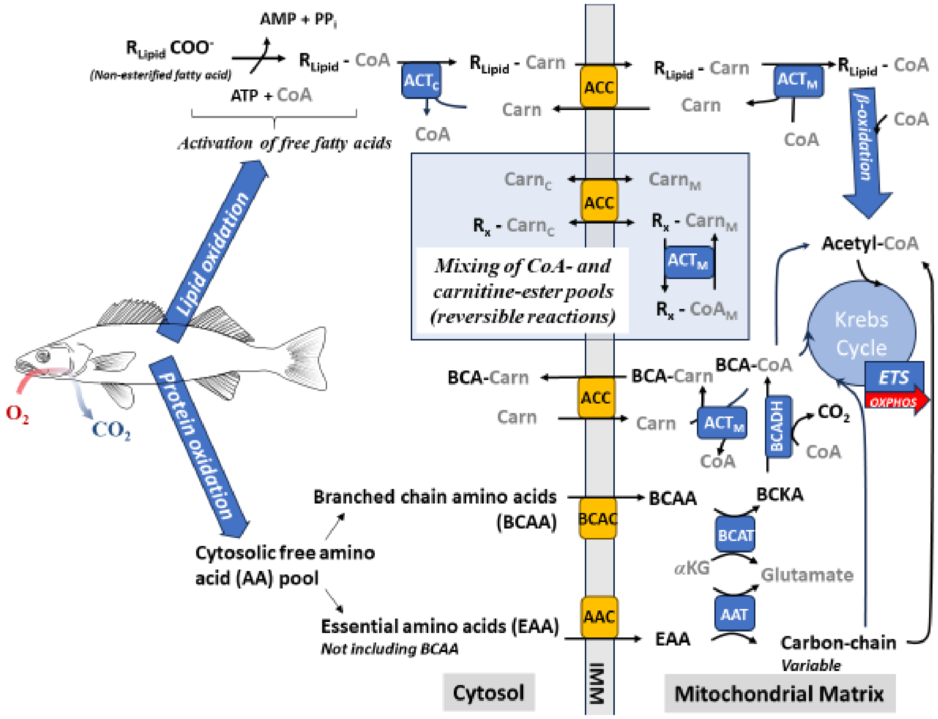

Figure 2.8*. Convergence of lipid and protein oxidation as a metabolic fuel in animals. Whole animal gas exchange, at the gills in this case, is mostly due to mitochondrial oxidation of carbon, initially from the diet, to carbon dioxide (CO2) in the Krebs cycle and pathways that supply it. Lipids are predominantly stored as long-chain (carbon chain of 14 or more) fatty acids, which need to be activated in the cytosol before catabolism by β-oxidation in the mitochondrial matrix (upper pathway). Enzymes activate the fatty acids by adding a free co-enzyme A (CoA) at the cost of ATP hydrolysis, forming an acyl-CoA ester (Rlipid-CoA). Cytosolic acyl-carnitine transferases (ACTc) replace the CoA with carnitine, and the acyl-carnitine ester (Rlipid-Carn). Influx of the Rlipid-Carn is by exchange with an intramitochondrial carnitine using acyl-carnitine, carnitine carriers (ACC). Intramitochondrial acyl-carnitine transferases (ACTM) replace the carnitine with CoA, and the fatty acid can be broken down to acetyl-CoA (2 carbon), which is then consumed by the Krebs cycle, releasing CO2 and reducing electron carriers that supply electrons to the electron transport system (ETS) which powers oxidative phosphorylation (OXPHOS) and the re-phosphorylation of ADP to ATP for driving cellular work. Proteins are made up of amino acids (AA), and the hydrolysis of protein to free amino acids, and uptake from the extracellular fluid, provides the cytosolic free amino acid pool (lower branch). Essential amino acids (EAA) are those that the organism cannot endogenously synthesize sufficient amounts needed for growth and homeostasis, and we focus on this as dietary intake is necessary for organismal integrity. Among the EAA, the branched-chain amino acids (BCAA) are metabolized within the*

matrix where they compete with lipid oxidation for a shared pool of free CoA, and importantly, can also exchange their CoA with carnitine via ACTs. The net oxidation of EAA in animals begins transamination (various alpha-amino acid transaminases, AAT of branched-chain amino acid transaminase, BCAT), which leaves the carbon chain, or branched-chain ketoacid (BCKA) in the BCAAs, which enters the Krebs following further modification by matrix enzymes (omitted for clarity). Note: The grey-blue box in the middle of the figure illustrates how, because the ACTs and ACCs are reversible, the intramitochondrial and cytosolic pools of CoA-esters and carnitine esters can mix; therefore, carnitine esters that leak from the cytosol to the extracellular space are hypothesized to reflect the matrix assemblage of carnitine esters that are feeding the Krebs cycle.

The overexpression of mTOR regulators and molecular chaperones in Northern caught walleye is consistent with the presence of metabolites related to protein breakdown molecules and intermediates of protein breakdown pathways; mTOR pathway regulation is related to the insulin pathway control and signaling, therefore related to protein catabolism signaling (Li et al., 2020). Furthermore, molecular chaperones can represent signs of nutritional stress (Da-Silva et al., 2011), including insufficient nutrient intake (Ghani et al., 2022). Amongst other genes upregulated in the Dauphin River are transcripts involved in glycolysis regulation and protein transport. Specifically, there is an upregulation of mTOR genes in the year 2018 Dauphin River walleye, specifically the mTORC1 regulatory and specific targets (Jeffrey et al., 2023). The mTORC1 complex is a nutrient, redox sensor in the cell, which is involved in protein synthesis regulation and protein turnover. The mTOR pathway is modulated by certain amino acids, like leucine in mammals, as well as oxidative stress, rapamycin, growth factors, and insulin (Son et al., 2019). This pathway may represent the regulatory pathways that are currently inducing the protein breakdown differences found in the Dauphin River walleye. Downregulated genes in Dauphin River walleye 2018 also included transcripts of components of the mitochondrial electron transport chain, complexes I, III, and IV; as well as low ATP-synthase expression (Jeffrey et al., 2023). These transcripts emphasize the possibility that these Northern region fish are undergoing environmentally stressful conditions or at least showing regional differences in metabolic state.

It was previously reported that Northern walleye have a higher concentration of some essential amino acids, together with equivocal results for products of amino acid breakdown pathways in the blood, making conclusions on protein turnover and amino acid degradation challenging (Thorstensen et al., 2020). The current, more comprehensive, study of metabolites finds further support for elevated amino acid catabolism, particularly due to the higher carnitine-esters of the amino acid catabolism pathways coinciding with elevated levels of the precursor amino acids as well (Fig. 2.7). Our study includes a larger set of metabolites from amino acids to lipid molecules, in comparison to Thorstensen et al. (2020) and it extends to 2 years of sampling of the walleye from Lake Winnipeg. Overall, our findings support the contention that walleye from different regions of Lake Winnipeg may be under different metabolic states, with spawning fish distinct from post-spawning fish, and Walleye from Dauphin differing from those sampled in more Southern sites like Matheson and Sandy Bar (Fig. 2.6-2.7).

2.5.3 Implications

This study has demonstrated that metabolomic profiles from wild fish may be reproducible between sampling years and across some of the different sites sampled. Moreover, the metabolite patterns support the contention that metabolomics may provide insight into the physiological state of these wild walleyes. While we must acknowledge that interpretations are still somewhat speculative, our contentions are based on a reasonable extension of general metabolic concepts and are limited to metabolites where there are repeated divergences in blood concentration between sites across years of sampling. Therefore, future studies to confirm and expand our findings linking condition factor, or growth trends, with the specific metabolites identified with metabolomics, to better identify what significant metabolic traits vary spatially and temporally in Lake Winnipeg walleye. Furthermore, our study uses non-lethal sampling for the collection of walleye tissues. Metabolomics research presents itself for the utilization of non-lethal sampling. Non-lethal can provide material to be examined in laboratory and other scientific settings, and multiomics (performing gene expression analyses on mRNA and protein isolates along with metabolomics) would be a promising means of expanding on the current study. Such multiomic approaches may use populations like the Lake Winnipeg walleye as proving grounds for future work on endangered species or areas where minimizing ecological disruption are required, thanks to the non-lethal approach to sample acquisition.

Finally, in regard to the walleye of Lake Winnipeg, we found metabolite patterns consistent with elevated reliance on long-chain fatty acids as a fuel during spawning in the Red River fish relative to other Southern captured, but post-spawn, walleye (Fig.2.6). Similarly, Dauphin River site, have metabolite patterns (Fig 2.7) consistent with elevated protein catabolism in comparison to the more Southern caught walleye which, along with lower body condition (Fig. 2.2), may support previous observations that the Dauphin River walleye are under some degree of environmental challenge or stress (Thorstensen et al., 2020). Both metabolomics (current study) and others (Thorstensen et al., 2020) are useful tools for interrogating the internal state of wild fishes based on the results with Lake Winnipeg walleye. However, perhaps more importantly given the impending challenges aquatic ecosystems like Lake Winnipeg will face due to climate change, whole blood metabolomics has provided evidence that a pattern between sampling years appears to be more consistent compared to interannual patterns seen for transcriptomics on gill biopsies (Jeffrey et al., 2023). While further confirmation is warranted, our findings suggest blood metabolomics may provide particular advantages for establishing long-term data sets for environmental monitoring of wild-sampled walleye and has identified metabolites, including amino acids and acylcarnitines, which may act as possible indicators of variation in the metabolic state of wild sampled walleye future use.

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3. Chapter 3: Testing for Change in the Blood Metabolome with Age across Lake Winnipeg Walleye, *Sander vitreus*

3.1 Contribution of Authors

Lilian Maria Wiens, conceptualization, statistical analysis, writing and editing; E.C. Enders, D. Watkinson, and DFO staff, fish collection; M Gaudry, J. Jeffrey, measurement of relative telomere length, M Yusishena, second reader of spines for aging, J.R. Treberg, conceptualization, editing and funding.

3.2 Introduction

The comparative biology of aging involves the study of aging across all levels of biological organization, from whole organisms to changes at the cellular and subcellar level. Aging often is associated with the term senescence, which also applies to all levels of biological organization from the organism (actuarial senescence, which is a function of mortality risk, or age-related changes in metabolism) to cellular senescence which can be evaluated at the level of changes in biomarkers of aging (for example, relative telomere lengths). Cellular aging has been commonly evaluated in mammals and birds, both being groups of vertebrates that are typically endothermic, and some ectotherms that are common laboratory model species such as: *Drosophila melanogaster* (Gimenez et al., 2013), *Caenorhabditis elegans* (Martins et al., 2016), and short-lived vertebrates like zebrafish (*Danio rerio*) (Almaida-Pagán et al., 2014; Yu et al., 2006). Most vertebrate ectotherms, including reptiles, amphibians, and fish taxa, with some exceptions (Frazier et al., 2014; Hamady et al., 2014; Newman et al., 2011), are less studied in the context of aging in comparison to birds and mammals (Finch, 1990). Vertebrate ectotherms may present a different perspective in studies of the mechanistical process of aging. Temperature is a major factor for ectothermic metabolism (Lear et al., 2020), development, growth and life cycle (De Jong et al., 2023; Zuo et al., 2012). For instance, ectotherm metabolic processes are under the influence of environmental thermal changes that may negatively impact their longevity (Burraco et al., 2020). Increasing temperatures may take their metabolic rate to high threshold levels which can accelerate physiological processes and cause stress and thus may shorten their lifespan (Burraco et al., 2020).

In addition to their internal physiology being highly influenced by the external temperature, many ectotherm taxa also show an indeterminant growth life history, unlike groups like mammals, birds and most model invertebrate organisms which show determinant growth. In contrast to determinant growth, where size at maturity is similar to maximum size of the species, indeterminately growing animals continue to grow well after reaching maturity and may be under different selection pressures, especially post-maturity, than determinate growing species (Finch, 2009; Mumby et al., 2015). The capacity for continued growth post-maturity may intrinsically give a selective advantage to indeterminately growing animals, thus potentially selecting for some capacity for longevity (Finch 1998, 2009). Indeterminate growers may have the advantage of a size-dependent decline in their mortality (Finch, 1998). Indeterminate growers may also acquire increasing fecundity with larger sizes and older age, implying positive evolutionary advantage at later age and the possibility of negligible senescence (Finch, 1990). Coincident with the possibility of negligible senescence, mortality in wild populations of upper trophic level fishes may be comparable between young, mature and older individuals of the population (Kocovsky and Carline 2001; Purchase et al., 2022). Furthermore, as they become larger, indeterminate growers have lower mass-specific metabolic costs and have longer starvation tolerance between periods of nutritional abundance and they also have lower mass-specific costs of locomotion (Alexander, 2005; Ali et al., 2003).

While wild fishes have been important in the study of aging and senescence (Finch 1998, 2009; Reinke et al., 2022; Rechsteiner et al., 2025), they have been underused for investigating how metabolism may change with age. In humans (Roberts et al., 2020), other mammals (Uchitomi et al., 2019), birds (Urvik et al., 2016) and a small lab-held fish (Ruhl et al. 2015), aging appears to affect several metabolic pathways and physiological processes, indicating metabolism is subject to senescence in these animals. It has been shown that branched chain amino acid accumulation occurs during aging (Canfield & Bradshaw, 2019; Liang et al., 2023; Nakano et al., 2013). Amino acids are the main components of proteins and therefore they are main players in protein turnover which includes both protein degradation and synthesis, which is key to growth through the balance between anabolism and catabolism. Amino acids are also important as signaling molecules, they fluctuate with nutritional status and caloric restriction state, so changes in amino acid concentrations may pinpoint to metabolic imbalance through the insulin signaling pathway within the organism with aging (Canfield & Bradshaw, 2019). Products of amino acids, like biogenic and polygenic amines including spermine, spermidine, and putrescine, which regulate many biological processes including autophagy, have also been found to change with aging with their accumulation correlated to longevity. For example, spermidine increases with aging in mammals, implicating these amines with mechanisms that correlate to long-age (Madeo et al., 2018; Minois, 2014).

The concentration of lipids in serum and plasma tend to increase with aging in most laboratory and human models, especially saturated lipid molecules (Berkowitz et al., 2021; Montoliu et al., 2014). Lipids are part of the cellular membranes, key signaling molecules, and major energy storage for animals and some changes in lipid metabolism has been correlated with organismal senescence and aging (Mutlu et al., 2021). Specifically increases in sphingomyelins and sphingolipids, in serum and plasma are correlated with longevity (Gonzalez-Covarrubias et al., 2013; Montoliu et al., 2014).

Metabolomics presents a powerful tool to investigate novel species for aging related trends given the patterns seen for specific metabolites and metabolic pathways in common model systems for the biology of aging. Metabolomic data quantifies the whole or partial make up of metabolite levels in tissues sampled (Schimpe-Ruthledge et al., 2016). This method with proper analyses and molecular insight can be used to decipher pathways of importance at the time of sampling when comparing across groups of organisms. If a metabolite increases in the blood of one group of organisms relative to another group, we can deduce that the blood pool for this specific compound has shown some shift in the balance between rate of appearance or disappearance within the blood. While interpretation of metabolic responses from metabolomic data must be done with caution, demonstrable shifts in the balance of metabolites are strong indicators of some internal change in the balance of metabolic fluxes.

Importantly, the plasma metabolome could differentiate between a range of rapid, gradual and negligible senescence aging phenotypes in wild-sampled fishes from temperate Eurasia (Trifonova et al. 2018) emphasizing the potential of metabolomics as a tool to interrogate the biology of aging in wild fishes. This past study included Zander (Sander lucioperca) an upper trophic level predatory fish, which was determined as a gradual aging phenotype. The congeneric Walleye (*Sander vitreus*), from temperate regions of North America is an ecologically similar species to Zander and have been found to show limited increase in mortality between mature young and older individuals in some populations (Kocovsky and Carline 2001). Due to their regional ecological and economic importance, walleye in Lake Winnipeg have been studied for large scale movement via telemetry (Turner, 2020) and general metabolism (Thorstensen et al., 2020), as well as growth (Sheppard et al., 2018), and nutritional studies (Kaufman et al., 2007). It is a prized species, making knowledge of their physiology, life history and the general health of the population important. Traits like chronological age are essential to understand life history (Erickson, 1982). Age estimates show the structural development of the fish, age at maturity, and growth trends that can pinpoint how the species is faring in the face of environmental change (Hoxmeier et al., 2006).

Relative to laboratory-based studies, there is a major lack of information on metabolic changes with aging in wild animals, and fishes are a particularly understudied group of ectotherms in the comparative biology of aging field (Reinke et al., 2022; Rechsteiner et al., 2025). The walleye population of Lake Winnipeg has been determined to have regional, and possible temporal, differences in metabolite concentrations within their blood (Chapter 2), with fish in the northern basin having a high concentration of essential and branched-chain amino acids, and lower levels of specific long-chain acylcarnitines relative to southern walleye. Given that these same groups of metabolites show age-dependent patterns in other species, we investigated if there are metabolites that change with age across the sampled walleye. Our results will test if patterns seen in these wild walleyes may parallel changes seen with aging in typical model species (Nakano et al. 2013; Montoliu et al. 2014; Canfield and Bradshaw 2019; Berkowitz et al. 2021 Liang et al. 2023). To assess if the walleye were showing evidence of a gradual cellular senescence pattern, we also measured relative telomere length in the nucleated red blood cells which have been reported to show age-dependent shortening in other fish species (Simide et al., 2016).

3.3 Methods

3.3.1 Fish Sampling

The fish used in this study were the same as those in chapter two of this thesis and further details can be found there. Walleye caught in 2017 were not sampled for spines, thus only 2018 fish could be used to test for age-dependent patterns in the blood metabolome. Spines were collected during the surgery for acoustic telemetry transponders from May-7th to June-6th, in four sites: Red River, Sandy Bar, Matheson, and Dauphin River (see Fig. 2.1 for sites of capture). In all cases, the pectoral spine was collected and stored dry at room temperature until processed. In total, 54 walleyes were both aged using fin spines and assessed by NMR and LC-MS/MS for whole blood metabolome (as described in Chapter 2). Finally, walleye sampled and analyzed for relative telomere length (see below) included 67 walleyes in 2017 and a total of 80 in 2018. Note, funding limitations prevented metabolomic assessment of all fish; higher numbers of walleye were used for relative telomere length assays than were analyzed for metabolomics.

3.3.2 Age Estimation

The collected fin spines were processed by fixation in EpoxiCure® resin and hardener (Buehler Inc.©) and later cut using an Isomet Low Speed Precision Cutter (Buehler Inc.©) to 800 µm thickness. From the base of the spine, 2 sections were cut per walleye, polished from course to fine with lapping film sheets (30 µm, 9 µm, and then 3 µm) from GessWein Canada. Following cutting and polishing, spines were viewed under a Leica ICC50W compound microscope, with a series of pictures taken using total magnification of 100X. To estimate the chronological age, the rings found on the spines were counted by two individuals making independent assessments of each of the spines. All age estimates were performed in the most basal section of the spine. When disagreement between the two readers occurred, a second independent reading was performed by both individuals. After this procedure if disagreement was still present, a third reading was done with both readers present to determine a final consensus age for that fish. Samples of spines that had agreement in the first or second independent readings were accepted on the first or second try accordingly.

3.3.3 Metabolomics

These data are a subset of those from Chapter 2. Briefly, whole blood samples were used for determining the metabolome, which was performed by The Metabolomics Innovation Centre (TMIC), University of Alberta, with the following protocols:

NMR Method: Deproteinization steps were done for the samples before spectroscopy using the (Psychogios et al. 2011), method. NMR spectroscopy: All 1H-NMR spectra were collected on a 700 MHz Avance III (Bruker) spectrometer equipped with a 5 mm HCN Z-gradient pulsed-field gradient (PFG) cryoprobe. 1H-NMR spectra were acquired at 25°C using the first transient of the NOESY pre-saturation pulse sequence (noesy1dpr) (Saude et al. 2006). All 1H-NMR spectra were processed and analyzed using the Chenomx NMR Suite Professional software package version 8.1 (Chenomx Inc., Edmonton, AB).

TMIC Prime Assay LC-MS/MS Method: A targeted quantitative metabolomics approach was done using a combination of reverse-phase liquid chromatography and direct injection mass spectrometry. Mass spectrometric analysis was performed on an API4000 Qtrap® tandem mass spectrometry instrument (Applied Biosystems/MDS Analytical Technologies, Foster City, CA) equipped with an Agilent 1260 series HPLC system (Agilent Technologies, Palo Alto, CA). Data analysis was done using Analyst 1.6.2.

3.3.4 Relative Telomere Length Using qPCR

DNA extractions were performed using a DNeasy Blood and Tissue kit (Qiagen) according to the manufacturer's instructions starting with 7-10 µl of whole blood. DNA in the eluants was quantified with a Qubit DNA HS assay kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific). This qPCR-based approach to estimate relative telomere length, developed in (Cawthon 2002), produces a telomere repeat (T) to single gene (S) copy ratio (T/S) for each DNA sample. The telomere repeats were amplified with the universal primer pair Tel1b (5' CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). The following modifications were applied from the original method (Cawthon, 2002): Two single copy genes were selected which apply to our chosen species, elongation factor 1 alpha (Ef1) and pannexin 2 (Panx2), to verify consistency of T/S ratios. Primers were designed in Geneious 9.1.8 (Biomatters Ltd.) using Genbank contigs specific to the European perch (Perca fluviatilis; accession number: KC513785.1) and walleye (accession number: KC827895.1), for Ef1 and Panx2, respectively. The 117 bp Ef1 amplicon was amplified using the primer pair P. fluviatilis_ef1_F (5'-AAACTGAAGGCTGAGCGTGA-3') and P. fluviatilis_ef1_R (5'-CTTGATGAAGTCCCTGTGTCCA-3'). The 53 bp Panx2 amplicon was amplified using the primer pair S. vitreus_Panx2_F (5'-CCCAAAGATCGAGAAAGCAGAT-3') S. vitreus_Panx2_R (5'-CTCGCTCAGTTATGCCAGGT-3').
All qPCR reactions were performed on separate 384-well plates for each primer pair using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with 10 ng DNA per reaction.

