UiT The Arctic University of Norway

Department of Arctic and Marine Biology

The effect of endurance training on mitochondrial function in Siberian huskies and Alaskan huskies

Field study

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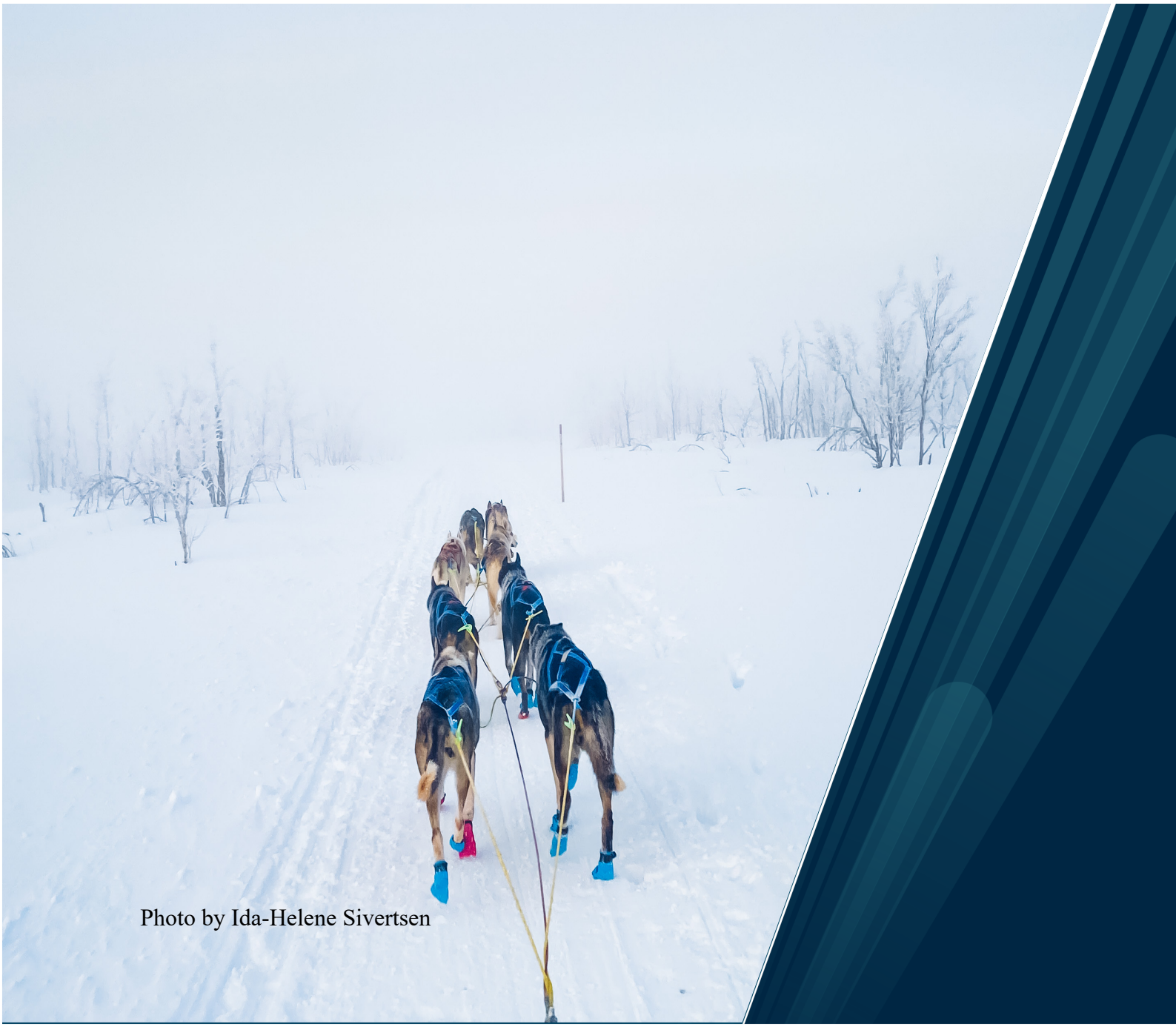

Photo by Ida-Helene Sivertsen

University of Tromsø
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Department of Arctic and Marine Biology

The effect of endurance training on mitochondrial function in Siberian huskies and Alaskan huskies

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Kennels:
Snykovet Siberian Husky Kennel
Mjaatvedt Husky

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Abstract

Teams of Alaskan huskies and Siberian Huskies are used to pull sleds and compete in long-distance races such as Finnmarksløpet (1200 km) and the Iditarod (1600 km). That is an elite athlete performance, and their ability to conduct such extreme endurance challenges under arctic conditions is poorly understood. A previous study has shown that Alaskan huskies possibly have the highest values of mitochondrial oxidative phosphorylation (255±38 pmol/s*mg) and electron transfer system (254±37 pmol/s*mg) ever recorded in a mammalian skeletal muscle. This current study aimed to determine respiratory capacity in skeletal muscles from Siberian huskies and Alaskan huskies, off-season (August) and at the end of the racing season (March/April) to highlight physiological adaptations in the respiratory capacity of skeletal muscles arising as a result of selective breeding amongst huskies, as well as potential difference in the acclimatization responses to endurance training. Micro biopsy samples from *M. biceps femoris* were taken from non-raced and raced Siberian huskies and Alaskan huskies (2022–2023), and tissue homogenates were prepared for high resolution respirometry analyzes as well as citrate synthase activity assays to determine mitochondrial density. Respiratory capacity in skeletal muscle mitochondria from Siberian huskies and Alaskan huskies was higher during racing season as compared to off-season. Group mean values of electron transfer system (E) and oxidative phosphorylation (P) for were (P: 175.2±89.5, E: 164.2±92.1 pmol/s*mg) for raced Alaskan huskies and (P: 190.4±52.9, E: 180.0±56.7 pmol/s*mg) for raced Siberian huskies. The increase in aerobic capacity possibly results from a measured increase in muscle mitochondrial density, indicating a clear acclimatization response to endurance training. Alaskan huskies were shown to have higher respiratory capacity in their skeletal muscles as compared to that of Siberian huskies, based on carbohydrates associated substrates, possibly due to difference in their genetics. This was shown by a higher carbohydrate associated mitochondrial capacity for ETS (E) and OXPHOS (P) for non-raced Alaskan huskies (E: 63.0±45.5, P: 56.0±36.3 pmol/s*mg) as compared to non-raced Siberian huskies (E: 24.39±18.30, P: 23.5±16.2 pmol/s*mg). Non-raced Alaskan huskies had large sex differences in oxygen consumption which may be explained by mitochondrial substrate preference. This study is the first to determine mitochondrial respiration in Siberian huskies, while the mitochondrial respiration determined in the Alaskan huskies are in line with the earlier findings. Future studies should aim at investigating sexual dimorphism preference as well as substrate preference for Siberian husky mitochondria.

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1 Introduction

Endurance is a commonly used term that most people understand but struggle to define consistently. In this thesis Endurance is defined as the capacity to sustain submaximal physical work for a prolonged period of time (Carey, 1983). Therefore, this study will primarily focus on submaximal work/speeds, and not max-speed, or sprinting. Endurance performance and its capacity in mammals is highly heritable and plastic (Bennett, 1991; Langlois, 1980). Underlying factors such as an individual's physiology, morphology and biochemical processes can limit or enhance their endurance capacity (Bennett, 1989). All these factors are needed to have the potential for being selected upon (Bennett, 1991). The underlying factors also determine acclimatizational processes as a response to life history and energetic and environmental challenges.

Sustained activity depends on the rate at which adenosine triphosphate (ATP) can be generated from metabolic pathways to support skeletal muscle contractions. With increased activity the oxygen demand rises because oxygen is essential for aerobic ATP production (detailed explanation in section 3.1.3). The increase in oxygen consumption primarily depends on the activity level or the locomotor mode (walking, running, swimming etc.) (Schmidt-Nielsen, 1972; Tucker, 1975). An animal's ability to sustain activity is regulated by the upper limit of oxygen consumption, which is termed maximal oxygen consumption (VO_2 max).

In general, there is a correlation between body size, body temperature and athletic ability to an individual's maximal oxygen consumption and endurance (Schmidt-Nielsen, 1972; Taylor, 1982). The evolutionary changes in an animal´s ability to consume oxygen therefore control the evolution of its endurance capacity (Bennett, 1991). Dogs and dog breeding represents an interesting case for the study of these mechanisms.

Domestic dogs are believed to be dated back to approximately 33,000 years ago, when dog-ancestors and wolf populations first started to diverge in southeast Asia, and secondly, 15,000 years ago dogs from southeast Asia were distributed worldwide and contributing to domestication (Savolainen, 2007; Wang et al., 2016). Selective breeding, or as Darwin stated "Man´s power of selection", is a process in which humans deliberately choose certain individuals with desirable traits to breed (Darwin, 1859). This process differs from natural evolution because it is driven by human intervention. Therefore, selective breeding, and not natural evolution, is a fitting term when speaking of specific changes within or amongst different dog breeds. Two Japanese dog breeds, Shiba and Akita, are perhaps the oldest and

are classified as ancient breeds, dating back between 14,500-300 BC (Tanabe, 1991). One lineage of dogs that traces back ≈ 9,500 years (Pitulko & Kasparov, 2017; Sinding et al., 2020) that is of particular interest with regard to endurance, is the arctic sled dogs.

Arctic sled dogs, bred to survive in harsh environments, were mainly used for hunting, transportation, and communication in remote areas of the Arctic (Chernova et al., 2016; Wendt, 1999). Their ability to conduct physical work over long distances in arctic climates made them the perfect tool for human societies because they could expand their reach into the wilderness by sledding (Chernova et al., 2016). These dogs did not need the luxury of clothes or other human-made tools to withstand the climatic conditions. Instead, they thrived in their own coat. Using a sled dog team to pull sleds was an efficient way to travel before airplanes, snowmobiles, and roads. Today, sled dogs are still used to travel into remote areas in Arctic and sub-Arctic regions and for recreational purposes, including racing. The Iditarod Trail Sled Dog Raced, more commonly known as The Iditarod, in Alaska is the longest sled dog race in the world, covering 1,600 km from Anchorage to Nome. The race was initially developed as a tribute to the mushers (someone who rides the sled behind a sled dog team) who conducted this journey on sleds to deliver diphtheria antitoxin for sick children, during the diphtheria epidemic in wintertime of 1925 (Thomas, 1998). Finnmarksløpet is the longest sled dog race in Europe, covering 1,200 km in the arctic landscape of Finnmark, Norway. During long-distance races such as these, dogs must be able to perform prolonged submaximal exercise in harsh environments with minimal rest and recovery periods. The fastest teams conduct the 1,200 km track in Finnmarksløpet in around six days, with a team of fourteen dogs from the start (https://portal.finnmarkslopet.no/results/list, 2022). This is an elite athlete performance, and their ability to conduct such extreme endurance challenges under the challenging arctic conditions is poorly understood.

1.1 Siberian husky

The Siberian husky (SH) is one of five modern sled dog breeds, originating from Siberia, where they were bred to pull sleds for the Chukchi people (Hinkemeyer et al., 2006). The exact timeline is not known, but the oldest Eskimo settlements containing dog bones dates back 8000 years (Zhokhov island) located on the Chukchi peninsula (Chernova et al., 2016).

In the early 1900s, sled dogs were imported to Alaska by the Russian William Gosak to race in the All-Alaska Sweepstakes Race 1909, the first sled dog race in the world with records back to 1908. In the following years, the swift, little foxy looking dogs imported from Siberia placed in the top and became famous for their speed and endurance while being smaller than their competitors (Thomas, 1998).

A team of the best dogs was put together for Roald Amundsen in 1913 to join him in his pursuit for the North pole. Another Norwegian, Leonhard Seppala, trained the team for Amundsen and ended up keeping and racing them after the expedition to the north pole was canceled. Seppala won the Sweepstakes race in 1915, 1916 and 1917 with his dogs, but became most famous for the Great serum race in 1925 (Thomas, 1998).

Seppala´s team of sled dogs continued to impress around the world by winning various long-distance races. Seppala bred his dogs based on performance, and in the 1930, the American Kennel Club officially recognized the Siberian husky as a new breed/pedigree dog: "Siberian husky, a thickly coated, compact sled dog of medium size and great endurance, was developed to work in packs, pulling light loads at moderate speeds over vast frozen expanses" (AKC, 1997, 2022). Seppala closed his kennel in 1931, and all the registered Siberian huskies today trace their lineage back to Leonhard Seppala and collaborating kennels (Thomas, 1998).

1.2 Alaskan husky

"Alaskan husky is a collective term for an animal with four legs and a tail that is willing to pull a sled" a musher once said (Hagen & Begerdal, 2000). But to narrow the definition down a bit, Alaskan huskies (AH) are not pedigree dogs with a breed standard, they are recognized as "a population of dogs with Northern breed ancestry specifically developed as working dogs to haul cargo-laden sleds across the Arctic terrain" (Collins 1991; Rennick 1987). Alaskan huskies are selected and bred based on performance, endurance, and work ethics, sharing a common genetic core established through generations of breeding for racing rather than appearance (Huson et al., 2010). Molecular studies show that within Alaskan huskies, increased number of genes from Siberian huskies and Alaskan Malamutes are associated with enhanced endurance, Pointer and Saluki with enhanced speed, and the Anatolian Shepherd demonstrates a positive influence on work ethics (**Fig. 1**) (Huson et al., 2010).

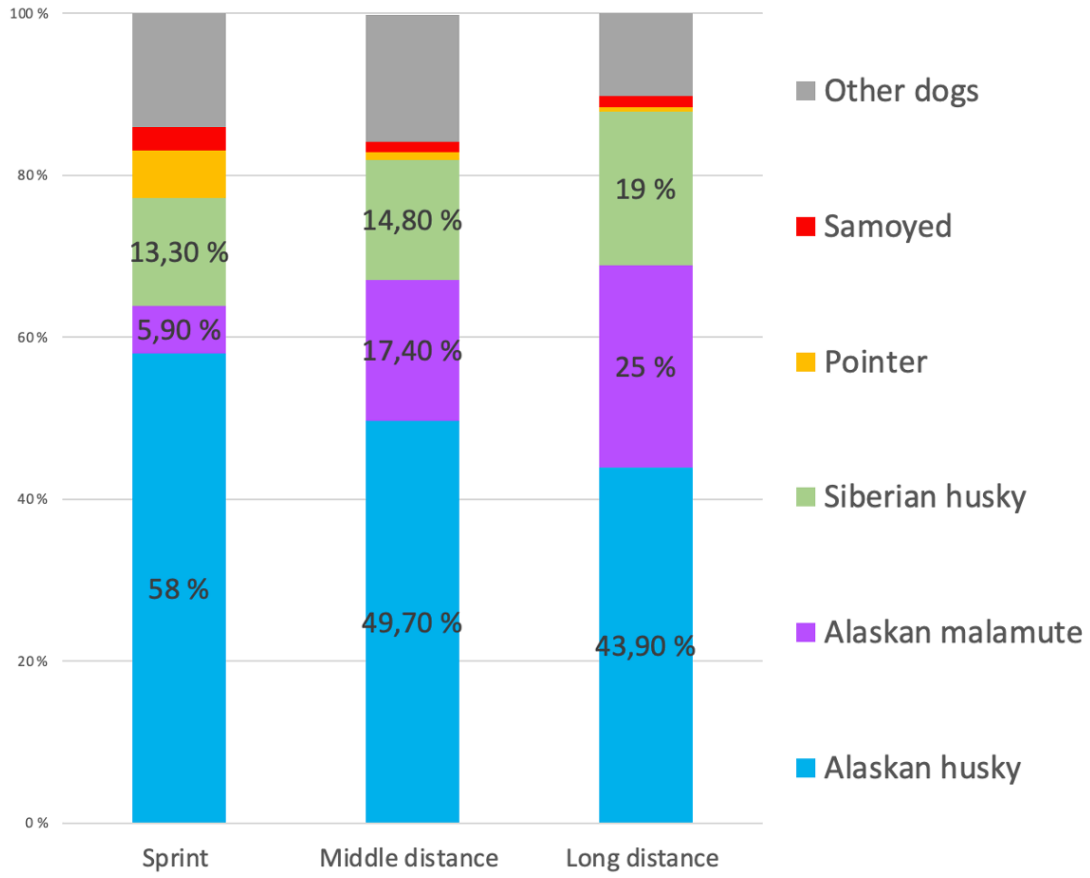

Figure 1. *Genetic composition/makeup of three populations of Alaskan huskies (n=199 blood samples from Alaskan huskies in high performance racing kennels), based on DNA cluster (similar gene expression) analysis of 96 previously described microsatellite-based markers. Huson et al. (2010) compared Alaska husky genotyping data in a cluster to related breeds, and their data did not align with any specific breed but instead aligned within their own group of Alaskan huskies, which they called "Alaskan husky signature". The three populations are divided by racing style, sprint, middle distance, and long-distance. The percentage of different breeds/dog type in the genetics of these three populations of Alaskan huskies are shown on the y-axis in different colors, and the x-axis represents the three groups. The Alaskan husky signature is displayed as "Alaskan husky". Figure modified from Huson et al. (2010).*

1.2.1 Alaskan huskies and Siberian huskies

Alaskan huskies are elite endurance athletes, as evidenced by their ability to maintain high speeds, up to about 16 km/h on long distance races such as Finnmarksløpet (http://results.finnmarkslopet.no/rs/results.jsp). Siberian husky teams also compete in long-distance races but are nowadays outcompeted by the Alaskan husky athletes, as demonstrated in **Table 1**.

***Table 1**. Finnmarksløpet 600 km, results from 2023, 2022 and 2020 to compare the fastest Alaskan huskies (AH) and Siberian huskies (SH) teams. *Difficult to extract the exact number of Siberian husky teams. https://portal.finnmarkslopet.no/results/list*

Year	First place	First SH team, RNB	Total number of AH and SH teams that finished
2023	AH: 2 d, 15 h, 33 min	SH. 6th place, 2 d, 20 h, 26 min	AH: 33 SH: 9
2022	AH: 2 d, 15 h, 46 min	SH. 18th place, 3 d, 2 min	AH: 35 SH: 4
2020	AH: 2 d, 19 h, 34 min	SH. 30th place, 3 d, 7 h, 41 min	AH: 51 SH: 2*

There may be several reasons why Alaskan husky teams are outcompeting the Siberian husky teams in most races. Few mushers compete in long distance races with teams of Siberian huskies, and they often have smaller kennels/fewer dogs to choose between compared to the professional, winning Alaskan husky mushers. There is no accessible database providing detailed information about the actual number of Alaskan huskies and Siberian huskies in Norway, which makes it difficult to compare the two.

Siberian huskies are purebred pedigree dogs, and even though the racing lines of the breed varies quite a lot in exterior – as breeding has focused on function more than "looks" – they still originate from the same "closed" gene pool and relate to the same breed standard as the show lines of the breed. Alaskan huskies on the other hand, are a mixture of different dog breeds selected primarily on performance – some polar breeds and some faster types of dogs including Greyhound, Pointers, and Australian Shepard – resulting in larger variations in genetics and hence exterior and function, as compared to Siberian huskies (Thorsrud & Huson, 2021). It appears that 100 years of selection has greatly influenced endurance and speed in these two "populations" of sled dogs, and their physiology and gene composition might reveal answers behind their extraordinary capacity.

1.3 Muscle physiology

Skeletal muscles consist of muscle fibers/cells, which are the force behind an animal's voluntary movements (**Fig. 2**). The movement is a result of the contractile elements of the muscles, called sarcomeres (Hill et al., 2016). The sarcomeres, which are the basic contractile

units of muscle fibers, are connected end-to-end in large numbers and make up the myofibril. The myofibril, in turn, spans the entire length of the muscle fiber and is responsible for the muscles ability to contract and generate force (Hill et al., 2016). The contractile sarcomeres are an assembly of proteins and protein fibers, and the movement of these filaments requires both Ca^{2+} and ATP. Filaments of myosin is one of the protein fibers, and it contains the binding site for ATP. Myosin therefore serves as an enzyme, hydrolyzing ATP into ADP and inorganic phosphate, thereby converting the chemical energy from ATP into mechanical force in the muscle contraction. Actin is another protein fiber in the sarcomere, which also consists of regulatory proteins, troponin, and tropomyosin. Troponin is the binding site for Ca^{2+}, which is released from the sarcoplasmic reticulum after the muscle fibers receive an action potential. With ATP present on the myosin filament, Ca^{2+}bound to troponin through tropomyosin will expose the binding site for myosin on the actin filament. Myosin can bind to actin, which allows the sarcomere to shorten/contract (Hill et al., 2016).

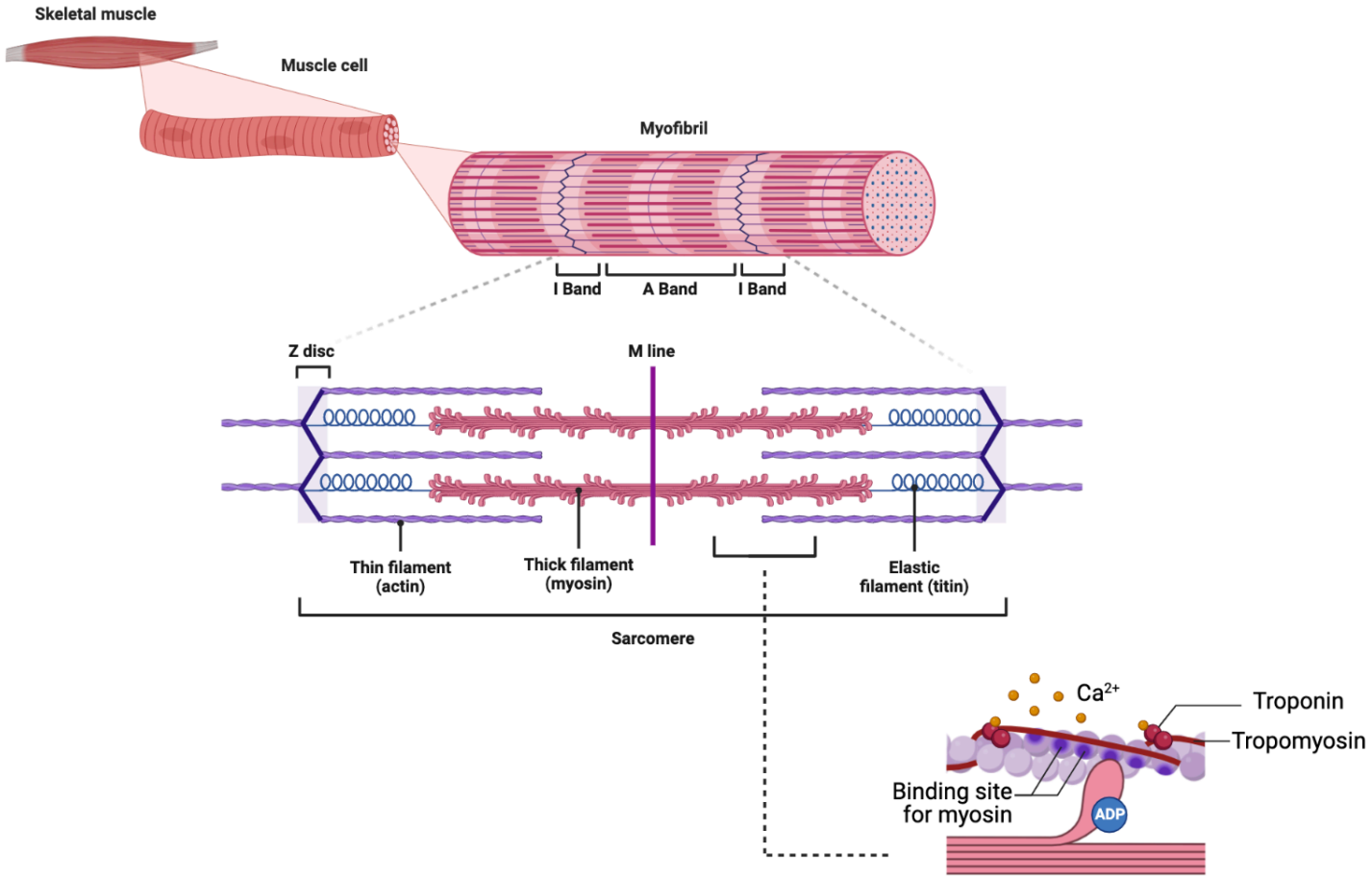

Figure 2. *Skeletal muscle and its structural components. A muscle fiber contains several myofibrils, which are made of repetitive sections of contractile elements called sarcomeres. Sarcomeres contain actin (thin filament) with regulatory proteins tropomyosin and troponin, which contain the binding site for calcium. Myosin (thick filament) contain the binding site for ATP. Figure created in BioRender.com*

In other words, for a muscle to contract, ATP and Ca^{2+} must be available, and for a muscle to sustain contractions, both are required in large amounts. In general, muscles contain large numbers of mitochondria that are responsible for various functions in the cell including oxidative phosphorylation and maintaining Ca^{2+} homeostasis, and are connected to the surrounding bundles of myofibrils in a network structure (Hill et al., 2016).

1.3.1 Muscle fiber types and characteristics

Skeletal muscles are composed of different types of fibers, which support functions like posture and static and explosive movements. These fibers are divided into Slow oxidative (Type I), Fast oxidative glycolytic (Type IIa) and Fast glycolytic (Type IIx) fibers, based on their different physiological abilities in ATP production (Hill et al., 2016). Slow oxidative fibers (Type 1) make up enduring muscles which have a high ATP demand and a large number of mitochondria compared to the more explosive, fast glycolytic fibers (Type IIx) (Hill et al., 2016). The enduring muscle fibers are thinner, have a higher density of surrounding capillaries, higher myoglobin content and are more fatigue resistant, compared to the other two types of muscle fibers. The fast oxidative glycolytic fibers (Type IIa) are most easily explained as an intermediate fiber type, between enduring (Type I) and explosive (Type IIx) fibers. This range of muscle fibers allows for different capabilities of the muscle (Hill et al., 2016).

Sled dog skeletal muscle composition is dominated by highly oxidative Type I and Type IIa fibers, which stores less glycogen compared to fast fibers and more triglycerides (Guy & Snow, 1981; McKenzie et al., 2005; van Boom et al., 2023). *Musculus biceps femoris* (**Fig. 3**) is a large and superficial muscle located on the hindlimb of the dog, highly involved in locomotor activity. It consists of a larger proximal part which originates from the *Ischial tuberosity* and the *Sacrotuberous* ligament of the hip. It extends towards the knee with its distal part, attaching to the *Fascial lata*, the *Patella* and the *Tibial crest*. The *M. biceps femoris* also projects a tendon to the *Calcaneus* (Achilles) (Carpenter Jr & Cooper, 2000).