The final concentration of each primer was 800 nM. The primer thermocycling conditions were 50°C for 20 min, 95°C for 2 min, and 27 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 60 s. The single copy gene thermocycling conditions were 50°C 2 min, 95°C for 2 min, and 40 cycles of 95°C for 20 s, and 60°C for 20 s. Both were followed by default melt curve conditions of 95°C with a ramp rate of 1.6°C/s for 15 s, 60°C with a ramp rate of 1.6/s for 1 min, 95°C with a ramp rate of 0.15°C/s for 15 s. Amplicon sizes were visually inspected by electrophoresis of a subset of four reactions on a 2% agarose gel. A pooled sample was created from the 67 individual DNA samples in 2017 and the 80 samples in 2018 DNA samples and diluted in a 4-fold serial dilution producing 5 concentrations ranging between 40–0.157 ng/µl. This serial dilution was used for efficiency tests (results of which ranged between 99-104%, data not shown). Non-target controls were also performed in triplicate on each plate where the sample DNA was excluded from the reaction. Ct values for these non-target control samples matched the background fluorescence values.

3.3.5 Statistical Analysis

Statistical Analyses were performed on the R software version 4.4.1 (R Core Team, 2020) and In all cases $p < 0.05$ was considered significant. Initial exploration performed to detect regional differences in the age and size relationships of the sampled fish. For age versus site, a one-way ANOVA was used while a two-way ANOVA was used initially to compare the size of walleye across sites. For the two-way ANOVA, the wet mass (W) and fork length (FL), in kg and mm, respectively, were ln transformed prior to analysis. Data were tested initially assuming an interaction term (using equation $\ln(W) \sim \ln(FL) * \text{Site}$) but if no significant interaction was found the data would be re-evaluated using an additive equation ($\ln(W) \sim \ln(FL) + \text{Site}$). Tukey post hoc tests were performed on the significant results on fish size prior to a multivariate approach using Principal Component Analysis to find relationships between the blood metabolome and chronological age of walleye.

Of note, sex was not incorporated into the statistical analysis although data was collected during surgery to implant acoustic telemetry devices. While the surgeon attempted to sex the individual walleye, this was not always possible or definitive and was not prioritized for animal welfare reasons. Because sex was not a definitively confirmed trait of each fish, we report the details for completeness but did not incorporate sex into any of the statistical analysis.

3.3.5.1 Principal Component Analysis (PCA):

To assess the structure and trends within the metabolite data a PCA was performed using packages FactomineR (Lê et al. 2008) for analysis, and FactoExtra (Kassambara and Mundt 2020) for visualization, on a set of 159 metabolites from 54 walleye sampled in 2018. Correlation analysis was performed comparing the age of the walleye versus the loadings. Note, in Chapter 2, where fish from 2017 and 2018 were analyzed, dimensions 1 and 2 were most influenced by year of sampling and site of sampling respectively. Because site differences were expected to also occur in the current chapter, it was determined to focus on the dimensions most influenced by the age of the walleye to prioritize metabolites for further interrogation by linear model (see 3.2.6) to determine if site or age is the predominant explanation of variation. In Chapter 2 the top 10% of metabolites that influenced the PCA results in dimensions 1 and 2 also had high correlation, ranging from approximately 50% to over 70% (Chapter 2). For the current analysis, the inclusion criteria for metabolites were a correlation of > 60% for dimensions that best explained variation with age (Dimensions 2 and 3) based on the PCA results. Note, site differences were found in the previous chapter; however, the bulk of the influence of site was in Dimension 1 of the PCA, therefore this chapter focused on the Dimensions that described variation with age (2 and 3).

3.3.6 Linear Mixed Models to test for patterns with age:

Metabolites which were found to correlate at or greater than 60% on the PCA dimensions 2 and 3 were further evaluated with linear models to evaluate specific patterns between blood metabolites and age. Model selection was based on first testing for an interaction between age and site (Metabolite ~ Age * Site). If the interaction was significant, indicating the relationship for the metabolite and age varied by site, further comparisons were made by Bonferroni post-hoc comparisons. Alternatively, when no significant interaction was found then an additive model (Metabolite ~ Age + Site) followed by Bonferroni post-hoc tests were used. The choice of a Bonferroni post-hoc correct was based on this being an even more conservative test, relative to a Tukey, to minimize the likelihood of Type I errors (false positives) when associating blood metabolites with age. The following we used to evaluate each metabolite:

Metabolite ~ Age * Site, which included Site as a random effect: ~1 | to test metabolite level relative to chronological age while including interaction across sites;

Metabolite ~ Age + Site, which included Site as a random effect only: ~1 | to test metabolite level relative to chronological age while excluding interaction across sites of capture; site as random effect. Model selection for each metabolite was also based on having no collinearities and or singularities within the residuals, while also having a significant prediction of age versus the metabolite. In cases where multiple models (1-2 above) fit this criterion, the one with the highest predictive power (using R2) was selected for that metabolite.

3.4 Results

3.4.1 Comparison of size across Walleye from different sites of capture and age.

Fish with chronological age determined by spines were initially compared across capture sites (Table 3.1). The linear models between age and site found significant differences ($R^2 = 0.76$, F (4,49) = 38.6, $p < 0.001$). Emmeans Tukey post-hoc contrasts found walleye caught in the Dauphin River were younger than those from the Red River: (t=0.113, p = 0.04), while all other comparisons were not significant.

Initial comparison of fish length by linear models in R found a significant relationship between natural log mass and natural log fork-length, (F(7,52) = 2.838, $p < 0.001$) as well as site differences being present, with significant interaction (F= 304.6 on 7 and 52 DF, p-value: < 0.001). The interaction term with significant includes only ln(FL): Site Red River (t= 2.85, $p<0.01$). The model indicated significance for Red River (t=-2.85, p= 0.03) versus Dauphin River, the intercept. Emmeans with Tukey post-hoc comparisons on the model (ln(FL)~ ln(W) * Site) showed Matheson had lower adjusted marginal means on fork-length than Red River walleye (t=-2.81, P=0.03), there is a trend toward Matheson being lower versus Sandy Barr (Table 3.1).

Further comparison of fish mass by linear models in R found a significant relationship between natural log fork-length and natural log mass, by site with interactions (F= 304.6 (7, 52), p= < 0.001). The interaction term with significance includes only log (W): Site Red River (t= 2.837, $p<0.01$). The model indicated significance for Red River (t=-2.849, p= 0.03) versus Dauphin River, the intercept. Emmeans with Tukey post-hoc comparisons on the model (ln(W)~

ln(FL) * Site) showed Matheson lower adjusted marginal means on mass than Red River walleye (t=-2.809, p= 0.034), there is a trend toward Matheson being lower versus Sandy Bar (Table 3.1).

Table 3.1. *Size, Age and Sex distribution Lake Winnipeg walleye.*

Site	N	Fork-Length (mm)	Wet mass (kg)	Age	Females	Males	Indeterminate
Dauphin River	15	476±721	1.39±0.681	6.33±2.0*	8	1	6
Matheson	15	509±104	1.68±1.11	7.87±2.9	13		2
Sandy Bar	9	469±103	1.50±1.1	6.67±2.0	5	4	
Red River	15	526±1262	2.34±1.72	9.53±3.9*	8	7	

Data for fork length, Wet mass and Age are mean ± standard deviation. For comparison of age across sites with ANOVA, * indicates groups that are significantly different ($p<0.05$). Differing superscript numbers indicate differences ($p < 0.05$) for Fork length or Wet mass by the linear models run.

Comparisons of walleye size using linear models (Fig 3.1) also found a robust relationship between W and FL across all sites, ($n = 60$, residual degrees of freedom= 52, $R^2 = 0.98$, adjusted $R^2 = 0.97$, Residual SE = 0.11, F = 304.6, $p<0.001$) and, similar to the ANOVA, a significant interaction between FL and site (Fig. 3.1), with Red River fish having a steeper relationship than those from Dauphin River (Estimate -4.09, t= -2.84, p = 0.006).

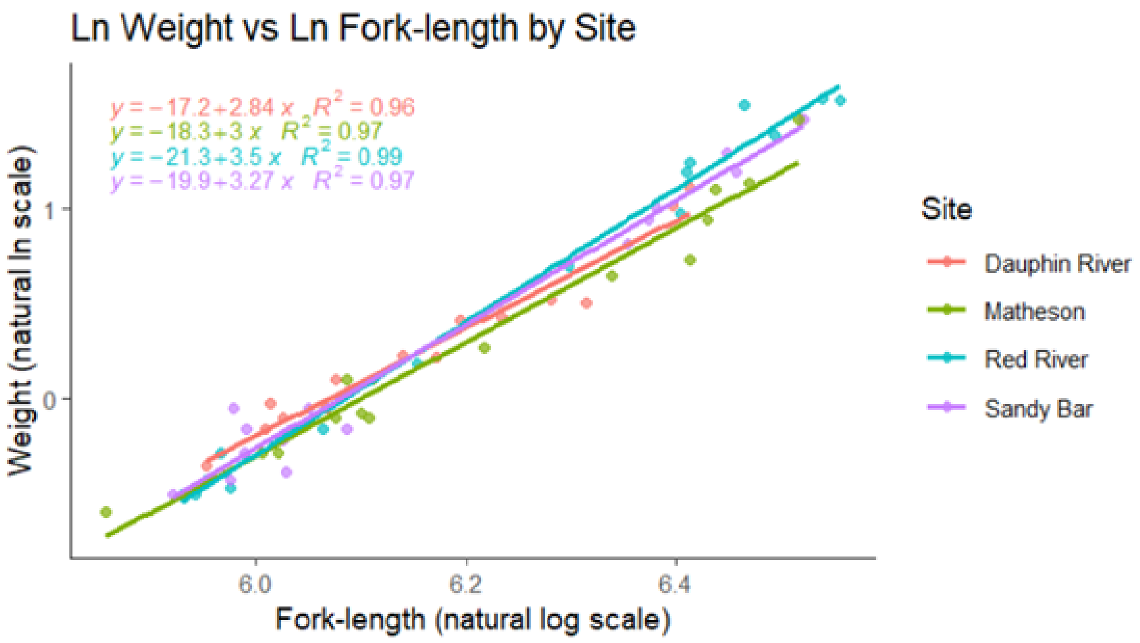

Figure 3.1 *Relationship between wet mass and fork length across the walleye sampled in Lake Winnipeg. Wet mass (kg.) relationships by fork-length (mm)and sites, data transformed into the natural log for mass and fork length, n=60. The only significant difference (p < 0.05) found was that the slope in Red River fish was steeper than the relationship found in walleye from Dauphin River.*

3.4.2 Initial comparison of metabolites across sites and age with PCA

The PCA variance and eigenvalues for the top five dimensions are shown in Table 3.2. The first dimension had an eigenvector of 36.13 with 22.87% of the variance explained. However, the data that was related to the age estimation was better explained by the second and third dimensions. The second dimension shows a 30% Pearson Product-Moment value (p = 0.025) on a correlation test with age while the third dimension has a Pearson Product-Moment of 61% (p <0.001) in relation to age. The biplot of the higher influencing metabolites that are 1 value and higher on the contribution to dimensions 2 and 3 illustrates several classes of metabolites are influential (Fig 3.2). The metabolites most influential to the second and third dimensions of the

PCA include amino acids, acyl-carnitines, lysophospholipids and phosphocholines, representing most of the contributing metabolites. Furthermore, the chronological age breakdown across individuals on the PCA axes 2 and 3 shows that the walleye is separated by age along both dimensions 2 and 3 (Fig. 3.3A). When comparing across sites, eigenvalues on average, separate sites mostly along dimension 2 (Fig. 3.3B).

Table 3.2. *Major eigenvalues and variance percent explained in Principal Components Analysis on Walleye whole blood metabolome.*

PCA Dimension	*Eigenvalue*	*Variance Explained*	*Cumulative Variance*
1	36.13	22.87%	22.87%
2	21.63	13.70%	36.55%
3	16.14	10.21%	46.77%
4	12.44	7.88%	54.64%
5	7.60	4.81%	59.45%

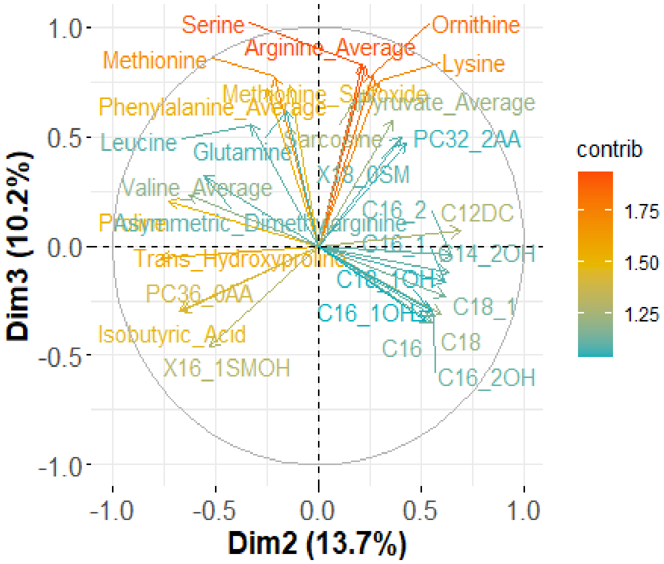

***Figure 3.2**. Principal Components Analysis biplot of influential metabolites from the whole blood metabolome of Lake Winnipeg walleye in relation to chronological age of individual fish. Influence of metabolites on estimation of chronological age of each individual and its place in comparison to Dimensions 2 and 3 on the PCA analysis. n = 54.*

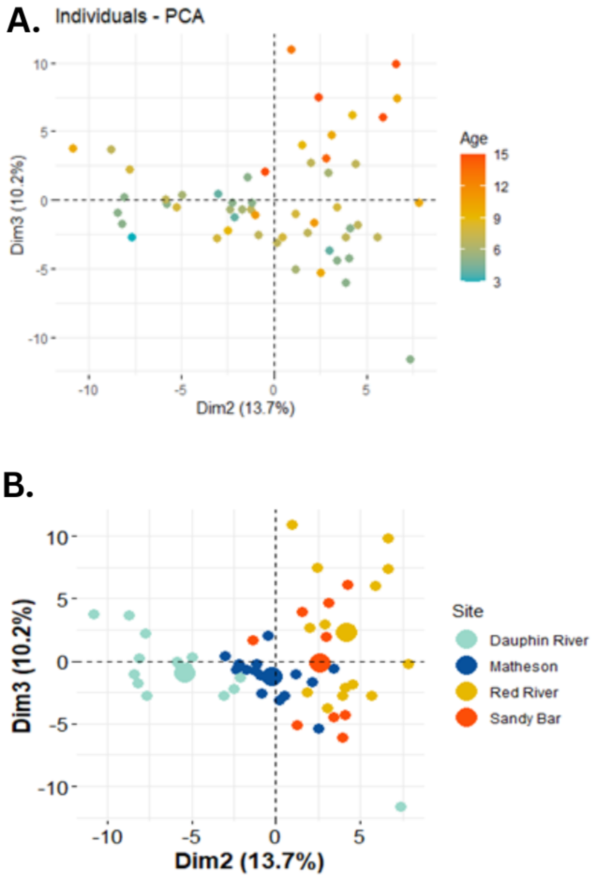

Figure. 3.3. *PCA biplot showing the individual walleye sampled in 2024 for age and metabolites, n=54. Data show all individual walleye in comparison to their age (A) or site of capture (B). Fig 3.3B. shows individual fish as small dots and the mean of the eigenvectors for all individuals from each site as a large symbol plotted against dimensions 2 and 3 of the PCA.*

3.4.3 Linear Mixed Models

The metabolites that correlated substantially (correlation of 60% above in the PCA) with the second and third PCA dimensions were further analyzed by mixed models to test for age-related patterns for individual metabolites. The selected metabolites could be broadly sorted into either amino acids and their metabolic derivatives (Table 3.2) or acylcarnitine esters along with a single phosphatidylcholine and isobutyric acid (Table 3.3). While most metabolites showed differences with site, which was anticipated (see Chapter 2), many also showed age-dependent differences beyond the effect of site (Tables 3.2 and 3.3). All amino acids that varied with age increased with chronological age, above and beyond the effect of site (Table 3.2). Likewise, the acylcarnitines C6:1 and C12DC and the phosphatidylcholine PC36:0AA decreased with age while the acylcarnitine C18:2 decreased with age (Table 3.3).

Table 3.3*. Linear mixed model results of the analyses by metabolite with 60% correlation with dimensions 2 and 3 of the PCA containing amino acids and related molecules. The table shows the intercept (effect of capture site) and the fixed effect of age within the models. N= 54 per metabolite. CI, confidence interval. R2, conditional R square value of the model.*

	Intercept[1]				**Fixed Effects for Age[1]**				
Metabolite	R2	Estimate	p-value	CI	Estimate	P-value	CI	Model Type	Transformation
Trans-hydroxyproline	0.72	2.47	0.003	0.88, 4.06	-0.11	0.017	-0.20, -0.02	a	ln
Proline	0.51	3.67	< .001	2.91, 4.44	0.03	0.262	-0.03, 0.09	a	ln
Arginine	0.23	3.19	< .001	2.77, 3.62	0.1	0.001	0.05, 0.15	a	ln
Glutamine	0.30	2.26	=.004	0.78, 3.75	0.22	0.005	0.05, 0.29	a	√2
Glycine	0.31	5.62	< .001	5.33, 5.91	0.06	< .001	0.03, 0.09	a	ln
Lysine	0.20	1.20	0.016	0.23, 2.17	0.18	0.002	0.12, 0.34	a	ln
Ornithine	0.28	0.44	0.317	-0.84, 1.59	0.21	< .001	0.12, 0.37	a	√2
Phenylalanine	0.54	3.52	< .001	3.14, 3.91	0.07	< .001	0.11, 0.27	a	ln
Methionine	0.34	3.35	< .001	2.16, 4.55	0.23	< .001	0.11, 0.36	a	√2
Methionine sulfoxide	0.25	1.97	0.001	1.47, 2.48	0.11	0.001	0.05, 0.17	a	√2
Sarcosine	0.53	2.87	< .001	1.92, 3.82	0.02	0.82	-0.13, 0.16	b	ln
Serine	0.36	3.89	< .001	3.60, 4.17	0.09	< .001	0.05, 0.12	a	ln
Valine	0.61	4.48	< .001	3.87, 5.08	0.07	0.002	0.02, 0.11	a	ln

[1] The alpha value for significance is 0.05 and the confidence interval is 95%
Bolded numbers highlight significance on the fixed effect of chronological age
[a]Metabolite ~ Age + Site, which included Site as a random effect only: ~1 | excluding interaction across sites of capture; site as random effect;
[b]Metabolite ~ Age * Site, Site as a random effect: ~1 | including interaction across sites;

Table 3.4. *Linear mixed model results of the analyses by metabolite with 60% correlation with dimensions 2 and 3 of the PCA containing lipid molecules. The table shows the intercept and the fixed effect of age within the models. N= 54 per metabolite CI, confidence interval. R2, the conditional R-squared value of the model.*

	Intercept				Fixed Effects for Age1				
Metabolite	R2	Estimate	p-value	CI	Estimate	P-value	CI	Model Type	Transformation
C10:2	0.16	4.05	< .001*	3.60, 4.51	0.02	0.240	-0.01, 0.06	b	Ln
C6:1	0.22	0.24	< .001*	0.21,0.27	-.00597	0.001*	-0.68, -0.18	a	None
C16:2	0.18	2.4	< .001*	2.08, 2.72	0.04	0.048*	0.00031, 0.07	a	ln (x 1000)
C12DC	0.17	-3.52	< .001*	-4.11, -2.94	-0.11	0.014*	-0.20, -0.02	a	Ln
C18:1	0.29	4.81	< .001*	4.12, 5.49	0.02	0.506	-0.04, 0.09	a	ln (x 1000)
C14:2OH	0.22	2.8	< .001*	2.34, 3.26	0.04	0.147	-0.01, 0.08	a	ln (x 1000)
C16:1	0.28	-2.33	< .001*	-2.95, -1.72	0.03	0.334	-0.03, 0.09	a	Ln
C5	0.61	-2.02	< .001	-2.90, -1.15	-0.15	0.495	-0.04, 0.08	b	Ln
PC36:0AA	0.57	4.92	< .001*	4.45, 5.40	-0.08	< .001*	-0.12, -0.04	a	Ln
Isobutyric Acid	0.73	1.07	< .001*	0.57, 1.57	-0.02	0.18	-0.05, .009	a	Ln

1 The alpha value for significance is 0.05 and the confidence interval is 95%
Bolded numbers highlight significance on the fixed effect of chronological age
aMetabolite ~ Age + Site, which included Site as a random effect only: ~1 | excluding interaction across sites of capture; site as random effect;
bMetabolite ~ Age * Site, Site as a random effect: ~1 | including interaction across sites

3.4.4 Relative Telomere Length

Having found age-dependent patterns for several blood metabolites, we investigated whether relative telomere lengths in the nucleated red blood cells may also show age-dependent patterns in walleye from Lake Winnipeg. Using the 2018 fish, the relationship between age and relative telomere length shows no significant correlation (Fig. 3.4). While we did not have age data for fish from 2017, we found a strong relationship between fork-length and age by site, across our samples from 2018 (Multiple R2= 0.71, F= 78.41 on 4 and 126 DF, p-value: < 0.001), meaning we could use fork-length as an approximation of chronological age in our fish, allowing for a larger dataset for walleye caught in 2017 and 2018. Relative telomere length comparison with fork-length did not show any significant correlation within any test for both years 2017

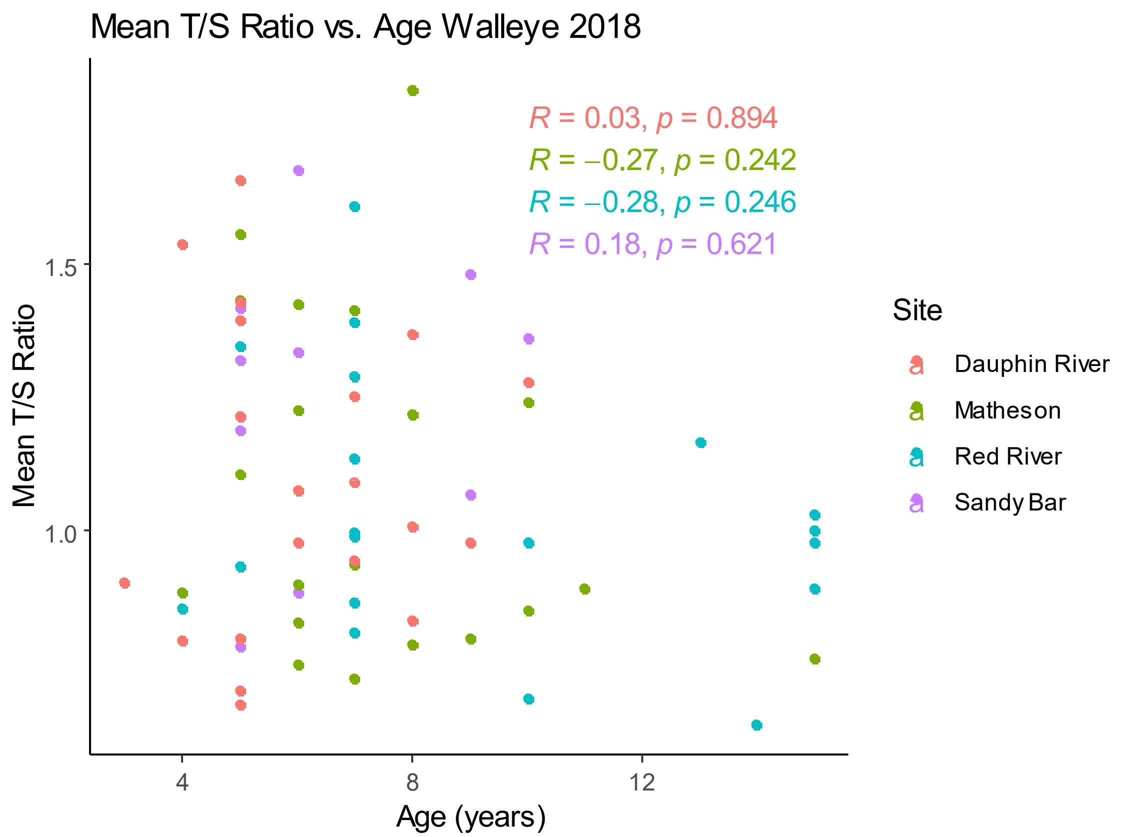

Figure 3.4. *Relative telomere length (T/S ratio) scatterplots for red blood cells from Lake Winnipeg walleye caught in 2018. Pearson correlation analyses found no significant relationship across or within sites (p > 0.05), as well as interaction linear models did not find any significance across age and sites as factors for chronological age on the walleye. Furthermore, a simpler model comparing telomere length versus age, show no significant age effects on telomere length.*

and 2018 on the walleye blood (Fig 3.4A and 3.4B). The relative telomere length also showed no significant differences (ANOVA, p>0.05 for all comparisons) by site for both years sampled (Fig. 3.5). Relative telomere length shows no significant correlation (Fig. 3.6)

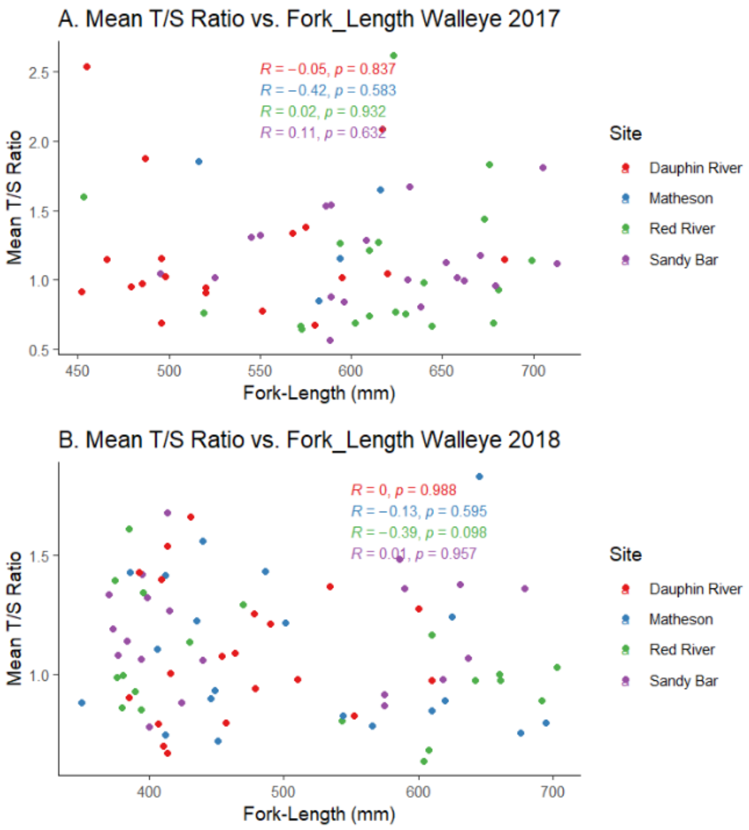

Figure 3.5. *Relative telomere length (T/S ratio) for walleye red blood cells for 2017 and 2018 Lake Winnipeg walleye. Fish were sampled in 2017 (A) or 2018 (B) with n=62 and 80, respectively. Analyses show no significance with age and the telomere length (p > 0.05). Furthermore, a simpler model comparing telomere length versus fork-length show no significant length effects on telomere length.*

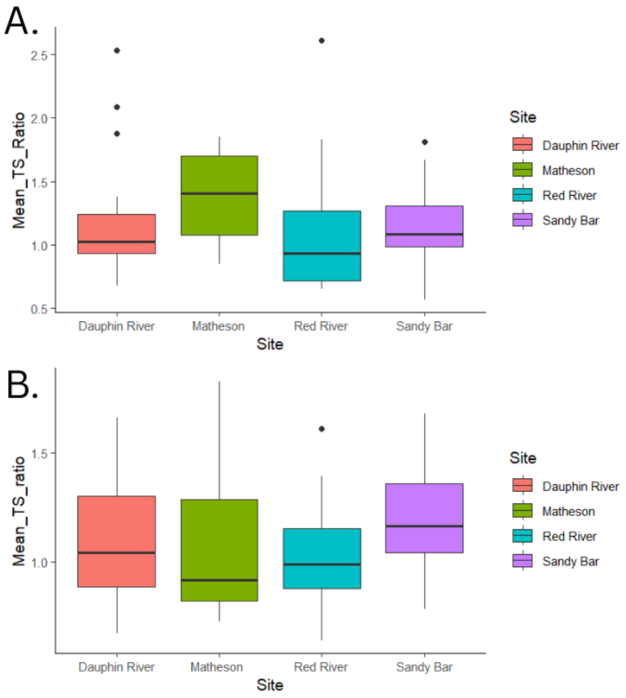

Figure 3.6. *Relative telomere length (T/S ratio) boxplots for red blood cells from walleye from regions of Lake Winnipeg. Data are from 2017 (A) and 2018 (B). None of the sites show significant differences in the mean in the ANOVA analysis. For (A), Red River n= 20, Sandy Bar n= 20, Matheson n= 4, Dauphin River n= 19. For (B.) Red River n=20, Sandy Bar n=20, Matheson n=20, Dauphin River n=20).*

3.5 Discussion

This chapter examined the metabolite profiles of Lake Winnipeg walleye in relation to chronological age. The study employed comparative approaches to identify age-related patterns in walleye blood across the four sites sampled in 2018 in Lake Winnipeg. The PCA analysis showed that age was better explained by the second and third dimensions. The influential metabolites found at higher concentrations with age in the whole blood of walleye from Winnipeg Lake included amino acids, acyl-carnitines, some lysophospholipids, and phosphocholines. The linear models performed on the influential metabolites versus age and site revealed age-dependent differences in metabolites, specifically amino acids, which increased with age. Acylcarnitines C6:1 and C12DC, with phosphatidylcholine PC36:0AA, decreased with age, but the pattern was not as striking as the amino acid tendencies seen in the walleye blood. Furthermore, no significant differences or patterns were found in the relative telomere length by site over the two years sampled for this assay.

Finch (Finch, 1990) proposed a broadly applicable generalization of senescence to encompass any age-dependent change in function across all levels of biological organization from molecules to whole organism traits. Finch also emphasized the importance of life history and how the potential for indeterminant growth may lead to very different consequences on biological aging, leading to the plausibility of negligible senescence (Finch, 1990, 2009), evidence for which has been greatly influenced by study of wild fishes. The current study's goal was to test this concept in a wild animal by measuring the whole blood metabolome of walleye from a range of ages. While a previous study argued a congeneric species displays gradual senescence in the wild (Trifonova et al. 2018), our focal species was considered appropriate as it is an upper trophic level predator (Sheppard et al., 2018; Steward & Watkinson, 2007; Turner, 2020) whose access to large energy-dense prey increases with body size, and thus increasing gap size to access larger prey (Costa, 2009). Together these traits lead to the capacity for indeterminate growth and greater potential fecundity with increasing size, and thus age (Porch et al., 2007; Sebens, 1987), for Lake Winnipeg walleye, all of which may favour negligible senescence. Unlike the pattern with age in humans and other more intensively studied mammals where many classes of metabolites may change (Angelini et al., 2017; Hulbert, 2005; Pamplona, 2008; Pelantová et al., 2023), we found in walleye that the majority of the age-related changes were limited to amino acids and other metabolites linked to protein metabolism (Table 3.3). Additionally, while lipids and their metabolites tend to increase with age in mammals (Johnson & Stolzing, 2019) in walleye most acylcarnitines, declined with increasing age, as did the phosphatidylcholine metabolite (PC36:0AA) and isobutyric acid (Table 3.4). The single lipid exception to age-related declines in metabolite concentration being the long-chained acylcarnitine C16:2, a polyunsaturate.

Importantly, while we had to account for region differences in metabolite levels in the Lake Winnipeg walleye whole blood metabolome (see Chapter 2), there when no significant differences in the size of walleye across the sites in this study, but the Northern most site (Dauphin River) did have fish of a younger age (Table 3.1) on average than the Southern mostly fish (Red River). If site effects were confounding our analysis, the Dauphin River fish being both typically higher in amino acid concentrations and being the youngest group tested should have led to a pattern of decreasing levels of amino acids with age; however, the patterns seen with chronological age are the inverse of those seen across sites of capture (Chapter 2) with the singular exception of trans-hydroxyproline (Table 3.3). For example, while arginine, lysine, methionine, phenylalanine and valine are all higher in the Dauphin River walleye, the more Northern site, the mixed linear models show no significant interaction between site and age and thus can demonstrate these essential amino acids all significantly increase with age (Table 3.3). This pattern was also seen for most acylcarnitine metabolites which increase with age (Table 3.4). Most of the specific short and medium chain acylcarnitines that change with age were not found to change across sites when measured over 2 years (Chapter 2). The singular exception, C16:2, both decreased with age and was highest in the Red River fish (Chapter 2), which are also the oldest. Taken as a whole, the majority of our results indicate the patterns seen with age are not biased or compromised by the changes in metabolites with sites of capture as patterns between site and age overwhelmingly occur in opposing direction.

Senescence brings a complex change in catabolism, anabolic processes and turnover of proteins, making amino acids, which are the building blocks of protein, potentially informative of these changes. Changes in circulating essential amino acids provide particularly valuable insight because their appearance is limited to exogenous (dietary) and endogenous protein degradation; therefore, differences in blood concentrations indicate a shift in the balance between supply and removal from the circulation by protein synthesis and other biosynthetic pathways or oxidation of the amino acid for energy metabolism. In most animal models so far studied, there is a tendency for essential and branched-chained amino acids to increase in serum and plasma with age, and this was also seen in walleye whole blood. Increasing amino acid concentration in aging models may be correlated to changes in muscle breakdown and function that come with senescence, as with increasing age a, loss of muscle mass is a common occurrence in humans (Yamada et al., 2018), non-human primates (McKiernan et al., 2011), and mice (Crombie et al., 2023; Huang et al., 2025). For example, phenylalanine was found to increase in blood of walleye and humans with age. In humans, increased phenylalanine is correlated with adiposity, cardiovascular disease, and higher insulin resistance in humans (Würtz et al., 2015), a shorter telomere length in human men (Eriksson et al., 2017). Valine also increased with age in walleye and increases in branched-chained amino acids are known in humans with age (Muilwijk et al., 2018).

In relation to protein turnover in the walleye, trans-hydroxyproline decreased with age in the blood of walleye (Table 3.3). Trans-hydroxyproline is predominantly found in collagen, thus the turnover of endogenous collagen or consumption of collagen containing food (like forage fish) should account for the vast majority of trans-hydroxyproline (Segrest & Cunningham, 1970), If protein turnover decreases with age or the walleye consume less food on a mass-specific basis as they grow larger, then trans-hydroxyproline would be expected to decline with age. Given the decline in growth rate observed as indeterminately growing fish approach their asymptotic size, it would be expected to see a pattern of dietary energy being allocated to somatic maintenance and reproductive output, and declining investment in growth with increasing age of the walleye. Taken together these patterns, suggests the elevated essential amino acids may reflect higher use and turnover of amino acids relative to protein intake, as the walleye age and this may coincide with lower mass-specific feeding rates as walleye age based on the trans-hydroxyproline pattern.

Arginine, also an essential amino acid in teleost fishes (Hoseini et al., 2020), increased with age in this study. Breakdown of arginine, specifically by the enzyme L-arginase, has been correlated with renal disease, arteriosclerosis and other diseases in humans (Jarrell et al., 2020). The increase of arginine with aging may imply changes in protein synthesis pathways, as well as stress control (Jacobs, 2011). Ornithine also increases with age in the Lake Winnipeg walleye. Ornithine is produced from arginine by L-arginase and then cleared via transamination or polyamine synthesis which uses decarboxylated S-adenosyl methionine in the process (Kern et al., 1999). S-adenosyl methionine is formed from methionine, which is another essential amino acid in teleost fishes (Wilson, 2003) that increased in concentration in blood with age in walleye. Methionine is involved in polyamine synthesis, acts as the primary methyl-donor through the methylation pathway and S-adenosyl methionine formation, and is cleared predominantly via trans-sulphuration which forms cysteine from homocysteine (Froese et al., 2019). As a sulfur containing amino acid, methionine is also involved in antioxidant pathways in the cells (Cabreiro et al., 2006). Excess methionine produces cysteine needed for antioxidants and protein synthesis (Bin et al., 2017). Methionine sulfoxide is an auto-oxidation product of methionine and high levels are associated with oxidative stress; however, in walleye blood the methionine sulfoxide is highly correlated with methionine levels (r2 = 0.73), suggesting the methionine sulfoxide increase with age simply reflects the higher levels of its precursor, methionine although we cannot discount the possibility of declining methionine sulfoxide reductase activity in walleye as they age.

3.5.1 Amino Acids Associated with 1-Carbon Metabolism

Taking into consideration increases in amino acid concentrations with age in the walleye, methionine, its auto-oxidative product methionine sulfoxide, along with serine and glycine are all notable as being able to generate metabolically available 1-carbon units. Interestingly, sarcosine, which is part of the glycine-N-methyltransferase pathway for disposing of excess 1-C units, did not change with age. These specific molecules may be a convergence sign and may represent the presence of an accumulation of metabolites that contribute to the 1-carbon pool with increasing age (Ducker & Rabinowitz, 2017). Metabolically accessible 1-carbon units are important in nucleotide synthesis, oxidative balance and methylation in the cell (Dang et al., 2024). Furthermore, glycine and serine contribute 1-carbon units via the folate cycle which is also important to biosynthesis and the methionine remethylation salvage pathway (Kory et al., 2018). A unifying idea that may explain the possible changes or differences with chronological age in walleye is the carbon-1 metabolism, as its pathways are involved in epigenetic modifications (Dang et al., 2024), which in turn will have an effect and influence metabolic processes like lipid metabolism.

3.5.2 Acyl carnitines and Phosphocholines

The lipid-containing acylcarnitines with medium and short chain fatty acyl tail tend to decrease in the blood with age, these acylcarnitine were C12DC, C5 and C6:1. The increase of C16:2 parallels the literature results for human blood metabolomics with aging (Giesbertz et al., 2023; Jarrell et al., 2020). The molecules C14:2, C12DC, and C5, and other acyl carnitines have all been known to correlate with age on humans (Jarrel et al. 2020). Furthermore, the long-chained acyl carnitines do not show a concrete senescence pattern. The phosphatidylcholine

PC36:0AA concentration trends downward versus age in the walleye blood, which parallels the pattern found for phosphocholines and other lipid-containing molecules that increase in humans and rodents with age (Chang et al., 2022). We found no similar relationship with age in walleye on the lipid molecules.

3.5.3 Relative Telomere Length is not a Marker of Age in Lake Winnipeg Walleye

Our results show no apparent changes with age and no differences across sites within Lake Winnipeg walleye for the relative telomere length value in red blood cells. The lack of pattern with age is of interest as it is speculated elsewhere that some fish may present negligible senescence (Finch 1990, 2009; Trifonova et al. 2018). Although most literature show shortening of telomeres with age in fish, it has also been shown that telomere shortening may better represent stress state conditions in fish (Espigares et al., 2021). Unlike most mammals and birds studied to date, fish express the telomerase enzyme complex in adult tissues (Klapper et al. 1998; Lund et al. 2009; Ocalewicz 2013; Pfeiffer and Lingner 2013), which may explain the lack of correlation between telomere length and age in the current study. A previous study also found that in lake trout, *Salvellinus namaycush*, a long-lived fish, telomeres do not show correlation with age in red blood cells or in sperm cells (Purchase et al., 2022). The accepted proxy for most studies currently is that telomere shortening may explain at least to some extent the causal reason for cells to start to showing signs of senescence making telomere shortening one of the more accepted markers of aging (Rossiello et al., 2022). Our study suggests that telomere shortening maybe a be a poor predictor of aging or senescence in wild fish, due to the probable presence of telomerase activity observed in other studies (Klapper et al. 1998; Ocalewicz 2013).

3.5.4 Summary

The concentration of specific amino acids in blood of humans increase with age (Muilwijk et al., 2018). Our results do parallel the literature in the matter of senescence compared to many mammalian models, with amino acid concentration in the blood increasing with age (Canfield & Bradshaw, 2019; Pitkänen et al., 2003), which may imply senescence or at least age-dependent changes in metabolism in our model (Nakano et al., 2013). Other metabolites which have high correlation with aging in the PCA analysis and show a decrease in concentration within the blood as the walleye grow older are: the short and medium chained acyl carnitines (C5, C6:1, C12DC), and the phosphocholine (36.0). This is contrary to work which found correlation between increase of phospholipid and other lipid-containing molecules in the blood with increasing age in humans (Collino et al., 2013; Montoliu et al., 2014).

Finally, our study emphasizes that caution is warranted when comparing metabolic and molecular markers of aging across phylogenetically diverse taxa. The process of aging in fish, especially at the metabolic and molecular level, is only starting to be deciphered and more studies like our work need to take place to fully understand fish senescence.

3.6 References

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4. Chapter 4: Lake Trout, *Salvellinus namaycush,* of Algonquin Lakes Comparison of Relative Telomere Length and Lipofuscin Accumulation of Algonquin Lakes: Senescence in the Wild or the Lack of It

4.1 Contribution of Authors

Lilian Maria Wiens, conceptualization, statistical analysis, writing and editing; M Yusishena, assistance with histological preparations; M Guzzo and T Durhack, animal and sample collection; J.R. Treberg, conceptualization, editing and funding.