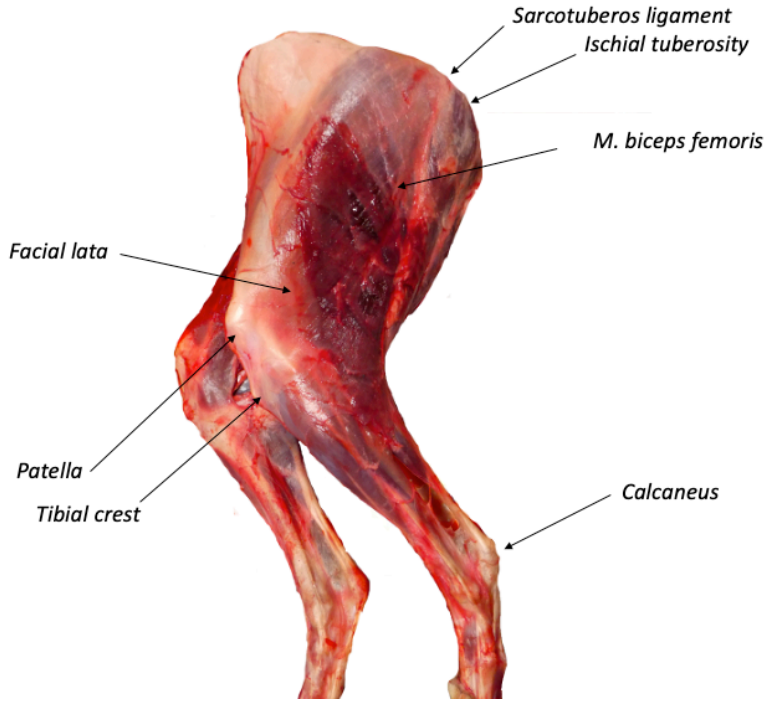

***Figure 3**. Modified necropsy picture from The Norwegian Veterinary Institute, showing the hindlimb of a Husky.*

The function of this muscle includes the extension of the hip joint, the innervation of the hindlimb and as a flexor of the tarsal joint (Carpenter Jr & Cooper, 2000; Evans & De Lahunta, 2013; Robins, 1990). In long distance sled dogs, the *M. biceps femoris* is an endurant muscle involved in every movement of the dog and is therefore of investigational interest in terms of mitochondrial respiration.

1.4 Mitochondrial respiration

Mitochondrial respiration is a key factor to understand how sled dogs handle extreme endurance challenges. Mitochondria were first discovered in the 19th century and were later found to have a key role in cellular energy production (Chance & Williams, 1956; Lewis & Lewis, 1915; Mitchell, 1961). The mitochondria can be described as a bioenergetic factory transferring energy from metabolic fuels into molecules of ATP (**Fig. 4**).

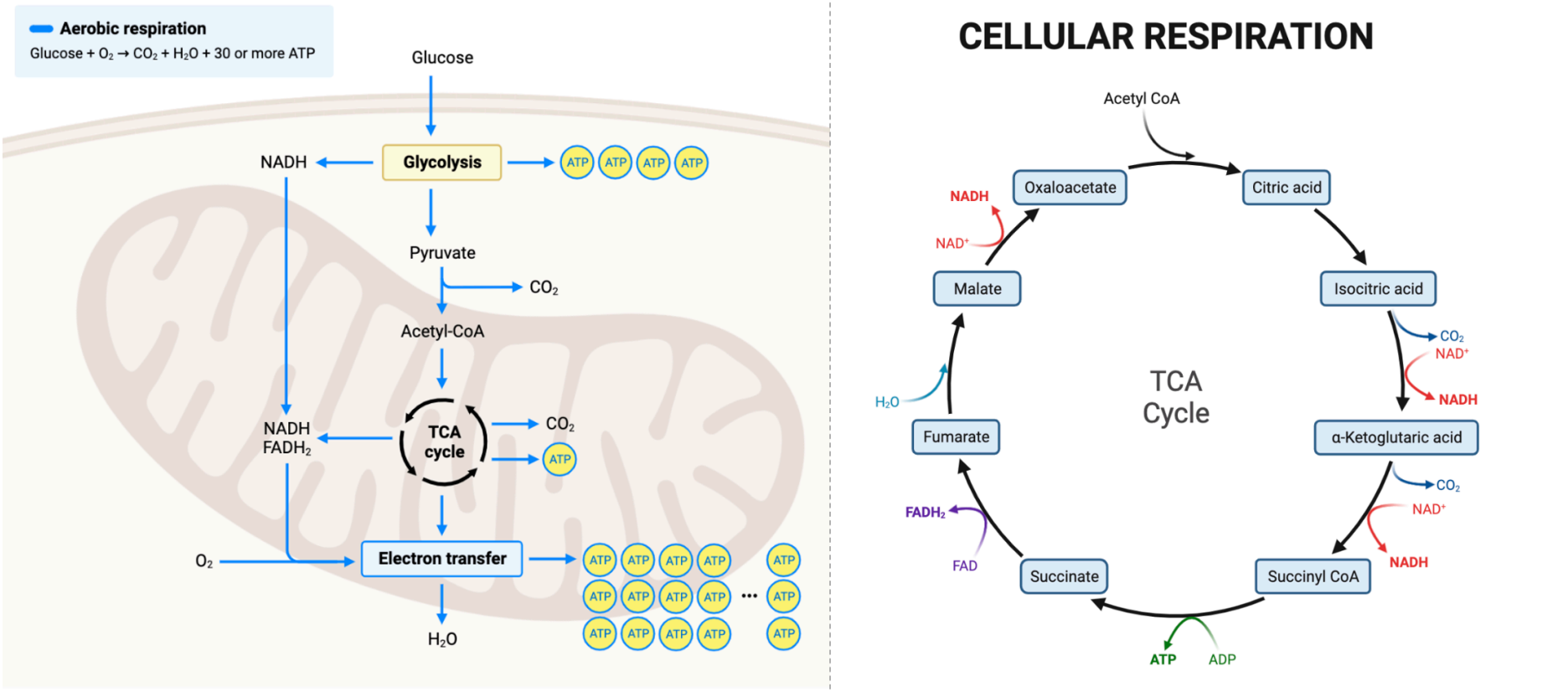

Figure 4. *Insulin released from pancreas directs muscle cells to take up glucose from the bloodstream, and one glucose molecule can be split into two molecules of pyruvate by a process called glycolysis. Glycolysis occurs in the cytosol and does not require oxygen. Pyruvate, a product of glycolysis, can then enter the mitochondria via a carrier protein, to be oxidized by a series of enzymatically catalyzed reactions called the tricarboxylic acid cycle (TCA-Cycle). Figure created in BioRender.com*

There are four major sets of reactions involved in aerobic catabolism; 1. Glycolysis, 2. Tricarboxylic acid cycle (TCA-Cycle), also called Krebs cycle, 3. Electron transport chain, and 4. Oxidative phosphorylation. These pathways can typically oxidize all major classes of foodstuff, meaning carbohydrates, amino acids, and fatty acids. Glycolysis is the first step, splitting glucose into two molecules of pyruvate by a series of enzymatically catalyzed reactions in the cytosol. Nicotinamide adenine dinucleotide (NAD+) is reduced by electrons and protons removed from glycolysis, serving as an intermediate electron acceptor (NADH) (**Fig. 4**). Pyruvate can then enter the mitochondria, where it will be oxidized into acetyl coenzyme A (A-CoA), which will continue in a cyclic series of enzymatically catalyzed reactions called the TCA-cycle (**Fig. 4**). Electrons and protons removed from the TCA-Cycle are transferred to NAD+ and Flavin adenine nucleotide (FAD+), reducing them to $NADH$ and $FADH_2$.

During aerobic respiration, oxygen is essential in the oxidation of the immediate electron acceptors, $NADH$ and $FADH_2$, because it serves as the final electron acceptor. Electrons from immediate acceptors feed into different respiratory complexes of the membrane bound **Electron Transfer System (ETS),** (**Fig. 5**). The oxidation of NADH and $FADH_2$ to NAD+ and FAD+ will make them available for accepting new electrons and protons. The cyclic series can therefore sustain, as long as oxygen is available (Hill et al., 2016).

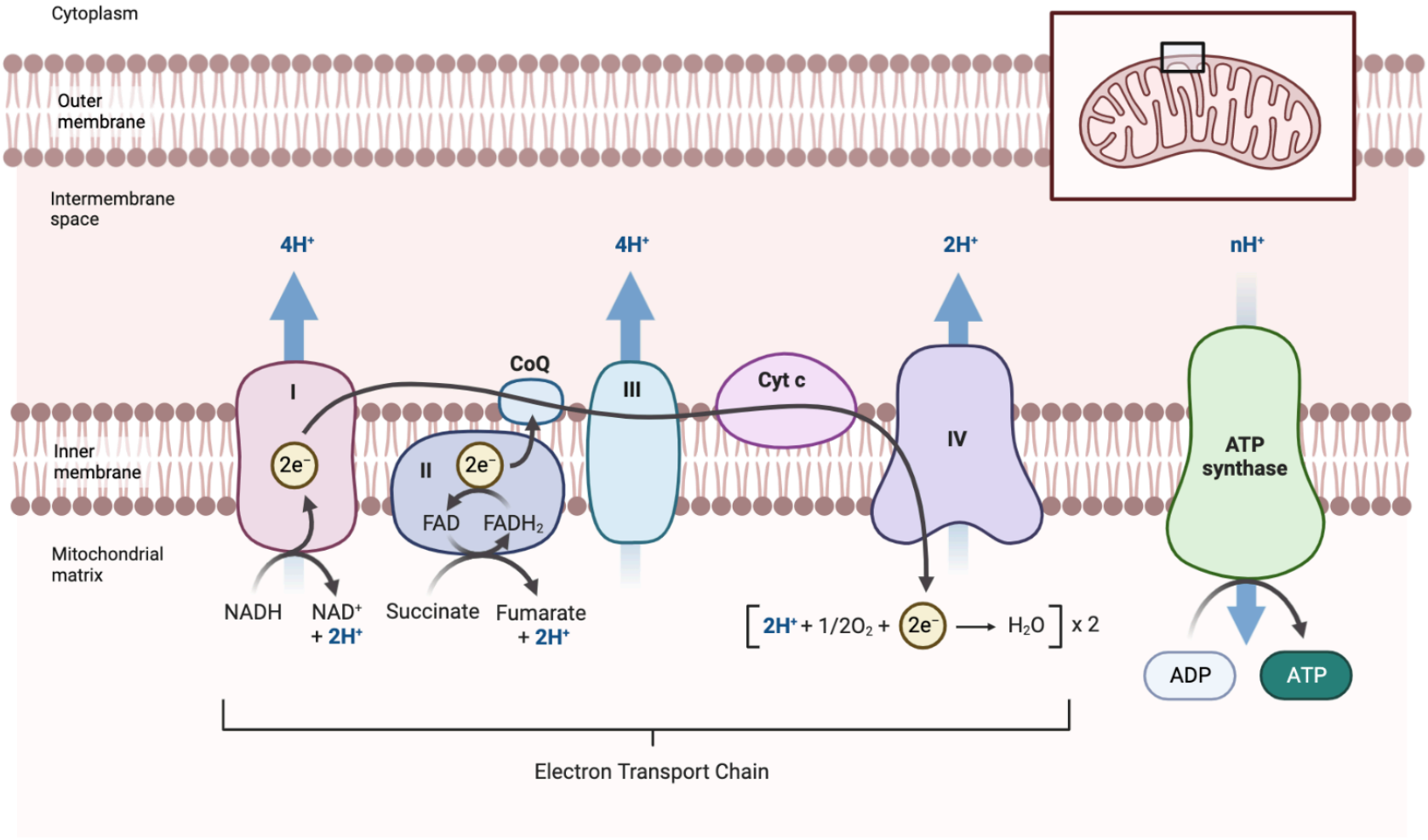

Figure 5. *The electron transport system including membrane bound complexes 1-4, molecules, ubiquinone, and cytochrome C, as well as the ATP-synthase (F1F0). Figure created in BioRender.com*

The electron transfer system is bound to the inner mitochondrial membrane and consists of different complexes and molecules (**Fig 5**). **Complex 1 (CI)** is a NADH-dehydrogenase that accepts electrons from NADH, regenerating NAD+ for a new electron pickup. CI transfers electrons to ubiquinone (CoQ), which then freely diffuses within the membrane, enabling CI to translocate protons away from equilibrium from the mitochondrial matrix to the intermembrane space, creating a proton gradient (Mitchell, 1961). The NADH electron transfer pathway (N-pathway) is linked to CI, and NADH generating substrates include pyruvate, malate, glutamate, oxaloacetate, oxoglutarate, and citrate from the TCA-Cycle, and hydroxybutyrate from fatty acid metabolism (Chance & Williams, 1956; Gnaiger, 2020; Mitchell, 1961).

Complex 2 (CII) is a succinate dehydrogenase and oxidizes succinate from the TCA-Cycle. The succinate-linked pathway (S-pathway) oxidizes succinate to fumarate, which includes FAD+ as an immediate electron acceptor. Electrons are passed to CoQ via $FADH_2$. Unlike CI, CII does not transfer protons to the intermembrane space (Chance & Williams, 1956; Gnaiger, 2020; Mitchell, 1961).

Complex 3 (CIII) cytochrome b-C_1 transfers electrons from CI and CII via cytochrome c, an electron carrier within the membrane, to complex 4. CIII contributes to the proton gradient by translocating protons with energy gained from passing electrons (oxidation) (Chance & Williams, 1956; Gnaiger, 2020; Mitchell, 1961).

Complex 4 (CIV) cytochrome c oxidase passes electrons to molecular oxygen, which, together with protons, will form water. Protons are also translocated to the inner mitochondrial membrane, taking part in the proton gradient. Oxygen is the final electron acceptor; without oxygen, the immediate electron acceptors cannot be reused (Chance & Williams, 1956; Gnaiger, 2020; Mitchell, 1961).

The Oxidative phosphorylation (OXPHOS) system is driven by the electrochemical proton gradient produced by the ETS in the ATP production by the ATP-synthase (F1F0) to phosphorylate ADP into ATP (**Fig. 5**). The F1F0 acts as an ion channel, allowing protons to diffuse back to the mitochondrial matrix. The reflux of protons releases energy which is used to drive the ATP-synthetase.

LEAK respiration allows system adjustments by proton and electron leakage across the membrane, which is related to heat production and does not contributie to biochemical work (Chance & Williams, 1956; Gnaiger, 2020; Mitchell, 1961).

In some cases, an uncoupled system can be beneficial. An example is found in brown adipose tissue, where the uncopling protein termogenin allows for an alternative proton flux back to the mitochondrial matrix, producing heat, rather than ATP, that is used for thermogenesis (Mitchell, 1961; Nicholls & Locke, 1984). An example of a partially uncoupled system is found in the skeletal muscles of sea otters, where a high proton LEAK contributes to maintaining body temperature (Wright et al., 2021).

The Electron transfer systems capacity (E) is a measurement of the maximum oxygen flux in the respiratory pathway of the mitochondria in an uncoupled state. **OXPHOS capacity (P)** is a measurement of the respiratory capacity in the ADP-activated state, meaning that there is a coupling to the phosphorylation of ADP to ATP. OXPHOS capacity (P) needs to be corrected for residual oxygen consumption (ROX), which is the instrumental background levels of oxygen (Benard et al., 2007).

1.5 Acclimatizational effects of training

Acclimatization is the physiological and or behavioral adjustments of an organism as a response to a different environment or climate, as stated in Henderson's dictionary of Biology (Lawrence, 2008). A physiological response to exercise refers to acute (occurring immediately during, or after exercise) changes, while acclimatization refers to chronic (long-term) alteration as a response to repeated exercise.

All movement require energy expenditure above the resting values, and all this energy is provided through oxygen usage, at least during aerobic work. Long term submaximal aerobic exercise is commonly performed by sled dogs with minimal rest and recovery. To sustain long exercises, the respiratory and cardiovascular systems must work together to supply the muscles with oxygen (Plowman & Smith, 2013). The respiratory system is the first step in the oxygen cascade, obtaining oxygen for aerobic work. The respiratory system includes external and internal respiration, together with pulmonary ventilation, and the respiration frequency must respond to the demand of the exercise. Acute alterations include external ventilation adjustments, to maintain both ventilation and perfusion, as well as enhanced alveolar ventilation. The internal ventilation can respond by increasing the oxygen extraction for the muscles to match oxygen with the work performed. Acclimatizational alterations of the respiratory system includes increased lung capacity, which allows for more efficient oxygen uptake and delivery, as well as improved breathing efficiency due to

improved strength in respiratory muscles (Plowman & Smith, 2013). The respiratory system is generally not a limiting factor for oxygen uptake in healthy individuals. The oxygen need depends primarily on the training intensity and duration.

Acute cardiovascular responses involve increased cardiac output, which refers to the amount of blood pumped per minute, and increased stroke rate (heart beats per minute), which will plateau and be maintained relatively constant throughout exercise. On longer durations, stroke rate will decrease close to, but above resting rates simultaneal as the heart rate increases. The cardiac output can therefore be maintained through the proportional relationship between stroke volume and heart rate (Plowman & Smith, 2013). Cardiovascular acclimatization to endurance training involves an increase in overall cardiac output, improvements in the hearts pumping action and hypertrophy (increased size or thickness), which can lead to a lower heart rate at rest together with reduced blood pressure.

Neuromuscular acclimatization is divided into resistance training and aerobic endurance training. Sled dogs are however conducting both to some extent, by pulling the sled with equipment and musher for long distances. Resistance training can increase muscle strength and power by alterations in both fiber size and motor unit recruitment. Regarding fiber size, regular endurance exercise can increase the size and number of slow oxidative (Type I) muscle fibers. During endurance exercise there is an increased demand for energy, which requires the recruitment of more motor units to contract the muscle fibers. Regular endurance exercise can lead to adaptations in the nervous system, which improves the recruitment and coordination of motor units during exercise. This can improve the efficiency of muscle contractions, leading to improved endurance performance (Plowman & Smith, 2013). Acclimatizational response to endurance training increases capillary density and mitochondrial density, which will improve the maximal oxygen consumption (VO_2 max) (Plowman & Smith, 2013). Hemoglobin, which is a protein found in red blood cells, binds to oxygen and transports it from the lungs to the muscles. Endurance acclimatization involve an increase in red blood cell numbers, as well as increase of hemoglobin amount, which will lead to increased oxygen carrying capacity of the blood. Myoglobin is another protein found in muscles cells that binds to oxygen and transports it from the blood to the mitochondria, where it is used to produce energy. Acclimatizational alterations to myoglobin includes increase in myoglobin content in the muscle fibers, which can improve oxygen delivery and utilization during exercise (Plowman & Smith, 2013).

In addition to the range of fiber types, the fibers can adapt its size or composition of fiber types to meet a changing demand. Both muscle fibers and the skeletal muscle mitochondria are plastic and can change in response to energetic challenges. Protein synthesis and degradation controls the regulation of muscle mass, and the skeletal muscles can adapt by changing size, metabolic properties, and composition (Hill et al., 2016). Endurance training favors change of muscle fibers composition towards the slow direction, while detraining usually favors a change in composition from slow to the fast direction (Scott et al., 2001). The number and the content of mitochondria varies between different muscle fibers, and the mitochondrial morphology is therefore dependent on the fiber type of the muscle, with a longer mitochondrial shape found in oxidative fibers (Mishra et al., 2015).

In 1945, Palladin demonstrated that mitochondria can increase their respiration in response to electrical signals (simulating a state of exercise) applied to skeletal muscles. This increase in mitochondrial respiration, together with an increase in volume are two important acclimatizational aspects of endurance (Hill et al., 2016; Palladin, 1945). Mitochondrial function and muscle fiber type is genetically determent to some extent, but is highly plastic and trainable toward both directions (Holloszy, 1967; Simoneau & Bouchard, 1995).

1.5.1 Mitochondrial plasticity

Mitochondria are highly plastic and can undergo remodeling changes depending on the energetic challenges met. Mitochondrial remodeling includes fission and fusion, mitophagy (removes damage mitochondria) and de novo biogenesis (formation of new mitochondria from preexisting ones) (Holloszy & Coyle, 1984; Pernas & Scorrano, 2016). Mitochondrion change shape and size by fusing together (fusion) or by dividing (fission) from each other, and this change is linked to the metabolic activity of the cell (Lewis & Lewis, 1915).

Mitochondrial growth is linked to fission because it must be replicated and inherent and can therefore only be generated from a pre-existing mitochondrion. Both mitochondrial fission and fusion are processes governed by proteins and allow adaptation to energetic demands (Pernas & Scorrano, 2016). The balance between fission and fusion is determined by the cellular condition, which can induce one process over the other. Fission can for example be induced by changes in metabolic demand, such as high levels of glucose or cellular stress, while fusion can be induced by cellular starvation to meet a higher energy demand of the cell

by increasing the mitochondrial respiration to mediate DNA damage (Gomes et al., 2011; Trudeau et al., 2011; Youle & Van Der Bliek, 2012). Fusion of mitochondria will allow its network to grow by mixing the contents, while fission can isolate networks and allow damaged mitochondria to go through mitophagy.

Mitochondrial length is also regulated by fission and fusion, and the events occur cyclic to control each other. Small mitochondria are likely to fuse to extend their network, and larger mitochondrion are more likely to go through fission. These events are balanced and allow for adaptation and repair (Cagalinec et al., 2013; Jones & Naylor, 2022). The shape and the configuration of the mitochondria have been found to be distinctive for skeletal muscle fiber types in humans (Ogata & Yamasaki, 1997). Small or fragmented networks of mitochondria are in general associated with more glycolytic activity, while larger networks are associated and more dependent on oxidative phosphorylation activity (Kraus et al., 2021).

Endurance training influences the mitochondria in many ways and can also improve the aerobic capacity by increasing the mitochondrial content and composition (protein to lipid ratio). Mitochondrial proton leakage can also be optimized by endurance training, the leakage goes through the inner mitochondrial membrane and bypasses the phosphorylation, thereby slipping away. Less leakage will optimize the system to use more energy available from the H+. Endurance training improves aerobic capacity by increasing O_2 uptake in the muscles, increasing oxidation and limiting lactate production. This effect can occur by larger muscle mass in content and size, by increasing mitochondrial size and number, but also by improving the OXPHOS system (Conley, 2016; Holloszy, 1967; Hood et al., 2006; Huertas et al., 2019).

Alaskan huskies have a significant upregulation of substrate driven skeletal muscle capacities during endurance competition, which indicates mitochondrial remodeling. This means that the skeletal muscle mitochondria reprogram to meet a higher energy demand. This remodeling pattern allows a higher coupled respiration at an unchanged mitochondrial volume. During periods of reduced endurance training, the demand for protein synthesis is reduced, and one could hypothesize that the respiratory capacity is decreasing together with lower intensity of training. However, this decrease has been found to be quite marginal (Davis et al., 2014; Miller et al., 2017), perhaps because of a limited resting period. It is unknown if this plasticity is unique to Alaskan Huskies or if other breeds/species share this capacity (Miller et al., 2015b).

1.6 Genome alterations

Ancient canid bone remains have been found at Zhokhov island in eastern Siberia. Genomic studies have identified these remains as an ancestor to modern sled dogs, tracing back at least 9500 years (Pitulko & Kasparov, 2017; Ramos Madrigal et al., 2021; Sinding et al., 2020). The genome from this ancient dog contains several mutations related to the cold environment, which is still under positive selection pressure in modern sled dogs (Sinding et al., 2020). Modern sled dogs are divided into five breeds: The Greenland dog, Siberian husky, Alaskan malamute, Samoyed, and the Canadian Eskimo dog (FCI, 2021). Sinding et al. (2020) investigated genes in which the Zhokhov dog and modern sled dogs carry the same haplotypes, a set of DNA variants that are close to each other on the same chromosome and therefore tend to be inherent together. They found three genes that may play an essential role in physical activity in the Arctic: the CACNA1A, TRPC4, and TRPV2.

CACNA1A is involved in skeletal muscle contractions by coding for a calcium channel subunit, which mediates neurotransmitter release necessary in the motor nerve connection to skeletal muscles (Kaja et al., 2007). Within the muscle, calcium is used as a messenger essential for contraction, which relies on ATP. Calcium also controls the cellular energy production itself by maintaining the mitochondrial membrane potential. It might also play a potential role in response to exercise-induced hypoxia because of its role in response to hypoxic conditions (Wang et al., 2005).

TRPC4 codes for a protein that makes up the transient receptor channel involved in mechanisms such as vasodilation and microvascular permeability in the lung (Lezama-García et al., 2022). TRPV2 codes for a thermosensitive receptor that may also be involved in pain sensation (Ferreira et al., 2015).

Sinding et al. (2020) described a gene, APOO, involved in the transport and metabolism of fatty acids (Turkish et al., 2014) that was under positive selection in sled dogs. Polar bears, another species adapted to the Arctic, also indicate a possible adaptation to fat-rich diet by another gene within the apolipoprotein family called APOB, which has been reported to be under selection (Liu et al., 2014). This is an example of convergent evolution, where different species independently evolve similar traits or adaptations to cope with similar environments or ecological niches.

Sinding et al. (2020) also found that sled dogs have fewer copies of genes (AMY2B and MGMA) that encodes for enzymes that contribute to the breakdown of starch produced and excreted by the pancreas and the small intestine called alpha-amylase and maltase-

glucoamylase, respectively, as compared to more recently domesticated dog populations following the development of agriculture such as the Saluki, English springer spaniel and Labrador retriever (Arendt et al., 2016; Arendt et al., 2014; Axelsson et al., 2013; Freedman et al., 2014; Ollivier et al., 2016; Reiter et al., 2016; Tonoike et al., 2015). This may indicate that sled dogs are less adapted to utilize a diet rich in carbohydrates. However, the number of AMY2B copies also varies within breeds, as seen in for example Siberian huskies and Alaskan malamutes (Axelsson, 2018; Sinding et al., 2020; Tosi et al., 2021). Balto, a historical Siberian sled dog, had fewer copies of AMY2B compared to most modern-day breeds. Similarly, modern Siberian huskies also have fewer copies of the amylase gene, which implies that these dogs did not evolve on a starch-rich diet (Moon et al., 2023). Lower copy numbers of AMY2B together with the positive selection of APOO and SLC25A40, both involved in fatty acid metabolism indicated that modern sled dogs may be more adapted to utilize a fat-rich diet, as compared to a starch rich diet (Sinding et al., 2020).

In summary, the sled dog genome contains several mutations related to their environment, such as running and pulling a sled under low-oxygen conditions, regulating body temperature, and the digestion of fats instead of carbohydrate-rich diets (Sinding et al., 2020).

1.7 Metabolism

The traditional diet for sled dogs consists of around 20–30 % fat, 30 % protein and 10–15 % carbohydrates in the form of starch (Davis, 2021). Previous studies have found evidence that Alaskan huskies are in a negative energy balance during long distance races (Hinchcliff et al., 1997; McKenzie et al., 2008), and that the main metabolite is carbohydrates, not fat or proteins as one might suggest based on the diet (Miller et al., 2015a). Generally, the relative contribution of glucose, fatty acids and proteins to the oxidative metabolism is a matter of the intensity of the exercise, and during prolonged submaximal exercise it almost fulfills the rate in which the cell needs ATP (Plowman & Smith, 2013).

Human metabolism is mainly fat driven during rest and low to mid intensity training. While performing high intensity training, fat metabolism is suppressed by carbohydrates as the new main source of metabolite (Spriet, 2014). Contrary to humans, Alaskan huskies rely on carbohydrate metabolism earlier than humans, and even light to modest phase exercising is driven mainly by carbohydrates (Miller et al., 2015a). It has also been shown that exercised

Alaskan huskies increase their carbohydrate metabolism while performing the same modest exercise as untrained Alaskan huskies (Miller et al., 2015a).