4.2 Introduction

The process of aging and related underlying mechanisms have been debated for decades, with hypotheses ranging from the free radical theory (Harman, 1956) and subsequent mitochondrial free radical theory (Harman, 1972), the mutation genome theory (Szilard, 1958) and antagonistic pleiotropy, as first proposed by Medawar (1952) and expanded on by Williams (1957), all still having great influence on the field. As a field, the biology of aging has had remarkable advancements recently because the field is going beyond the typical model organisms and incorporating more comparative approaches (Rechsteiner et al., 2025), as well as resurgent interest in ectotherms, where the canonical patterns of typical laboratory-based model organisms are not always recapitulated (Reinke et al., 2022). Over the last 70+ years of research on aging in animals, fishes have periodically played prominent roles; for example, Gerkinès's work in a state-of-the-art synthesis of research on aging from the 1950's (Gerkin, 1959), Craig's review in the 1980s (Craig, 1987) and in the 1990s certain long-lived fishes were paramount to Finch's arguments in support of negligible, if not negative, senescence occurring in some species (Finch, 1990). More recently, fishes have become notable model organisms for the study of aging the laboratory settings, with the zebrafish and several short-lived killifishes showing notable parallels with rodent models of aging (Bergmans et al., 2023; Van houcke et al., 2015), including some hallmarks of aging like telomere erosion, (Ocalewicz, 2013); however, long-lived fishes have emerged as a group that may not consistently conform to these hallmarks of aging and senescence (Costantini et al., 2017; Nielsen et al., 2016; Reinke et al., 2022). Despite the impact long-lived fishes in the wild had on the field decades ago (Finch, 1990), the reasons that short-lived and long-lived fishes show highly disparate patterns of aging are unclear and are at best still speculative based on differences in life history patterns (determinant or indeterminant growth, single or repeated spawning). In many cases, understanding of aging in long-lived fishes relies on sampling from the wild, (reviewed in Finch, 1990, 2009; Gerking, 1957, 1959; Woodhead, 1980; 1998), although recent findings have begun to re-establish wild fishes as important tests of hypotheses associating particular cellular responses with long life-spans. For example, no patterns of age-related oxidative stress markers were found in the Greenland shark (Costantini et al., 2017), which is arguably one of the longest living vertebrates (Nielsen et al, 2016). A long-lived teleost, the bigmouth buffalo was found to show no decrease in relative telomere length over a range of ages from 2-99 years old, while also showing patterns of possible improved immune capacity with increasing age (Sauer et al., 2021). Additionally, while some salmonids are known for senescence associated with their semelparous life history, the lake trout (*Salvelinus namaycush*) is a long-lived iteroparous member of the family (Kennedy, 1954) that has also been used to test for aging-related patterns in the wild (Purchase et al., 2022). Before addressing the knowledge gaps this research will address and the study design, it is important to briefly introduce growth and biological aspects of the study species, as well as review the use of cellular markers for comparative studies of aging.

4.2.1 Study species: the Lake Trout

Lake trout is the largest non-anadromous Salmonid in North America, found in temperate to Arctic freshwater systems that are relatively cool or year-round cold (Steward & Watkinson, 2007), and is an important species in northern lakes due to their ecological influence as an apex aquatic predator and as a targeted fisheries species (Johnson et al., 2012). Lake trout is a long-lived fish, having a potential lifespan of up to 50 years (Schram & Fabrizio, 1998), and is also a slow-growing species (Froese & Pauly, 2018), making lake trout susceptible to overfishing (Steward & Watkinson, 2007). Growth in wild lake trout can vary, depending on the forage available, and may display indeterminate or determinant patterns, meaning they may increase in size substantially after maturing or largely cease growing post maturity respectively. For instance, when large forage fishes are available, there is indeterminate growth while when only invertebrates are available as prey for adult lake trout the growth potential is limited and fish show a more determinant type pattern of growth (Pazzia et al., 2002). Similarly, very small and oligotrophic lakes may impose a determinant-like growth pattern on lake trout as well, even when they have forage fish, but the ecosystem shows marked nutrient limitation on productivity (Purchase et al., 2022). Determinant growth in lake trout appears to be imposed on populations by nutrient limitation because indeterminant growth may come with the implication of increasing reproductive fitness with size or age as fish reach a larger size, as larger fish may have greater fecundity, and the potential predators become fewer as the trout get larger (Koenigbauer & Höök, 2023; Uusi-Heikkilä, 2020).

4.2.2 Comparative Biology of Aging and Senescence

In their systematic review Finch highlighted some time ago that the process of biological aging, and cellular senescence, has not been investigated sufficiently in long-lived ectotherms, and especially in long-lived fishes (Finch 1990). As of now, some studies examine the process of aging in the wild and in non-model organisms (Oudes et al., 1998); however, this topic is still far behind what we know for mammalian and common invertebrate laboratory models like nematodes and fruit flies. Knowledge of aging across animals in a comparative context can be important to understanding how the processes of aging are shaped by natural selection. Furthermore, cellular senescence is also an important topic in human health research (Newman et al., 2011), and a comparative view of the aging process can give a better understanding of factors that are unique to human studies of age-related declines in physiology. Therefore, studying long-lived fish aging phenotypes may help to understand the senescence process better, specifically, understanding aging across long-lived organisms, including humans. We also know there is high diversity in the animal Kingdom in how senescence influences organismal function, or perhaps not in apparently immortal species (Petralia et al., 2014), which stresses the necessity of further study of non-model organisms to broaden the understanding of the underlying mechanisms that influence senescence.

In commonly used laboratory model organisms, the process of senescence at the cellular level has been extensively studied, leading to some characteristic markers of aging. A non-extensive list of cellular senescence, and thus evidence of cellular aging, includes lipofuscin accumulation, (Munnell & Getty, 1968), cell-type-specific arrest of the cell cycle (Childs et al., 2023), increased beta-galactosidase expression in aged tissues (Choi et al., 2000), and telomere shortening (Anchelin et al., 2011).

Comparisons of cellular characteristics or markers of aging in fish are less comprehensive but are necessary to clarify if there are different dynamics of senescence across the main taxa of vertebrates. In zebrafish, Danio rerio, telomere shortening occurs; therefore, this marker displays a similar pattern to that found in humans and mice (Henriques et al., 2013). Unlike most mammals, zebrafish express telomerase activity as adults, although this expression declines along with the length of telomeres with aging in this species (Anchelin et al., 2013).

However, while many examples of telomere shortening with increasing age in vertebrate species can be found, notable exceptions like zebra finches, *Taenopygia guttata*, show no relationship with age on any tissue sampled, which included red blood cells, spleen, bone marrow, muscle, heart, liver, and brain (Reichert et al., 2013). Telomere dynamics appeared to vary across tissues, with shorter telomeres in tissues that show high turnover, over as the liver and spleen, versus other tissues that do not show the same rate of cellular division (Reichert et al., 2013).

4.2.3 Rationale for the Current Study

Different animal taxa may express different traits that are related to senescence (Finch 1990). Differences in senescence traits may correlate with life history across taxa. One example is the indeterminate growth of some animal species, where many ectotherm vertebrates have continued growth post-maturity, like some fish and reptiles (Blanco & Sherman, 2005). Indeterminately growing animals may show no apparent decrease in reproductive capacity as they age (Finch 1990). The combination of indeterminant growth, with no age-dependent decline in reproductive output, raises the possibility that such animals could show negligible senescence (Finch, 1998).

However, there is diversity in growth patterns due to environmental conditions, such as food availability, and lake trout present a valuable model to test how growth potential interacts with biological aging and cellular senescence. A study of lake trout in Great Slave Lake, which has a substantial range of sizes in the forage fish resources for lake trout to exploit as prey, shows an age-dependent increase in mortality in lake trout (Kennedy, 1954). Conversely, in small lakes in Western Ontario where lake trout growth is limited by low food-web complexity, leading to only small forage fishes as the most energy dense prey, there was little to no change in mortality with increasing age (Purchase et al., 2022). In the latter study, there was also no evidence for cellular senescence based on relative telomere length or sperm swimming performance between young and old lake trout, raising the possibility that food restriction at the ecosystem level may have relevance to the aging process.

Competition is an intraspecific and interspecific interaction that may confer detrimental effects in populations; for example, smallmouth bass (*Micropterus dolomieu*) has been shown to affect prey consumption by lake trout. (Morbey et al., 2007). The introduction of smallmouth bass in Lake Opeongo of Algonquin Park decreased the quantity of stomach contents in lake trout, which implies competition for prey between these two predators (Morbey et al., 2007). Therefore, the presence of competing predators may influence feeding success and add additional nutritional stress on a population of lake trout.

The current study re-examines aging in wild lake trout to test if the lack of aging phenotype (Purchase et al., 2022) may be related to the nutritional limitation of the specific ecosystems they were sampled from. To do so, two common aging markers that have been used to show a progressive aging phenotype in humans and other mammal models: lipofuscin accumulation (Di Guardo, 2015) and telomere shortening (Gopalakrishnan et al., 2013; Ocalewicz, 2013; Olsson et al., 2018; Shammas, 2011) were measured in tissues from wild lake trout populations. Four separated populations of lake trout in the Algonquin Parks area of Ontario, Canada, were sample to test for signs of aging at the cellular level. This study allows for evaluating if life history dynamics coincide with differences in senescence phenotypes in lake trout by comparing fish from large bodies of water with indeterminate growth potential

(Lake Hogan and Lake Openogo) and smaller lakes with more determinant growth patterns (Lake Shirley and Lake of Two Rivers, LOTR) (Table 4.1). The study design also allows to compare if the presence of smallmouth bass as competitors influence patterns associated with aging.

4.3 Methods

4.3.1 Sampling and Biological Data:

Lake trout were caught (depths ranging between 6–20 m) with a mixture of short-duration sets (<20 min) of gill nets and angling with barbless hooks. Fish were held at the Harkness Laboratory for Fisheries Research as described elsewhere (Guzzo et al, 2024). Briefly, fish were held, without food, in tanks at ∼9 °C for ∼48 h and underwent a respirometry protocol before euthanasia. Fish were euthanized by an overdose of buffered tricaine methanesulfonate (60 mg L−1). Blood was collected into heparinized syringes with subsequent severing of the spinal cord, followed by tissue collection. All biological measurements (lengths, body and specific organ masses and sex) along with chronological age were determined post-mortem. Age was determined by otolith structure and is the same as provided elsewhere (Guzzo et al., 2024). Red blood cells were collected by centrifuging at 5000 x g for 5 minutes at room temperature, followed by the removal of the plasma and 'buffy' coat (interphase layer) containing the white blood cells, with the remaining red blood cells (RBCs) being collected for relative telomere measurement. Samples of liver and heart, along with RBCs, were flash frozen with liquid nitrogen and stored at approximately -70°C (or colder) until processing. Tissues that were used for histological lipofuscin measurements were introduced into a 10% neutral buffered formalin liquid solution immediately post-dissection and stored at 4°C until processed for histology.

Table 4.1. *Lake characteristics of the four lake trout populations sampled for this study*

	Lake			
Aspect	Hogan	LOTR*	Opeongo	Shirley
Smallmouth Bass	Absence	Presence	Presence	Absence
Cisco	Presence	Absence	Presence	Absence
Area (Ha)	1303	292	5154	481
Mean Depth (m)	7.4	15.4	6.7	14.6

* Lake of Two Rivers. Note, data are from Guzzo et al., 2024.

4.3.2 Histological Measurement of Lipofuscin

Processing: The liver and heart tissues were washed with PBS solution and passed through a series of increasing concentrations of ethanol for clearing, with the following order (all ethanol concentrations are v/v): 80% (45 min), 90% (45min), 100% (60 min) three times. This was followed by two steps with a clearing solution (Slide Brite™ from NewComers Supply), one of 30 min and a second of 60 min. The tissues were then embedded in paraffin at 60°C in two steps: one of 30 minutes and a second of 2 hours. Tissues were immersed in melted paraffin and then placed on embedded rings, previously sprayed with a releasing agent and released from the metal rings after a cold bath. Embedded tissues were sectioned with a BIOBASE BK rotary microtome. Sectioning of tissue was to 5-7 µm of thickness. Sections were placed on a 40 °C bath and mounted on glass slides, then allowed to dry until staining protocols were performed.

Lipofuscin Staining with Sudan Black B: Staining of prepared slides was performed following instructions and modifications from the supplier, Newcomer Supply Laboratory©, based on (Carson & Hladik, 2009; Luna, 1992; Sheehan & Hrapchak, 1980; Survana et al., 2019). Tissues were stained with Sudan Black B, Fisher BioReagents™, to specifically target and quantify lipofuscin, known to accumulate with age in model organisms (Jacobson et al., 2010; Oudes et al., 1998). Slides of liver or heart tissues were dehydrated in a wash of 100% propylene glycol before being stained. The slides were immersed in the Sudan Black B stain, according to the manufacturer’s instructions, for one hour, agitating occasionally, followed by placing the slide into 85% propylene glycol (v/v with water) for 3 min. The slides were then washed in distilled water and counterstained using Nuclear Fast Red Stain N3020™ (Sigma Aldrich). For counterstaining, the slides were placed in the Nuclear Fast Red Stain for 5 min, washed gently with tap water, blotted for excess water and sealed under a coverslip mounted with Quick Aqueous Mounting Medium™ before optical density measurements with ImageJ. (Ferreira & Rasband, 2003).

Images were taken with a LEICA ICC50W compound camera microscope with the following settings: 100x total magnification, brightness 50%, gamma 60% and saturation 65%. To determine the intensity of the yellow-brown lipofuscin stain (Davan-Wetton & Montero-Melendez, 2024), the software ImageJ was used to quantify the optical density of the image, which was then converted to 8-bit. Adjusting and calibrating for the yellow hue of lipofuscin was done by setting the threshold range of 99-151.36 on the distribution curve, which isolates the yellow stain from the rest of the image. After focusing on the specific area of interest and excluding background noise, parameters were set by: Set Measurements, Measure, Analyze, to mark specific measurements taken and quantify the area’s intensity of staining (Ferreira & Rasband, 2012; Mezei et al., 2011). The value of the mean grey intensity was collected and converted to optical density with the formula: log10(255/255-x), where x=mean grey intensity calculated from the picture (Bassingthwaighte et al., 1964; Myers et al., 2013).

4.3.3 Relative Telomere Length by Quantitative PCR

Quantitative PCR was performed using the method of Cawthon (2002) to determine the relative telomere length of the RBC, liver and heart. The DNA isolation was performed using the QIAGEN® Blood and Tissue Kit, following these steps: For RBCs, 20 µl of proteinase K is added to a microcentrifuge tube, 7µl of RBCs are added, and the volume is finally adjusted to 220 µl with PBS buffer, pH 7.2 (50 mM potassium phosphate, 150 mM NaCl). This mixture was incubated at 56°C for 10 min. For the heart and liver tissue, a small amount of frozen tissue was cut and pulverized while being kept cold with liquid nitrogen. Frozen tissue was added to a microcentrifuge tube and topped with 180 µl of Buffer ATL (tissue lyser), sodium dodecyl sulphate at 2.5-10% plus, non-hazardous proprietary ingredients at balance. The tube was left to incubate for 1.5 hr at room temperature, with periodic vortex mixing during the process After the incubation process, a final vortex of 15s at maximum speed was performed, followed by addition of 200 µl of buffer AL (lysis solution, guanidine hydrochloride 30-50%, non-hazardous proprietary ingredients at balance) and vortex mixed followed by addition of 200 µl of 100% ethanol and vortex mixed again. This mixture was then transferred to a DNeasy Mini® spin column placed on a 1.5ml tube, and 200 µl of Buffer AW1 (wash buffer with guanidine hydrochloride 30-50%, non-hazardous proprietary ingredients at balance). This mixture was centrifuged at 8000 x g for 75 s at room temperature, and the eluent was discarded. The column is placed in another 1.5ml microtube, 200 µl Buffer AW2 (ethanol wash buffer TRIS-based) is added, the mixture is centrifuged at 8000 x g for 3 min, twice with eluent again being discarded both times. The column was transferred to a new 1.5ml microcentrifuge tube, and the DNA was eluted by the addition of 200 µl of Buffer AE, left to incubate at room temperature for 1 min, then centrifuged at 8000 x g for 3 min twice. DNA elution was collected, pooled and then quantified with a Qubit DNA HS assay kit and Qubit 2.0 Fluorometer (Thermo Fisher Scientific) and subsequently diluted to 10 ng/µl.

The qPCR-based approach (developed by Cawthon, 2002), which produces a telomere repeat (T) to single gene (S) copy number ratio (T/S) for each DNA sample, was used to quantify relative telomere length with the following modifications (from Purchase et al., 2022). The telomere repeats were amplified with the universal primer pair Tel1b (5'CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT-3') and Tel2b (5'-GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT-3'). Two different single-copy genes were selected, orexin (OX) and follicle-stimulating hormone (FSH), to be able to verify the consistency of T/S ratios depending on which single-copy gene was targeted. Primers were designed in Geneious 9.1.8 (Biomatters Ltd.) using Genbank contigs specific publicly available mRNA sequences of lake trout, with accession numbers HQ656804.1 and HM057170.1 for OX and FSH, respectively (See Appendix I for full sequences and molecular information). The primers were manufactured by Integrated DNA Technologies. qPCRs were performed on separate 384-well plates for each primer pair using the QuantStudio 5 Real-Time PCR System (Thermo Fisher Scientific) with 8ng DNA for RBCs and 10 ng DNA for liver and heart per reaction. The final volume of each qPCR reaction was 10 µl, consisting of 5 µl 2x PowerUp SYBR Green Master Mix (Thermo Fisher Scientific), 1 µl primer master mix, 3 µl water, and 1 µl of isolated DNA extract. The final concentration of each primer was 800 nM. The telomere primer pair thermocycling conditions were 50°C for 20 min, 95°C for 2 min, and 27 cycles of 95°C for 15 s, 56°C for 15 s and 72°C for 60 s. The single copy gene thermocycling conditions were 50°C for 2 min, 95°C for 2 min, and 40 cycles of 95°C for 20 s, and 60°C for 20 s. Both of these qPCR thermocycling programs were followed by default melt curve conditions of 95°C with a ramp rate of 1.6°C s for 15 s, 60°C with a ramp rate of 1.6 s for 1 min, 95°C with a ramp rate of 0.15°Cs-1 for 15 s. A pooled sample for each tissue was created by combining equal amounts of DNA from all individual DNA extracts and diluting them in a 4-fold serial dilution, producing 5 concentrations ranging between 40–1.25 ng µl-1 for the three tissues. This serial dilution was included in triplicate on each plate and used for efficiency tests, which ranged between 97-99% for heart and liver 96-97% for the RBCs. Non-target controls were also performed in triplicate on each plate, where the DNA in each reaction was replaced with 1 µl of water, and the critical threshold (Ct) values for these non-target control samples matched the background fluorescence values. The formula for the Ct values at the end for each tissue was: ((2Telb)/(2^single copy gene))-1 was calculated for each single copy gene combination, and the values were averaged between the two single copy genes to get one Ct total value per tissue.

4.3.4 Statistical Analyses

Correlations, and linear models were performed using the R software version 4.4.1 (R Core Team, 2020). The linear models transformed the predictors to a mean of zero and a variance of one, as part of the analysis package, before fitting the model. 95% Confidence Intervals (CIs) and p-values were computed using a Wald t-distribution approximation. When necessary, data were Ln transformed to meet assumptions of homoscedasticity and a $p < 0.05$ was considered significant in all cases. Note, sex was determined for all fish (distribution of females, males and indeterminant reported in table 4.2) but due to unbalanced distribution across lakes sex was not accounted for as a factor in statistical analyses.

4.3.4.1 Comparisons of fish size, age and relative organ masses across lakes.

The relationship between body mass and fork length across the lakes sampled for this study was compared, following the linearization of data by taking the Ln of mass in relation to a linear axis for fork length. To understand how fork length relates to age across lakes sampled, a linear model was used because exploratory examination indicated a linear relationship between age and fork length. To test for differences in size-at-age, the following was used: fork length (FL) ~ age * lake.

To test if lake trout differentially partition nutritional resources between cardiac capacity, metabolic turnover or reproductive preparation, we compared the relative organ size of the ventricle, liver and gonad across lakes. To do so, we compared the following: organ mass ~ FL * lake and if no significant interaction was found, an additional test of organ mass ~ FL + Lake was also evaluated. Note, ventricle tends to scale isometrically in teleost fishes, including lake trout (Edmunds et al., 2018) as does liver in lake trout (Cyr et al., 2018), and we initially tested relative gonad mass with the assumption that this organ would also scale isometrically with body size.

4.3.4.2 Comparison of cellular markers of aging

To evaluate if there are patterns with age and the markers of cellular senescence (relative telomere length as T/S and lipofuscin staining density), a linear model was used based on: Aging marker value ~ age * lake, where the aging marker was the T/S or lipofuscin staining intensity for each organ or tissue tested. If no significant interaction was found between age and lake, an additive model was also used to evaluate differences across lakes. Note, relative telomere lengths were measured in RBC, liver and heart while lipofuscin levels were only tested in liver and heart. Note that the linear models performed in R take the intercept on the first variable alphabetically, and treats this parameter as the reference for the y-intercept. To compare across variables, the package emmeans was used, and emtrend was used to compare slopes on the linear models, when pertinent. Note that the linear models performed in R take the intercept on the first variable alphabetically and treat this parameter as the reference for the y-intercept. ANOVAs were performed to compare means across lipofuscin and optical density data across lakes, and a Tukey post hoc performed if differences were found.

4.4 Results

4.4.1 Morphological Data

Almost all fish were mature and could be definitively identified for sex and significant relationships and differences in the morphological data (Table 4.2), particularly involving body mass and specific lakes. Linear Models Comparing Fork Length to Morphological Variables The model of the data for fork length vs. mass, ln transformed both variables to make a linear line, and account for differences in variance, the model predicts fork length significantly for this data, with $p < 0.0001$, degrees of freedom (DF) = 64, and $R^2 = 0.96$ for the natural logarithm of fork length on the analyses. Furthermore, the next linear model with interaction, including fork length vs. mass and lake, showed differences across lakes ($R = 0.97$, $F = 311.8$ on 7 and 58 DF, $p < 0.0001$). Breakdown of the model shows: fork-length vs mass found with positive estimate=2.98, t=0.21, p<0.001, therefore length predicts mass; LOTR estimate=8.25, t=3.70, p<0.001; Opeongo and Shirley are found with no difference versus Hogan in adjusted size (the y-intercept); the significant interaction term is because LOTR slope of

Table 4.2. *Biological aspects of lake trout, Salvelinus namaycush, from the four populations used in this study.*

	Lake			
Aspect	Hogan	LOTR	Opeongo	Shirley
Sample Size	17	17	16	16
Mass (g)	1920±667a	694±152	1792 ±547a	596±188
Fork-Length (mm)	556±67b	403±35	531±54b	382±37
Age (yr)	13.82±6.00	7.47±3.62c	12.07±5.76	12.75±4.37
Gonad Mass (g)	140.39 ±124.68d	33.32±37.87	109.82+80.03d	23.33 ±14.47
Liver Mass (g)	24.31±9.81e	7.65±3.50	22.83±8.14e	6.44±2.77
Ventricle Mass (g)	1.57±0.60f	0.56±0.11	1.74±0.69f	0.51±0.20
Sex	F=13, M=3, UN=1	F=7, M=10	F=8, M=8	F=4, M=12

F, female. M, male. UN, undetermined sex. a significantly higher mass in comparison to smaller lakes. b Significantly higher fork-length than in smaller lakes. c Significantly lower or younger age for LOTR lake trout than the three other lakes. d Significantly higher gonad mass than for smaller lakes. e Significantly higher liver mass than for smaller lakes. Significantly higher ventricle mass for bigger lakes' lake trout. Data with "±" are mean ± standard deviation.

ln FL in relation to ln M is lower than that for Hogan, estimate=-1.38, t=-3.78, $p<0.001$. The additive model, as there were found interactions, is not reported.

The relationship between fork-length (ln transformed) and age differs by lake (Fig. 4.1B). The model with age and site interactions (R= 0.83, F= 38.82 on 7 and 55 DF, $p < 0.001$) found site and age differences but did not show significant interactions. Therefore, we move to analyze linear model of fork length models vs age and lake, with no interaction (R= 0.81, F= 63.48 on 4 and 58 DF, $p < 0.001$): found significant effects of age (estimate= 0.01, t=5.06 $p< 0.001$), and there are lake effects of Shirley Lake with, estimate= -0.36, t= -11.84, $p < 0.001$; and LOTR has been found within the model, with estimate= -0.26, t=-7.54 $p<0.001$, figure 4.1B.

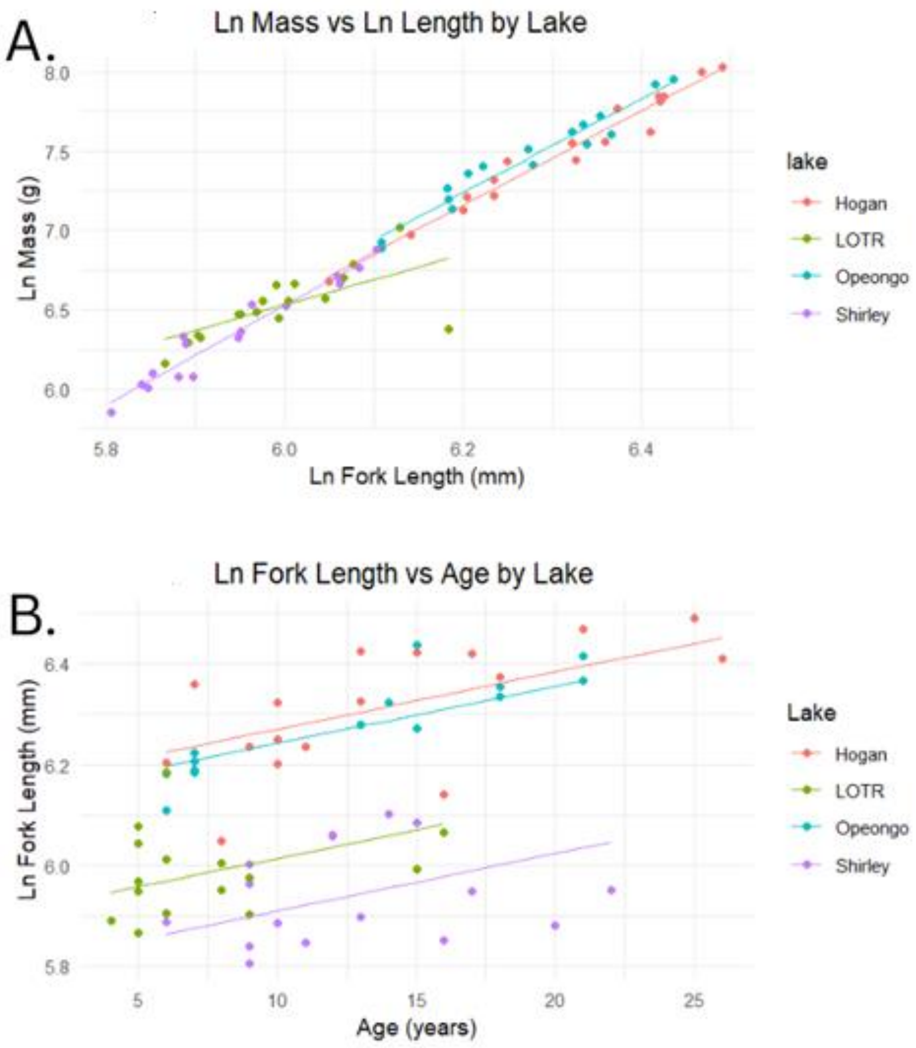

Figure 4.1*. Fork length and mass relationships of lake trout from four different populations. A. Graph of linear model of the fork-length vs mass and lake with interaction, lake effects were found for LOTR Lake versus the reference lake Hogan, and the other two lakes. n = 16, 17, 16, and 17 for Lakes Hogan, LOTR, Opeongo and Shirley, respectively, the data was back-transformed to return them to the raw mass scale, as model was done on the ln data, ensuring they align with the scatterplot of observed mass, plots the raw data, while overlaying the model predictions as regression lines, grouped by lake. B. Graph of the linear model of the ln fork-length vs the effects of age and lake with interaction. Significant effects of age are found, and some differences are significant for Shiley Lake, and LOTR has been found within the model. n = 16, 15, 15, and 17 for Lakes Hogan, LOTR, Opeongo and Shirley, respectively.*

The marginal mean pairwise comparison (Tukey, df=58) on adjusted fork-length (Ln transformed for homoscedasticity) indicates lake trout from Hogan and Lake Opeongo were not different in size, and trout from both smaller lakes, LOTR and Lake Shirely, were also not different in size from each other (p> 0.05). Fish from Lake Hogan were longer than the trout from both LOTR (estimate 0.26, t= 7.54, p<0.001) and Lake Shirley (estimate 0.36, t= 11.84, p<0.001). Likewise, trout from Lake Openogo were also larger than fish sampled from LOTR (estimate -0.23, t -6.81, p<0.001) as well as fish from Lake Shirely (estimate 0.33, t 10.60, p <.0001).

4.4.1.1 Ventricle Mass vs Body Mass

The size of the ventricle, relative to total body mass (Fig. 4.2A), varied by lake (R= 0.95, F= 175.1 on 7 and 58 DF, p < 0.001) with the ventricle in large fish from Lake Opeongo being larger than those in fish from Lake Hogan (t=3.64, p< 0.001). A post hoc analysis using marginal means, however, found no significance in the comparison analysis of the interaction model per lake. All the other comparisons across the lakes within the model were not significant.

4.4.1.2 Liver Mass vs Body Mass

Body mass was a significant predictor of liver mass (estimate 0.01, t= 8.334,p<0.001) when using a model with interaction Fig 4.2B, but there were no differences in the slope of this relationship between lakes. The post hoc analysis with marginal means comparison showed significant differences with relative liver size being larger in fish from Lake Opeongo the trout from Lake Hogan (t=-4.66, p< 0.001).

4.4.1.3 Gonad Mass vs Mass

The linear model comparing the gonad mass with total fish mass found no significant interaction and shows no difference between lakes (Fig. 4.2C) when using a simple additive model (model without interaction) (R= 0.54, F= 18.17 on 4 and 61 DF, p< 0.001).

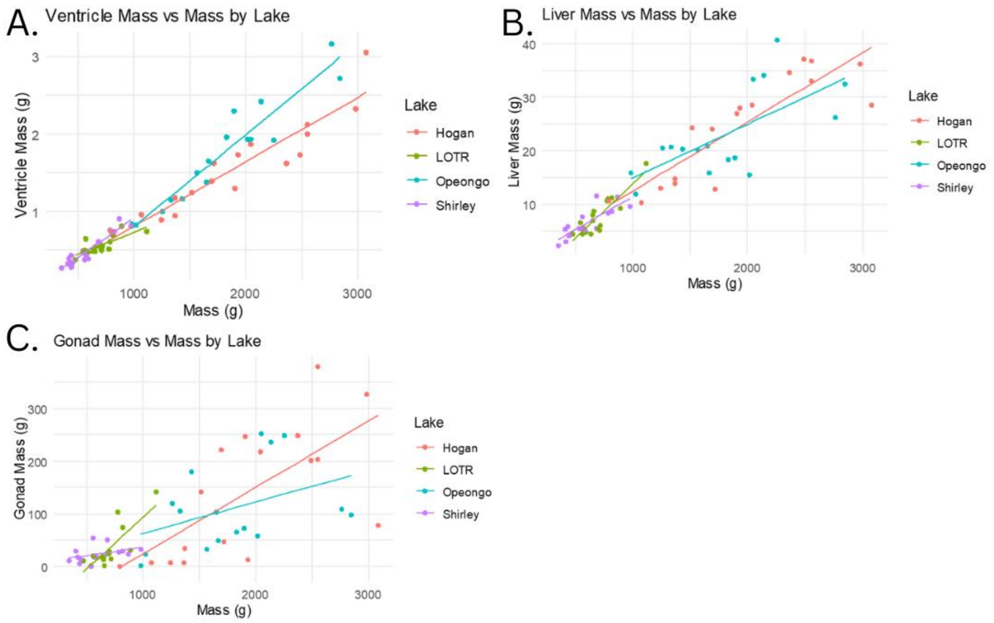

Figure 4.2. *Organ relative to body mass relationships for (A) ventricle, (B) liver and (C) gonad in four populations of lake trout. In all cases the relationship with body mass is significant ($p < 0.05$) but no significant effect of lake ($p > 0.05$) was found. n = 16, 17, 16 and 17 for Lakes Hogan, LOTR, Opeongo and Shirley, respectively.*

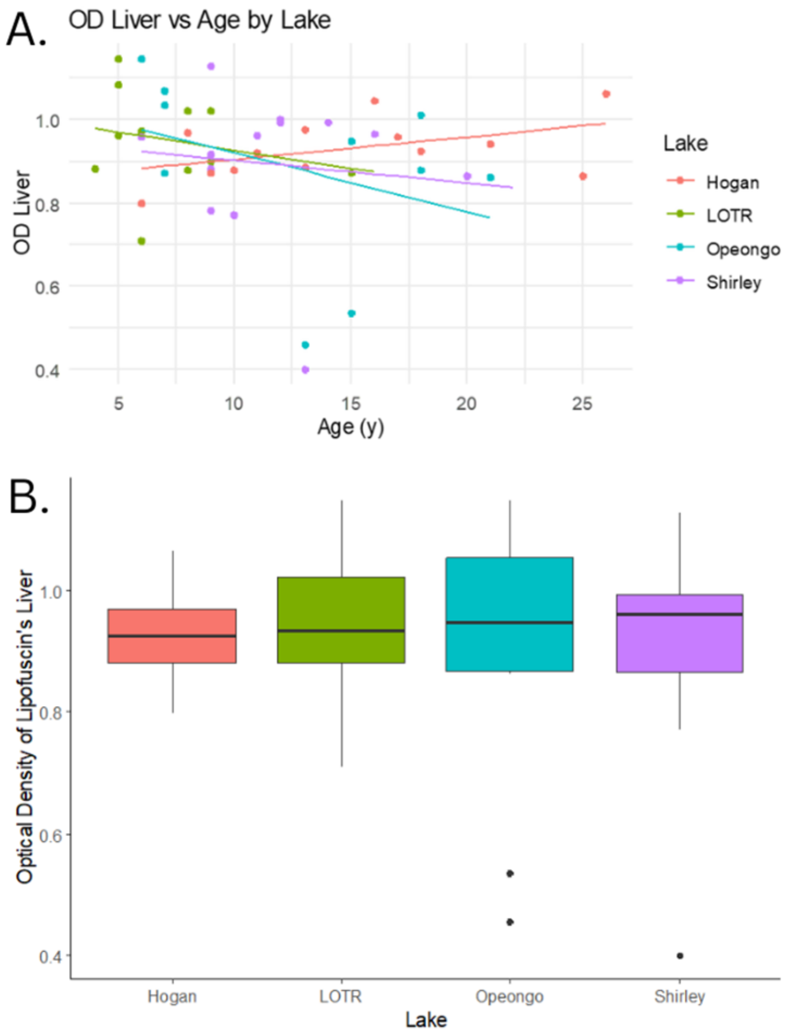

***Figure 4.3**. Boxplot from lake trout liver tissues showing the spread and median of values of the four lakes included in this study. A. Optical density of liver tissue vs age by lakes linear model with interactions dotplot. The model explains a statistically not significant and weak proportion of variance with R2 = 0.11, and p =0.689, with no significant lake effects or age effects in the model. B. Comparison of mean liver lipofuscin across populations. Data are boxplots showing mean (horizontal line) and upper to lower quartile (box) and 95% confidence interval (vertical line), with outliers shown as individual dots. Hogan n=17, LOTR n=17, Opeongo n=17, Opeongo n=16, Shirley n=16. One-way ANOVA show no significant values for lake effects on the average of the optical density values for liver tissue on lake trout.*

4.4.2 Lipofuscin Accumulation

The study found no significant correlation between lipofuscin concentration and age in any of the tissues; however, we have found some lake effects in some of the tissues examined.

Optical Density in the Liver Tissue

The lipofuscin staining intensity in liver (Fig 4.3) and associated ANOVA (formula: OD_liver ~ lake) suggests that the main effect of lake is statistically not significant and small (F(3, 45) = 0.30, p = 0.822; Eta2 = 0.02, 95% CI [0.00, 1.00]). Linear model of optical density (OD) ~ age + lake (F= 0.44 on 4 and 42 DF, p-value: 0.78), for liver tissues, shows no age influence or significance on the linear model of optical density of liver tissue versus lake and age Also noteworthy, there was no correlation between liver and heart lipofuscin (data not shown).

Heart tissue optical density by lake: The ANOVA (formula: OD_heart ~ lake) suggests that the main effect of lake is statistically not significant (F(3, 62) = 2.21, p = 0.10; Eta2 = 0.10, and 95% CI (0.00, 1.00)). The linear model (Fig. 4.4) with interaction between age and lake (F= 1.35 on 7 and

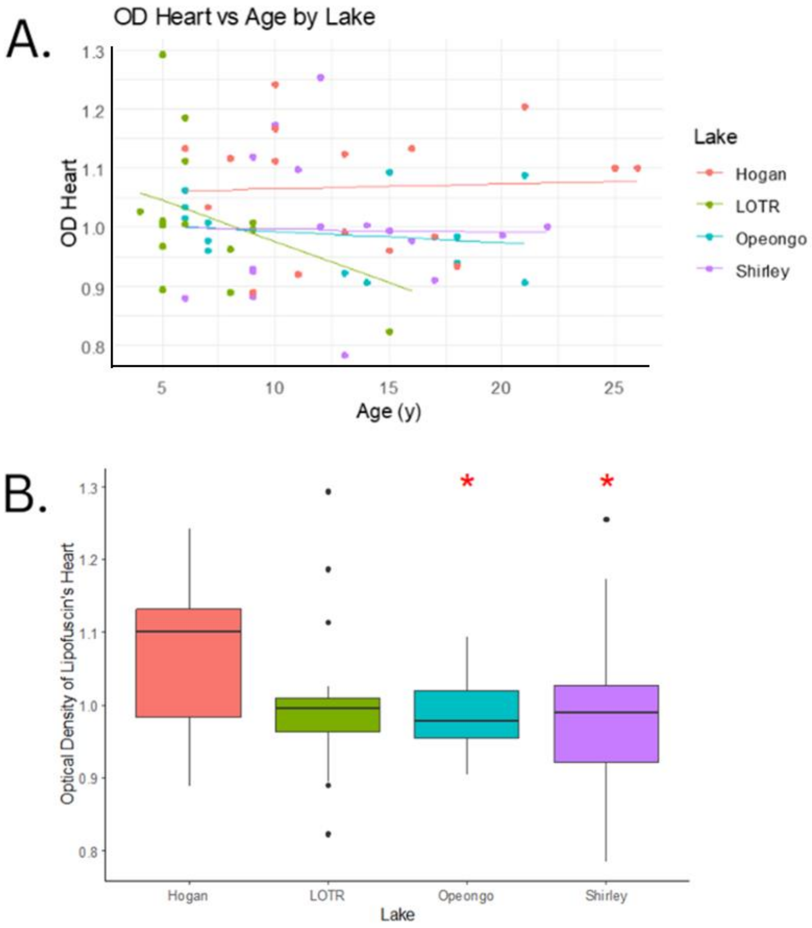

Figure. 4.4. *Lipofuscin accumulation in heart tissue from four populations of lake trout. A. Relationship between lipofuscin staining in heart tissue with Sudan black measured as optical density (OD) and fish age across lakes with all individual values plotted ($p > 0.05$ in all cases). B. Comparison of mean heart lipofuscin across populations. Data are boxplots showing mean (horizontal line) and upper to lower quartile (box) and 95% confidence interval (vertical line), with outliers shown as individual dots. * Significantly lower than Lake Hogan (One-way ANOVA, $p < 0.05$). Lake of Two Rivers (LOTR). n = 17, 17, 16 and 16 for Lake Hogan, LOTR, Lake Opeongo and Lake Shirley respectively.*

55 DF, p= 0.25) shows no significant effects of lake on the lipofuscin-related optical density, as well the age effect is not significant within the model (F= 1.636 on 4 and 58 DF, p-value: 0.18). Using a simpler addition model, marginal lake effects are found (F= 2.15, p= 0.10) and age is not significant (F= 0.09, 0.76).

4.4.3 Relative Telomere Length and Age

The T/S ratios from the telomere PCR assays also did not show any significant correlation with age for all three tissues sampled (Figs. 4.5-4.7). A linear mixed model comparing T/S ratios to age and lakes indicated differences between some lake environments. A correlation between heart and liver T/S values was found, but no other correlations were found (Appendix 3).

4.4.3.1 Linear Models for RBC vs Age and Lake

The interaction model (estimated using OLS) of T/S RBC ratio with age and lake (formula: T/S RBC ratio ~ age * lake) was not statistically significant (F= 1.34 on 7 and 20 DF, p-value: 0.28) for any effect of lake or age within the interaction (Fig. 4.5). There were additive effects in the non-interaction model significant (R= 0.39, F= 4.06, p-value: 0.0113), with Opeongo (estimate 40245, t=2.39, 0.025) having higher average estimate versus the reference lake Hogan; the age effect is slightly negative but it is not significant. The marginal means analysis showed only marginal effects, however. Using an ANOVA to test for mean averages across lakes was also not significant (Fig.4.5).

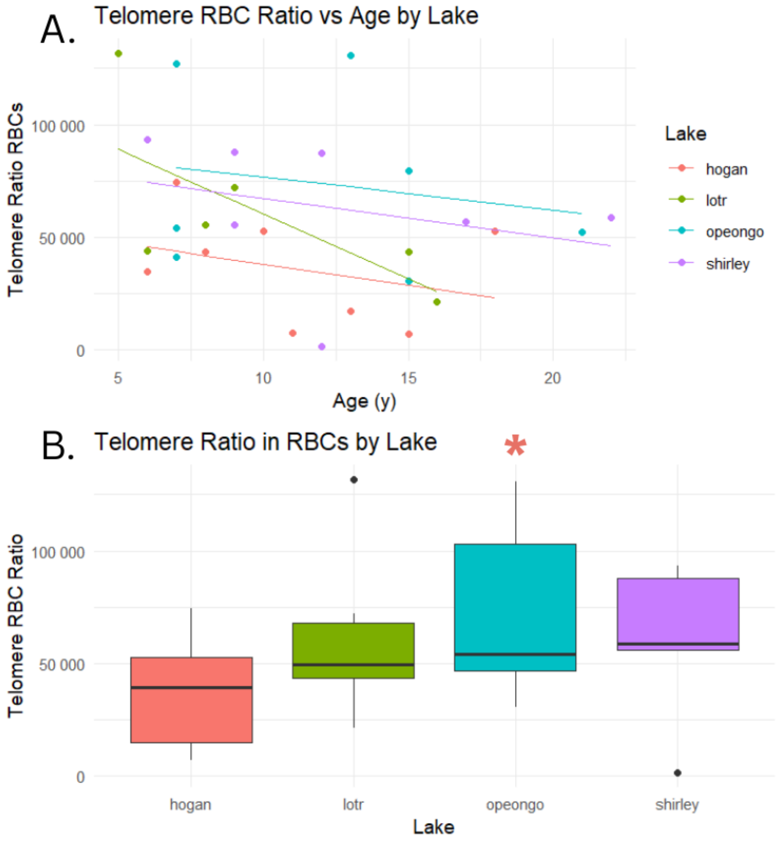

Figure 4.5. *Relative telomere length (T/S ratio) in red blood cells (RBC) from four lake trout populations. A. Relationship between the estimate of telomere length (T/S ratio) and fish age across lakes with all individual values plotted. No significant relationships were found ($p > 0.05$ in all cases) on the interaction model shown in the graph. B. Comparison of mean RBC relative telomere length (Telomere ratio) across populations. Data are boxplots showing mean (horizontal line) and upper to lower quartile (box) and 95% confidence interval (vertical line), with outliers shown as individual dots. * Significant difference from Lake Hogan (One-way ANOVA, $p < 0.05$ did not find significant differences across lakes). The additive linear model run found also no significance. Lake of Two Rivers (LOTR). n = 8, 6, 7 and 7 for Lake Hogan, LOTR, Lake Opeongo and Lake Shirley respectively.*

4.4.3.2 Linear Models for Heart vs Age and Lake

We fitted a linear model (formula: T/S heart ratio~ age * lake) to predict T/S heart ratio with age by lake (F= 1.47 on 7 and 22 DF, p= 0.23) which was also statistically not significant (Fig. 4.6). For additive effects the non-interaction model shows no significance for any of the variables in the formula T/S heart ratio~ age + lake (R= 0.18, F= 1.37 on 4 and 25 DF, p= 0.27).