Returning to the composition of sled dog food, it seems that Alaskan huskies are oxidizing more glucose compared to what they obtain from their diet, indicating that they must have evolved a very efficient system to transfer other substrates into glucose (Davis, 2021). Some evidence points towards gluconeogenic substrates, such as glycerol and amino acids, which are generated into glucose from non-carbohydrate substrates, but this machinery is complex and requires more investigation in sled dogs (Tosi et al., 2021). The sparing and replenishment of the glycogen stores have long been thought to be associated with muscle fatigue. However, studies have shown that there is minimal to no changes related to ATP concentrations during fatigue and low muscle glycogen concentrations and that fatigue might be associated with other nonmetabolic factors instead such as neuromuscular control, temperature, or dehydration (Green, 1991). Alaskan husky skeletal muscles appear to be highly fatigue resistant, and based on their high fat diet they seem to rely on hepatic (related to/or associated with the liver) glucose to support prolonged submaximal exercise (Davis et al., 2020).

1.8 Mitochondrial respiration in Alaska husky

The relationship between VO_2 max and mitochondrial respiration is that VO_2 max is primarily limited by the capacity of the cardiovascular and respiratory systems to transport oxygen to the muscles, as well as the ability of the muscles to extract and utilize oxygen to produce energy. Mitochondrial respiration plays a critical role in the latter process, as it is the primary pathway by which cells utilize oxygen to produce ATP (Banse et al., 2007; Conley, 2016; Holloszy, 1967; Hood et al., 2006; Huertas et al., 2019). Therefore, individuals with a higher VO_2 max generally have a higher capacity for mitochondrial respiration, which allows them to produce ATP more efficiently and sustain aerobic exercise for longer periods of time. Foxhound and Greyhounds are two dog breeds known for high burst speeds (72 km/h). They have mass specific VO_2 max ranging from 114–149 ml/min/kg (Musch et al., 1987; Staaden, 1984). Human elite endurance athletes are reported to have mass specific VO_2 max of 94 ml/min/kg (Astrand & Rodahl, 1986; Bergh et al., 1978; Poole & Erickson, 2011), and thoroughbred racehorses a VO_2 max of 217 ml/min/kg (Langsetmo et al., 1997; Poole & Erickson, 2008). Alaskan huskies completing the Iditarod have a VO_2 max of 240 ml/min/kg

(Miller et al., 2017). Endurance horses and dogs deviate from the mass specific VO_2 max prediction for mammals, which reflects extreme development of cardiorespiratory fitness and muscular endurance capacities in both (Poole & Erickson, 2011).

Up till now, mitochondrial respiration has only been studied in one type of dog, the Alaskan husky (Davis & Barrett, 2021; Miller et al., 2017). Miller et al. (2017) used high-resolution respirometry to investigate mitochondrial respiration in Alaskan huskies. They compared a raced group of Alaskan huskies that had completed the 1,600 km long Iditarod race (n=6 males) with a non-raced group (n=5, two females and three males) of Alaskan huskies during two different seasons (raced and non-raced). The aim of the study was to examine mitochondrial respiration in permeabilized skeletal muscle fibers (*M. biceps femoris*) of Alaskan huskies in the off-season (non-raced) and following the 1600 km Iditarod Sled Dog raced (Raced). They found that the maximal oxygen flux in the respiratory pathway of the mitochondria was 255±38 pmol/s*mg of tissue for OXPHOS capacity P, and 254±37 pmol/s*mg for ETS capacity E (Miller et al., 2017). This is believed to be the highest mean values of OXPHOS and ETS ever recorded in a mammalian skeletal muscle (Miller et al., 2017).

For comparison, humans have OXPHOS values ranging from sedentary to elite endurance athletes of 85–180 pmol/s*mg, measured in *M. vastus lateralis,* located at the lateral side of the thigh that promotes movement, strength and stability for the hip and knee (Boushel et al., 2007; Gnaiger, 2009; Mettauer et al., 2001; Mogensen et al., 2006; Rasmussen et al., 2001a). Another comparison can be the mixed breed racing horses, which displayed a mean OXPHOS value of 150 pmol/s*mg of tissue, measured in the *M. triceps branchii*, located in the front leg (antagonist elbow flexor) (Votion et al., 2012). The muscle fiber composition of these different endurance trained muscles is similar, with a domination of Type 1 fibers (Poole & Erickson, 2011).

Alaskan huskies are selected and bred for sustained exercise in extreme conditions and might exemplify the adaptive potential of mitochondrial function in mammalian species. Alaskan huskies bred for extreme endurance seem to have a large overlapping genetic signature with Siberian huskies (**Fig. 1**). Therefore, it would be interesting to compare the Alaskan husky with the pure breed Siberian husky. To examine the potential of mitochondrial function in the Alaskan huskies and Siberian huskies, one can use high-resolution

respirometry to look at the substrate-specific changes in respiratory capacity for carbohydrates (Miller et al., 2015).

1.9 Aims and hypothesis

The aim of this study is to determine respiratory capacity in skeletal muscles from Siberian huskies and Alaskan huskies off-season (August) and at the end of the racing season (March/April). This may highlight physiological adaptations in the respiratory capacity of skeletal muscles arising through selective breeding of huskies, as well as potential differences in the acclimatization responses to endurance training. Knowledge about Alaskan husky and Siberian husky-athletes can contribute to performance, as well as improvements of health and wellbeing for these sled dogs.

Hypothesis to be tested in this study:

1. Respiratory capacity in skeletal muscles from both Alaskan huskies and Siberian huskies is higher during racing season as compared to off-season.
2. Training-induced increase in aerobic capacity results from an increase in muscle mitochondrial density.
3. Selective breeding aimed to enhance race performance and endurance in Alaskan huskies may have led to a higher respiratory capacity in their skeletal muscles compared to that of Siberian huskies bred within a particular breed standard.

2 Materials and methods

2.1 Animal welfare statement

The field project was approved by the Norwegian Food safety authority (NFSA; FOTS ID 28932). Two changes regarding numbers of animals were applied for in the field project and were approved by NFSA between the first and after the second sampling. The project complies with the Norwegian and European legislation for animal research. Participants involved in the sampling process have had training in experimental animal science and were supervised and aided by an authorized veterinarian.

2.1.1 Animals included in the study

Originally, eight sled dogs (four Siberian huskies and four Alaskan huskies) were included in the project and believed to provide sufficient data for statistical comparisons between groups. Using parallel samples in experiments would partly compensate for the small group sizes, but lack of consistency and the fact that some samples did not pass the analyses gave a smaller data set than anticipated. Therefore, the number was later increased to 10 dogs and later 13 dogs as approved by NFSA.

Included in the study were seven Alaskan huskies (two males born 2019, two females born in Aug 2019, a female born in 2012, and two males borne in 2016) and six Siberian huskies (one female born in 2019, two sisters born in 2016, one female born in 2015, and two brothers born in 2021) (Appendix Table 1) from two different collaborating kennels located outside Tromsø, Norway (Mjaatvedt husky and Snykovet Siberian Husky Kennel). Eight of the dogs (AH1–4 and SH1–4) trained and raced together as one team during competition season 2021/2022. For the following season, 2022/2023, the Alaskan huskies raced in one team (AH1–7), and the Siberian huskies in another team (SH1–6), this time, trained by two different mushers.

2.1.2 The sled dog team

The dogs lived outdoor all year round in secure dog yards. One to three dogs shared yard, and all dogs had access to proper housing with wooden fibers or dried hay. During training season all dogs were provided with 2–3 meals per day, a meat soup consisting of dry protein kibbles together with raw meat mixed with water. Siberian husky kibbles contained 35 % protein, 23.5 % carbohydrates, and 21 % fat (Virbac veterinary HPM baby, Virbac, France). Alaskan husky kibbles contained 28 % protein, 28 % carbohydrates, 21 % fat (Sporting life energy 4300, Royal Canin, France). In addition, both groups received raw meet consisting of 15 % protein and 10% fat (VOM Active, VOM og hundemat, Norway). In supplement, they were also getting vitamin B, omega 3 oil and digestive support powder. During the off-season in summer, the dogs were fed two times a day with less raw meat. In addition to regular feedings, they also received treats and chewing stimuli such as dried fish and whole meat during all seasons.

Racing season: The autumn training started when the temperature was low enough (usually below 12 °C), for the 2021/2022 season the training started in July. The training

distance, duration and intensity increased during the season. The training was conducted with a cart during autumn and sled during winter. To keep track of the progression and recovery needed, the training runs were logged by the mushers. During July–February in the 2021/2022 season the dogs conducted 2,700 km in total on dry land and snow. For 2022/2023 season, the Siberian Huskies conducted 3,700 km, and the Alaskan huskies a total of 2,500 km, both on dry land and snow prior to sampling in March.

Off-season: The off-season started in April/May and training intensity was gradually decreased. During warmer days activities were restricted to the afternoon when temperatures were lower. Summer activities included swimming, hiking, biking, cuddles and playing.
A canine physiotherapist was used to check the dogs when needed, as well as veterinary checkups and vaccinations. Massages were also used after training to ensure proper muscle recovery.

2.1.3 Sampling of the dogs

Skeletal muscle biopsies (approximately 50 mg) were taken from the *M. biceps femoris* from AH1–4 and SH1–4 in May 11–20, 2022 (**Appendix table 1**). This sampling was after the Beaskádas, Bergebyløpet, and Finnmarksløpet FL600 (**Table 2**).

The second sampling was in August 14–18, 2022 (**Appendix Table 1**) during the off-season with lower activity. Alaskan huskies AH1–4 and SH2–6 was included during the second sampling, and SH1 was excluded due to a planned pregnancy.

The third sampling was conducted between March 16–25, 2023 (**Appendix Table 1**) during the racing season. AH1–7 and SH1–4 + SH6 were included in this sampling, SH5 was excluded due to rehoming. The SH team had finished four races prior to sampling, Beaskádas, TromsQuest, Bergebyløpet, and Herringen Trail. The AH team participated in one race this season, TromsQuest (**Table 2**).

***Table 2**. Participation in sled dog races during the season of 2022 and 2023, with a combined team of Alaskan and Siberian huskies (AH/SH), or teams divided into Alaskan huskies (AH), and Siberian huskies (SH)*

Races	Distance	Average speed	Year	Team
Beaskádas 300 homerun race	160 km		January 2022	AH/SH
Bergebyløpet	240 km	11.9 km/h	February 2022	AH/SH

Finnmarksløpet FL600	600 km	9.5 km/h	March 2022	AH/SH
Beaskádas 300	245 km	11.4 km/h	January 2023	SH
TromsQuest	60 km	15.1 km/h	January 2023	SH
Bergebyløpet	240 km	10.6 km/h	February 2023	SH
Herringen Trail	50 km	12.3 km/h	March 2023	SH
TromsQuest	180 km	13.8 km/h	January 2023	AH

2.2 Micro biopsy procedure

In the current study, all dogs were given the NSAIDs firocoxib (Previcox 227 mg, 5 mg/kg per oral) at least one hour before the biopsy procedure and on the following two days to ensure appropriate analgesia (total of three days). On the day of the procedure, dogs were given deksmedetomidinhydroklorid (Sileo mouth gel 0.1 mg/ml, 125 µg/m2 on the mouth mucous membrane) 30 minutes prior to sampling. The dogs stood during the following procedure while they were gently held in place by two people from the kennel, including the musher. The proximal part of *M. biceps femoris*, located between the knee and hip on the hindleg, was shaved (approximately 2.5 x 2.5 cm), washed with chlorhexidine (HiBiSCRUB, 40 mg/ml) or cetylpyridin (Pyrisept, 1 mg/ml) and disinfected with 70 % ethanol using sterile compresses. Thereafter the skin and overlying fascia was infiltrated with lidokainhydroklorid/adrenalin (Xylocain-Adrenalin 10 mg/ml + 5 µg/ml, 4–6 ml in an L-Block, 1,5 ml in each direction, and 1 ml in the middle of the L-shape, penetrating a little deeper). We thereafter waited approximately 10 minutes to ensure appropriate local analgesia (**Fig. 6**).

***Figure 6**. Injection of the local anesthetics to the left, and the core biopsy needle on the right. Photos: Silje Sælen-Helgesson.*

The skin was disinfected again with 70 % ethanol prior to the micro biopsy procedure. The device used for the micro biopsy was a version of a spring-loaded one-handed automated system (Merit medical systems, USA, CTT1411 TEMNO Evolution Coaxial Bundle 14 G. 11.0 cm, Coax Bundle 16.0 cm 13.5 G.,) (**Fig. 8**). The introducer needle was set by the veterinarian on the proximal *M. biceps femoris*. The penetration depth was pre-set to 1 cm. The introducer also consisted of a core biopsy needle. The introducer needle was removed, leaving the core biopsy needle in its place (**Fig. 6**). The trigger needle was then introduced through the core needle and pre-set to obtain approximately 20 mg of tissue. Unloaded with a spring, the needle fired into the muscle, obtaining a small piece of tissue. The trigger needle was introduced twice for most dogs, collecting 40-50 mg of tissue and three times for two dogs, to yield enough muscle tissue. The core needle was then removed, and the puncture site examined, cleaned, and documented with photographs (**Fig. 7**). The puncture site was thereafter examined daily for the two following days.

The muscle tissue was immediately transferred to a tube, containing ice cold BIOPS solution (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine) which was mixed and prepared earlier, and put on ice awaiting homogenate preparations.

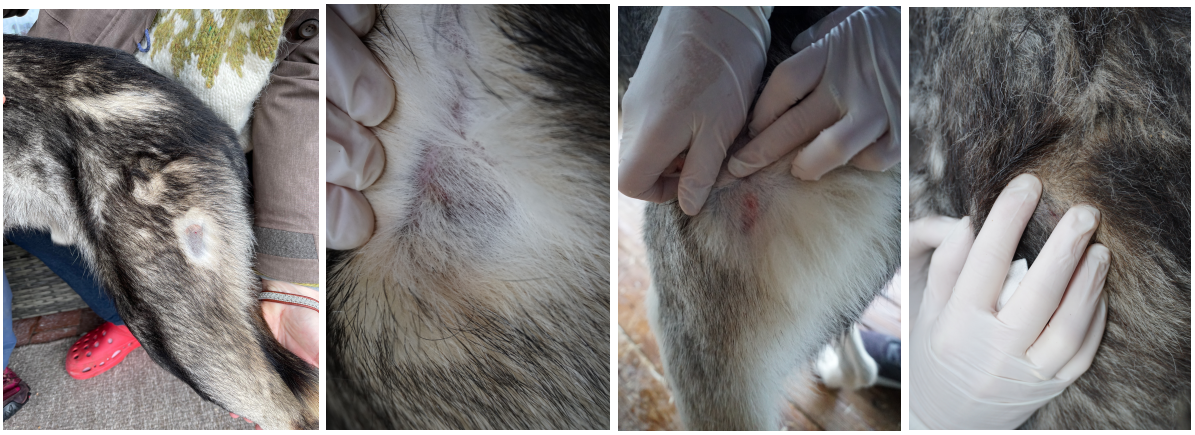

Figure 7. Post-Biopsy wound. *Pictures taken directly after the core biopsy needle was removed, and the puncture site cleaned. Photos: Chiara Ciccone and Silje Sælen-Helgesson.*

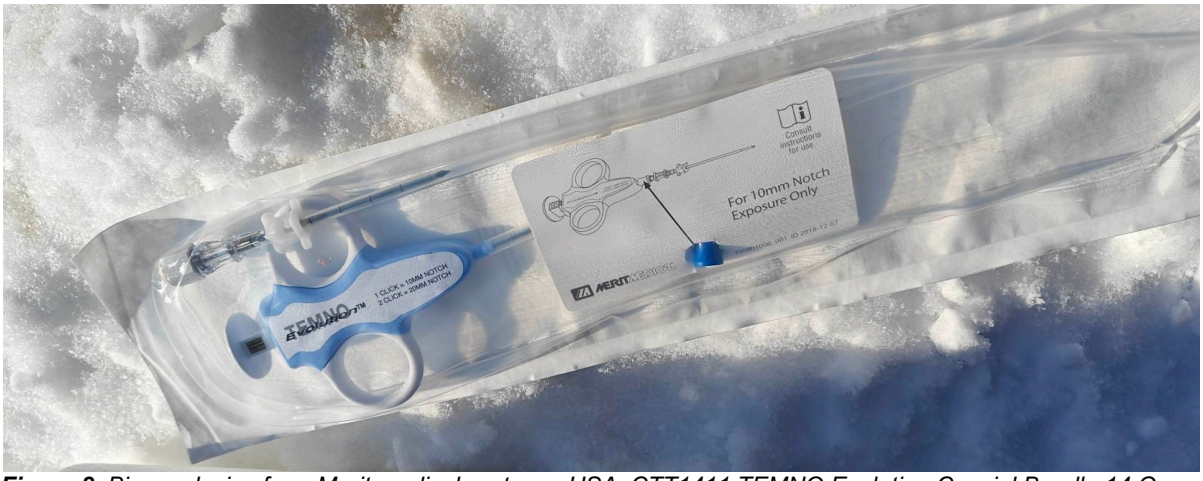

Figure 8. *Biopsy device from Merit medical systems, USA; CTT1411 TEMNO Evolution Coaxial Bundle 14 G, 11.0 cm, Coax Bundle 16.0 cm 13.5 G.*

2.3 Homogenate

2.3.1 Non-raced 2022 and Raced 2023 samples

Skeletal muscle samples were taken as described in section 4.2 and transferred to a vial containing 500 µl of BIOPS (10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine). All samples were kept on ice and brought to the research facilities at the Department of Arctic and Marine Biology, UiT. Samples were dried carefully on paper, weighed, and adjusted to 50 mg of tissue, before placing it back in a new container with 500 µl of fresh BIOPS. All further steps were conducted on ice (**Fig. 9**). A small scissor was used to cut the tissue into small pieces, before adding 50 µl of 2,5 % trypsin (Cat. No: 15090046, Thermo Fisher Scientific, Massachusetts, USA) and mixing it for a final concentration of 0.25 %. The sample was then incubated for 20 minutes.

The liquid containing BIOPS and trypsin was carefully removed and the sample was washed two times with BIOPS (500 µl) using a glass pipette, without removing the tissue from the container. After, the tissue was placed on the small disc of a shredder tube (**Fig. 10**). The small chamber was then closed with a tube tool, turned upside down, and the big chamber filled with 500 µl of premade respiratory medium that was preserving the mitochondrial function in the sample, MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$-6 H_2O, 60 mM Lactobionic acid, 20 mM Taurine, 10 Mm KH_2PO_4, 20 mM HEPES, 110 mM D-Sucrose, 1 g/l BSA) (Gnaiger, 2020). The amount of MiR05 was adjusted from sample to sample, with regards to the amount of tissue, to make a 10 % homogenate. The shredder tube was closed and placed in the shredder base. The PBI shredder was then placed on the shredding base, in position 1, and stirred for 10–12 seconds. After that, the sample was collected with a pipette and transferred to an Eppendorf tube. The Eppendorf tube was placed in a centrifuge (Himac CT15RE centrifuge, Hitachi Koki Co., Ltd) Ltd for 1 minute at 2000 rpm, at 4 °C. The supernatant was then transferred to a new Eppendorf tube. For each O2k Oroboros chambers (x4 chambers), 20 µl of the same samples were used. After addition of the sample, the chambers were left 10 minutes for stabilization.

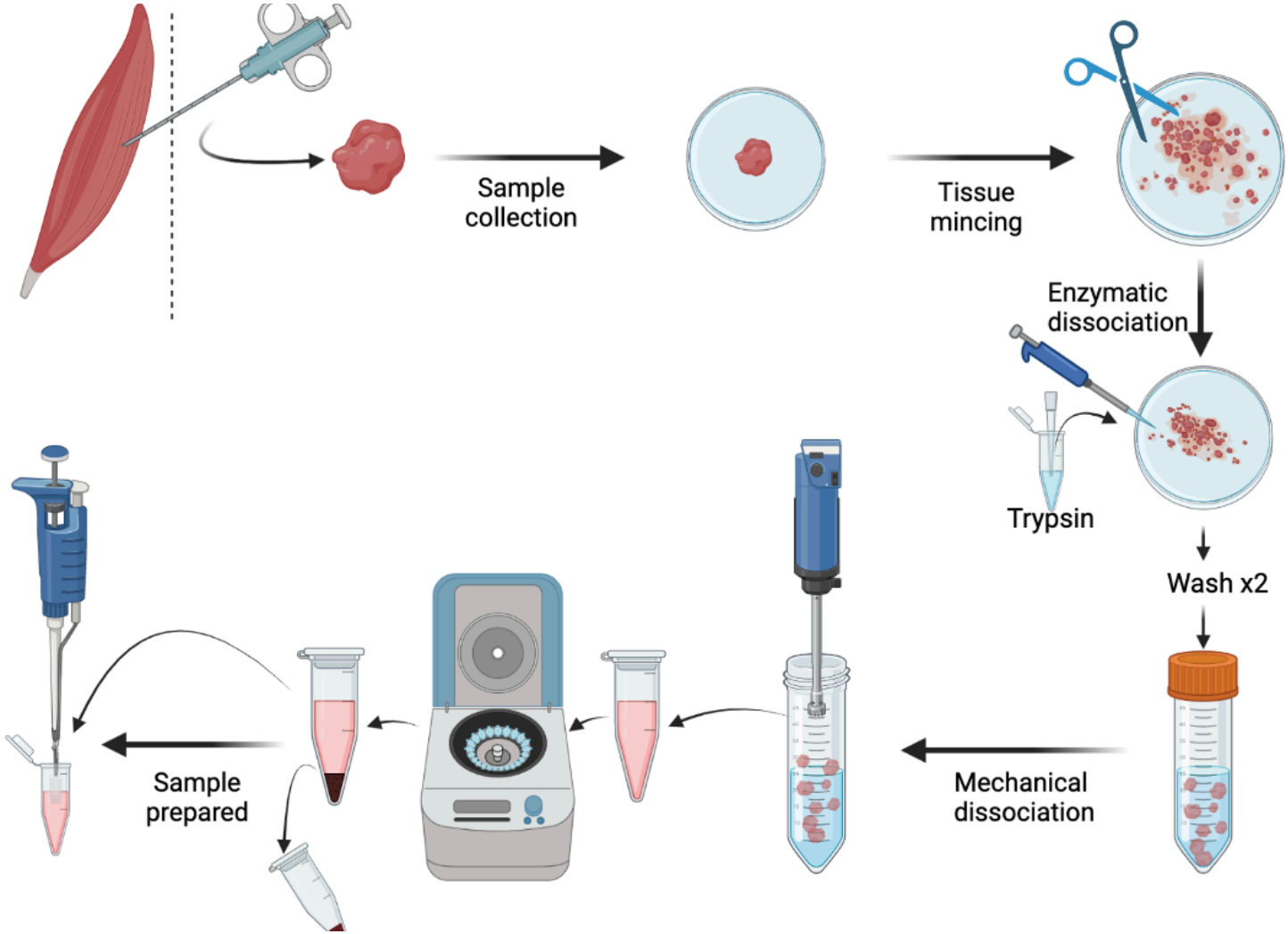

Figure 9. *Simplified version of the homogenate method, including sample preparation, enzymatic and mechanical dissociation.*

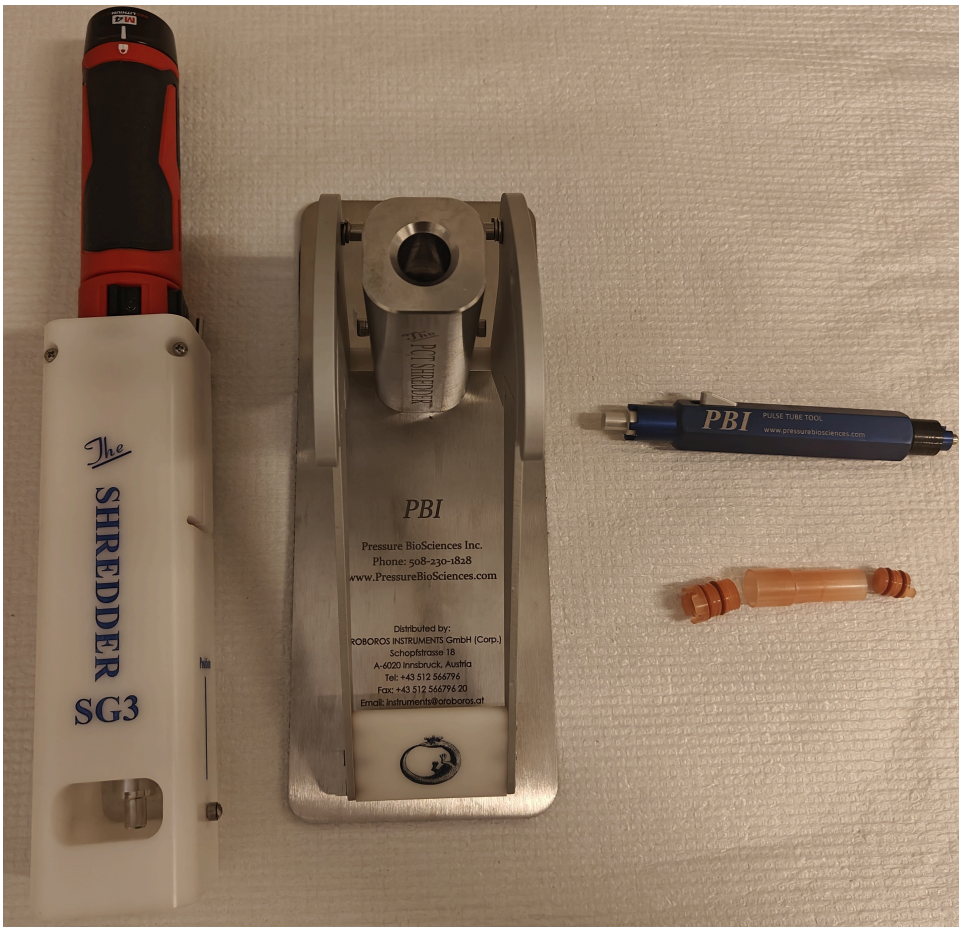

***Figure 10**. From the left, SG3 Shredder, PBI shredder base, PBI tube tool, and shredder tube with two lids. Oroboros Auxiliary HRFR-Tools (Draxl et al.)*

2.3.2 Raced 2022 samples

Raced samples from 2022 differs in the homogenate preparation compared to the other two seasons in trypsin incubation concentration. Raced 2022 samples were prepared with a final concentration of trypsin of 0.5 %, (50 µl of 2.5 % trypsin in 250 µl of BIOPS) which were previously used for frozen samples. For fresh samples, this concentration turned out to be too high and may have led to mitochondrial damage. Besides trypsin concentration, the homogenate method is the same for all seasons.