Comparison across lakes by ANOVA also found no significant differences in the ventricle relative telomere length between lakes (Fig. 4.6).

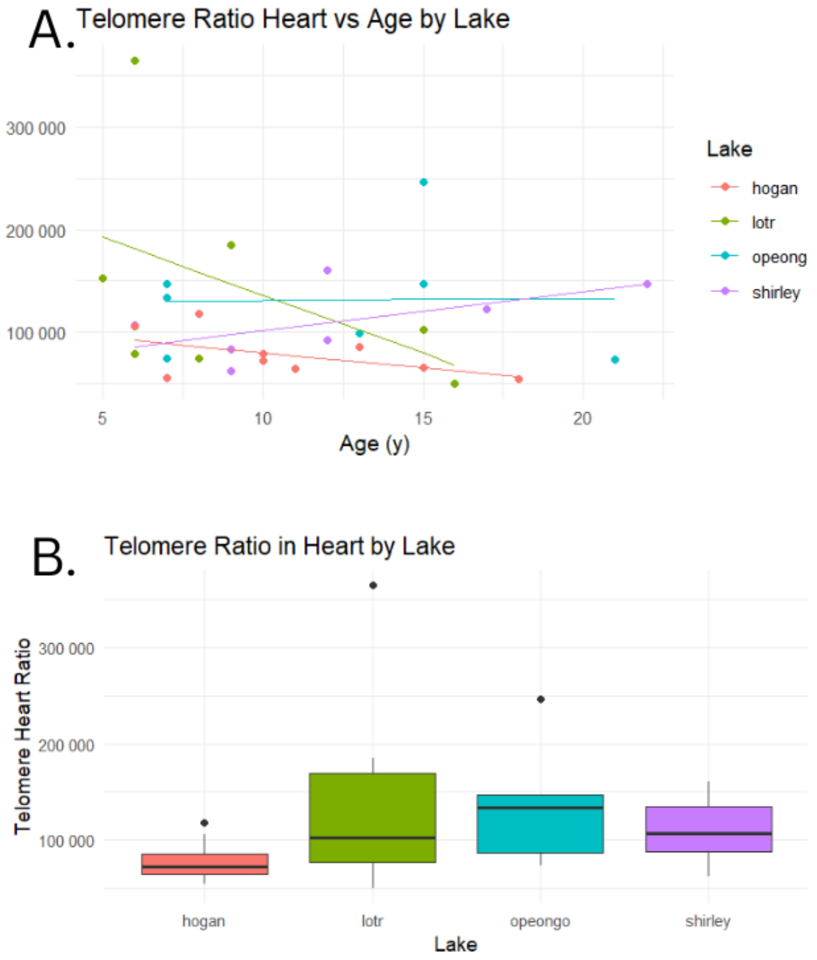

Figure 4.6. *Relative telomere length (T/S ratio) in heart tissue from four lake trout populations. A. Relationship between the estimate of telomere length (T/S ratio) and fish age across lakes with all individual values plotted. No significant relationships were found (p > 0.05 in all cases) on the interaction model. B. Comparison of mean relative telomere length in heart tissue (Telomere ratio) across populations. Data are boxplots showing mean (horizontal line), with upper to lower quartile (box) and 95% confidence interval (vertical line). Outliers are shown as individual dots. No significant differences found (One-way ANOVA, p > 0.05), and no significance on the linear models. Lake of Two Rivers (LOTR). n = 9, 7, 7 and 7 for Lake Hogan, LOTR, Lake Opeongo and Lake Shirley respectively.*

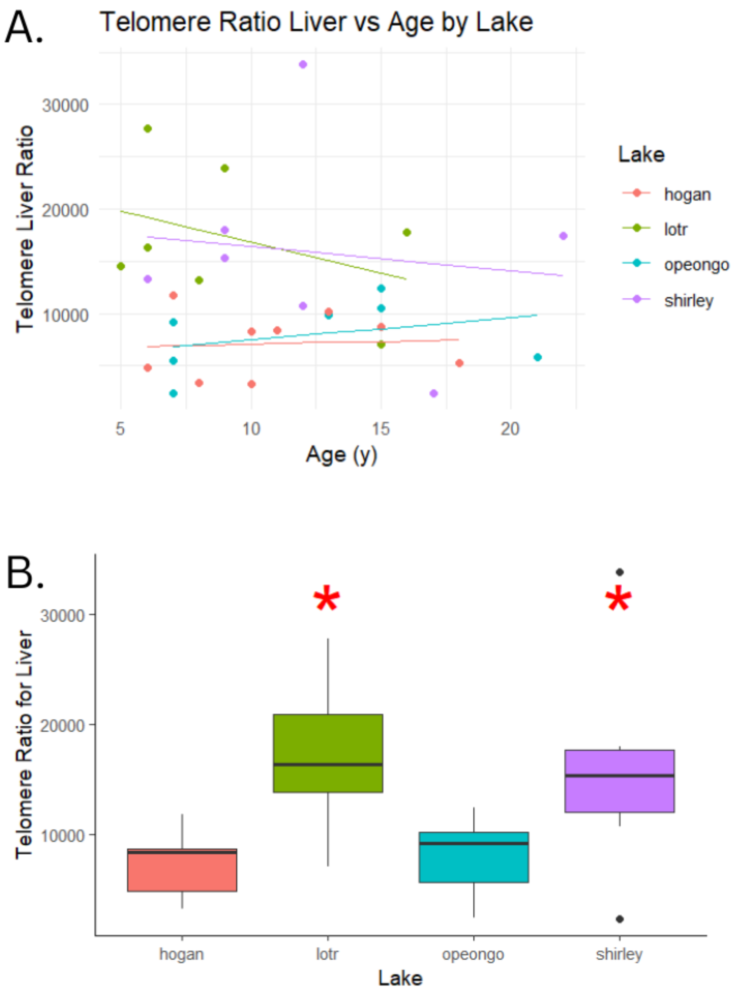

Figure 4.7. *Relative telomere length (T/S ratio) in liver tissue from four lake trout populations. A. Relationship between estimate of telomere length (T/S ratio) and fish age across lakes with all individual values plotted. No significant relationships were found ($p > 0.05$ in all cases). B. Comparison of mean relative telomere length in liver tissue (Telomere ratio) across populations. Data are boxplots showing mean (horizontal line), with upper to lower quartile (box) and 95% confidence interval (vertical line). Outliers are shown as individual dots. * Significant differences from Lake Hogan (One-way ANOVA, $p < 0.05$). Lake of Two Rivers (LOTR). n = 9, 7, 7 and 7 for Lake Hogan, LOTR, Lake Opeongo and Lake Shirley respectively.*

4.4.3.3 Linear Models for Liver vs Age and Lake

For liver, the linear model (formula: T/S liver ratio ~ age * lake) for relative telomere lengths was non-significant (F= 2.34 on 7 and 22 DF, p= 0.06), Fig 4.7. There is a tendency for the T/S to decrease with age, but not significantly, when the lake is controlled, and the lake effect is not significant; there were no significant interactions on this model. Therefore, we moved to the additive model, which found significant differences across lakes (F= 4.06 on 4 and 25 DF, p-value: 0.01), with the post hoc analysis of marginal means presenting Shirley (estimate= -8904, t=-2.812 and 0.04), and LOTR (estimate= -9913, t=-3.13 and p=0.02) with lower estimated marginal means versus the intercept, Lake Hogan. ANOVA of liver telomere lengths also show lake differences (lake estimate=3 61, t=4.86, p= 0.01) but no significant effect of age. The ANOVA found differences between Hogan and LOTR (estimate=-9913, t= -3.13, p=0.02), and Lake Shirley (estimate= -8904, p=-2.816 p=0.04). The LOTR showed a tendency towards higher marginal means than Opeongo (estimate= 8954, t=2.63, p=0.06).

4.5 Discussion

4.5.1 Standard Markers of Age and Long-Lived Fish

This study compared lipofuscin and relative telomere lengths as potential markers of senescence in four populations of lake trout, two from large lakes and two from smaller lakes. It was found that wild sampled lake trout show no age-dependent lipofuscin accumulation in liver or heart, and relative telomere lengths do not shorten with age in RBCs, liver or heart. The populations were selected due to differing growth potential and the presence or absence of a particular competitor, the smallmouth bass (Table 4.1). As anticipated, the large lakes have lake trout with indeterminate growth that reach much larger size than the two populations from smaller lakes with a more determinate type of growth pattern (Fig. 4.1). The presence of competing smallmouth bass in the lake had no clear effect on the lake trout size potential senescence marker levels.

While confirming past work on the effect of age on relative telomere length in lake trout (Purchase et al, 2022), the lack of differences with age in two commonly used markers of cellular senescence do not show follow the anticipated patterns found in laboratory models (Munnell & Getty, 1968; Nakano et al., 1989), humans (Di Guardo, 2015; Shammas, 2011) and some wild species of animals, including some short-lived fish (Arslan-Ergul & Adams, 2014; Ng'oma et al., 2014). In most laboratory models (Shammas, 2011) and some wild species (Anchelin et al., 2011; Gopalakrishnan et al., 2013), markers of aging like telomere length tend to correlate negatively with age, and the lipofuscin accumulations are positively correlated with age (Whiteford & Getty, 1966), a pattern which we do not see in this work. This finding is intriguing, as it may show that lake trout indeed do not show signs of decline with biological aging, at least within the ranges in the present study. The continuous cellular turnover that will be found with indeterminately growing animals, even though rates will slow as the lake trout approaches their asymptotic size for the available resources, may come with the possibility of continued cellular maintenance and adult expression of telomerase activity across several tissues in adult lake trout tissues (Klapper et al., 1998). Telomerase in humans and other mammals tends to not be expressed in adult tissues (González-Suárez et al., 2002; Heist, 2003). This continued growth, or at least growth potential, may also explain the lack of correlation of lipofuscin, which is accumulation of glycation products, as cell turnover and apoptosis may still be occurring in the adult fish (El Maï et al., 2023; Panasiak et al., 2023).

The Age Factor or Marker of Stress: Relative differences across values in relative telomere length can be related to stress, incurred by reactive oxygen species (ROS) overproduction (Dunn et al., 2024; Evans et al., 2021) or rapid turnover rates of the cells within tissues (Dlouha et al., 2014). Comparing across tissues, the lowest values for the relative telomere length estimates were in the liver, a tissue with high cellular turnover rates in comparison with RBCs and the heart. Telomere length is considered a marker of age for humans; however, this specific cellular characteristic has been argued to be a better representation of stress in fishes instead solely being an aging marker (Debes et al., 2016; Schreck & Tort, 2016; Sopinka et al., 2016). Previous work in this thesis (Chapter 3) has shown that metabolite composition of the blood changes with aging in walleye, Sander vitreus, which parallels findings in mammals; however, relative telomere length did not show any correlation with age in walleye (Chapter 3). Given that both walleye and lake trout are both temperate fish with potential for indeterminate growth, the current study confirms that relative telomere length may not correspond to a marker of cellular senescence, at least not in the age range of the animals in these studies. Furthermore, lake trout are considered indeterminate growers; therefore, telomere length may be a good marker of stress instead, due to the possibility of telomerase expression in adult tissues in fish (Anchelin et al., 2011; Panasiak et al., 2023). This may not be as improbable; in some theories of aging, it is said that evolution will have a decreased effect of selection on the aging phenotypes of an organism; therefore, deleterious traits will accumulate with advanced age as they are not selected out (Williams, 1957). However, this effect also needs to account for the probability of decreasing reproductive capacity, which may be present in most mammal models so far studied, but may not decrease in some indeterminate growers, like some fish (Wallace, 1925). Not many studies have addressed whether tissue-level markers of senescence change with age in long-lived fish species like lake trout. Although there is evidence of an increase in mortality in the wild in untouched lake trout populations (Kennedy, 1954), the possibility of increasing reproductive capacity in this species is here, and with this possibility, of larger size, decrease in predation probability in the wild and higher reproductive ability, evolution may preferably select for longevity, or negligible senescence.

Differences Across Lakes

This study did uncover some differences in relative telomere lengths across populations of lake trout in the Algonquin region. Relative telomere length differences across lakes were found in liver and red blood cells. Heart telomeres in lake trout show no differences across lakes. However, RBCs telomere length is higher in the Lake Opeongo in comparison to the other large body of water, Lake Hogan (Fig. 4.5). Conversely, liver telomeres of lake trout form the two smaller lakes, Shirley and LOTR, have longer relative telomere lengths relative to fish from larger lakes (Fig. 4.7). For the two much smaller lakes, Shirley and LOTR, if the growth of the lake trout is limited due to a shorter food web, this may imply a possibility of caloric restriction for these upper trophic level predators in these lakes. Diet composition and availability for fish affect the growth of fish species and, therefore, may have an effect on the aging and longevity of a lake trout population in this study. Therefore, there may be possible diet effects on growth across populations, if different prey is available across a population of lake trout or across geographical environments that present different prey species composition in the waters. It is known that caloric restriction models show that decreased ingestion of foodstuffs increases longevity in vertebrates and invertebrate animals (Merry, 2002). One prey species known to affect its predator, like lake trout, is cisco (Coregonus artedi), which is one native prey species for lake trout and known to influence trophic dynamics in cold water systems, cisco is present in two of our sampling lakes, Lake Hogan and Lake Opeongo (Ebener et al., 2008; Parks & Rypel, 2018; Trippel & Bearnish, 1989).

The accumulation of lipofuscin in heart and liver tissue across lakes had some additive effects of the lake on the heart tissue for Shirley and Opeongo lakes, with a much lower average of lipofuscin accumulation versus Lake Hogan, but those effects were not present after post hoc analysis of the model. This is intriguing as Opeongo is a much larger lake than Shirley, and Shirley Lake does not have the presence of cisco, which is found on Lakes Opeongo and Hogan. Possibly, some other effects and variables may not be accounted for in this study, leaving some clarification for further study. However, in general, even with some significant results for possible lake effects, the patterns of telomere length and lipofuscin accumulation across lakes were inconsistent across tissues, with no clear or striking environmental pattern that may explain these differences.

Investigation on a large sample of lake trout growth and mortality traits, showed that in an unexploited population lake trout has an increased in mortality with age, and tend to be fairly resilience to exploitation and show a diverse of seasonal and geographical growing rates and patterns (Kennedy, 1954); however, the untouched population of lake trout in this study did show increase mortality with age. Recent study on smaller lakes and samples on the

Experimental Lake Area of Ontario on lake trout, show the possibility that lake trout growth patterns in small fish maybe different, and that lake trout may not become as large, as they may be energy deficient, as the small environment and expected short food chain, will restrict lake trout growth (Purchase et al., 2022).

4.5.2 Limitations and Further Research

Our study had a relatively small sample size (a total of 66 fish for lipofuscin optical density and 30 for relative telomere length analyses) when compared to most ecological studies. Further research should look at expanding the sample size and include a wider range of ages. Lake trout is an ectotherm, and environmental factors will have an influence in the rate of aging, which we did not include in this study (Fernandes et al., 2021; Flouris & Piantoni, 2015; Pal & Tyler, 2016). Studies on a different geographic scope or work to account for the diet (food web) environment can be fully explored and may clarify specific patterns found that could not be accounted in this study. The investigation-specific mechanisms behind the observed lack of correlation of age with telomere length can be of use to decipher these results. A good approach can be investigating the telomerase enzyme expression in the tissues of lake trout, as it is known that the presence of telomerase may be found in adult tissues of indeterminate growers, like some fish (Klapper et al., 1998). Exploration of a bigger range of ages in lake trout will be of benefit as well as our study only included fish with maximum age of just over 20 years, lake trout has been recorded to live until 50 years (Pauly, Daniel and Froese, 2001). This may confirm if the trend of no correlation of age with the aging markers studied in this work will extend to late age stages for late trout.

4.5.3 Conclusion

This study examined lake trout populations across four different lakes in Algonquin Park and analyzed telomere length analysis and lipofuscin accumulation and compared their relationship with chronological age. Our study did not find any significant trend for both age markers assayed, for telomere length, liver tissues showed lower relative values, showing a potential relationship with cellular turnover rates. However, no age relationship was found for age on the relative telomere length; this marker may be a possible indicator of oxidative stress.

We found some lake effects across both markers of aging with the lipofuscin stain of Opeongo and Shirley Lakes exhibiting lower optical density in heart tissue, versus Hogan. Relative telomere length shows lake effects as well, with Lake Opeongo having higher lipofuscin accumulation on the liver and red blood cells. Perhaps this represents a lower turnover rate in the larger lake ecosystem versus the smaller lakes. Effects of environmental variations can significantly impact organism physiology, like diet type through web food interactions, intrinsic effects like oxidative stress and cell turnover rates as well, can also explain to some extent telomere length and lipofuscin differences across lakes in lake trout.

The lack of age significance on the candidate markers of aging across tissues can also be explained by the possible expression of the telomerase enzyme system in adult tissues of lake trout. Future studies should address the expression of this enzyme in the tissue of this species. Furthermore, our work has reiterated the assessment of aging markers on lake trout, does not follow the expected pattern of aging versus laboratory and human studies done previously.

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5. Summary

5.1 Contribution of Authors

Lilian Maria Wiens, conceptualization, writing and editing.

My thesis work has examined two species of temperate fish: walleye, *Sander vitreus*, and lake trout, *Salvelinus namaycush.* The study focused on metabolic and molecular protocols to investigate naturally occurring variation in the blood metabolome of walleye and aging in both animals. This new knowledge may help to better understand the physiological function of fish, particularly in relation to regional or temporal changes in metabolism and the aging process in general.

5.2 Walleye Metabolomics Geographical Patterns

The second chapter compared the blood metabolites in walleye of Lake Winnipeg sampled non-lethally over 2 years (2017-2018) across regions of Lake Winnipeg. Our study confirmed geographical patterns of variation in molecular markers across sites of capture found in gill transcriptomes (Jeffrey et al., 2023) and blood metabolites (Thorstensen et al. 2020). The work confirmed specific higher concentrations of amino acids within the blood of walleye that were caught in the Northern basin of the lake. On the other hand, the Southern basin walleye presents a higher concentration of specific lipid molecules. Both year patterns showed specific regional differences in metabolite levels were repeated; therefore, the specific patterns for some metabolites was seen in both years in this study. The conclusions of this thesis chapter parallel a geographical pattern of differences found across this same population where the same walleye, shows geographical differences of expressed genes in their gill transcriptome of Northern versus Southern fish (Jeffrey et al., 2023). The Northern fish in the parallel study of this population (Jeffrey et al., 2023) show higher expression of genes that are related to hypoxia and certain signaling pathways of protein breakdown and remodeling. Which raises the question as to the origin of the difference across basins of walleye in Lake Winnipeg. Northern walleye may be going through an external or internal stress, as shown in the transcriptomics study and the results of this thesis. The possibility of nutritional differences across food web composition can be a reason for the divergence across basins in the walleye of Lake Winnipeg. The Northern walleye may show a lower feeding regime relative to the Southern walleye. Reproduction, which is an important evolutionary force and cannot be discounted in any population, can also be an explanation for the presence of elevated long-chain lipids on the Southern walleye, as it is the Southern walleye that were in spawning season time during the sampling of this study. Lastly, there may be some other effects, like environmental influences and anthropogenic effects that were not defined in our study. More research to understand the cause of geographical differences in metabolic molecules in the blood should be undertaken, as walleye is indeed an important predator in this lake, as well as a prized species by fisheries and subsistence in the region.

Limitations for chapter 2 will come from the diversity of fishes, and indeed, on the study organism, it is known that fishes have high adaptability when it comes to phenotypes, due to life stage and environmental constraints, which may introduce confounding variables which may render the interpretation of results difficult (Huang et al., 2022; Späth et al., 2022). Environmental influences are of importance and known to influence ectothermic species. Due to constraints of time, our study did not compare the temperature differences between basins of the lake temperature differences; water quality or contaminants have not been accounted for either. This information may have given some clarity to differences across basins on the presence of propylene glycol (data not shown), for example, which was in higher concentration in the Northern basin’s walleye of the lake. Furthermore, another limitation comes from the nature of the metabolomic assays. Metabolomics has advantages as it gives a large amount of information to the researcher; however, interpretation and analysis of the results therefore becomes more complex. There is no standard workflow in the field of metabolic research; some studies do show similar methods of analysis and collection of the samples, but diversity in results may be one drawback of this lack of standardization across studies (Hajnajafi & Iqbal, 2025).

5.3 Walleye Metabolites versus Chronological Age

The third chapter set out to investigate if chronological age has an effect on the concentrations of major metabolic molecules sampled in a wild fish, the walleye, of Lake Winnipeg, to further understand understand the process of aging. Walleye were sampled in 2018 for spines, age determined, and the whole blood metabolome of the aged fish from chapter 2 was scrutinized for age-related patterns. The metabolites were compared by statistical methods to the chronological age of the walleye. The study found some correlations, especially with essential and branched-chain amino acid concentration within the blood; these metabolites tend to increase with chronological age. Results also show some effect on the concentrations of short-chained and medium-chained acylcarnitines, with decreases in concentration of these metabolites in the blood of the walleye. These results parallel some mammal studies, where amino acid concentration and their breakdown pathways increase with age. Furthermore, some lipid molecules are shown to decrease in humans with age in the blood.

Possible limitations of this study include: the complexity of the metabolome, as many molecules are sampled and data interpretation can be difficult (Di Minno et al., 2022; Nguyen et al., 2024). As well, the age of the fish was only determined by spines for the 2018 caught fish;

it only represents a one-time picture of the possible changes taking effect for this species. We must also consider possible divergences across organisms due to taxa and life stage (Huang et al., 2022; Späth et al., 2022), nutrition (Housley et al., 2018), and environmental influences, especially in ectotherms, a good example is the temperature influence on metabolism amongst other factors (Aguilar et al., 2022).

Furthermore, amino acids are also used as signaling molecules within the organism; their presence in the blood can reflect nutrient intake state, certain diseases present and underlying stress responses (Canfield & Bradshaw, 2019), increase in amino acid concentration with age may also be an effect of changing in signaling pathways involved in senescence as well. The essential animo acids' high concentration in the blood of walleye, can either represent excess food intake or a high-level protein catabolism, we must note for the majority of fish as indeterminate growers there is no evidence that increase age represents lower condition. Therefore, the pattern of amino acids in the walleye blood pattern will favour oxidation of amino acids. For the walleye model in this project, on the second chapter the amino acid concentration is higher as well on the Dauphin River site, Northern fish, these fish tend to be smaller in size compared to the Southern walleye, maybe signifying stunted growth in the North for walleye.

5.4 Lake Trout Aging in the Wild

This study investigated lake trout, a long-lived fish, within four different lakes in Algonquin Park, where food-web interactions and limitations to growth can be investigated. More specifically, the fourth chapter looked at the presence and differences in aging markers at the cellular level in lake trout from four lakes in Algonquin Park. A study by Purchase et al. 2022) found no evidence of senescence in a male lake trout model, and other workers have found negligible senescence in some fish (Finch 1998; Froehlich et al. 2013; Maslov et al. 2019).

This chapter tested for biochemical senescence markers in any of the lake trout of the four lakes sampled. This is of importance especially as some of the study's fish reside in larger lakes, where bigger food items may lead to bigger adult lake trout size, therefore bringing the possibility of a growth trajectory that will reflect indeterminate growth. The markers used in this study consisted of histological determination of lipofuscin accumulation and relative telomere length assays.

The histological analyses show no concrete correlation between the chronological age of the lake trout in this study versus lipofuscin accumulation in the heart and the liver of lake trout. Therefore, there is no concrete evidence of a specific pattern or differences in optical density across lake trout of different lakes in lipofuscin accumulation as well. Furthermore, the three tissues (heart, red blood cells, and liver) assayed for relative telomere lengths by quantitative PCR and no pattern or correlation with age and relative telomere length.

The current study has confirmed the previous work (Purchase et al., 2022) that lake trout do not show cellular senescence patterns with increasing age, patterns that are seen in mammal models of aging. However, this chapter expands the results of a former study, which only included males, as this chapter of the thesis includes both sexes in the sampling (Purchase et al., 2022). Furthermore, the result of this chapter is consistent with the idea of the possible variance in the senescence process that medium and long-lived indeterminate growers may present (Finch 1998, 2009).

Effects of food web constitution may influence the diet of lake trout in different environments. It is accepted that environmental factors may influence aging, including diet. (Merry, 2002), temperature regimes for ectotherms (Carvalho et al., 2017), overcrowding (Benesh et al., 2016) All may affect the aging process, and may have been an influence on the lake trout in this study. One notable fact is that there were some differences across lakes, which may be present for relative telomere length; the trends are inconclusive.

Limitations in this study: our sample size only included 30 fish for the relative telomere length and 66 for the lipofuscin accumulation; the total number was small. Further study of other tissues and including more fish to make the power of the statistical tests stronger. Furthermore, the range of ages across the sample includes fish younger than 30 years. Further work, including larger and older trout, in which a higher range of ages is included, can be useful to discern if the patterns seen in this chapter will be repeated in larger age trout. However, caution needs to be taken into consideration as a larger sample and older, bigger fish have to be used with caution, as it can affect the population, as lethal sampling to include other tissues is necessary.

5.5 Take Home Message

This thesis used biochemical, histological and genetic methods to assess physiology and aging markers on walleye and lake trout. In the first two chapters, the study sampled non-lethally and analyzed whole blood in walleye of Lake Winnipeg, to identify metabolic markers of physiological stress and chronological age influences in this population. The results of the second chapter can be used to better understand the health of the walleye population, specifically the Northern basin cohort, which is important for subsistence and commercial fisheries. The third chapter opens the possibility of adding more wild species, especially fish, non-determinate growers, to the field of aging, as the results show some confirmation of metabolite patterns of aging that are found in mammal models. This adds to knowledge in the aging process and that ectotherms do have in some part some similar patterns of aging versus human and mammal senescence. The fourth chapter investigated whether lake trout in the Algonquin Lakes present signs of cellular senescence in their tissues; however, the results show no correlation of age with two standard markers of aging in mammals, including humans. These results may represent the possibility of negligible senescence in lake trout, therefore implying added evidence which backs the antagonistic pleiotropy theory of aging.

5.6 References

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6. Appendices

6.1 Chapter 2 Appendices

ANOVA Analysis Results for Metabolites

Appendix Table 2.1. Results of the ANOVA Interaction Model of the Metabolites with a .65 Value of the first two Axes.

Term	Df	sumsq	meansq	statistic	p.value	Metabolite
Site	3	18.79888	6.2662948	13.792381	1.06E-07	Methionine
Year	1	43.35576	43.355757	95.427862	1.33E-16	Methionine
Site: Year	3	4.86904	1.6230133	3.5723213	0.0163866	Methionine
Residuals	110	49.97632	0.4543302	NA	NA	Methionine
Site	3	41.92324	13.974414	25.263731	1.65E-12	Valine
Year	1	11.79596	11.795962	21.325403	1.06E-05	Valine
Site: Year	3	2.435245	0.8117483	1.4675241	0.2273712	Valine
Residuals	110	60.84555	0.5531414	NA	NA	Valine
Site	3	35.37865	11.792882	18.494036	8.73E-10	Isoleucine
Year	1	9.235279	9.2352791	14.483108	0.0002329	Isoleucine
Site: Year	3	2.243626	0.7478752	1.1728457	0.3234858	Isoleucine

Residuals	110	70.14245	0.6376586	NA	NA	Isoleucine
Site	3	51.91103	17.303677	32.849504	3.07E-15	Trans.Hydroxyproline
Year	1	4.584179	4.5841786	8.7026585	0.0038828	Trans.Hydroxyproline
Site:Year	3	2.561615	0.8538718	1.6210003	0.1886304	Trans.Hydroxyproline
Residuals	110	57.94317	0.5267561	NA	NA	Trans.Hydroxyproline
Site	3	45.75964	15.253213	24.215456	4.16E-12	Asparagine
Year	1	0.182894	0.1828945	0.2903567	0.5910809	Asparagine
Site:Year	3	1.768937	0.5896457	0.9361004	0.4258788	Asparagine
Residuals	110	69.28853	0.6298957	NA	NA	Asparagine
Site	3	44.6837	14.894568	24.498311	3.23E-12	Tryptophan
Year	1	2.423055	2.4230548	3.9853959	0.0483668	Tryptophan
Site:Year	3	3.015063	1.0050209	1.65304	0.1813774	Tryptophan
Residuals	110	66.87818	0.6079835	NA	NA	Tryptophan
Site	3	31.36042	10.453474	17.628432	2.05E-09	Proline

Year	1	20.06084	20.060841	33.830016	5.99E-08	Proline
Site:Year	3	0.349901	0.1166337	0.1966876	0.8984555	Proline
Residuals	110	65.22884	0.5929894	NA	NA	Proline
Site	3	40.79042	13.596808	22.039883	2.99E-11	C5
Year	1	0.07095	0.0709504	0.1150078	0.7351599	C5
Site:Year	3	8.277613	2.7592042	4.47256	0.0052901	C5
Residuals	110	67.86101	0.6169183	NA	NA	C5
Site	3	21.2081	7.0693676	11.541547	1.23E-06	Leucine
Year	1	20.05113	20.051127	32.735747	9.23E-08	Leucine
Site:Year	3	8.36415	2.78805	4.551809	0.004791	Leucine

term	Df	sumsq	meansq	statistic	p.value	Metabolite
Site	26.94286	26.34742	4.2532456	27.377462	2.18E-02	Methionine
Year	26.94286	26.24669	4.1113156	22.267432	3.00E-02	Methionine
Site:Year	26.94286	26.14595	3.9693856	17.157402	0.0382352	Methionine
Residuals	26.94286	26.04522	3.8274557	NA	NA	Methionine

Site	26.94286	25.94448	3.6855257	-7.777321	3.03E-01	Valine
Year	26.94286	25.84375	3.5435957	-19.67542	4.17E-01	Valine
Site: Year	26.94286	25.74301	3.4016657	-31.57353	0.5305363	Valine
Residuals	26.94286	25.64227	3.2597358	NA	NA	Valine
Site	26.94286	25.54154	3.1178058	-5.93786	4.31E-01	Isoleucine
Year	26.94286	25.4408	2.9758758	-14.59846	0.5931349	Isoleucine
Site: Year	26.94286	25.34007	2.8339458	-23.25905	0.7548778	Isoleucine
Residuals	26.94286	25.23933	2.6920159	NA	NA	Isoleucine
Site	26.94286	25.13859	2.5500859	-16.83745	2.53E-01	Trans.Hydroxyproline
Year	26.94286	25.03786	2.4081559	-32.4517	0.3471167	Trans.Hydroxyproline
Site:Year	26.94286	24.93712	2.2662259	-48.06595	0.4414319	Trans.Hydroxyproline
Residuals	26.94286	24.83639	2.124296	NA	NA	Trans.Hydroxyproline
Site	26.94286	24.73565	1.982366	-14.79872	7.65E-01	Asparagine
Year	26.94286	24.63492	1.840436	-26.4384	0.9778047	Asparagine

Site:Year	26.94286	24.53418	1.698506	-38.07807	1.1907441	Asparagine
Residuals	26.94286	24.43344	1.5565761	NA	NA	Asparagine
Site	26.94286	24.33271	1.4146461	-12.79969	2.58E-01	Tryptophan
Year	26.94286	24.23197	1.2727161	-24.22232	0.3486475	Tryptophan
Site:Year	26.94286	24.13124	1.1307861	-35.64496	0.4393362	Tryptophan
Residuals	26.94286	24.0305	0.9888562	NA	NA	Tryptophan
Site	26.94286	23.92977	0.8469262	-0.213366	1.20E+00	Proline
Year	26.94286	23.82903	0.7049962	-8.929239	1.65E+00	Proline
Site:Year	26.94286	23.72829	0.5630662	-17.64511	2.0963961	Proline
Residuals	26.94286	23.62756	0.4211363	NA	NA	Proline
Site	26.94286	23.52682	0.2792063	-8.691506	2.52E-01	C5
Year	26.94286	23.42609	0.1372763	-17.47517	0.2547517	C5
Site:Year	26.94286	23.32535	-0.004654	-26.25883	0.2573968	C5
Residuals	26.94286	23.22461	-0.146584	NA	NA	C5

Site	26.94286	23.12388	-0.288514	9.2866294	6.39E-03	Leucine
Year	26.94286	23.02314	-0.430444	5.7917603	8.78E-03	Leucine
Site:Year	26.94286	22.92241	-0.572374	2.2968911	0.011177	Leucine

term	Df	sumsq	meansq	statistic	p.value	Metabolite
Site	26.94286	22.82167	-0.714304	12.047372	4.64E-02	Methionine
Year	26.94286	22.72094	-0.856234	6.9373422	5.46E-02	Methionine
Site:Year	26.94286	22.6202	-0.998163	1.8273123	0.0628149	Methionine
Residuals	26.94286	22.51946	-1.140093	NA	NA	Methionine
Site	26.94286	22.41873	-1.282023	-43.47163	6.44E-01	Valine
Year	26.94286	22.31799	-1.423953	-55.36973	7.58E-01	Valine
Site:Year	26.94286	22.21726	-1.565883	-67.26784	0.8715931	Valine
Residuals	26.94286	22.11652	-1.707813	NA	NA	Valine
Site	26.94286	22.01579	-1.849743	-31.91965	9.17E-01	Isoleucine
Year	26.94286	21.91505	-1.991673	-40.58024	1.0783636	Isoleucine
Site:Year	26.94286	21.81431	-2.133603	-49.24084	1.2401065	Isoleucine

Residuals	26.94286	21.71358	-2.275533	NA	NA	Isoleucine
Site	26.94286	21.61284	-2.417463	-63.68021	5.36E-01	Trans.Hydroxyproline
Year	26.94286	21.51211	-2.559393	-79.29446	0.6300622	Trans.Hydroxyproline
Site:Year	26.94286	21.41137	-2.701323	-94.90871	0.7243774	Trans.Hydroxyproline
Residuals	26.94286	21.31064	-2.843253	NA	NA	Trans.Hydroxyproline
Site	26.94286	21.2099	-2.985183	-49.71775	1.40E+00	Asparagine
Year	26.94286	21.10916	-3.127113	-61.35743	1.6166228	Asparagine
Site:Year	26.94286	21.00843	-3.269043	-72.99711	1.8295622	Asparagine
Residuals	26.94286	20.90769	-3.410973	NA	NA	Asparagine
Site	26.94286	20.80696	-3.552903	-47.06759	5.30E-01	Tryptophan
Year	26.94286	20.70622	-3.694833	-58.49023	0.6207136	Tryptophan
Site:Year	26.94286	20.60548	-3.836763	-69.91287	0.7114024	Tryptophan
Residuals	26.94286	20.50475	-3.978693	NA	NA	Tryptophan
Site	26.94286	20.40401	-4.120623	-26.36098	2.55E+00	Proline

Year	26.94286	20.30328	-4.262553	-35.07686	2.99E+00	Proline
Site:Year	26.94286	20.20254	-4.404483	-43.79273	3.4440793	Proline
Residuals	26.94286	20.10181	-4.546413	NA	NA	Proline
Site	26.94286	20.00107	-4.688343	-35.04249	2.60E-01	C5
Year	26.94286	19.90033	-4.830273	-43.82615	0.2626868	C5
Site:Year	26.94286	19.7996	-4.972203	-52.60981	0.2653318	C5
Residuals	26.94286	19.69886	-5.114133	NA	NA	C5
Site	26.94286	19.59813	-5.256063	-1.197978	1.36E-02	Leucine
Year	26.94286	19.49739	-5.397993	-4.692847	1.60E-02	Leucine
Site:Year	26.94286	19.39666	-5.539923	-8.187716	0.0183616	Leucine
term	Df	sumsq	meansq	statistic	p.value	Metabolite
Site	26.94286	19.29592	-5.681853	-3.282718	7.10E-02	Methionine
Year	26.94286	19.19518	-5.823783	-8.392748	7.92E-02	Methionine
Site:Year	26.94286	19.09445	-5.965713	-13.50278	0.0873947	Methionine
Residuals	26.94286	18.99371	-6.107643	NA	NA	Methionine

Site	26.94286	18.89298	-6.249573	-79.16594	9.85E-01	Valine
Year	26.94286	18.79224	-6.391503	-91.06404	1.10E+00	Valine
Site:Year	26.94286	18.69151	-6.533432	-102.9621	1.2126499	Valine
Residuals	26.94286	18.59077	-6.675362	NA	NA	Valine
Site	26.94286	18.49003	-6.817292	-57.90143	1.40E+00	Isoleucine
Year	26.94286	18.3893	-6.959222	-66.56203	1.5635923	Isoleucine
Site:Year	26.94286	18.28856	-7.101152	-75.22262	1.7253352	Isoleucine
Residuals	26.94286	18.18783	-7.243082	NA	NA	Isoleucine
Site	26.94286	18.08709	-7.385012	-110.523	8.19E-01	Trans.Hydroxyproline
Year	26.94286	17.98635	-7.526942	-126.1372	0.9130078	Trans.Hydroxyproline
Site:Year	26.94286	17.88562	-7.668872	-141.7515	1.007323	Trans.Hydroxyproline
Residuals	26.94286	17.78488	-7.810802	NA	NA	Trans.Hydroxyproline
Site	26.94286	17.68415	-7.952732	-84.63679	2.04E+00	Asparagine
Year	26.94286	17.58341	-8.094662	-96.27646	2.255441	Asparagine

Site:Year	26.94286	17.48268	-8.236592	-107.9161	2.4683804	Asparagine
Residuals	26.94286	17.38194	-8.378522	NA	NA	Asparagine

Appendix Table 2.2. Results of the ANOVA Additive Model of the Metabolites with .65 Value of the first two Axes.

term	Df	sumsq	meansq	statistic	p.value	Metabolite
Site	3	18.79888	6.2662948	13.792381	1.06E-07	Methionine
Year	1	43.35576	43.355757	95.427862	1.33E-16	Methionine
Site:Year	3	4.86904	1.6230133	3.5723213	0.0163866	Methionine
Residuals	110	49.97632	0.4543302	NA	NA	Methionine
Site	3	41.92324	13.974414	25.263731	1.65E-12	Valine
Year	1	11.79596	11.795962	21.325403	1.06E-05	Valine
Site:Year	3	2.435245	0.8117483	1.4675241	0.2273712	Valine
Residuals	110	60.84555	0.5531414	NA	NA	Valine
Site	3	35.37865	11.792882	18.494036	8.73E-10	Isoleucine
Year	1	9.235279	9.2352791	14.483108	0.0002329	Isoleucine
Site:Year	3	2.243626	0.7478752	1.1728457	0.3234858	Isoleucine
Residuals	110	70.14245	0.6376586	NA	NA	Isoleucine
Site	3	51.91103	17.303677	32.849504	3.07E-15	Trans.Hydroxyproline
Year	1	4.584179	4.5841786	8.7026585	0.0038828	Trans.Hydroxyproline
Site:Year	3	2.561615	0.8538718	1.6210003	0.1886304	Trans.Hydroxyproline
Residuals	110	57.94317	0.5267561	NA	NA	Trans.Hydroxyproline

Site	3	45.75964	15.253213	24.215456	4.16E-12	Asparagine
Year	1	0.182894	0.1828945	0.2903567	0.5910809	Asparagine
Site:Year	3	1.768937	0.5896457	0.9361004	0.4258788	Asparagine
Residuals	110	69.28853	0.6298957	NA	NA	Asparagine
Site	3	44.6837	14.894568	24.498311	3.23E-12	Tryptophan
Year	1	2.423055	2.4230548	3.9853959	0.0483668	Tryptophan
Site:Year	3	3.015063	1.0050209	1.65304	0.1813774	Tryptophan
Residuals	110	66.87818	0.6079835	NA	NA	Tryptophan
Site	3	31.36042	10.453474	17.628432	2.05E-09	Proline
Year	1	20.06084	20.060841	33.830016	5.99E-08	Proline
Site:Year	3	0.349901	0.1166337	0.1966876	0.8984555	Proline
Residuals	110	65.22884	0.5929894	NA	NA	Proline
Site	3	40.79042	13.596808	22.039883	2.99E-11	C5
Year	1	0.07095	0.0709504	0.1150078	0.7351599	C5
Site:Year	3	8.277613	2.7592042	4.47256	0.0052901	C5
Residuals	110	67.86101	0.6169183	NA	NA	C5
Site	3	21.2081	7.0693676	11.541547	1.23E-06	Leucine
Year	1	20.05113	20.051127	32.735747	9.23E-08	Leucine
Site:Year	3	8.36415	2.78805	4.551809	0.004791	Leucine
Residuals	110	67.37662	0.6125147	NA	NA	Leucine
Site	3	27.34659	9.1155293	14.350149	5.89E-08	Phenylalanine
Year	1	14.62471	14.62471	23.022993	5.07E-06	Phenylalanine
Site:Year	3	5.154289	1.7180965	2.7047185	0.048906	Phenylalanine