2.4 Substrate-uncoupler-inhibitor titration protocol (SUIT) for High resolution respirometry

2.4.1 Raced 2022 and Non-raced 2022 samples

High-resolution respirometry (HRR) using an Oroboros oxygraph is a technique used to measure mitochondrial respiration in biological samples, such as tissue homogenates. The technique involves measuring the oxygen consumption rate of a sample in a closed chamber while gradually adding substrates and inhibitors (SUIT) to different mitochondrial respiratory complexes. This provides detailed information on mitochondrial function and bioenergetics (**Fig. 11**). All samples were analyzed with high resolution respirometry with the Oxygraph-2k Oroboros at 37 °C. Approximately 50 mg muscle fibers from each biopsy were homogenized and prepared for respirometry (Oroboros) and Citrate synthase analysis. The experiments were run in 3–4 parallel chambers in two different respirometers (Oxygraph O2K, Oroboros instruments, Innsbruck, Austria).

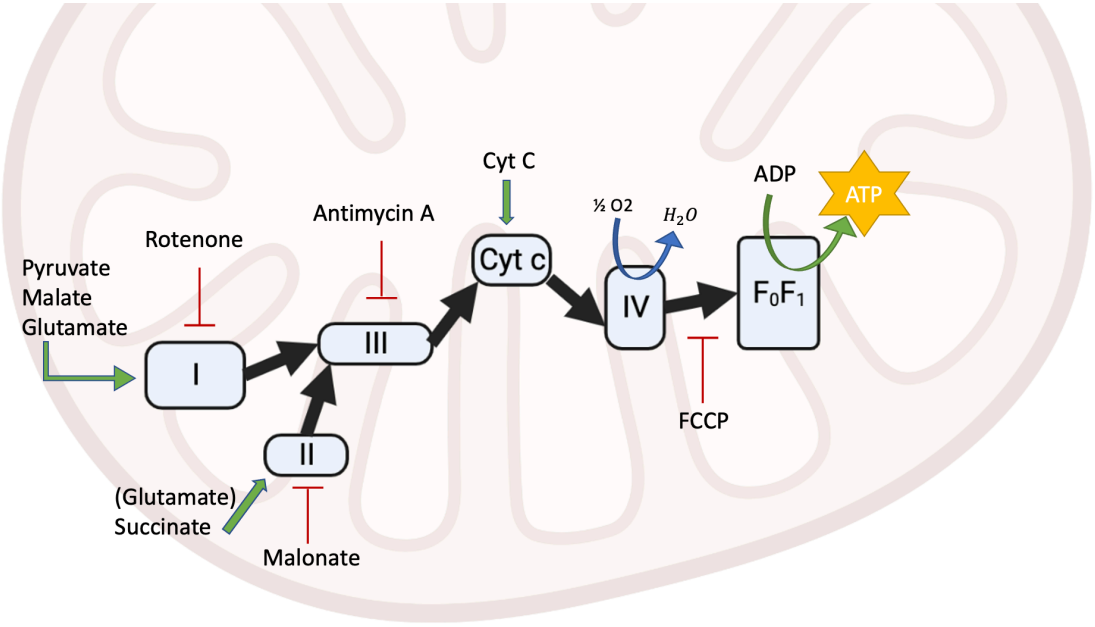

***Figure 11**. A visual image of the SUIT-protocol, with substrates added in green, and inhibitors added in red, following the protocol from table 2.*

All chambers were washed, and oxygen sensors calibrated each day, prior to experiments. Prior to calibration, the respiration medium (2.1 ml of MiR05) was added to each individual chamber at a set temperature of 37 °C. When all four chambers were stabilized after the addition of the sample, the SUIT protocol was initiated (**Table 3**). Initially, 5 µl Pyruvate (5 mM), 2.5 µl of Malate (0.5 mM) and 10 µl of Glutamate (10 mM) was

titrated into each chamber to produce NADH, to stimulate LEAK respiration through CI. After this, 20 µl of ADP (5 mM) was added, to stimulate OXPHOS through CI, followed by 5 µl of Cytochrome C (10 µm) to investigate the mitochondrial membrane integrity, ensuring that respiration was not limited by the loss of Cytochrome c. Usually, if there is an increase in respiration larger than X % after the Cytochrome c addition, indicating possible mitochondrial damage, results are excluded. To look at the OXPHOS through both CI and CII, 20 µl of Succinate (10 mM) was added, followed by 1 µl of FCCP (0.5 µM) to uncouple OXPHOS from ETS. During the first experiment with samples from both AH and SH, FCCP was added in small quantities to assess uncoupling effect and making sure that respiration was not limited by ATP synthase, and the final volume of 1 µl was determined for both dog-samples. To inhibit CI, 1 µl of Rotenone (0.5 µM) was added leaving the remaining oxygen flux from CII and CIII. Then 2.5 µl of Malonate (5 mM) was added to inhibit CII, with the remaining respiration through CIII and in the end, 1 µl of Antimycin A (2.5 µM) was added to inhibit CIII, which left the residual oxygen consumption.

***Table 3**. Substrate-uncoupler-inhibitor-titration protocol for skeletal muscle homogenate*

Substrate	**Concentration**	**Quantity [µl]**	**State**
Pyruvate	5 mM	5	LEAK
Malate	0.5 mM	2.5	LEAK
Glutamate	10 mM	10	LEAK
ADP	5 mM	20	P CI
Cytochrome C	10 µm	5	Mt integrity
Succinate	10 mM	20	P CI + CII
FCCP	0.5 µM	1	ETS CI + CII
Rotenone	0.5 µM	1	ETS CII
Malonate	5 mM	2.5	ETS CIII
Antimycin A	2.5 µM	1	Residual oxygen consumption (ROX)

Concentration in the Oroboros chamber can be calculated by **Eq.1** and consists of the homogenate concentration (0.1 g/ml), the amount of sample in the chamber (0.02 ml), and the volume of respiration medium MiR05 (2.1 ml).

$$Eq.1 \qquad 0.1\frac{g}{ml}x\frac{0.02\,ml}{2.1\,ml} = \frac{0.00095g}{ml} = 0.95\,mg/ml$$

The P/E control ratio is used to express OXPHOS limitation by ETS and was calculated from P CI + CII over ETS. The limitation is expressed in values from 0–1, and a result of P/E>1.0 usually indicates the presence of experimental artefacts (inhibitory uncoupler concentration or inhibition of ET pathway from OXPHOS inhibitors). The coupling efficiency of ETS (E) to OXPHOS (P) determines the amount of ATP produced by OXPHOS, P/E ratio. If P=E, the ATP synthetase by OXPHOS matches or exceeds the capacity of the ETS. If P<E, Phosphorylation limits OXPHOS, and OXPHOS is less than ETS, and is P>E; the ETS is less than OXPHOS. Protons slipping away or electron leakage forming reactive oxygen species can reduce the coupling between oxygen and ATP turnover. P/E control ratio can be calculated from high resolution respirometry measurements of OXPHOS (P CI + CII) and ETS, **Eq. 2**.

$$\text{Eq. 2} \qquad \text{P/E}\,\frac{OXPHOS}{ET\ pathway} = control\ ratio$$

The coupling control ratio, CCR ranges from 0–1, and combines the effects of coupling and limitation by the phosphorylation system. Low CCR indicates high efficiency in ATP production, and high CCR indicates less efficient ATP production. Coupling control ratio can be calculated from **Eq. 3** and LEAK/ OXPHOS refers to measured LEAK and OXPHOS from P CI.

$$Eq.3 \qquad CCR\text{:}\ \frac{LEAK}{OXPHOS} = coupling\ control\ ratio$$

2.4.2 Raced 2023 samples, moderate hypothermia 25° C

Due to an Oroboros machine default setting, all Raced samples from 2023 were mistakenly processed at a temperature of 25 °C instead of 37 °C, as used for raced 2022 and non-raced 2023 samples. This default setting was luckily discovered, after a long period of

trouble shooting. Mitochondrial respiration is highly temperature dependent (Sechi et al., 1973), and a temperature of 25 °C is therefore reflecting a state of moderate hypothermia and cannot be directly compared to the other two seasons. A hypothermic respiratory state is not reflecting the actual respiratory capacity of the animals measured. To compensate for this mistake, theoretical calculations were made from the hypothermic data to indicate theoretical values for a temperature state of 37 °C (Gnaiger, 2009; Pesta & Gnaiger, 2012).

> "The temperature coefficient, Q_{10} can be used to explain an exponential relationship between a physiological rate (mitochondrial respiration) and temperature, to specify the multiplicative factor by which the rate increases when the body temperature is increased by a standardizer increment of 10 °C " (Hill et al., 2016).

This factor is called the temperature coefficient Q_{10} (Hill et al., 2016). Arrhenius Model is provided in **Eq. 4**, where R_T is the physiological rate at any given body temperature T, and $R_{(T-10)}$ is the rate at a body temperature 10 °C lower than T.

Eq. 4 $$\boldsymbol{Q_{10}} = \frac{R_T}{R_{(T-10)}} \quad Q_{10}$$

This relationship is illustrated in **Fig. 12**, where the red line has a $\boldsymbol{Q_{10}}$=1 which means that that reaction is temperature independent. Most biological reactions fall within the scope of the blue and green line, with a $\boldsymbol{Q_{10}} = 2 - 3$. A $\boldsymbol{Q_{10}}$ of 2 doubles the reaction rate for every 10 °C rise in temperature, while a $\boldsymbol{Q_{10}}$ of 3 triples the reaction every 10 °C rise in temperature.

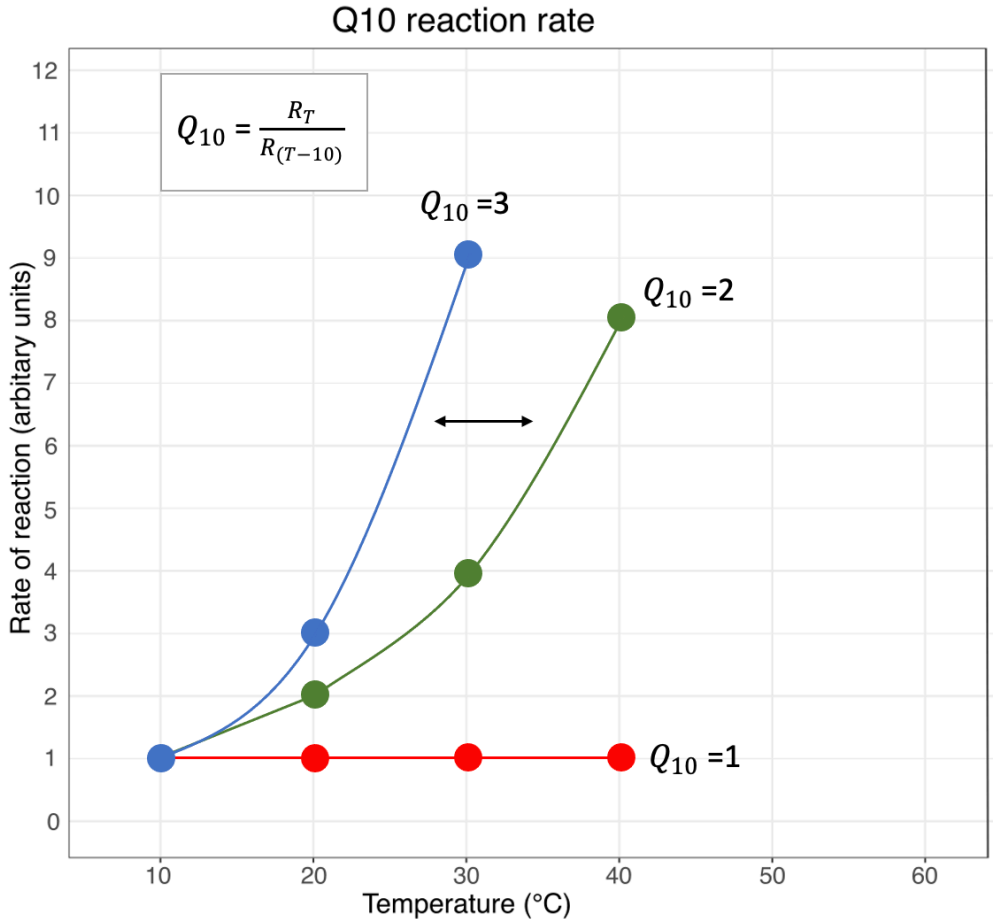

Figure 12. *Figure Shows the temperature sensitivity of a physiological reaction/process due to an increase in 10 °C. If Q_{10}=1, the reaction is not temperature sensitive. Q_{10}=2 doubles the reaction rate each 10 °C rise in T. Q_{10}=3 triples the reaction rate each 10 °C rise in T. Most biochemical processes fall between the range of 2-3 showed by the black arrow in the figure. Modified from:* ***2017 SILO Inc (https://silo.tips/download/temperature-regulation).***

A Q_{10}=2, was chosen as a basis for calculations, based upon human predictions from T25 °C -T35 °C, which gives a Q_{10}=2.3 to convert data measured at 25 °C, to respiration at 37 °C (Q_{10} $2^{(T12/T10)} = 2.3$) (Gnaiger, 2009; Pesta & Gnaiger, 2012).

2.5 Citrate synthase activity

Mitochondrial density was determined by measuring citrate synthase activity (CS). CS is an enzyme within the TCA-cycle which catalyzes the irreversible reaction of Acetyl CoA + Oxaloacetate to form Citrate and has been widely used as a quantitative marker for mitochondrial density due to its high sensitivity to changes in mitochondrial content (Vigelsø et al., 2014). CS activity was measured in the homogenate of tissue initially prepared for the Oroboros to track training-induced changes in mitochondrial density (Eigentler et al., 2015; Wiegand & Remington, 1986).

During the CS activity assay, the sample Acetyl CoA reacted with DTNB (5,5´-Dithiobis (2-nitrobenzoic acid), Elleman's regent) which formed TNB (5-thio-2-nitrobenzoic acid). TNB formation could be measured at 412 nm in the spectrometer, and it correlated with the CS enzymatic activity within the sample.

Approximately one week prior to absorbance measurements, stock solutions were prepared and stored at 4 °C: Tris-HCI buffer (1.0 M, pH 8.1), Tris-HCI buffer (0.1 M, pH 7.0), Triethanolamine-HCI buffer (0.5 M, pH 8.0) + EDTA (5 mM), and Triton X-100 (10 %). Acetyl-CoA (12.2 mM) was aliquoted into small tubes containing 250 µl, and stored at -20 °C. On the day of measurements, fresh buffers of Triethanolamine-HCI buffer (0.1 M, pH 8.0), Oxalacetate (10 mM, pH 8.0) and DTNB (1.01 mM, pH 8.1) were prepared and diluted from stock solutions previously prepared.

The Epoch microplate reader with program Gen5 (Epoch and Gen5, BioTek Instruments, Inc. Vermont USA.,) was turned on and programmed to read absorbance of 412 nm, in 15 sec intervals, for a total time of 2 minutes.

All the following preparations were conducted on ice.

Samples from the homogenate preparations were diluted 1:3 in 0.1 M Tris-HCI buffer (5 µl homogenate in 10 µl buffer), and a total of 4 µl of the diluted homogenate were transferred into three wells of a 96-well microplate, so that each sample was divided into three replicates (**Table 3**). Standard solutions were also added to verify correct readings by the microplate reader. In the wells C1–3, 200 µl of Tris-HCI buffer (0.1 M, pH 7.0) was added as a negative control. Citrate synthase (Prod. No: C3260, Sigma Aldrich, Germany) was diluted two times, the first dilution (D1) was made by adding 2 µl citrate synthase in 998 µl of 0.1 M Tris-HCI buffer pH 7.0 (Diluted 1:500). The second dilution 1:4 (D2) was made with 100 µl of D1 in 300 µl 0.1 M Tris-HCI buffer pH 7.0, for a final dilution of D2=1:2000. After, 200 µl of D2 was transferred to wells A1-3, and 4 µl to wells B1-3, as a positive control (Table 3).

A Reaction mix (RM) was made by adding 195 µl distilled H_2O, 12.5 µl of Oxaloacetate (10 mM, pH 8.0), 6.25 µl of Acetyl CoA (12.2 mM), 6.25µl of Triton x100 (10 % solution), and 25µl of DTNB (1.01 mM, pH 8.1) for a total volume of 245 µl per sample. In each well, 196 µl of the RM was added with an 8-channel pipette to the diluted sample (4 µl), giving a final volume of 200 µl.

The total number of samples used during citrate synthase measurements varied, because samples from raced and non-raced groups were run separately (example in **Table 4**).

DTNB (1.01 mM, pH 8.1) was added as the last component of RM, and when added, the RM was immediately pipetted into all wells of the microplate, which was thereafter run in the microplate reader at room temperature (20–25 °C).

Table 4. *Microplate sample overview: containing diluted homogenate from samples SH1–4 and AH1–4 Raced 2022 (Green color), AH1–4 and SH 2–6 non-raced 2022 (Blue color) and SH1–6, AH1–7 (Orange color). Replicated three times + RM. D2= 200µl, D2+ RM= 4µl of D2 dilution and 196 µl reaction mix), and 0.1B =200µl 0.1 M Tris-HCl buffer.*

	1	2	3	4	5	6	7	8	9	10	11	12
A	D2	D2	D2	AH2	AH2	AH2	SH3	SH3	SH3	SH6	SH6	SH6
B	D2+RM	D2+RM	D2+RM	AH3	AH3	AH3	SH4	SH4	SH4	AH1	AH1	AH1
C	0.1B	0.1B	0.1B	AH4	AH4	AH4	SH5	SH5	SH5	AH2	AH2	AH2
D	SH1	SH1	SH1	AH1	AH1	AH1	SH6	SH6	SH6	AH3	AH3	AH3
E	SH2	SH2	SH2	AH2	AH2	AH2	SH1	SH1	SH1	AH4	AH4	AH4
F	SH3	SH3	SH3	AH3	AH3	AH3	SH2	SH2	SH2	AH5	AH5	AH5
G	SH4	SH4	SH4	AH4	AH4	AH4	SH3	SH3	SH3	AH6	AH6	AH6
H	AH1	AH1	AH1	SH2	SH2	SH2	SH4	SH4	SH4	AH7	AH7	AH7

Specific CS activity is calculated with **Eq. 5**, where V is the specific CS activity in IU (international units), 1 IU represents activity where 1 µmol of citrate is transformed per minute, r_A represents the delta chance in absorbance per minute of time, and l is the cuvette length. Eb is the excitation coefficient to TNB at 412 nm, and VB is the stoichiometric value for TNB. *V cuvette* represents the total volume (200 µl), and *V sample* the sample volume (4 µl).

$$Eq.5 \qquad \mathcal{V} = \frac{r_A}{l * \mathcal{E}_B * V_B} * \frac{V\ cuvette}{V\ sample} = \text{IU/mL}$$

To convert IU/mL from **Eq. 5**, one can divide the specific CS sample activity by the sample concentration from the Oroboros chamber **Eq. 1**, to obtain the CS activity expressed per mass of the sample **Eq. 6**.

$$Eq.6 \qquad \frac{IU/mL\ (\ spesific\ CS\ sample\ activity)}{0.95\ mg/mL\ (Sample\ concentration\ in\ Oroboros\ chamber)} = IU/mg^{-1}$$

2.5.1 Optimizing the protocol

Prior to sampling the dogs, the protocol was tested out and improved using samples from other projects running in the lab including frozen seal tissue and fresh reindeer muscle. Basic Oroboros machine handling was practiced by learning the cleaning and calibration procedures. This was repeated several times to ensure confidence and proper understanding of the machine and interpretation of the oxygraphs.

Operation of the biopsy equipment was practiced on a chicken breast fillet purchased from the supermarket. This was done to test out two different types of biopsy guns, as well as to figure out how much muscle it was obtaining.

To test whether a prolonged incubation in BIOPS would damage the samples, and hence whether two dogs could be sampled in parallel, two samples were taken from one dog during the first day of the sampling of the Siberian huskies. One sample was left resting in BIOPS solution on ice while the other sample was homogenized and used in the Oroboros experiment. The results from the two samples were similar, and therefore it was decided that one could carry on with obtaining samples from two dogs per day.

The result from the dogs sampled in March 2022 was uneven and lacked a consistency between the different replicates and between different samples. To understand how to solve this problem, at first a new batch of ADP was used in two of the chambers, together with an old batch in the last two chambers. In all four chambers the ADP lacked a response, so it was decided to test different concentrations of pyruvate. Changing the pyruvate concentration had no effect, pointing to an error in the BIOPS solution. Homogenates with a BIOPS solution that was re-frozen several times, were compared with homogenates with a fresh solution from the freezer. The fresh solution gave more even results, and it was therefore decided that refreezing the leftover solution should be avoided in the following experiments, and that a fresh one should be used each time. However, the freshness of the BIOPS solution did not improve the stability of the results as much as expected, which lead us to the discovery that the enzyme trypsin was the factor causing the unexpected variations between the samples. The homogenate had been prepared with a final concentration of trypsin of 0.5 %, (50 µl of 2.5 % trypsin in 250 µl of BIOPS) which we previously used for frozen samples. For fresh samples, this concentration turned out to be too high and may have led to mitochondrial damage. The concentration was therefore changed to 0.25 % (50 µl of 2.5 % trypsin in 500 µl of BIOPS), and tested with dog-samples, as well as with reindeer samples, resulting in better evenness amongst and within the sample replicates.

2.6 Statistical analyses

Cytochrome c difference of 30 % was accepted and included, although most samples were within a 10 % difference (**Appendix figure A3**).

For data analysis of high resolution respirometry data, DatLab computer program (Oroboros Instruments, Innsbruck, Austria) was used. From there, mean values from stable Oroboros levels were used for each individual and organized in Excel (Microsoft (2023). Microsoft Excel (Version 16.72) Microsoft Corporation). Welch´s two sample t-test was performed between groups in R-studio. P-values with $p<0.05$ were considered statistically significant. Mean values are presented, including standard deviation, unless otherwise stated. Graphs are made in R-studio and modified in Inkscape (RStudio Team. PBC. 2023 (Version 2023.03.0+386), The Inkscape Team (2022), Inkscape (Version 1.2.2)).

3 Results

3.1 Micro biopsies were minimally invasive

Micro biopsy of AH and SH was performed during three seasons, May (raced 2022), August (non-raced 2022) and March (raced 2023). The Sileo mouth gel, given to each dog 30 min prior to sampling yielded no changes in dog behavior (**Appendix table 1**), although it was supposed to have a sedative effect. One (SH3) out of thirteen dogs showed some distress over the sound from the biopsy gun clicking during the first sampling/field experiment (raced 2022), and one dog (SH1) displayed a small reaction to the injection of the local anesthetic during the same season. Besides these two dogs, none of the other dogs reacted to the injection or biopsy sampling. During the third sampling/field experiment (raced 2023) we had a small blood assembly in the insertion tube on several dogs, but no hemorrhage was detected after removal of the tube. Two of the thirteen dogs sampled March 2023, however, got a minor hemorrhage after removal of the insertion tube, which stopped after a few seconds, and three minutes of digital press, respectively. Post sampling, there were no signs of infection, limping or general discomfort, and the small wounds healed up quickly within the first few days.

3.2 Mitochondrial density increases with endurance training

Individual measures of citrate synthase activity for AH raced 2022 (AHR22) ranged between 0.43–0.87 [IU·mg^{-1}], with a mean group value of 0.66±0.2 [IU·mg^{-1}]. The same individual values from raced 2023 (AHR23) were ranging between 0.41–3.24 [IU·mg^{-1}], with mean 1.57±0.9 [IU·mg^{-1}] (Fig. 13). There was a statistically significant difference between AHR22 and AHR23 (p=0.04*, Appendix Table 2). AHR23 also had a larger variation within the group compared to the other groups (**Fig. 13**).

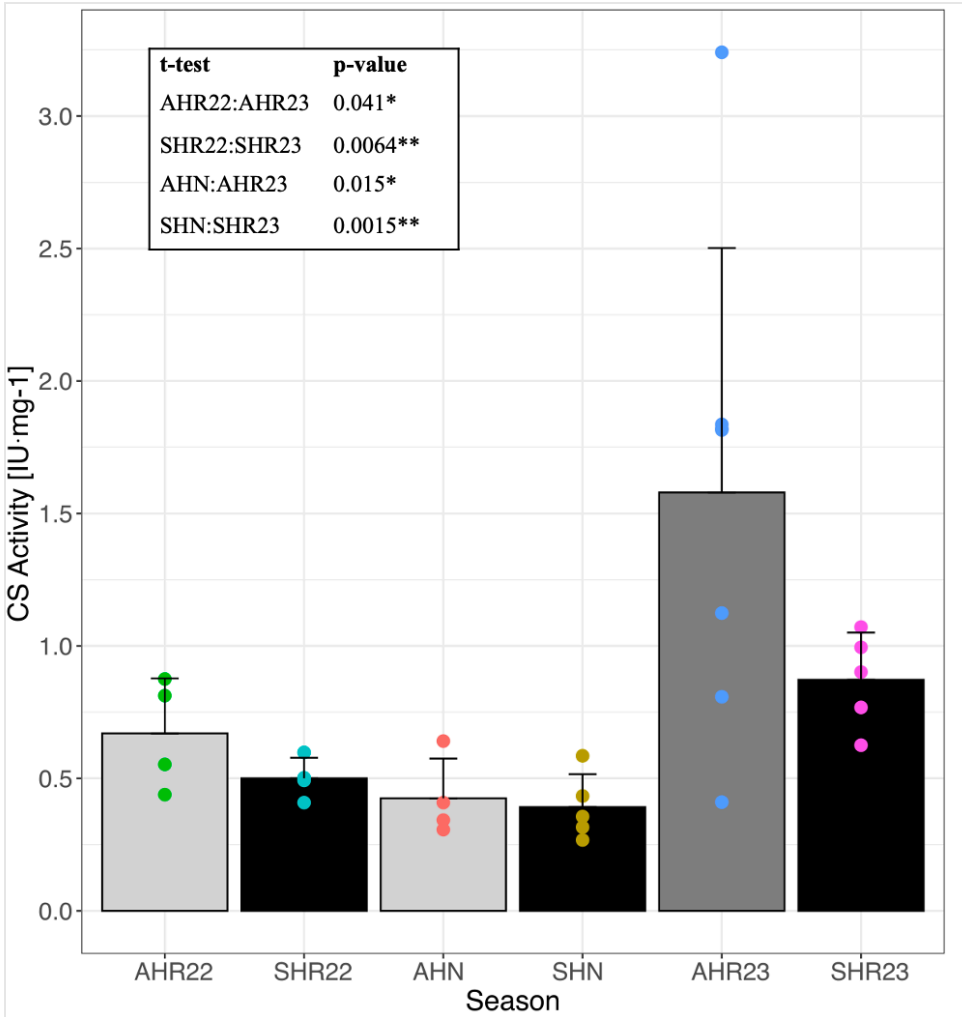

***Figure 13**. Group mean citrate synthase (CS) activity [IU·mg^{-1}] for Alaskan huskies (AH) and Siberian huskies (SH) per season. Non-raced and Raced 2023 prepared with the same protocol, while Raced 2022 samples differs in regard to trypsin incubation concentration. Mean and standard deviations: Alaskan huskies Raced 2022 (AHR22) = 0.66±0.20, n=4. Siberian huskies Raced 2022 (SHR22) = 0.50±0.07, n=4. Alaskan huskies Non-raced (AHN)= 0.40±0.15, n=4. Siberian huskies Non-raced (SHN)=0.39±0.12, =5. Alaskan huskies Raced 2023 (AHR23) =1.57±0.90, n=7. Siberian huskies Raced 2023 (SHR23) =0.87±0.17, n=5. P.values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

Citrate synthase activity measured for SH raced 2022 (SHR22) were ranging from 0.4–0.59 [IU·mg^{-1}], with group mean of 0.50±0.07, while the same values from raced 2023

(SHR23) ranging from 0.62–1.07 [IU·mg^{-1}], with group mean of 0.87±0.17 (**Fig. 13**). As for the AH, there was a statistically significant difference between SHR22 and SHR23 (p=0.0063**, **Appendix Table 2**).