Residuals	110	69.87441	0.6352219	NA	NA	Phenylalanine
Site	3	31.30477	10.434922	16.861514	4.42E-09	C3
Year	1	10.29799	10.297987	16.640244	8.58E-05	C3
Site:Year	3	7.322609	2.4408695	3.9441363	0.0102628	C3
Residuals	110	68.07464	0.6188603	NA	NA	C3
Site	3	8.808668	2.9362227	8.5954288	3.55E-05	C16.2
Year	1	69.32064	69.320639	202.9276	9.99E-27	C16.2
Site:Year	3	1.294384	0.4314612	1.2630494	0.2906849	C16.2
Residuals	110	37.57631	0.3416028	NA	NA	C16.2
Site	3	7.137715	2.3792382	8.1830009	5.76E-05	Arginine
Year	1	76.29922	76.299223	262.41871	6.68E-31	Arginine
Site:Year	3	1.58015	0.5267168	1.8115562	0.149257	Arginine
Residuals	110	31.98291	0.2907537	NA	NA	Arginine
Site	3	6.793565	2.2645216	7.7766539	9.33E-05	Lysine
Year	1	73.35737	73.357365	251.91848	3.24E-30	Lysine
Site:Year	3	4.817636	1.6058787	5.5147908	0.0014482	Lysine
Residuals	110	32.03143	0.2911949	NA	NA	Lysine
Site	3	3.836785	1.2789284	3.9830375	0.0097732	Taurine
Year	1	77.56274	77.562744	241.55795	1.61E-29	Taurine
Site:Year	3	0.28016	0.0933867	0.2908392	0.8319362	Taurine
Residuals	110	35.32031	0.3210937	NA	NA	Taurine
Site	3	16.44055	5.4801827	12.330106	5.16E-07	C18_2
Year	1	49.49112	49.491125	111.35228	2.10E-18	C18_2

Site:Year	3	2.17823	0.7260766	1.633632	0.1857384	C18_2
Residuals	110	48.8901	0.4444554	NA	NA	C18_2
Site	3	9.55252	3.1841733	8.6171519	3.46E-05	Malate
Year	1	64.96743	64.967431	175.81776	1.50E-24	Malate
Site:Year	3	1.833316	0.6111055	1.6538009	0.1812084	Malate
Residuals	110	40.64673	0.3695158	NA	NA	Malate
Site	3	13.81068	4.6035613	9.8112539	8.67E-06	C14.2OH
Year	1	50.40128	50.401278	107.41678	5.69E-18	C14.2OH
Site:Year	3	1.174681	0.3915604	0.8345057	0.4777101	C14.2OH
Residuals	110	51.61336	0.4692123	NA	NA	C14.2OH
Site	3	3.297489	1.0991631	1.9449568	0.1265484	C9
Year	1	50.49449	50.494488	89.349429	7.04E-16	C9
Site:Year	3	1.043174	0.3477247	0.615295	0.6065183	C9
Residuals	110	62.16485	0.565135	NA	NA	C9
Site	3	8.944712	2.9815708	10.297832	4.98E-06	Xanthine
Year	1	70.95849	70.958486	245.0784	9.29E-30	Xanthine
Site:Year	3	5.24808	1.74936	6.0419884	0.0007574	Xanthine
Residuals	110	31.84872	0.2895338	NA	NA	Xanthine
Site	3	4.310016	1.4366719	3.0173381	0.0329858	C5_1
Year	1	59.2319	59.231897	124.40047	8.78E-20	C5_1
Site:Year	3	1.082813	0.3609377	0.7580513	0.5200062	C5_1
Residuals	110	52.37527	0.4761389	NA	NA	C5_1
Site	3	6.346376	2.1154588	5.1698571	0.0022192	O.Phosphocholine

Year	1	62.76034	62.760341	153.37666	1.38E-22	O.Phosphocholine
Site:Year	3	2.882279	0.9607595	2.3479491	0.0765552	O.Phosphocholine
Residuals	110	45.011	0.409191	NA	NA	O.Phosphocholine
Site	3	5.818407	1.9394689	3.3344011	0.0221164	C5MDC
Year	1	46.00218	46.002179	79.088515	1.33E-14	C5MDC
Site:Year	3	1.197434	0.3991447	0.6862232	0.5623269	C5MDC
Residuals	110	63.98198	0.5816544	NA	NA	C5MDC
Site	3	13.35367	4.4512235	9.0663078	2.05E-05	Glutamate
Year	1	46.25654	46.256542	94.215905	1.84E-16	Glutamate
Site:Year	3	3.383835	1.1279451	2.2974128	0.0815566	Glutamate
Residuals	110	54.00595	0.4909632	NA	NA	Glutamate
Site	3	9.120932	3.0403107	5.8281056	0.0009846	C14.2
Year	1	48.44132	48.441317	92.859296	2.67E-16	C14.2
Site:Year	3	2.054753	0.6849175	1.3129486	0.2738782	C14.2
Residuals	110	57.383	0.5216636	NA	NA	C14.2
Site	3	4.67448	1.5581602	4.0537897	0.008942	Ornithine
Year	1	64.66022	64.660223	168.22337	6.64E-24	Ornithine
Site:Year	3	5.384461	1.7948203	4.669497	0.0041361	Ornithine
Residuals	110	42.28084	0.3843712	NA	NA	Ornithine
Site	3	10.20219	3.4007291	5.7976347	0.0010222	C16.2OH
Year	1	41.1712	41.171197	70.189523	1.95E-13	C16.2OH
Site:Year	3	1.103716	0.3679052	0.6272125	0.5989309	C16.2OH
Residuals	110	64.5229	0.5865718	NA	NA	C16.2OH

Site	3	7.601137	2.5337124	6.6697439	0.0003525	Hypoxanthine
Year	1	66.23234	66.232339	174.34999	1.99E-24	Hypoxanthine
Site:Year	3	1.379557	0.4598523	1.2105152	0.3093984	Hypoxanthine
Residuals	110	41.78697	0.3798815	NA	NA	Hypoxanthine
Site	3	6.292265	2.0974215	6.1065012	0.0006999	IMP
Year	1	72.25682	72.256823	210.37086	2.72E-27	IMP
Site:Year	3	0.668824	0.2229414	0.6490788	0.5851783	IMP
Residuals	110	37.78209	0.3434735	NA	NA	IMP
Site	3	7.015736	2.3385788	3.7796714	0.0126212	X18.1SM
Year	1	39.69536	39.695363	64.156671	1.30E-12	X18.1SM
Site:Year	3	2.229104	0.7430347	1.2009119	0.3129359	X18.1SM
Residuals	110	68.0598	0.6187254	NA	NA	X18.1SM
Site	3	1.544625	0.5148749	0.7822519	0.5063102	C6
Year	1	42.46359	42.463589	64.515132	1.16E-12	C6
Site:Year	3	0.590248	0.1967494	0.2989223	0.82611	C6
Residuals	110	72.40154	0.6581958	NA	NA	C6
Site	3	7.797532	2.5991773	6.2405798	0.0005942	Guanidoacetate
Year	1	63.15061	63.150614	151.62353	2.00E-22	Guanidoacetate
Site:Year	3	0.237281	0.0790935	0.1899022	0.9030814	Guanidoacetate
Residuals	110	45.81457	0.4164961	NA	NA	Guanidoacetate

Appendix Table 2.3. Model Analysis Comparison Table of Lake Winnipeg Walleye ANOVA Models

Metabolite	AIC Interaction	AIC_additive	delta_AIC	Interaction_p

Methionine	251.492	256.462	4.970	0.016
Valine	274.713	273.343	-1.369	0.227
Isoleucine	291.491	289.206	-2.285	0.323
Trans.Hydroxyproline	268.945	268.050	-0.895	0.189
Asparagine	290.046	287.020	-3.025	0.426
Tryptophan	285.868	285.071	-0.797	0.181
Proline	282.921	277.552	-5.369	0.898
C5	287.589	295.170	7.581	0.005
Leucine	286.744	294.552	7.808	0.005
Phenylalanine	291.039	293.437	2.398	0.049
C3	287.960	294.016	6.056	0.010
C16:2	217.841	215.837	-2.004	0.291
Arginine	198.823	198.513	-0.310	0.149
Lysine	199.001	209.535	10.533	0.001
Taurine	210.535	205.467	-5.068	0.832
C18:2	248.899	248.042	-0.856	0.186
Malate	227.109	226.315	-0.794	0.181
C14:2OH	255.295	251.950	-3.345	0.478
C9	277.244	273.208	-4.036	0.607
Xanthine	198.326	210.325	11.999	0.001
C5:1	257.024	253.439	-3.585	0.520
O-Phosphocholine	239.144	240.468	1.324	0.077
C5MDC	280.644	276.832	-3.812	0.562

Glutamate	260.642	261.813	1.171	0.082
C14:2	267.799	265.950	-1.849	0.274
Ornithine	231.760	239.905	8.145	0.004
C16:2OH	281.637	277.639	-3.999	0.599
Hypoxanthine	230.374	228.206	-2.167	0.309
IMP	218.485	214.556	-3.929	0.585
X18:1SM	287.934	285.737	-2.197	0.313
C6	295.232	290.190	-5.042	0.826
Guanidoacetate	241.232	235.841	-5.390	0.903

6.2 Chapter 3 Appendices

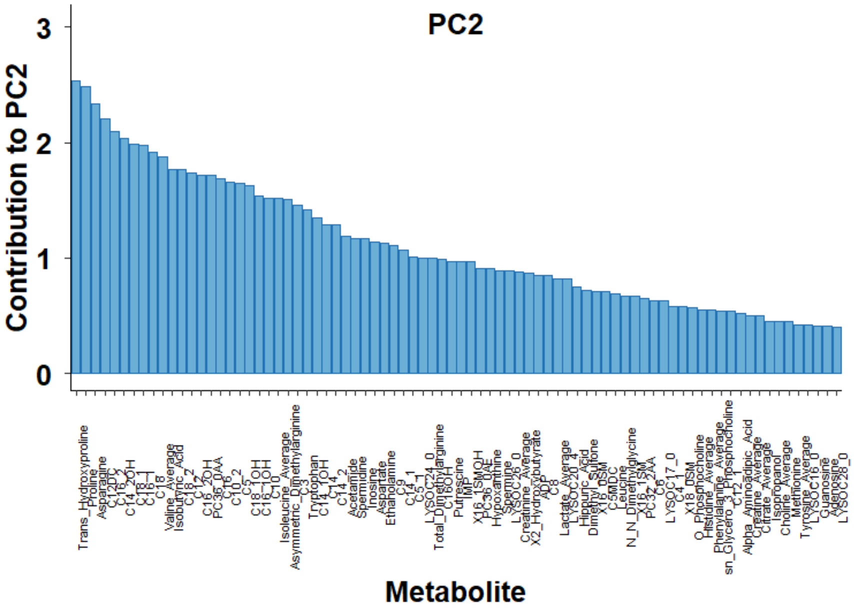


Fig. 3.1. Screeplot of metabolites1 found to have the highest contribution percent to the second dimension of the PCA analysis. The metabolites within this axis that showed a significant p-value on the Pearson test and had a correlation of >0.60. List of metabolites in graph: Dodecanedioylcarnitine, C12DC; phosphatidylcholine(36:0), PC36_0AA; hexadecadienoylcarnitine, C16_2; (Hydroxytetradecadienylcarnitine), C14_2OH;

(valerylcarnitine), C5; (hexadecenoylcarnitine), C16_1; (octadecenoylcarnitine), C18_1; (decadienylcarnitine), C10_2; (octadecanoylcarnitine), C18; (octadecadienylcarnitine), C18_2; (propionylcarnitine), C3; (hydroxyhexadecadienylcarnitine), C16_2OH; (hexadecanoylcarnitine), C16; (dodecanoylcarnitine), C12; (hydroxyoctadecenoylcarnitine), C18_1OH; (decanoylcarnitine), C10; hydroxysphingomyelin (16:0), (16:1SMOH), X16_1SMOH; (hydroxyhexadecenoylcarnitine), C16_1OH; (tiglylcarnitine), C5_1,; phosphatidylcholine (36:3), PC36_0AE; (tetradecenoylcarnitine), C14_1OH; sphingomyelin (16:0), sphingomyelin (16:0), X16_0SM; (tetradecanoate), C14; (nonaylcarnitine), C9; inosine monopphosphate, IMP; lysophosphocholine (20:4), LYSOC20_4; (tetradecadyenylcarntine), C14_2; sphingomyelin (16:1), x16_1SM; (octanoylcarnitine),C8; LYSOC24_0; (hydroxyhexadecanoylcarnitine), C16OH; phosphatidylcholine (32:2), PC32_2AA; (butenylcarnitine), C4_1; sphingomyelin (12:0) X12_OSM; lysophosphocholine (26:0), LYSOC26_O; (methylglutarylcarnitine), C5MDC; (hexanoylcarnitine), C6; lysophosphocholine (17:0), LYSOC17_O; phosphatidylcholine (38:0), PC38_OAA; lysophosphocholine (16:0), LYSOC16_O; hydroxysphingomyeline (C22:2), X22_2SMOH. 2Hexenoylcarnitine, C6_1; lysophosphocholine (18:0), LYSOC18_O; sphingomyelin (18:0), X18_OSM; phosphocholine (0-32:2), PC32_2AA; phosphocholine (40:6), PC40_6AA; X16-1SMOH; hydroxysphingomyelin (22:2), X22_2SMOH; sphingomyelin (20;2), X20_2SM; hydroxysphinmyelin (14:1), X14_1SMOH; sphingomyelin, (16:1), X16-1SM; sphingomyelin (16:0), X16_OSM. Hexenoylcarnitine, C6_1; lysophosphocholine (18:0), LYSOC18_O; sphingomyelin (18:0), X18_OSM; phosphocholine (0-32:2), PC32_2AA; phosphocholine (40:6), PC40_6AA; X16-1SMOH; hydroxysphingomyelin (22:2), X22_2SMOH; sphingomyelin (20;2), X20_2SM; hydroxysphinmyelin (14:1), X14_1SMOH; sphingomyelin, (16:1), X16-1SM; sphingomyelin (16:0), X16_OSM.

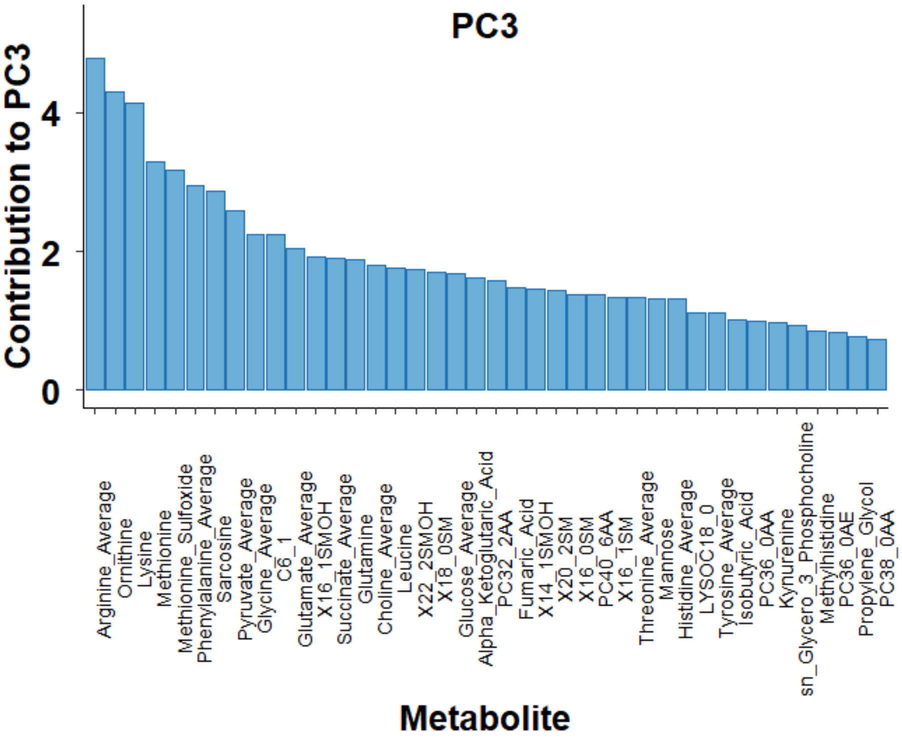


Figure 3.4. Screeplot of metabolites found to have the highest contribution percent to the third dimension of the PCA analysis. List of new metabolites shown in this graph: Hexenoylcarnitine, C6_1; lysophosphocholine (18:0), LYSOC18_O; sphingomyelin (18:0), X18_OSM; phosphocholine (0-32:2), PC32_2AA; phosphocholine (40:6), PC40_6AA; X16-1SMOH; hydroxysphingomyelin (22:2), X22_2SMOH; sphingomyelin (20;2), X20_2SM; hydroxysphinmyelin (14:1), X14_1SMOH; sphingomyelin, (16:1), X16-1SM; sphingomyelin (16:0), X16_OSM.

6.3 Chapter 4 Appendix 1

Primer Sequences for the Quantitative PCR

Sn_FSH_F1
Sequence (5' to 3'): GGCATGTAACTTCAAGGAGTGG
Type: Primer
Length: 22
%GC: 50.0
Hairpin Tm: None

Self Dimer Tm: None
Tm: 59.3
created by: primer3

Sn_FSH_R1
Sequence (5' to 3'): TTGGCTACGGGTATGAAGAAGG
Type: Primer
Length: 22
%GC: 50.0
Hairpin Tm: None
Self Dimer Tm: None
Tm: 59.8
created by: primer3

Sn_Ox_F1
Sequence (5' to 3'): TTGCAGACAGAAATCCCACTCC
Type: Primer
Length: 22
%GC: 50.0
Hairpin Tm: None
Self Dimer Tm: None
Tm: 60.6
created by: primer3

Sn_Ox_R1
Sequence (5' to 3'): CCGTCCCATCACCTGAGC
Type: Primer
Length: 18
%GC: 66.7
Hairpin Tm: None
Self Dimer Tm: None
Tm: 59.8
created by: primer3

Tel1b
Sequence (5' to 3'): CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTGGGTT
Type: Primer
Length: 39
%GC: 48.7
Hairpin Tm: None
Self Dimer Tm: None
Tm: 68.1

Tel2b

Sequence (5' to 3'): GGCTTGCCTTACCCTTACCCTTACCCTTACCCTTACCCT
Type: Primer
Length: 39
%GC: 53.8
Hairpin Tm: None
Self Dimer Tm: 1.2
Tm: 67.4

6.4 Chapter 4 Appendix 2

Tissue PCR Differences and PCR Efficiency and Slopes

Correlation Tests

Appendix Table 4.3. Table of correlation coefficients and p-values for telomere length value results comparison. * Shows significant p-value in the correlation test.

	Liver values	**P-values**	**RBC values**	**P-values**	**Heart values**	**P-values**
Liver values	1.00	NA	0.12	0.53	-0.38	0.038*
RBC values	-0.12	0.53	1.00	NA	0.13	0.50
Heart values	0.38	0.038*	0.13	0.50	1.00	NA

Standard Curves	**RBC**		
	Slope	Efficiency	Eff %
FSH	-3.3	2.01	100.92
TELB	-3.3	2.01	100.92
OX	-3.3	2.01	100.92

Standard Curves	**LIVER**

	Slope	Efficiency	Eff %
FSH	-3.4	1.97	96.84
OX	-3.4	1.97	96.84
TELB	-3	2.15	115.44

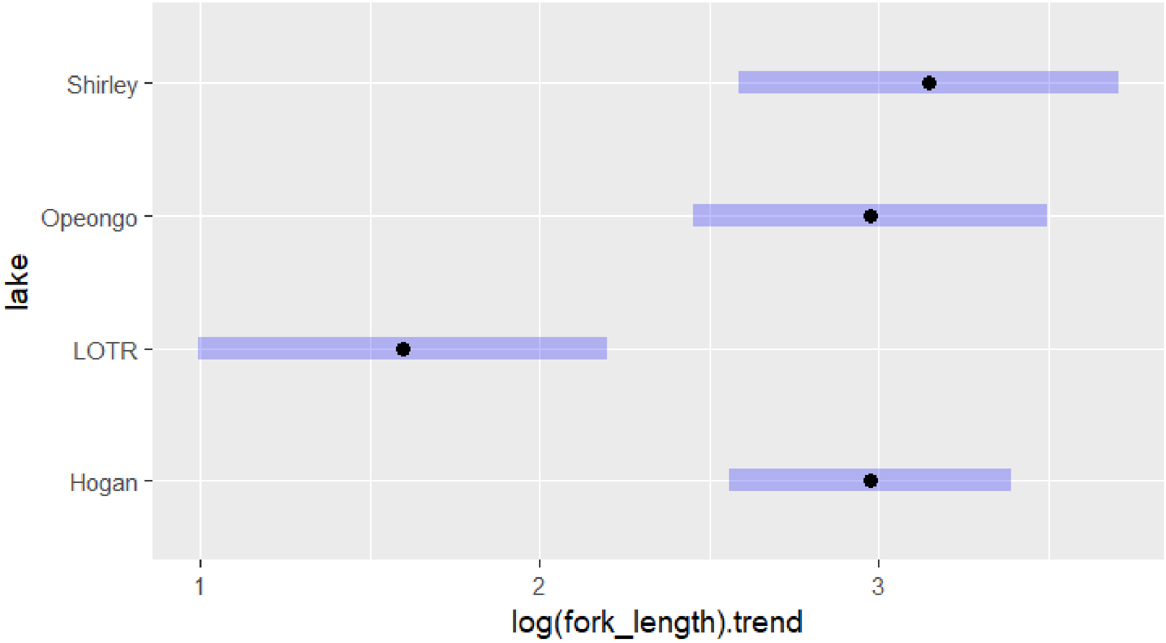
Standard Curves	**HEART**

	Slope	Efficency	Eff %
TELB	-3.4	1.97	96.84
FSH	-3.3	2.01	100.92
OX	-3.3	2.01	100.92

Tissue Optical Density Differences

Correlation Test Optical Density

Pearson's product-moment correlation showed a p-value = 0.16, and the R2=0.21



Appendix Fig 4.13, Emtrends graphs of the r plot(emtrends(lnmodel_growth_mfli, ~ lake, var = "log(fork_length)")), showing the slope of fork-length per lake pair differences to the intercept, Lake Hogan, for the interaction model.