The significant difference between citrate synthase activity in muscle biopsies from raced AH and SH sampled May 2022 and March 2023 (**Fig. 13**) gave further evidence for potential mitochondrial damage from trypsin incubation (Section 4.5.1). Results from the R22 samples were consequently excluded from further analysis.

Citrate synthase activity measured for AH non-raced (AHN) ranged from 0.3–0.64 [IU·mg^{-1}], with mean value 0.40±0.15 [IU·mg^{-1}], and when compared to their AHR23 values in **Fig. 13,** 1.57±0.90 [IU·mg^{-1}], there was a significant difference in measured mitochondrial CS activity between seasons (with a p-value of 0.01*), with the highest activity found in AHR23. For SH, statistical difference was found between non-raced (SHN) (0.39±0.12 [IU·mg^{-1}]), and SHR23 (0.87±0.17 [IU·mg^{-1}]), with a p-value of 0.0015** (**Appendix Table 2**).

3.3 High resolution respirometry

High resolution respirometry (HRR) data is presented in mass specific oxygen flux, JO_2/m [pmol·s^{-1}·mg^{-1}], of the group mean including standard deviation.

3.3.1 Samples from raced 2022 dogs excluded

Raced 2022 samples were, as stated above, exposed to potential mitochondrial damage due to the trypsin treatment (Section 2.5.1.) HRR data for this group are presented in **Appendix figure. 4**.

3.3.2 Non-raced AH show a consistently higher mitochondrial oxygen consumption compared to non-raced SH

AH from the non-raced group (measured at T 37 °C) showed a higher mitochondrial oxygen consumption, but with a larger standard deviation, compared to that of the SH (**Fig. 14**). The highest individual datapoints within the AH group were those of the two males AH1 and AH2. The lowest datapoints within the AH group belonged to AH3, the oldest dog included (a female born in 2012). The mitochondrial oxygen consumption for the SH group was in general lower compared to AH, and the data points were less spread. The highest

points within the SH group belonged to SH2 and SH5, one female and one male. SH3, SH4 and SH6 were more similar in their individual means.

There was a significant difference in LEAK respiration (p=0.001**), with the highest mean LEAK found in the Alaskan husky group (8.2±2.1[pmol·s^{-1}·mg^{-1}],). Oxidative phosphorylation also differed significantly between the two groups, for both P CI and P CI + CII, (p=0.01 and p=0.02, respectively). The highest mean oxygen flux was found in the AH group, 31.8±25.7 [pmol·s^{-1}·mg^{-1}], (P CI) and 56.0±36.3 [pmol·s^{-1}·mg^{-1}], (P CI + CII). Significant difference was also found for the uncoupled state (ETS) between groups (p=0.02*) with the highest mean oxygen flux found in the AH group 63.0±45.5 [pmol·s^{-1}·mg^{-1}]. There was no difference between ETS CII and ETS CIII between groups (p=0.05, p=0.1) with ETS CII of mean AH and SH being 32.4±15.0 [pmol·s^{-1}·mg^{-1}] and 21.0±11.3 [pmol·s^{-1}·mg^{-1}] respectively. Mean flux for ETS CIII of AH was 4.5±1.3 [pmol·s^{-1}·mg^{-1}], and for SH it was 3.8±1.0 [pmol·s^{-1}·mg^{-1}],

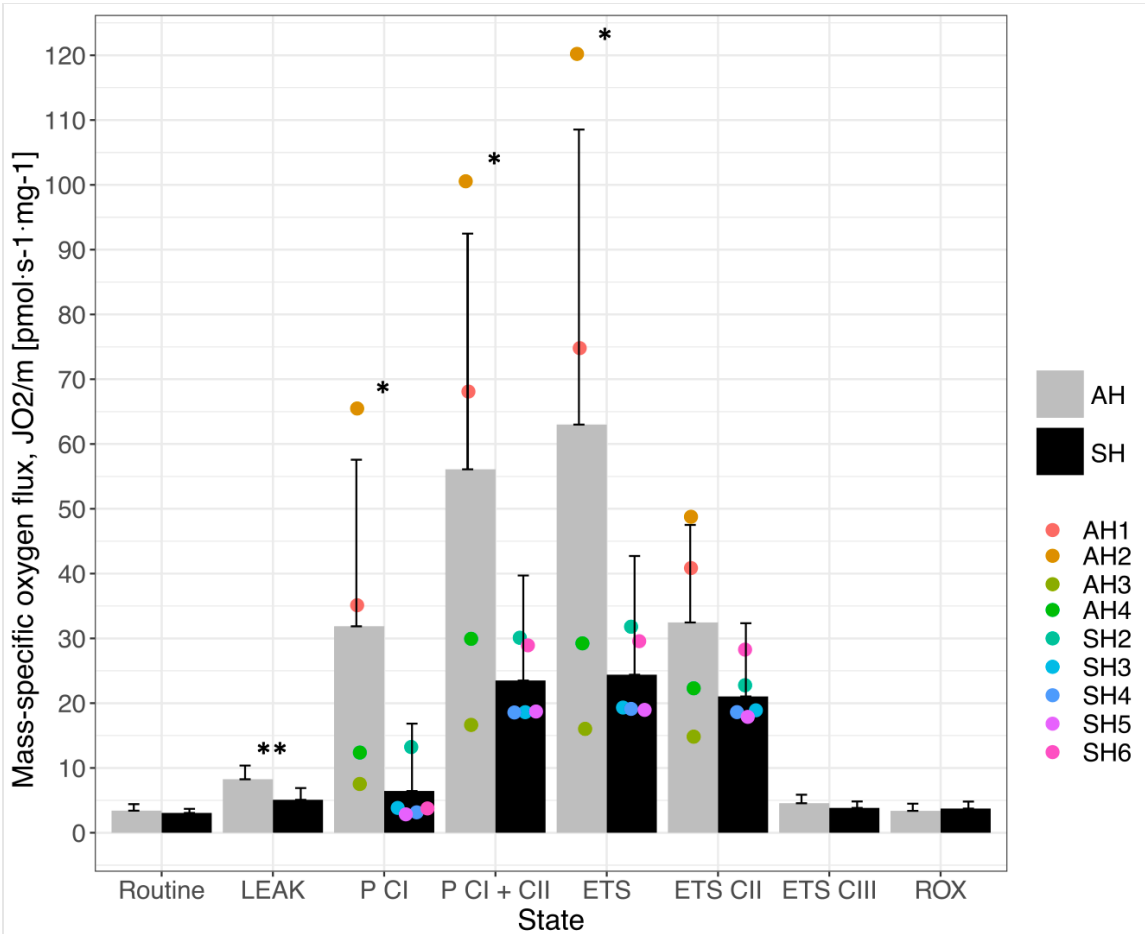

***Figure 14**. Alaskan huskies compared to Siberian huskies non-raced are plotted with group means, including mean of individual dog data points to account for the variation within the data. Only bars containing a large deviation include the individual data points. Alaskan husky mean values and standard deviation; Routine:3.4±1.0, LEAK:8.2±2.1, P CI 31.8±25.7, P CI+CII 56.0±36.3, ETS 63.0±45.5, ETS CII 32.4±15.0, ETS CIII 4.5±1.3, ROX*

*3.3±1.1. Siberian huskies mean values and standard deviation; Routine 3.0±0.6, LEAK 5.0±1.8, P CI 6.4±10.3, P CI+CII 23.5±16.2, ETS 24.39±18.30, ETS CII 21.0±11.3, ETS CIII 3.8±1.0, ROX 3.7±1.0 [pmol·s^{-1}·mg^{-1}].p-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

The AH group displayed a significant sex difference in mitochondrial respiration, with the highest oxygen consumption found in the male group for both OXPHOS, ETS and ETS CII (**Fig. 15**). No difference was found in LEAK or ETS CIII. This data supports the variation found within the AH group presented in **Fig. 14**. Sex differences were not found in the SH group, and these data were therefore not included.

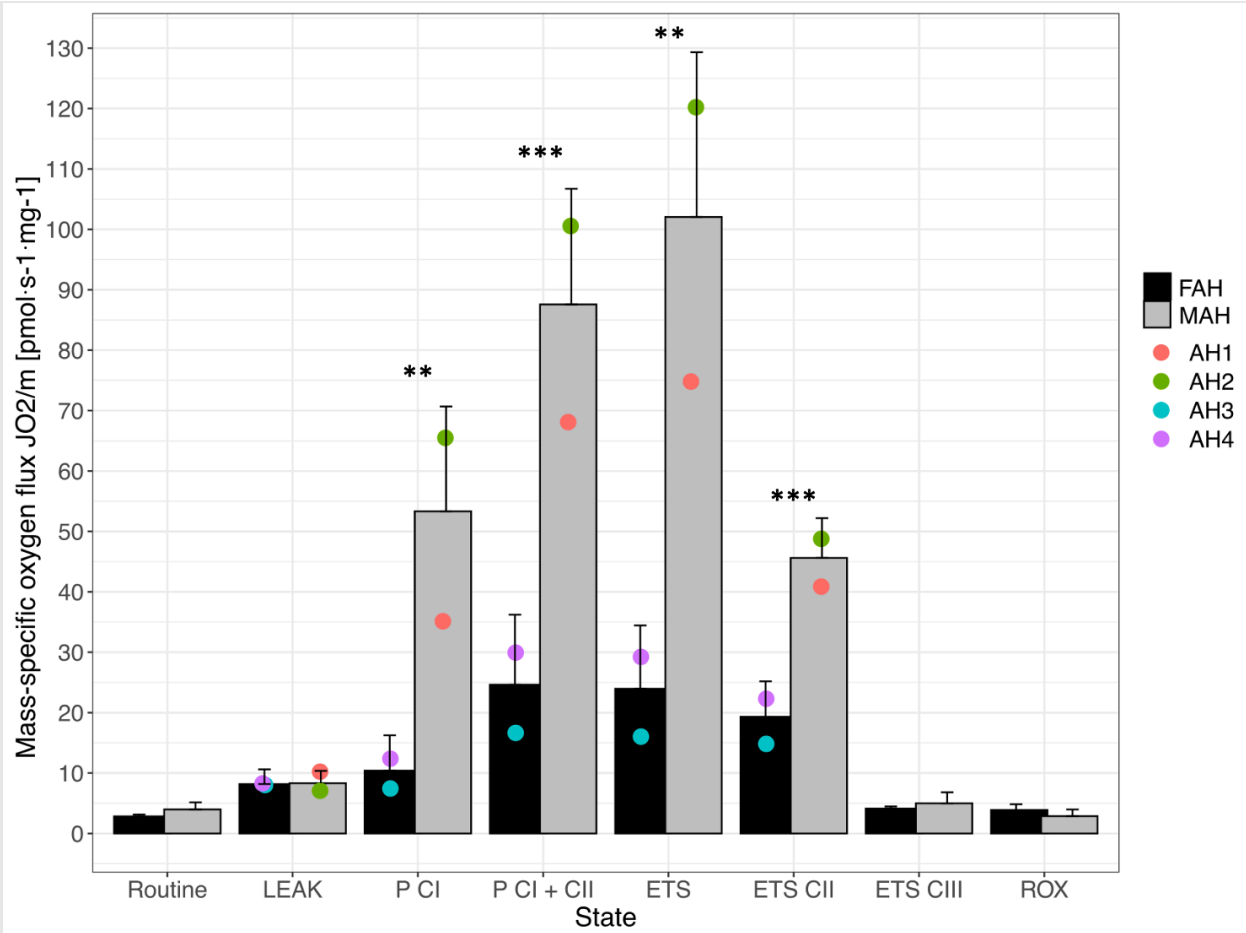

Figure 15. *Alaskan huskies non-raced divided into males and females. Alaskan huskies Female group mean values and standard deviation; Routine:2.8±0.3, LEAK:8.1±2.4, P CI 10.3±5.8, P CI+CII 24.6±11.5, ETS 23.9±10.4, ETS CII 19.3±5.8, ETS CIII 4.1±0.3, ROX 3.8±0.9. Alaskan huskie Male group mean and standard deviation values; Routine 3.9±1.1, LEAK 8.3±2.0, P CI 53.3±17.3, P CI+CII 87.5±19.1, ETS 102.0±27.2, ETS CII 45.6±6.5, ETS CIII 4.9±1.8, ROX 2.8±1.1 [pmol·s^{-1}·mg^{-1}]. P-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

3.3.3 No significant differences in oxidative phosphorylation between raced groups

Raced samples from 2023 for AH and SH were similar in their oxygen consumption for most of the states measured, and both groups contained large standard deviations, measured at 25 °C (**Fig. 16**). The highest individual datapoint within the AH group belongs to the female AH5, and the two males AH1 and AH2, who are all related. The lowest datapoints within the AH group belonged to AH3, AH4 and AH7, the oldest female, together with a female and a male. The trend for the SH group was similar compared to the AH group, and the data points were less spread. The highest points within the SH group belonged to SH1, SH2 and SH6, two females and one male. There was a significant difference in LEAK (p=0.003**) and ETS CIII (p=0.02*) between AH and SH, with the highest LEAK found in the AH group (17.6±5.2 [pmol·s^{-1}·mg^{-1}]), and the highest oxygen flux of ETS CIII found in the SH group (3.7±0.9 [pmol·s^{-1}·mg^{-1}]). No difference was found between groups for oxidative phosphorylation or the electron transport system, besides ETS CIII, for raced 2023. Results measured at T25 °C (**Fig. 16**), reflected a situation of moderate hypothermia, and not the actual values at body temperature.

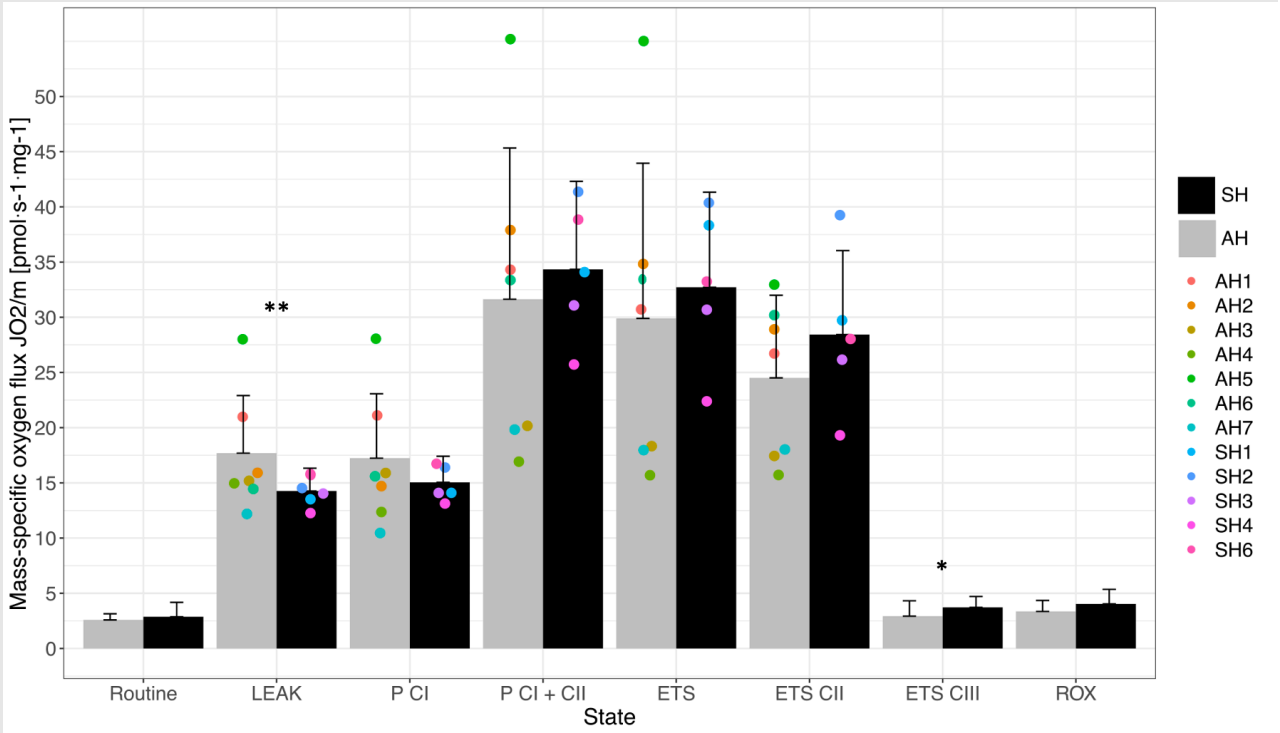

***Figure 16**. Mass specific oxygen flux of raced Alaskan huskies (AH) and Siberian huskies (SH) 2023 measured at **T 25℃**. Mass specific oxygen flux of group means presented including standard deviation, for each state. Individual sample means are included to present the variation found within the data, for the states containing the largest deviation. Alaskan huskies mean values; Routine 2.5±0.5, LEAK: 17.6± 5.2, P CI 17.2±5.8, P CI+CII 31.6±13.7, ETS 29±14, ETS CII 24.5±7.4, ETS CIII 2.9±1.3, ROX 3.3±0.9. Siberian huskies mean values; Routine 2.8±1.3, LEAK 14.2±2, P CI 15.0±2.3, P CI+CII 34.3±7.9, ETS 32.7±8.6, ETS CII 28.4±7.6, ETS CIII 3.7±0.9,*

*ROX4.0±1.3 [pmol·s-1·mg-1]. P-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

Theoretically calculated values for mass specific oxygen flux at 37 °C assuming a Q_{10} of 2.3 (**Fig. 17**) (Gnaiger, 2009; Pesta & Gnaiger, 2012), show the same trend as for the values measured at 25 °C (**Fig. 16**), and individual values increased with temperature. The maximal theoretical OXPHOS capacity for AH (n=7) was 175.2±89.5 [pmol·s^{-1}·mg^{-1}], and for SH (n=5) 190.4±52.0 [pmol·s^{-1}·mg^{-1}], with the largest deviation found within the AH group. The theoretical maximum induced uncoupled respiration was 164.2±92.1[pmol·s^{-1}·mg^{-1}] and 180.0±56.7 [pmol·s^{-1}·mg^{-1}] for AH and SH, respectively. **Appendix figure 5 and 6**, shows the difference between T25 and T37 for AH and SH.

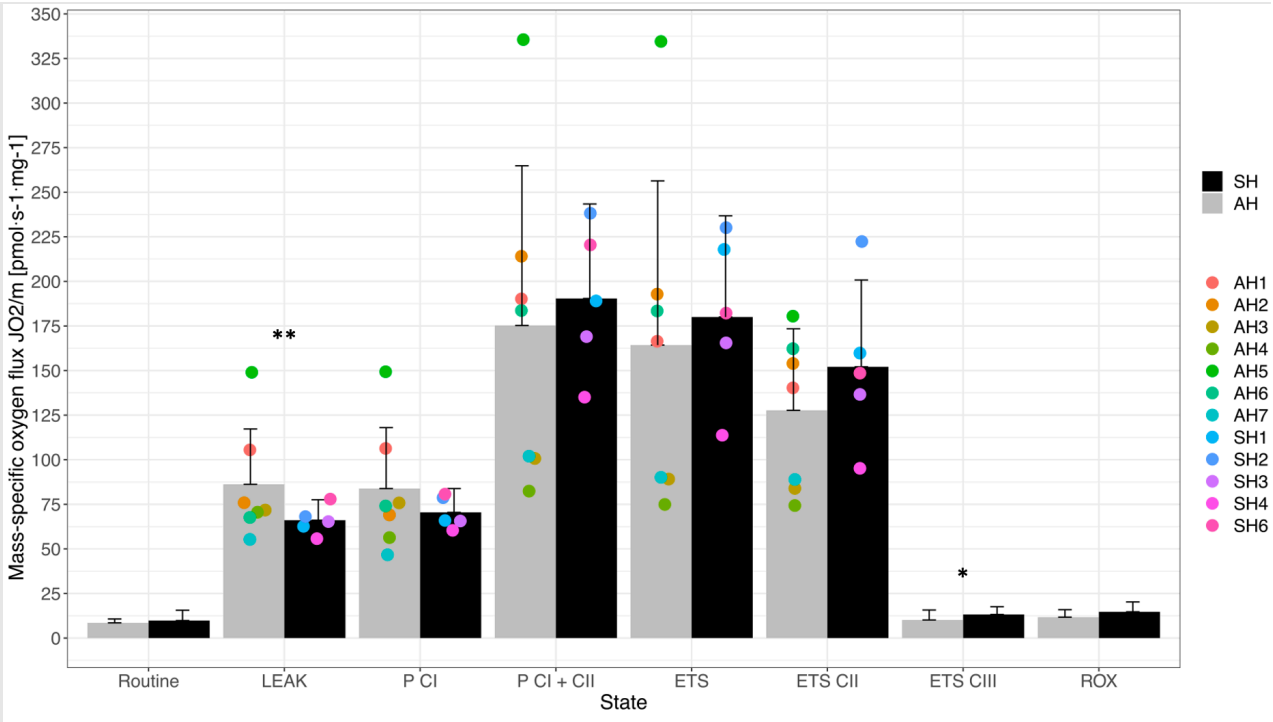

***Figure 17**. Theoretical Q10 (2.3) T37 values of mass specific oxygen flux of group means presented including standard deviation for Raced 2023. Individual sample means are included to present the variation found within the data, for the states containing the largest deviation. Alaskan huskies (AH) mean and SD values; Routine 8.5±2.1, LEAK: 86.1±30.0, P CI 83.8±34.1, P CI+CII 175.2±89.5, ETS 164.2±92.1, ETS CII 127.6±45.7, ETS CIII 10.1±5.5, ROX11.7±4.1. Siberian huskies (SH) mean and SD values; Routine 9.8±5.8, LEAK 66.1±11.4, P CI 70.4±13.3, P CI+CII 190.4±52.9, ETS 180.0±56.7, ETS CII 152.1±48.6, ETS CIII 13.2±4.3, ROX 14.6±5.5 [pmol·s^{-1}·mg^{-1}]. P-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

3.3.4 Endurance training increase the mitochondrial oxygen consumption and decrease group differences

SH had a similar trend in mitochondrial oxygen consumption between seasons, with higher oxygen consumption and larger standard deviation in the raced group (**Fig. 18**). SH

raced 2023 T37 had a significantly higher LEAK compared to non-raced 2022, with a p value of p= <0.0001 (**Fig. 18**). The raced group also had a significantly higher oxidative phosphorylation, for both P CI and P CI + CII, with p values of p= <0.0001. There was also statistically significant difference in ETS, ETS CIII, and ETS CIII between the season, with a higher oxygen flux in the raced group. The highest individual oxygen fluxes were seen in SH2 (raced 2023) for the states P CI + CII, ETS and ETS + CII (**Fig. 18**).

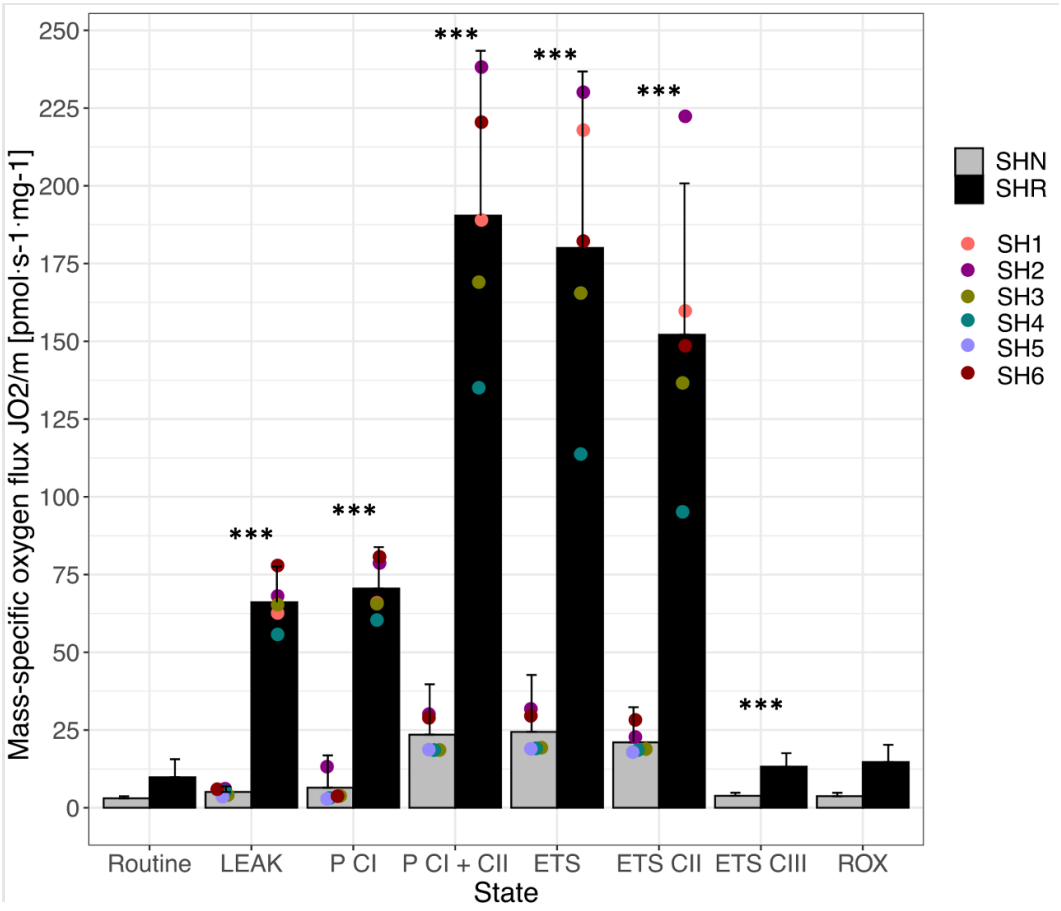

Figure 18. *Mass specific oxygen flux of group means presented including standard deviation, for each state and season. Raced 2023 presented with Theoretical Q10=2.3 (T37) values. Individual sample means are included to present the variation found within the data, for the states containing the largest deviation. SHN mean values; Routine 3.0±0.6, LEAK 5.0±1.8, P CI 6.4±10.3, P CI+CII 23.5±16.2, ETS 24.39±18.30, ETS CII 21.0±11.3, ETS CIII 3.8±1.0, ROX 3.7±1.0. SHR T37mean values; Routine 9.8±5.8, LEAK 66.1±11.4, P CI 70.4±13.3, P CI+CII 190.4±52.9, ETS 180.0±56.7, ETS CII 152.1±48.6, ETS CIII 13.2±4.3, ROX 14.6±5.5 [pmol·s^{-1}·mg^{-1}]. P-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

AH had a higher mitochondrial oxygen consumption in the raced 2023 T 37 group compared to the non-raced group (**Fig. 19**). The mass specific oxygen consumption of the male outliers in the non-raced group (AH1 and AH2) were higher in the raced group, but closer to the group mean. In the raced group the largest outlier was AH5, who is a female. Raced 2023 T37 had a higher LEAK respiration compared to non-raced 2022, p= <0.0001.

The raced group also had a statistically higher oxygen consumption for both OXPHOS and ETS, with p<0.0001. Individual dog datapoints for the two seasons show that the female AH3 was among the individuals with the lowest oxygen flux over the two seasons, while the two males, AH1 and AH2 was among the highest. The general trend was higher amongst the males, and lower within the female group. AH5 was an outlier compared to the other females.

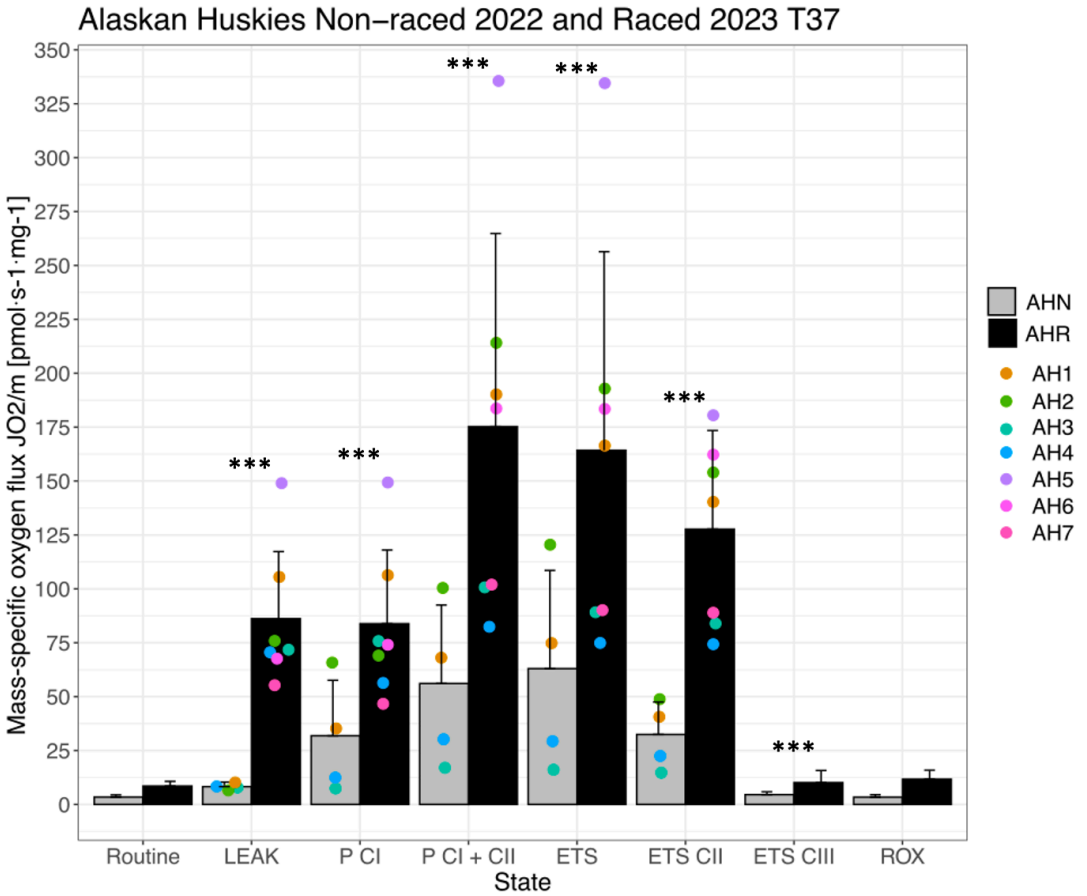

Figure 19. *Mass specific oxygen flux of group means presented including standard deviation. Raced 2023 presented with Theoretical Q10=2.3 (T37) values. Individual sample means is included to present the variation found within the data, for the states containing the largest deviation. AHN mean values; Routine 3.4±1.0, LEAK: 8.2± 2.1, P CI 31.8±25.7, P CI+CII 56.0±36.3, ETS 63.0±45.5, ETS CII 32.4±15.0, ETS CIII 4.5±1.3, ROX 3.3±1.1. AHR T37 mean values; Routine 8.5±2.1, LEAK 86.1±31.0, P CI 83.8±34.1, P CI+CII 175.2±89.5, ETS 164.2±92.1, ETS CII 127.6±45.7, ETS CIII 10.1±5.5, ROX 11.7±4.1[pmol·s^{-1}·mg^{-1}]. P-values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

3.3.5 P/E control ratio and coupling control ratio (CCR)

The results from AH and SH non-raced were both close to 1, (AHN: 0.96±0.10, SHN: 0.97±0.05), with no significant difference between the two groups (**Fig. 20**). For the raced groups, the P/E was slightly higher than 1 (AHR: 1.07±0.10, SHR: 1.06±0.12), with no significant difference between the two. Results from the raced groups indicate experimental

artefact. Also, results from raced group are the same, if calculated from either T25 or theoretical T37, due to the usage of a coefficient equal for all states. There was a significant difference between Siberian husky non-raced and raced, p=0.01*. The same results were found in the Alaskan husky non-raced compared to raced, p=0.02*.

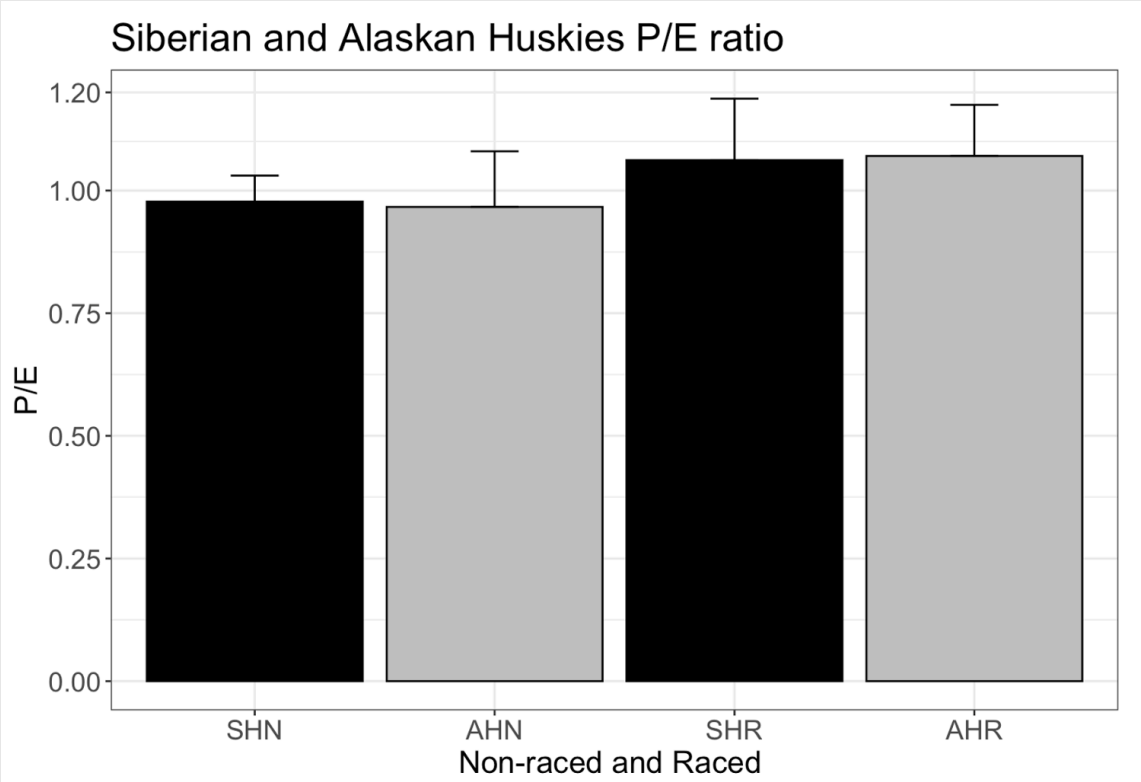

Figure 20. *Coupling control efficiency (P/E) of group mean values, SHN (0.97±0.05), AHN (0.96±0.11), SHR (1.06±0.12), AHR (1.07±0.10). Significant differences between SHN and SHR (p=0.01531*), and significant difference between AHN and AHR (p=0.02324*). No significant difference between SHR and AHR, nor SHN and AHN.*

The highest group mean of CCR was found in the SHN group (1.2±0.4) and the lowest in AHN (0.52±0.40) (**Fig. 21**). There was a significant difference in CCR between non-raced and raced for Siberian huskies (p=0.009**), as well as Alaskan huskies non-raced and raced (p=0.0016**). There were also significant differences in CCR between Alaskan and Siberian huskies non-raced (p=0.00024***) and for Alaskan and Siberian huskies raced (0.011*). However, all groups contained large standard deviations. Also, results from Raced group were the same, if calculated from either T25 or theoretical T37, due to the usage of a coefficient equal for all states.

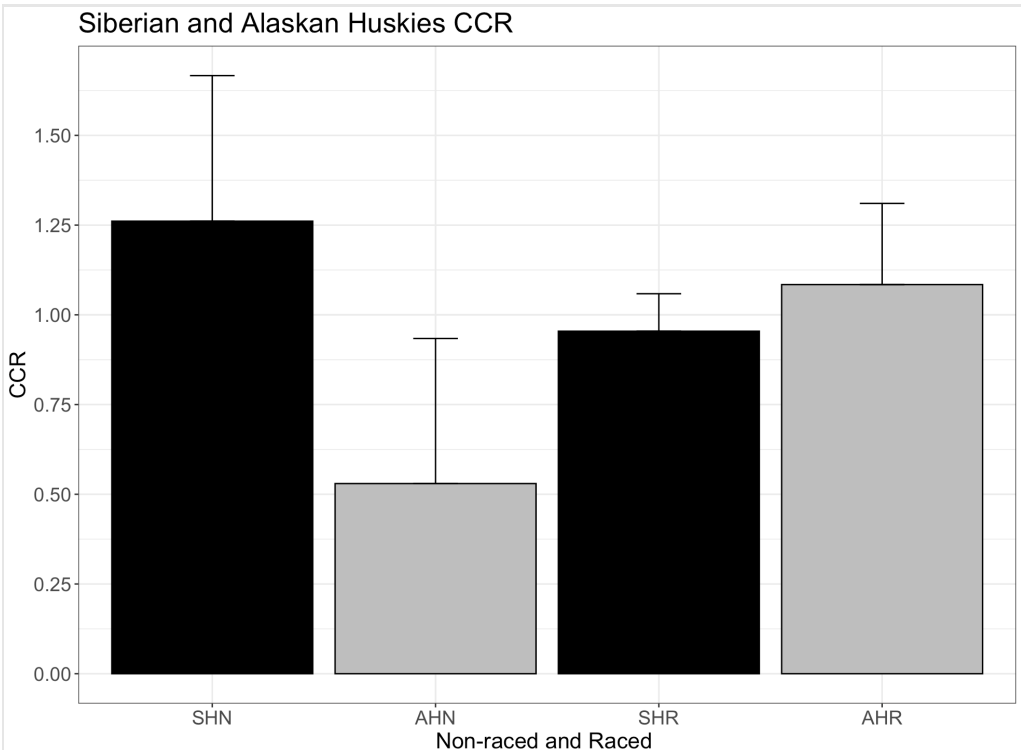

Figure 21**. CCR (LEAK/P CI) For Siberian huskies non-raced (SHN:1.2±0.4) and raced (SHR:0.9±0.1), with a significant difference between the two seasons (p=0.009**). Alaskan huskies Non-raced (AHN:0.52±0.40) and Raced (AHR:1.0±0.2) with a significant difference between the two seasons (p=0.0016**). There are also significant differences between SHN and AHN (p=0.00024) as well as for SHR and AHR (0.011*).*

4 Discussion

Non-raced Alaskan huskies had a higher carbohydrate associated capacity in their ETS-activity and produced ATP at a higher rate compared to that of Siberian huskies. This means that their mitochondria can consume oxygen at a higher rate and therefore produce ATP faster compared to the Siberian huskies in this study. Alaskan huskies consequently seem to have a more efficient mitochondrial respiration based on carbohydrates associated substrates, possibly due to difference in their genetics. Non-raced Alaskan huskies also had large differences between sexes in oxygen consumption (**Fig. 15**), which may be explained by mitochondrial substrate preferences (Horton et al., 1998; Montero et al., 2018). A significant difference in LEAK respiration was found between Alaskan and Siberian huskies in our study (**Fig. 14**), Alaskan huskies having the highest LEAK-values in the non-raced samples.

Comparing the theoretical values for the raced 2023 dogs, respiratory capacity in skeletal muscle mitochondria were similar in electron transfer system and ATP-production between Alaskan huskies and Siberian huskies (**Fig. 17**). However, Alaskan huskies had a higher LEAK and ETS-activity in CIII. This finding supports general knowledge about exercise acclimatization and is similar to what was reported in the paper by Miller et al.

(2017). Our results showed a significant increase in mitochondrial density between non-raced and raced groups, with the highest density found in the muscle biopsies from the raced 2023 group, showing that endurance training in fact does increase muscle mitochondrial density for both Siberian huskies and Alaskan huskies (**Fig. 13**).

4.1 High resolution respirometry non-raced

A carbohydrate associated substrate-uncoupler-inhibitor titration (SUIT) protocol was chosen for experimental real-time mitochondrial analysis as a component of metabolic phenotyping. This decision was based on previous findings from skeletal muscle respirometry in Alaskan Huskies indicating that carbohydrates are the preferred substrate during exercise in these dogs (Miller et al., 2017), and to our knowledge, the absence of any evidence for a preferred energy substrate in Siberian Huskies. Results from the tissue homogenate high respirometry analyses for non-raced groups were overall lower than expected based on previous studies. The OXPHOS capacity P for non-raced Alaskan huskies was 56±36.3 [pmol·s^{-1}·mg^{-1}], and 23.5±16.2 [pmol·s^{-1}·mg^{-1}] for the Siberian huskies (**Fig. 14**). The OXPHOS capacity for the non-raced group in Miller et al. (2017) which used permeabilized muscle fibers (five months without training over the summer season), was 204±27 [pmol·s^{-1}·mg^{-1}], which is substantially higher compared to our results. The same goes for ETS capacity E, where the non-raced Alaskan huskies in the present study had a value of 63±45.5 [pmol·s^{-1}·mg^{-1}], the Siberian huskies a value of 24.3±18.3 [pmol·s^{-1}·mg^{-1}] (**Fig. 14**), while Miller et al. (2017) had results in the non-raced group of 204±27 [pmol·s^{-1}·mg^{-1}] for ETS capacity E. Factors that might affect results from the present study includes both homogenate as a method, and the usage of local anesthetics, as discussed in section 6.6.1.

The higher LEAK found in non-raced Alaskan huskies compared to non-raced Siberian huskies, might be beneficial in terms of heat production, as the Alaskan huskies generally seem to have less dense fur than the Siberian huskies. Mitochondrial LEAK is not an all-or-nothing response and can, therefore, regulate long-term body temperature and energy expenditure. Shivering thermogenesis is an effective short-term solution for generating heat, which involves rapid contraction of skeletal muscles. This mechanism is costly and can lead to exhaustion, and it is therefore not sustainable as a long-term solution (Nedergaard & Cannon, 2014; Rolfe & Brown, 1997). Mitochondrial LEAK is, therefore, particularly important for animals that live in cold environments, such as sled dogs. Having a high LEAK will also counteract the production of reactive oxygen species (ROS), which is normally

produced by electron slip from CI and the ubiquinone cycle (CI + CII). Allowing some of the energy to slip away instead of contributing to the electrochemical gradient, will therefore limit ROS production, which is caused by higher mitochondrial activities (Korshunov et al., 1997).

Higher OXPHOS capacity found in the non-raced Alaskan huskies correlates with the knowledge that the Alaskan huskies in general can sustain activity at a higher speed compared to Siberian huskies, as seen during long distance-races such as Finnmarksløpet (Table 1). Endurance and speed can be influenced by many factors, such as genetics, angles and anatomy, size of the dogs, sex, amount of training, food and hydration, and length of legs (Bryce & Williams, 2017; Gregersen et al., 1998). External factors such as the weather, snow conditions, sled type, weight in sled, number of dogs in a team will also affect the speed of the team. Therefore, it is not as straight forwards as to just say that the Alaskan huskies may be faster than Siberian huskies. However, it is still a bit surprising, because it seems that the Siberian husky never runs to full exhaustion but keep a buffer of "energy" in case they need it on later occasions. Alaskan huskies do not to exhibit the same type of behavior and seem able to run to exhaustion, this ability could perhaps be compared to as the common psychological term of having "grit", which means that an individual can be especially tough mentally and are therefore able to push physical limits. However, this is highly speculative and lacks evidence.

As stated before, the preferred substrate usage of Siberian husky mitochondria is not known, and it would therefore be interesting to investigate the mitochondrial function with a fatty acid associated protocol to determine if there is a difference in metabolic phenotypes between Siberian huskies and Alaskan huskies. Miller et al. (2017) used a protocol for the mitochondrial fatty acid oxidation pathway (FAO) to test if the mitochondria works better with fat substrates compared to carbohydrates and found no difference in OXPHOS or ETS between the raced and the non-raced groups. The results from the two protocols were tested against each other (SUIT 1; FAO, vs SUIT 2; carbohydrates) and no difference was found in mean values for the raced group. The non-raced group had a significant difference in both OXPHOS and ETS, favoring the FAO protocol, which is closer linked to their diet. This means that the results from the non-raced group in the present study might have had different results for both groups if tested with a FAO protocol.

The large sex differences in oxygen consumption found in non-raced Alaskan huskies in our study may be due to the same physiological differences as seen in other species. In humans, females have been shown to have greater fat oxidation during exercise, independent

of aerobic exercise capacity compared to that of males, who are more carbohydrate dependent (Horton et al., 1998; Montero et al., 2018). This difference in substrate usage is likely due to the difference in hormone levels because estrogen produced in higher amounts in females has been shown to increase the amounts of fatty acids available for oxidation during exercise (Oosthuyse & Bosch, 2012). Males tend to have more muscle mass in general compared to females, which can be linked to higher testosterone levels (Auyeung et al., 2011). The proportion of body fat that make up the body composition also differs between sexes, and females sems to be more efficient in fat oxidation, possible due to the higher proportion of body fat (Tarnopolsky et al., 1990). However, this has not been investigated in sled dogs. Miller et al. (2017) tested both FAO and carbohydrate associated substrates, but their raced group contained only males. Two females and three males were included in the non-raced group in their study and might have contributed to their findings regarding substrate usage during exercise. Female Alaskan huskies might increase their oxygen consumption if tested with a FAO protocol. Siberian huskies might also increase their oxygen consumption if tested with fatty acids, and it would therefore be interesting to investigate this in future studies.

4.2 High resolution respirometry Raced 2023 samples

Muscle samples from Siberian huskies and Alaskan huskies raced 2023, which were homogenized and analyzed in HRR at 25 °C, were similar in group trends with significant higher LEAK in the Alaskan husky group, and significantly higher oxygen consumption by ETS CIII in the Siberian husky group. These results reflect a situation of moderate hypothermia and can therefore not be compared to data obtained at 37 °C for the non-raced groups. A Q_{10} factor of 2.3 was therefore used to assess theoretical values for T37. The Q_{10} factor chosen stems from human temperature values (Gnaiger, 2009; Pesta & Gnaiger, 2012) and might therefore not reflect the correct values for sled dogs because it can be both tissue and species dependent (Lemieux et al., 2017; Pesta & Gnaiger, 2012). Davis & Barret (2021) looked at the effect of temperature on mitochondrial oxygen consumption in Alaskan huskies and hypothesized that athletic conditioning would improve the mitochondrial tolerance to hyperthermia. The investigated temperatures were 38, 40, 42 and 44 °C. An attempt to calculate a sled dog specific Q_{10} from their data was done, however this turned out to be unsuccessful. Uncertainties regarding the findings in the study by Davis & Barret (2021) also made it difficult to assess data. They stated that their findings were unexpected and

counterintuitive, possibly stemming from an oversight in their methodology. They calculated the coupling-control ratio L/P (L: LEAK respiration, and P: The ratio between O2 consumption and ATP production) from the NADH- pathway and S-pathway for P, but only the NADH pathway for L. It seems that the S-pathway for L wasn't measured, and therefore their OXPHOS efficiency calculations (coupling-control ratio) seems to be incomplete (Gnaiger, 2020). They did not explain the reason behind this choice (Davis & Barrett, 2021).

Results from theoretically calculated values for 37 °C had the same trend as seen in the 25 °C groups, because the same coefficient was used to adjust for the temperature. The maximal theoretical OXPHOS capacity P for raced 2023 (T37) Alaskan huskies was 175.2±89.5 [pmol·s^{-1}·mg^{-1}], and for Siberian huskies 190.4±52 [pmol·s^{-1}·mg^{-1}]. This indicates that Siberian huskies are superior with regards to exercise acclimatization compared to Alaskan huskies. There are however many factors affecting these theoretical results. If Siberian husky mitochondria are better adapted to fatty acids, the respiration values may not be as high as that shown by the theoretical calculations. The amount of training also differed between the groups. The Siberian husky team conducted 3,700 km of endurance training compared to 2,500 km by the Alaskan husky team during the 2022/2023 season. This difference in 1,200 km might also affect the acclimatization seen in the two groups.

The theoretical OXPHOS capacities obtained for raced Alaskan huskies and Siberian huskies in this current study were lower compared to raced Alaskan huskies from Miller et al. (2017) (255±38 [pmol·s^{-1}·mg^{-1}]). However, the Alaskan huskies in the study by Miller et al. (2017) had trained 4,500 km – substantially more compared to the dogs in the current study. Compared to human elite endurance athletes (180 [pmol·s-1·mg-1]) and endurance racing horses (150 [pmol·s^{-1}·mg^{-1}]) described in the introduction, OXPHOS capacity P values from the present study on Alaskan huskies (175.2±89.5 [pmol·s^{-1}·mg^{-1}]), and Siberian huskies (190.4±52 [pmol·s^{-1}·mg^{-1}]), were similar, and slightly higher than that of the endurance horses (150 [pmol·s^{-1}·mg^{-1}]) (Boushel et al., 2007; Gnaiger, 2009; Mettauer et al., 2001; Mogensen et al., 2006; Rasmussen et al., 2001a; Votion et al., 2012). In general, these differences could be due to difference in anesthetics used (Miller et al. 2017 used general anesthesia) and in methods (homogenate vs. permeabilized muscle fibers). But most importantly, our Raced T37 values were based on predictions, and not experimentally measured at the correct temperature, which will influence the result. Sexual dimorphism in metabolic phenotypes could also affect the results, and Miller et al. (2017) used an all-male group. A still unknown substrate

preference for the Siberian husky group can also be a contributing factor. Calculating theoretical values for this might mask actual substrate use thresholds and can therefore indicate lower or higher use of the metabolite compared to experimentally obtained data for that temperature. However, data from the present study allows for group comparisons, since samples were treated equally, except for Raced 2022, and Raced 2023 T25. Obtaining multiple biopsies from the same wound could potentially collect a mixture of muscle fibers, and therefore it is common to normalize HRR data when using a tissue homogenate. HRR data is not independent of mitochondrial density and can therefore be normalized to mitochondrial density markers to obtain data reflecting mitochondrial quality, independent of density (Gnaiger, 2020). However, data from this study was not normalized to the mitochondrial marker and is therefore density dependent.

The theoretical values for raced 2023 (T37) ETS capacity E was 164.2±92.1[pmol·s^{-1}·mg^{-1}] and 180±56.7 [pmol·s^{-1}·mg^{-1}] for Alaskan huskies and Siberian huskies respectively and are again lower compared to Miller et al. (2017), with ETS value of 254±37 [pmol·s^{-1}·mg^{-1}].

4.3 High resolution respirometry Raced 23 T37 vs. Non-raced

The significantly higher OXPHOS and ETS values of theoretical raced 2023 compared to non-raced Siberian and Alaskan huskies with High Resolution Respirometry shows that endurance training and racing results in higher mitochondrial oxygen capacity, similar to the findings of Miller et al. (2017). OXPHOS and ETS are upregulated to match the increased demand for ATP in the raced samples compared to the non-raced samples. However, as already stated, theoretical values should not be compared to absolute values, and the results must be interpreted with caution.

The P/E ratio for the non-raced groups were both found to be close to 1, (AHN: 0.96±0.1, SHN: 0.97±0.05), which indicates a highly coupled system. For the Raced 2023 groups, the P/E was slightly higher than 1, indicating the presence of experimental artefacts. The Raced group P/E was calculated from T25 data, which was experimentally obtained, but no difference would appear in the P/E if T37 was used instead, because of adjustments to the same value for all individuals (Q_{10}: 2.3). Therefore, this result indicates experimental artifacts, from either inhibitors or uncouplers from the SUIT protocol, or possible contamination of the LA used. P/E is presented in means, and even so, no outlier from individual calculation can

account for the results, because the trend was similar for all individuals. There is no point in comparing P/E from raced T25 to non-raced group because of both temperature and artifact contributions.

CCR calculated for non-raced groups showed a value of 0.52±0.4 for the Alaskan huskies, indicating a high efficiency of OXPHOS through CI, while the Siberian husky value (1.2±0.4) was above the value of 1, indicating high LEAK and or low activity of P CI. The CCR indicates the system's efficiency by determining the proportion of total pumped protons used to create ATP, as opposed to being dissipated as heat or other types of energy. There is some artefact interfering because the value is outside of the range. The Siberian huskies had lower activity of P CI compared to the Alaskans non-raced, which might indicate some sort of damage to the outer mitochondrial membrane, or perhaps a difference in substrate preference. Raced 2023 CCR values were calculated from T25 and would be the same from theoretical T37. Both Siberians and Alaskans had values close to, or above 1 (SHR: 0.9±0.1, AHR: 1±0.2), which means that the relationship between activity of P CI and LEAK is inefficient, and possibly stemming from measured temperature (raced 2023 T25), or possible experimental artifacts.

4.4 Mitochondrial density, measured as CS activity

CS activity measured in the tissue homogenate was significantly different between muscle biopsies from raced 2022 and raced 2023 Alaskans huskies and Siberian huskies, indicating potential mitochondrial damage from trypsin (0.5 %) incubation in the Raced 2022 samples. The homogenate protocol was the same for samples from both the non-raced and raced 2023 dogs (0.25 % trypsin), and samples from those two seasons are therefore comparable. The results of increased mitochondrial density after endurance training found in this study are in line with previous studies as endurance training is known to improve mitochondrial function and density (Memme et al., 2021). Endurance training results in a higher CS activity in the muscle mitochondria, not only in dogs, but also in other mammals, such as mice and humans (Siu et al., 2003; Vigelsø et al., 2014). Wakshlag et al. (2002) investigated CS activity from skeletal muscle biopsies taken from *M. biceps femoris* from English pointers and Labrador retrievers prior to (September), and during the peak of activity during hunting season (March). Their study included males and females, and a total of 11 samples were used to investigate CS activity. Results showed an increase in CS activity from

pretraining to peak activity, which is in line with our findings from the sled dogs (**Figure 13**). Wakshlag et al. (2002) collected biopsies during general anesthesia, and their samples were obtained 24 hours after the last training session was completed. They too prepared a 10 % homogenate for analyses (Wakshlag et al., 2002). Reported findings went from pretraining CS 26.8±10.0 (µm substrate oxidized/min) to 32.7±11.1 (µm substrate oxidized/min) for the peak activity group for both breeds (Wakshlag et al., 2002), which is difficult to compare to our units presented in the mitochondrial marker unit: IU (µmol/min*mg of tissue). These units were chosen in the present study to allow for normalization to Oroboros data.

The exercise training effect on CS activity has been reported to vary from 0–100 % and can be influenced by the timing of muscle sampling in relation to the last exercise session (Leek et al., 2001). The last training was conducted 15–24 hours prior to sampling and should therefore not have an acute exercise effect (adaptations occurring immediately during or after exercise) on the sample CS activity, according to Wakshlag et al. (2002). Acute exercise effect on CS activity compared to trained-rested humans have been shown to increase the CS activity of the sample by 49.4 %, compared to an increase from non-trained resting individuals to trained-rested individuals by 18.2 % (Leek et al., 2001). Leek et al. (2001) also investigated mitochondrial structure by electron microscopy and found swollen mitochondria 1 hour after exercise (acute effect), that can lead to artificially elevated CS values.

Another variable affecting CS assays is the temperature at which the assay is run. Not all studies mention the temperature setting of their CS assay and a standardized analytical method should be used by all laboratories to compare absolute values of CS activity (Vigelsø et al., 2014). In our case the CS essay was run at room temperature, and this may have underestimated the values as compared to at 37 °C. Other methodical errors possibly effecting CS activity in the present study could be from pipetting errors, resulting in larger differences between the three sample replicates in AHR23 especially, as well as between different individuals, such as the AHR23 group. This led to repetitive trials to obtain data with low variability.

4.5 Animal welfare

A project involving animals must comply with the Norwegian and European legislation for animal research, but studying mitochondrial respiration in long-distance trained sled dogs requires privately owned dogs. This adds an additional layer to the ethical and moral perspective with regards to personal connection and communication with the animal

owners. Transparency and documentation are important for both, as well as having a good dialogue that keeps the owners well informed throughout the project.

Our aim has been to ensure the best possible welfare of the animals included in the project and to be available for questions and concerns throughout the process. The sampling of the animals was considered minimally invasive without expected long-lasting effects by the NFSA. This was confirmed throughout the sampling (**Appendix Table 1)**. To avoid injuries, training distances, duration and intensities increase gradually during the racing seasons, and were tracked by the mushers to ensure progression and recovery. Massages, veterinary and canine physiotherapists were used to prevent injuries and ensure good health.

The three R´s were also carefully considered and implemented as explained below.

Reduce: Each sample was used for several parallel experiments to ensure high quality data, and to minimize the total number of biopsies and individuals needed.

Refine: The previous study on Alaskan huskies by Miller et al. (2017) performed the biopsy under general anesthesia, including eleven dogs in their study (five off-season dogs and six dogs after racing the Iditarod). There is always a risk of adverse reactions in some individuals when using anesthetics, and Nordic breed dogs are reported to have a genetic polymorphism that predisposes them to opioid-related dysphoria (Claude). To minimize the invasiveness and risk of the biopsy procedure, we consequently decided to sample the dogs in our experiment without general anesthesia or sedation. An anti-inflammatory NSAID was also used as analgesic to ensure good animal welfare. Sileo oromucosal gel (dexmedetomidine) was used prior to the biopsy procedure but did not seem to have any notable effect on dogs behavior. The sampling environment was also adjusted for individual dogs needs. Some individuals were sampled indoors, while some were sampled in their dog yard, accompanied by other dogs or independently, depending on the comfort of the individual dogs. A micro biopsy procedure was chosen because it is minimally invasive and allows usage of local anesthetics instead of anesthesia or sedation. This minimizes the total strain on the individual dog compared to general anesthesia operations. Micro biopsy procedures are a commonly chosen method on humans, for both participant and ethical comfort, and is also preferable for a study that requires multiple muscle samples during various time (Hayot et al., 2005; Newmire & Willoughby, 2022).

Replace: There are no suitable *in vitro* or model approaches that can recapitulate the mitochondrial phenotype and yield information on respiratory capacity of skeletal muscles in sled dogs.

The Sileo oromucosal gel (dexmedetomidine) did not appear to have any notable effect on the behavior of the huskies, even though it has well documented calming effects in other dog breeds (Hauser et al., 2020). During the two seasons, there was a difference in muscle mass between the raced and non-raced groups. The raced groups had the largest muscle mass, as observed visually and by palpation, which was an expected response to endurance training. Better biopsies were taken from the raced groups, with more muscle mass obtained compared to non-raced group. Some samples from the non-raced group contained some fat, indicating that a depth (1 cm) was not sufficient for some of the dogs during that season. Additionally, it has been reported that taking multiple biopsies from the same muscle group can affect the mitochondrial biogenesis and induce oxidative stress, which might have contributed to large variations found within the homogenate (Newmire & Willoughby, 2022). Differences in blood assembly in the insertion tube were also clear between seasons and might be explained by denser vascularization to the muscles for the raced groups (Prior et al., 2004).

4.6 Sample preparations

Even though homogenate preparations can cause reduced maximal respiratory capacity and, potentially, mitochondrial damage, homogenate was chosen as a method over permeabilized muscle fibers to study mitochondrial respiration in this project (Larsen et al., 2014). Homogenate preparations have the advantage of being more user friendly, and less time consuming compared to permeabilized fibers (which also suffer from individual variability during preparation) making the homogenate a more suitable method for a master project. Permeabilized fibers also has the advantage of requiring small amounts of tissue for HRR analyses, while homogenate requires larger volume of tissue, both for evenness within the homogenate, and for obtaining high volume-specific oxygen fluxes because noise from the oxygen season can limit respiratory fluxes (Gnaiger, 2009; Rasmussen et al., 2001b; Rasmussen et al., 2004).

4.6.1 Possible Effects of local anesthesia

Another factor possibly affecting the homogenate mitochondria is the local anesthetics, previously mentioned to possibly induce alteration of muscle mitochondrial energetics (Hogan et al., 1994). This is however difficult to assess, due to many different local

anesthetic brands and active ingredients, as well as different body mitochondria assessed. For skeletal muscle mitochondria, there is not much information about the possible role of xylocaine adrenalin as a contaminant, but it has previously been reported to suppress the ETS and the mitochondrial membrane potential, studied in *in vitro* neuronal cells (Okamoto et al., 2016). There are also several studies pointing towards a direct effect of local anesthetics on mitochondrial function, and how the local anesthetics is administrated seems to be of importance. Ideally it should not be infiltrated into the muscle, only under the superficial fascia (Hogan et al., 1994; Pesta & Gnaiger, 2012; Votion et al., 2012). There are also studies using administration of local anesthetics only under the skin (Boushel et al., 2007; Newmire & Willoughby, 2022). During this study, however, local anesthesia was injected into the muscle, and therefore might have had a negative effect on the samples used. Exposure time to local anesthetics, as well as the dose administered might also affect the muscle mitochondria (Hogan et al., 1994; Mogensen et al., 2006; Newmire & Willoughby, 2022). If the local anesthesia dose used during this study was too high, it might also have had a negative effect on the samples used. The present study briefly looked into the effect of local anesthesia on muscle mitochondrial function presented in **Appendix figure 7**, which indicated lower respiration of the local anesthesia-treated muscle compared to non-treated muscle. There also seemed to be a difference in cytochrome c response, with only one accepted replicate from the local anesthesia treatment, compared to two accepted replicated from the non-treated muscle. However, this data is only indicative, and the sample size too low to be able to discuss anything regarding the effect of local anesthetics. Nevertheless, it might show that it would be interesting to assess this possible problem in the future.

5 Conclusion

Long distance sled dogs provide a unique model to examine the variability in mammalian aerobic exercise performance. This present study is the first to report on mitochondrial respiration in the pure breed Siberian husky, accompanied by data on Alaskan huskies previously reported in a few studies (Davis & Barrett, 2021; Miller et al., 2017). Respiratory capacity in skeletal muscle mitochondria from Alaskan huskies and Siberian huskies was higher during racing season than off-season. This increase in aerobic capacity possibly results from an increase in muscle mitochondrial density. Selective breeding aimed to enhance race performance and endurance in Alaskan huskies might have led to a higher respiratory capacity in their skeletal muscles measured with carbohydrates, as compared to that of Siberian huskies. Future studies should investigate sexual dimorphism, as well as substrate preference for Siberian husky mitochondria.

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Appendix

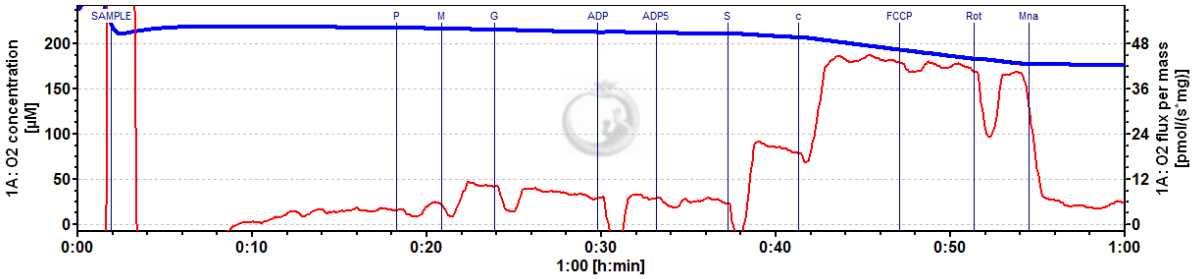
Sampling notes from individual dogs A. Table 1, including season, sex, and year of birth.

***A.Table 1**. Sampling notes for each individual dogs, over the three seasons sampled.*

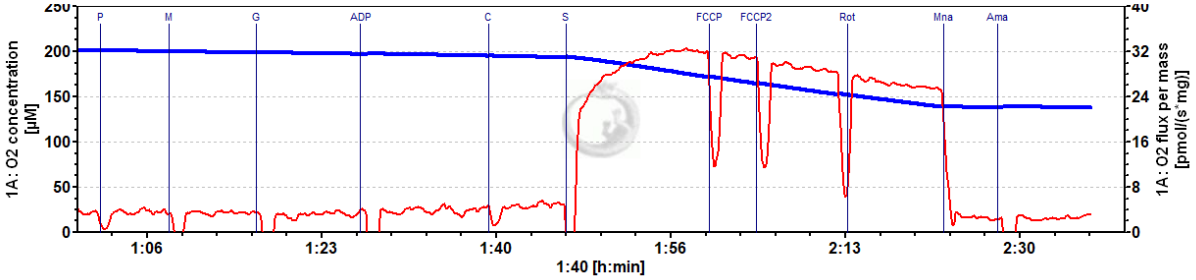
Season	Individual	Sex	Born	Biopsy	Sampling note
Raced 2022	SH1	Female	2019	X3	No notable effect of Sileo, small reaction to injection of LA
Raced 2023	SH1			X2	No notable effect of Sileo
Raced 2022	SH2	Female	2015	X4	Four biopsies taken from the same insertion point and sample quality tested throughout the day (*see protocol development), no notable effect of sileo, small amount of blood in insertion tube
Non-raced 2022	SH2			X3	Right side biopsy, small amount, no notable effect of Sileo
Raced 2023	SH2			X2	No notable effect of Sileo, imagined pregnancy and sampling postponed one week
Raced 2022	SH3	Female	2016	X2	Reacted to biopsy sound and showed some general stress. No notable effect of Sileo
Non-raced 2022	SH3			X2	No notable effect of Sileo, showed some distress
Raced 2023	SH3			X2	No notable effect of Sileo, small amount of blood in the insertion tube
Raced 2022	SH4	Female	2016	X2	No notable effect of Sileo
Non-raced 2022	SH4			X2	No notable effect of Sileo
Raced 2023	SH4			X2	No notable effect of Sileo, small amount of blood in the insertion tube
Non-raced 2022	SH5	Male	2021	X2	No notable effect of Sileo
Non-raced 2022	SH6	Male	2021	X2	No notable effect of Sileo
Raced 2023	SH6			X2	No notable effect of Sileo, small amount of blood in the insertion tube

Raced 2022	AH1	Male	2019	X2	No notable effect of Sileo
Non-raced 2022	AH1			X3	Small amount of sample obtained, no notable effect of Sileo
Raced 2023	AH1			X2	No notable effect of Sileo, minor hemorrhage stopped by 3 minutes of digital press
Raced 2022	AH2	Male	2019	X2	No notable effect of Siloe, small amount of blood in the insertion tube
Non-raced 2022	AH2			X3	Small amount of sample obtained, no notable effect of Sileo, minor hemorrhage stopped by seconds of digital press
Raced 2023	AH2			X2	No notable effect of Sileo
Raced 2022	AH3	Female	2012	X3	Small amount of sample obtained, no notable effect of Sileo,
Non-raced 2022	AH3			X2	No notable effect of Sileo
Raced 2023	AH3			X2	No notable effect of Sileo
Raced 2022	AH4	Female	2019	X2	No notable effect of Sileo
Non-raced 2022	AH4			X3	Small amount of sample obtained, no notable effect of Sileo
Raced 2023	AH4			X2	No notable effect of Sileo, small amount of blood in the insertion tube
Raced 2023	AH5	Female	2019	X2	No notable effect of Sileo
Raced 2023	AH6	Male	2016	X3	No notable effect of Sileo, minor hemorrhage post sampling, probably due to licking the wound
Raced 2023	AH7	Male	2016	X2	No notable effect of Sileo

Figure A1 shows one reading of an invalid response to cytochrome c addition. Figure A2 shows one reading with a valid response after cytochrome c was added.



***A 1**. Readings from one experimental chamber where sample AH4 had a difference after Cyt C was added larger than 30 % and was therefore excluded from the results.*



***A 2**. Graph from one experimental chamber containing sample SH1 who passed the Cyt C test.*

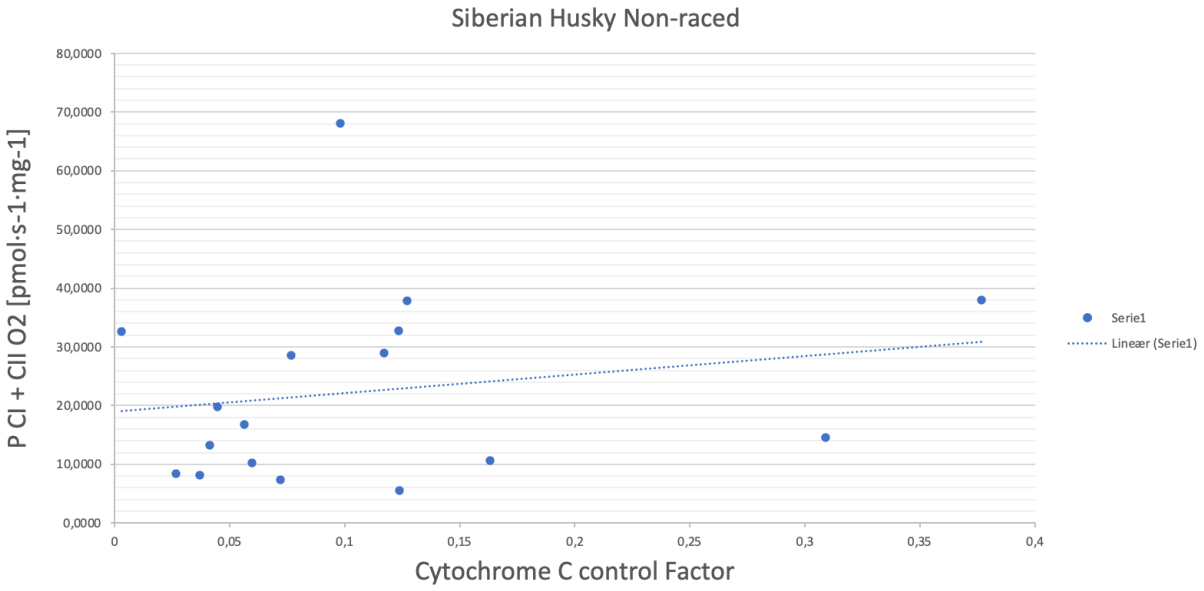
Citrate synthase activity p-values from welch two sample t.test, performed in R.studio, between seasons A.Table 2.

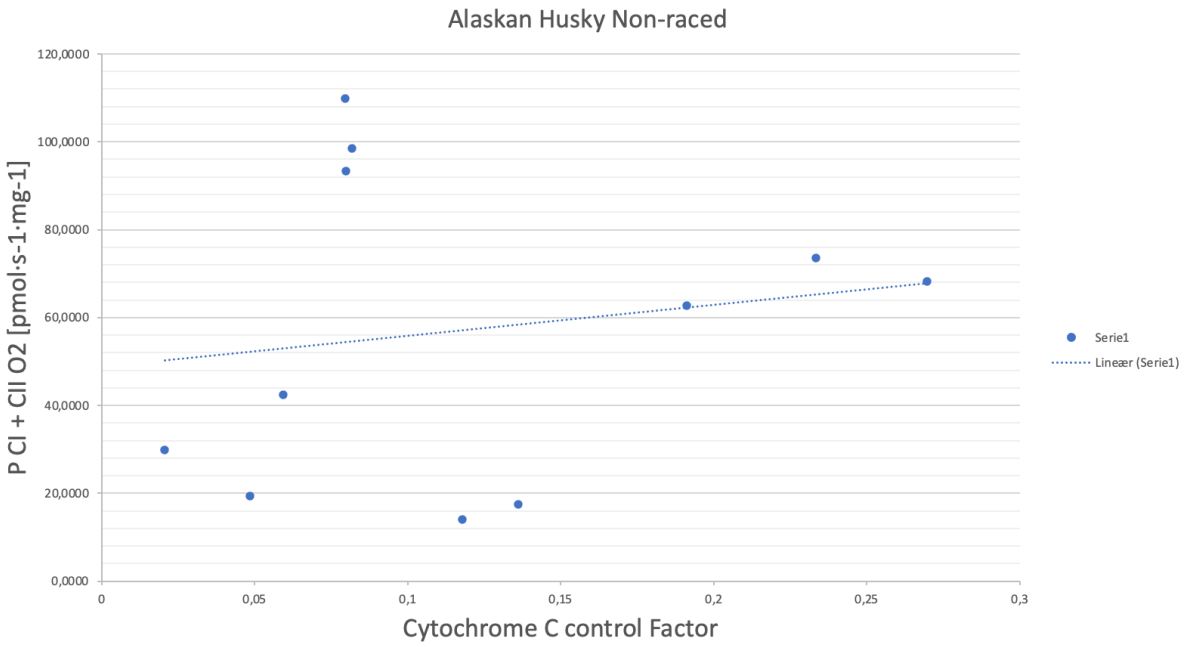
***A.Table 2** Welch´s t-test for CS activity comparisons between groups and seasons, including p-values. Significance is marked in red.*

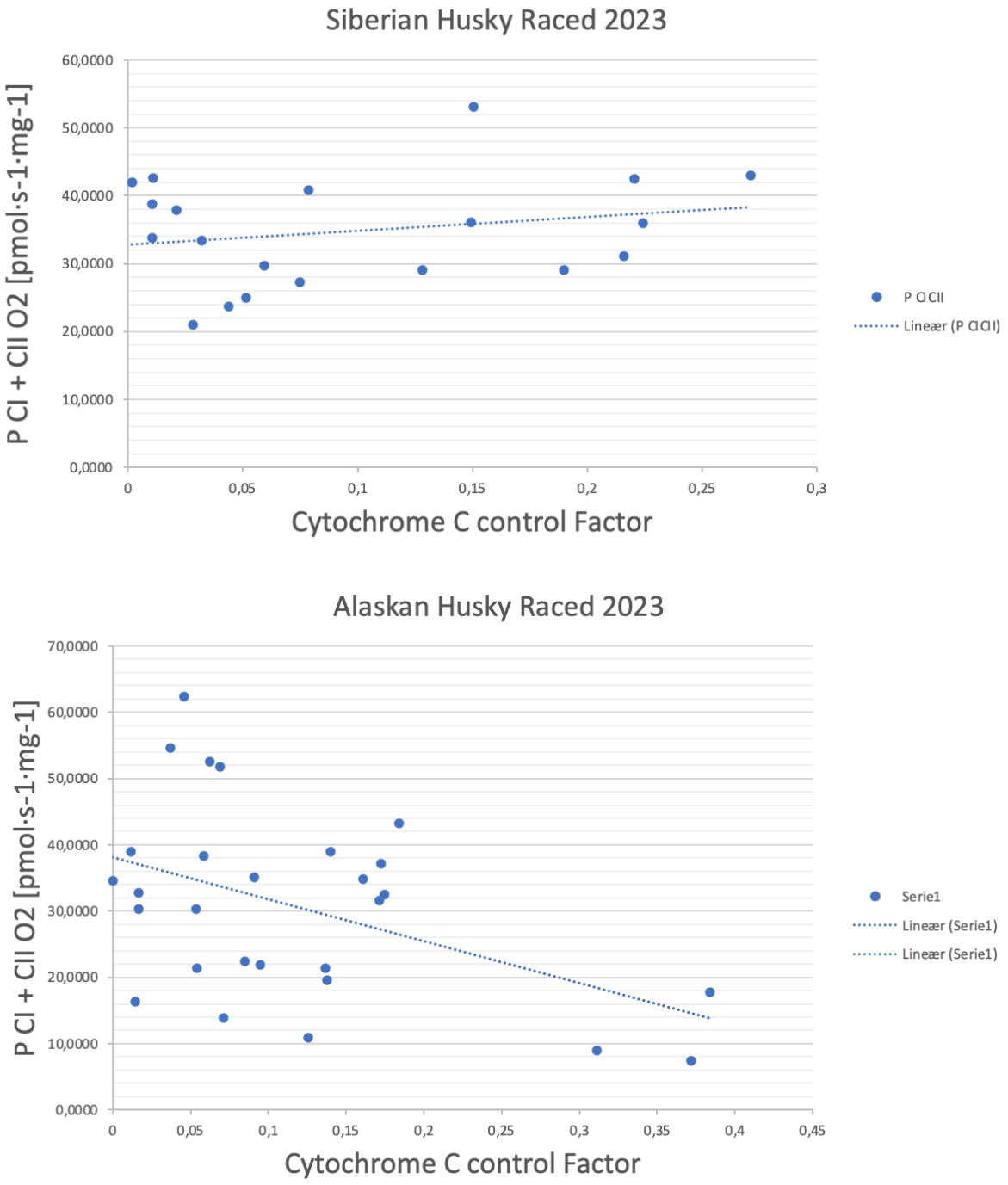
Difference in CS Activity by season	**p-value**
AH Raced 2022 vs. SH Raced 2022	p-value = 0.2047
AH Non-raced vs. SH Non-raced	p-value = 0.7355
AH Raced 2023 vs. SH Raced 2023	p-value = 0.09103
AH Raced 2022 vs. AH Raced 2023	p-value = 0.04102
SH Raced 2022 vs. SH Raced 2023	p-value = 0.006455
AH Non-raced vs. AH Raced 2023	p-value = 0.01574
SH Non-raced vs. SH Raced 2023	p-value = 0.001576

In the study by Miller et al. (2017) Cytochrome c induced respiration was considered valid until 30 % increase. Based on the study by Laner et al. (2014) who showed that there was no relationship between cytochrome C and phosphorylation and determined that the Cyt C threshold should be experimentally determined. Cytochrome C control factor was calculated for each sample, $FCF_c = (J_{CHOc}-J_{CHO})/J_{CHOc}$ where JCHOc is the respiration after addition of cytochrome c, and JCHO is the respiration before addition of cytochrome C,

plotted against OXPHOS capacity of P CI + CII. No linear relationship was found; therefore, it was concluded that cytochrome C control factor is independent from the O_2 flux (Fig.A3).



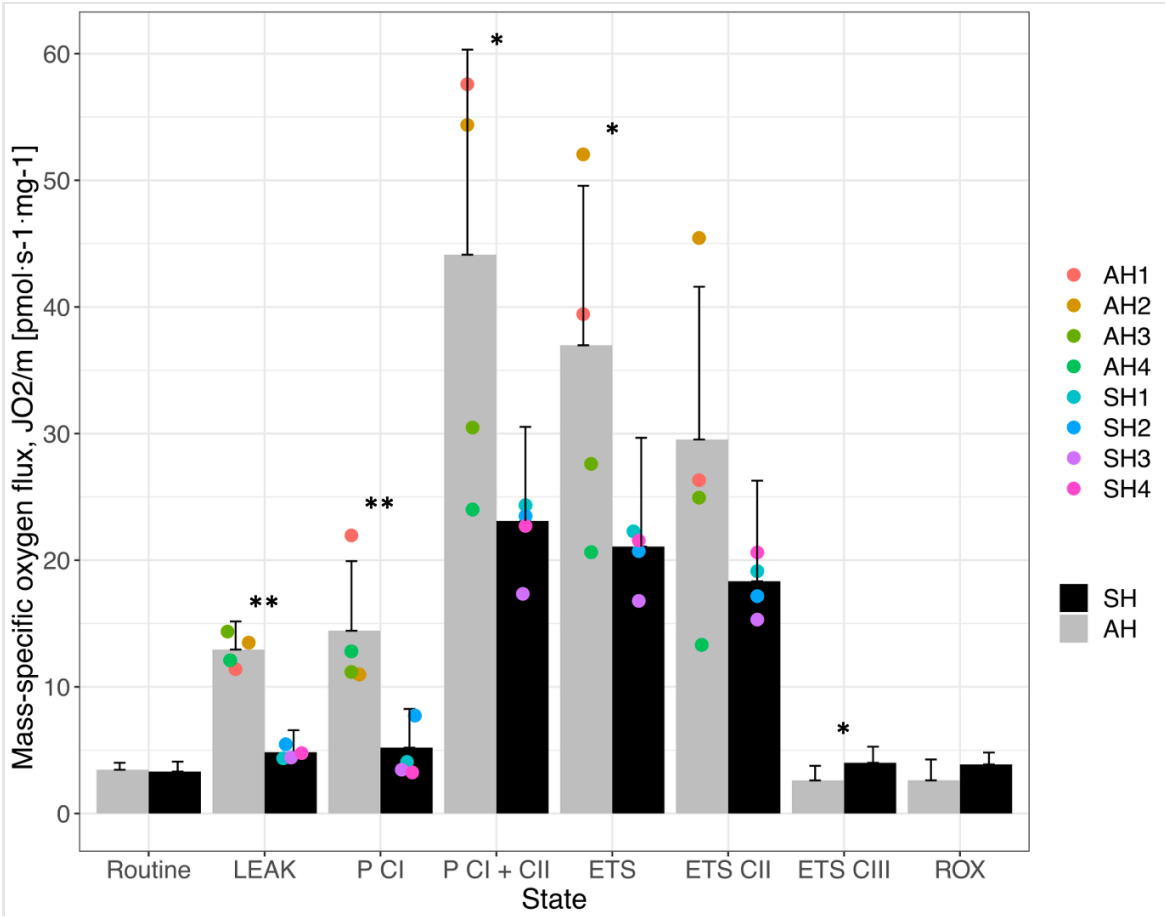




***A 3**. No linear relationship was found between Cytochrome C control Factor and O_2 flux for Alaskan and Siberian husky Raced and Non-raced samples.*

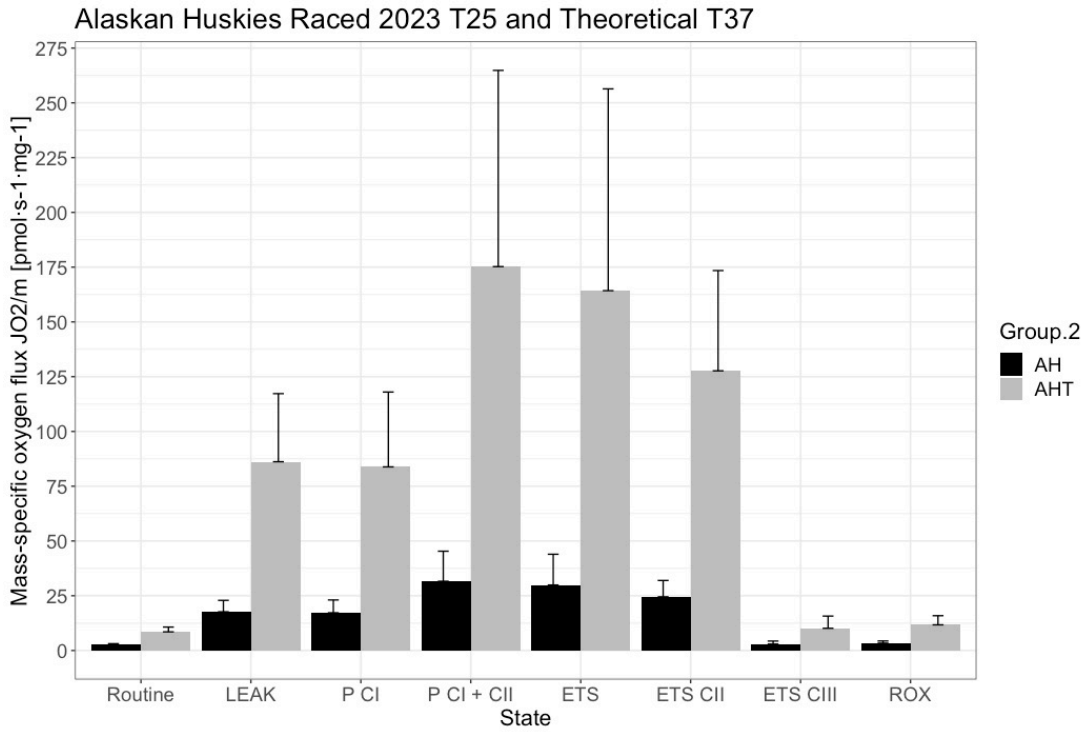
Trends in Fig. A4 suggest that the oxygen flux was higher in Alaskan huskies compared to Siberian Huskies. The two highest individual points within Alaskan Huskies were AH1 and AH2, both males. The Siberian husky group had a smaller deviance between data points and appeared to be more homogenous as a group. The Siberian husky group was an all-female group. There were significant differences in LEAK, P CI , P CI + CII, ETS, and

ETS CIII (Appendix Table 3). No difference was found between the groups in ETS CII p-value: 0.056.

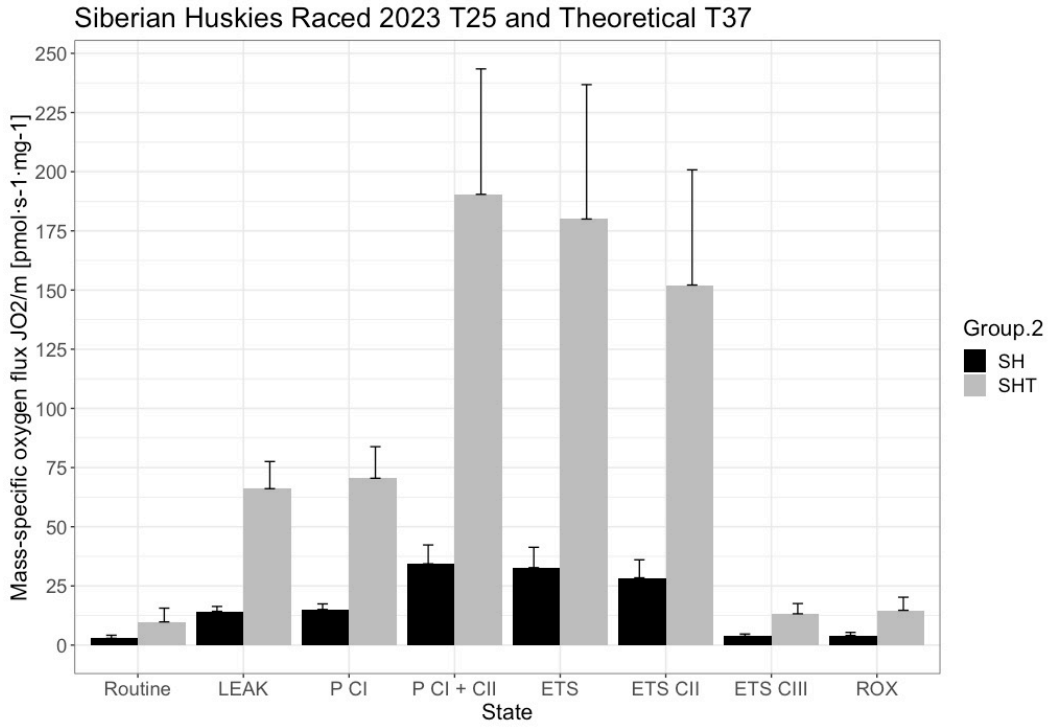


A 4. *Group mean values presented, including individual sample means as points in states that have a large sd. Alaskan husky: Routine: 3.45±0.50, LEAK: 12.9±2.2, P CI: 14.4±5.4, P CI + CII: 44.12±16.10, ETS:36.9±12.5, ETS CII:29.5±12.0, ETS CIII:2.6±1.1, ROX:2.6±1.6. Siberian huskies; Routine:3.3±0.7, LEAK:4.8±1.7, P CI:5.1±3.0, P CI + CII: 23.0±7.4, ETS:21.0±8.5, ETS CII:18.3±7.9, ETS CIII:4.0±1.2, ROX:3.8±0.9 [pmol·s^{-1}·mg^{1}]. P.values presented in level of significance, where * = <0.01, **=<0.001, ***=<0.0001.*

Raced 2023, T25 and theoretical T37 plotted in Figures A4-5.



A 5. *Mass specific oxygen flux of group means presented including standard deviation, for each state. Presented with Theoretical Q10=2.3 (T37= T) values and measured T25. Individual sample means is included to present the variation found within the data, for the states containing the largest deviation. Alaskan huskies T25 (AH) mean values; Routine 2.5±0.5, LEAK: 17.6± 5.2, P CI 17.2±5.8, P CI+CII 31.6±13.7, ETS 29±14, ETS CII 24.5±7.4, ETS CIII 2.9±1.3, ROX 3.3±0.9. Alaskan huskies T37 (AHT) mean values; Routine 8.5±2.1, LEAK: 86.1±31, P CI 83.8±34.1, P CI+CII 175.2±89.5, ETS 164.2±92.1, ETS CII 127.6±45.7, ETS CIII 10.1±5.5, ROX 11.7±4.1[pmol·s^{-1}·mg^{-1}].*



A 6. *Mass specific oxygen flux of group means presented including standard deviation, for each state. Presented with Theoretical Q10=2.3 (T37) values and measured T25. Individual sample means is included to present the variation found within the data, for the states containing the largest deviation. Siberian huskies T25 mean values; Routine 2.8±1.3, LEAK 14.2±2, P CI 15±2.3, P CI+CII 34.3±7.9, ETS 32.7±8.6, ETS CII 28.4±7.6, ETS CIII*

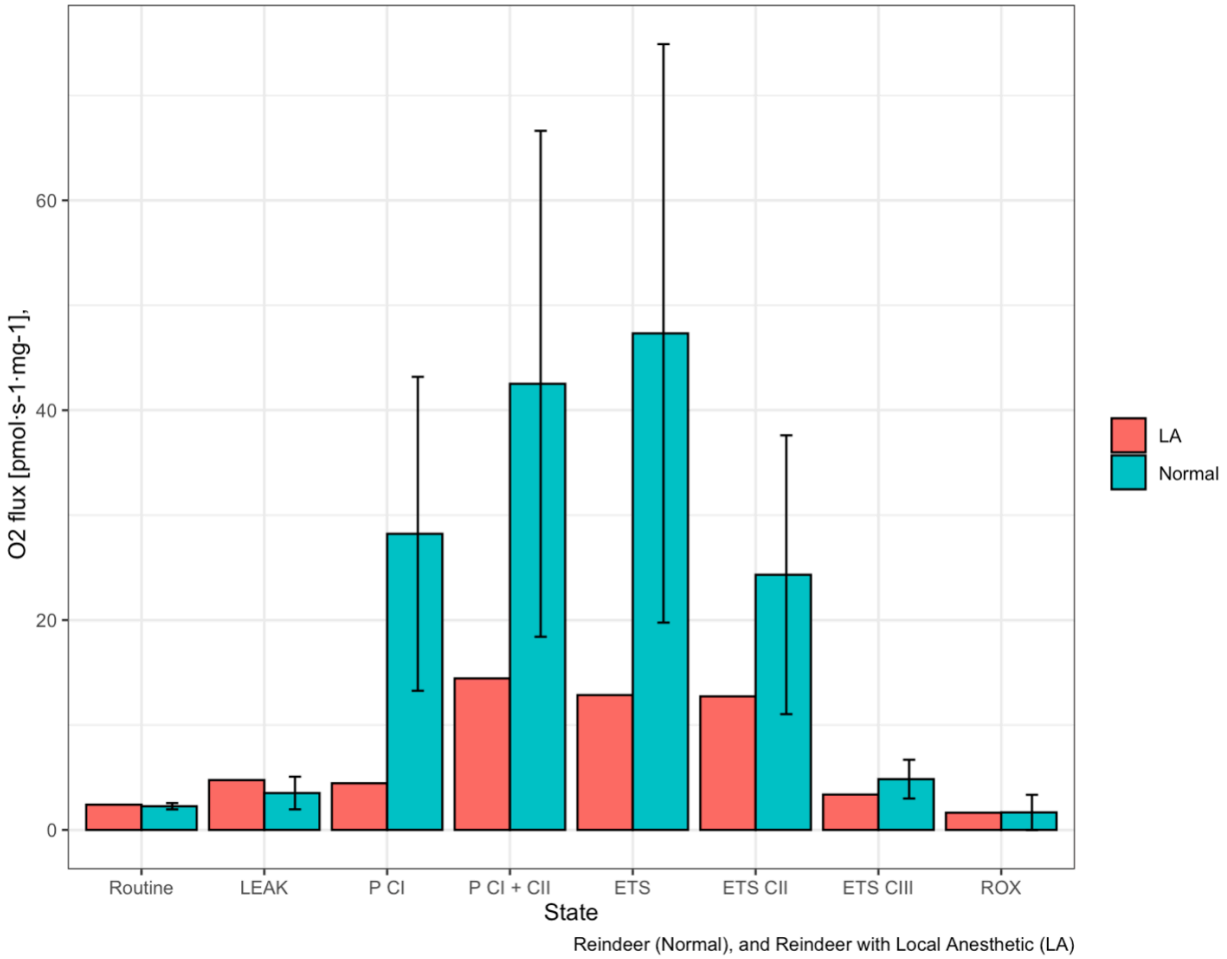
3.7±0.9, ROX 4±1.3.Siberian huskies T37 mean values; Routine 9.8±5.8, LEAK 66.1±11.4 , P CI 70.4±13.3, P CI+CII 190.4±52.9, ETS 180±56.7, ETS CII 152.1±48.6, ETS CIII 13.2±4.3, ROX 14.6±5.5[pmol·s^{-1}·mg^{-1}].

Welch two sample t.test performed between groups and seasons A.Table3.

***A.Table 3**. Welch two sample t.test on individual sample replicates within groups, for group comparisons over different seasons*

Season and groups	LEAK p-value	P CI p-value	P CI + CII p-value	ETS p-value	ETS CII p-value	ETS CIII p-value
AH Raced 22 vs. SH Raced 22.	6.506e-06	0.003268	0.01291	0.01565	0.05677	0.03068
AH Non-raced vs. SH Non-raced	0.001115	0.01271	0.02132	0.02703	0.05652	0.1534
AH Raced 23 vs. SH Raced 23	0.003887	0.08695	0.4031	0.4039	0.09105	0.02925
AH Non-raced vs. AH Raced 23 (T25)	3.8e-09	0.1071	0.06515	0.04838	0.1394	0.004246
SH Non-raced vs. SH Raced 23 (T25)	2.005e-15	0.005033	0.02367	0.1101	0.03491	0.7424
AH Non-raced vs. Raced 23 T37	5.178e-13	6.067e-05	1.719e-06	0.0001034	3.229e-11	3.131e-05
SH Non-raced vs. SH Raced 2023 T37	2.545e-15	2.2e-16	8.659e-12	1.1e-10	2.931e-10	1.165e-08

One reindeer euthanized for other scientific purposes was sampled post-mortem to investigate the effect of local anesthetics on mitochondrial function. 4 ml of the local anesthetic Xylocaine-adrenaline was injected immediately post-mortem, and a biopsy was sampled directly after injection from the *M. triceps branchii*, homogenized and analyzed in two chambers of HRR. One biopsy was also taken post-mortem from the other *M. triceps branchii,* without LA injection as a control, and analyzed in two chambers of HRR. Results are included in Figure A5. Only one chamber from the local anesthesia injected muscle passed the Cyt C control. Both samples from the non-treated muscle passed and are included in the figure with standard deviation between the two chambers. Trends in the results indicate that there might be a different in oxygen flux between a treated and a non-treated muscle, but the sample size is very small, and there is no proper way to compare the two with this method. However, it would be interesting to look further into the effect of local anesthetics on muscle mitochondrial function in the future the potential contaminating effects (figure A. 6).



A 7*. Samples from one reindeer, euthanized for other experiments were used and collected post-mortem.*

5.1.1.1 R codes for CS Activity T-test

```
# assigning Season for AH and SH
R22A <- subset(CS, Season=="R22A")
R22S <- subset(CS, Season=="R22S")
N22A <- subset(CS, Season=="N22A")
N22S <- subset(CS, Season=="N22S")
R23A <- subset(CS, Season=="R23A")
R23S <- subset(CS, Season=="R23S")
#bind R22, N22, R23
R22 <-rbind.data.frame(R22A,R22S)
N22 <-rbind.data.frame(N22A,N22S)
R23 <-rbind.data.frame(R23A,R23S)
#bind R22-R23 for both A and S
R2223A <- rbind.data.frame(R22A,R23A)
R2223S <- rbind.data.frame(R22S,R23S)
# bind R23 and N22 for both A and S
R23N22A <- rbind.data.frame(R23A,N22A)
R23N22S <- rbind.data.frame(R23S,N22S)
```

```
#p values for R22
PR22 <- aov(`CS activity`~Season, data = R22)
summary(PR22)
t.test(`CS activity`~Season, data = R22)
# p value: 0.2047, no difference between AH and SH

#p values for N22
PN22 <- aov(`CS activity`~Season, data = N22)
summary(PN22)
t.test(`CS activity`~Season, data = N22)
# p = 0.7355, no difference between AH and SH

#p values for R23
PR23 <- aov(`CS activity`~Season, data = R23)
summary(PR23)
t.test(`CS activity`~Season, data = R23)
#p = 0.09103, no difference between AH and SH

#p values for R22 R23 A
PR2223A <- aov(`CS activity`~Season, data = R2223A)
summary(PR2223A)
t.test(`CS activity`~Season, data = R2223A)
# P= 0.04102 Difference between R22 and R23 AH

#p values for R22 R23 S
PR2223S <- aov(`CS activity`~Season, data = R2223S)
summary(PR2223S)
t.test(`CS activity`~Season, data = R2223S)
# P= 0.0063455 Difference between R22 and R23 SH

#p values for R23 N22 A
PR23N22A <- aov(`CS activity`~Season, data = R23N22A)
summary(PR23N22A)
t.test(`CS activity`~Season, data = R23N22A)
# P= 0.01574 Difference between R23 and N22 AH

#p values for R23 N22 SH
PR23N22S <- aov(`CS activity`~Season, data = R23N22S)
summary(PR23N22S)
t.test(`CS activity`~Season, data = R23N22S)
# P= 0.001576  Difference between R23 and N22 SH
```

5.1.1.2 R-codes Raced 2023

```
# dividing state to dog, AH, SH

RoutineR <- subset(R, State=="Routine")
LEAKR <- subset(R, State=="LEAK")
PCIR <- subset(R, State=="P CI")
```

```
PCICIIR <- subset(R, State=="P CI + CII")
ETSR <- subset(R, State=="ETS")
ETSCIIR <- subset(R, State=="ETS CII")
ETSCIIIR <- subset(R, State=="ETS CIII")
ROXR <- subset(R, State=="ROX")

LER <- aov(`Raw data`~Dog, data = LEAKR)
summary(LER)

t.test(`Raw data`~Dog, data = LEAKR)

#P= 0.003887

#p values for P CI
PCIRR <- aov(`Raw data`~Dog, data = PCIR)
summary(PCIRR)
t.test(`Raw data`~Dog, data = PCIR)
# p value: 0.8695

#pvalue for P CI + CII
PCICIIRR <- aov(`Raw data`~Dog, data = PCICIIR)
summary(PCICIIRR)

t.test(`Raw data`~Dog, data = PCICIIR)
#pvalue: 0.4031

#pvalue for ETS
ETSRR <- aov(`Raw data`~Dog, data = ETSR)
summary(ETSRR)

t.test(`Raw data`~Dog, data = ETSR)
# pvalue 0.4039

#pvalue for ETS CII
ETSCIIRR<- aov(`Raw data`~Dog, data = ETSCIIR)
summary(ETSCIIRR)

t.test(`Raw data`~Dog, data = ETSCIIR)
# p value 0.09105

ETSCIIIRR <- aov(`Raw data`~Dog, data = ETSCIIIR)
summary(ETSCIIIRR)

t.test(`Raw data`~Dog, data = ETSCIIIR)
# 0.02925
```

5.1.1.3 Non-raced 2022

Non-raced 2022:

```
RoutineN <- subset(N, State=="Routine")
LEAKN <- subset(N, State=="LEAK")
PCIN <- subset(N, State=="P CI")
PCICIIN <- subset(N, State=="P CI + CII")
ETSN <- subset(N, State=="ETS")
ETSCIIN <- subset(N, State=="ETS CII")
ETSCIIIN <- subset(N, State=="ETS CIII")
ROXN <- subset(N, State=="ROX")

# Finding P values for LEAK for both groups Non-raced
# welch two sample t-test.
LE <- aov(`Raw data`~Dog, data = LEAKN)
summary(LE)
t.test(`Raw data`~Dog, data = LEAKN)
#P=0.001115

#p values for P CI
PCI <- aov(`Raw data`~Dog, data = PCIN)
summary(PCI)
t.test(`Raw data`~Dog, data = PCIN)
# p value:0.01271

#pvalue for P CI + CII
PCICII <- aov(`Raw data`~Dog, data = PCICIIN)
summary(PCICII)

t.test(`Raw data`~Dog, data = PCICIIN)
#pvalue:  0.02132

#pvalue for ETS
ETS <- aov(`Raw data`~Dog, data = ETSN)
summary(ETS)
t.test(`Raw data`~Dog, data = ETSN)
# pvalue  0.02703

#pvalue for ETS CII
ETSCII<- aov(`Raw data`~Dog, data = ETSCIIN)
summary(ETSCII)

t.test(`Raw data`~Dog, data = ETSCIIN)
# p value 0.05652

ETSCIII <- aov(`Raw data`~Dog, data = ETSCIIIN)
summary(ETSCIII)

t.test(`Raw data`~Dog, data = ETSCIIIN)
# 0.1534
```

5.1.1.4 Alaskan husky sex

```
AH <- subset(N, Dog=="AH")

LEAKSN <- subset(AH, State=="LEAK")
SEXPCIN <- subset(AH, State=="P CI")
SEXPCICIIN <- subset(AH, State=="P CI + CII")
SEXETSN <- subset(AH, State=="ETS")
SEXETSCIIN <- subset(AH, State=="ETS CII")
SEXETSCIIIN <- subset(AH, State=="ETS CIII")

t.test(`Raw data`~Sex, data = LEAKSN)
#P= 0.9158 No difference male femle

#p values for P CI
t.test(`Raw data`~Sex, data = SEXPCIN)
# p value: 0.003542 difference male female

#pvalue for P CI + CII
t.test(`Raw data`~Sex, data = SEXPCICIIN)
#pvalue: 0.000523 male female

#pvalue for ETS
t.test(`Raw data`~Sex, data = SEXETSN)
# pvalue 0.001688

#pvalue for ETS CII

t.test(`Raw data`~Sex, data = SEXETSCIIN)
# p value 0.00001693


t.test(`Raw data`~Sex, data = SEXETSCIIIN)
# 0.3408 male female
```

5.1.1.5 Raced 2022

```
MRoutineN <- subset(MAY, State=="Routine")
MLEAKN <- subset(MAY, State=="LEAK")
MPCIN <- subset(MAY, State=="P CI")
MPCICIIN <- subset(MAY, State=="P CI + CII")
METSN <- subset(MAY, State=="ETS")
METSCIIN <- subset(MAY, State=="ETS CII")
METSCIIIN <- subset(MAY, State=="ETS CIII")
MROXN <- subset(MAY, State=="ROX")

# Finding P values for LEAK for both groups
# welch two sample t-test.
MLE <- aov(`Raw data`~Dog, data = MLEAKN)
summary(MLE)
```

```
t.test(`Raw data`~Dog, data = MLEAKN)
#P= 6.506e-06

#p values for P CI
MPCI <- aov(`Raw data`~Dog, data = MPCIN)
summary(MPCI)
t.test(`Raw data`~Dog, data = MPCIN)
# p value: p-value = 0.003268

#pvalue for P CI + CII
MPCICII <- aov(`Raw data`~Dog, data = MPCICIIN)
summary(MPCICII)

t.test(`Raw data`~Dog, data = MPCICIIN)
#pvalue: p-value = 0.01291

#pvalue for ETS
METS <- aov(`Raw data`~Dog, data = METSN)
summary(METS)

t.test(`Raw data`~Dog, data = METSN)
# p-value = 0.01565

#pvalue for ETS CII
METSCII<- aov(`Raw data`~Dog, data = METSCIIN)
summary(METSCII)

t.test(`Raw data`~Dog, data = METSCIIN)
# p-value = 0.05677

METSCIII <- aov(`Raw data`~Dog, data = METSCIIIN)
summary(METSCIII)

t.test(`Raw data`~Dog, data = METSCIIIN)
# p-value = 0.03068
```

5.1.1.6 Non-raced 2022 vs. Raced 2023 T37

AHN vs. AHR23T37

```
t.test(`Raw data`~Dog, data = LEAKAH)
#P= 5.178e-13

#p values for P CI
t.test(`Raw data`~Dog, data = PCIAH)
# p value: 6.067e-05

#pvalue for P CI + CII

t.test(`Raw data`~Dog, data = PCICIIAH)
```

```
#pvalue: p-value = 1.719e-06

#pvalue for ETS
t.test(`Raw data`~Dog, data = ETSAH)
# pvalue p-value = 0.0001034

#pvalue for ETS CII

t.test(`Raw data`~Dog, data = ETSCIIAH)
# p value p-value = 3.229e-11

t.test(`Raw data`~Dog, data = ETSCIIIAH)
# p-value = 3.131e-05

SHN vs. SHR23T37

SHN to SHRQT
# welch two sample t-test.
LESH <- aov(`Raw data`~Dog, data = LEAKSH)
summary(LESH)

t.test(`Raw data`~Dog, data = LEAKSH)
#P= p-value  2.545e-15

#p values for P CI
PCISHH <- aov(`Raw data`~Dog, data = PCISH)
summary(PCISHH)

t.test(`Raw data`~Dog, data = PCISH)
# p value: p-value < 2.2e-16

#pvalue for P CI + CII
PCICIISSH <- aov(`Raw data`~Dog, data = PCICIISH)
summary(PCICIISSH)

t.test(`Raw data`~Dog, data = PCICIISH)
#pvalue:  p-value = 8.659e-12

#pvalue for ETS
ETSSSH <- aov(`Raw data`~Dog, data = ETSSH)
summary(ETSSSH)

t.test(`Raw data`~Dog, data = ETSSH)
# pvalue p-value = 1.1e-10

#pvalue for ETS CII
ETSCIISSH<- aov(`Raw data`~Dog, data = ETSCIISH)
summary(ETSCIISSH)

t.test(`Raw data`~Dog, data = ETSCIISH)
```

```
# p value  p-value = 2.931e-10

ETSCIIISSH <- aov(`Raw data`~Dog, data = ETSCIIISH)
summary(ETSCIIISSH)

t.test(`Raw data`~Dog, data = ETSCIIISH)
# , p-value = 1.165e-08
```

5.1.1.7 P/E ratio

```
#t.test P/E

SHNN <- subset(PE, Dog=="SHN")
SHRR <- subset(PE, Dog=="SHR")
PESH <-rbind.data.frame(SHNN,SHRR)

t.test(`P/EIND`~Dog, data = PESH)
# p value: p-value = 0.01531 SHN vs SHR
AHNN <- subset(PE, Dog=="AHN")
AHRR <- subset(PE, Dog=="AHR")
PEAH <-rbind.data.frame(AHNN,AHRR)

t.test(`P/EIND`~Dog, data = PEAH)
# p value:  p-value = 0.02324 AHN to AHR

PEAHSHN <-rbind.data.frame(AHNN,SHNN)

t.test(`P/EIND`~Dog, data = PEAHSHN)
# p value:  p-value = p-value = 0.7824 AHN to SHN
PEAHSHR <-rbind.data.frame(AHRR,SHRR)

t.test(`P/EIND`~Dog, data = PEAHSHR)
# p value: p-value = 0.8158 AHR to SHR
```

5.1.1.8 CCR

```
#t.test CCR

RSHNN <- subset(PE, Dog=="SHN")
RSHRR <- subset(PE, Dog=="SHR")
RPESH <-rbind.data.frame(RSHNN,RSHRR)

t.test(RCR~Dog, data = RPESH)
# p value: p-value = 0.009227 SHN vs SHR
RAHNN <- subset(PE, Dog=="AHN")
RAHRR <- subset(PE, Dog=="AHR")
RPEAH <-rbind.data.frame(RAHNN,RAHRR)
```

```
t.test(RCR~Dog, data = RPEAH)
# p value:  p-value = 0.001694 AHN to AHR

RPEAHSHN <-rbind.data.frame(RAHNN,RSHNN)

t.test(RCR~Dog, data = RPEAHSHN)
# p value:  p-value = p-value = 0.0002468 AHN to SHN
RPEAHSHR <-rbind.data.frame(RAHRR,RSHRR)

t.test(RCR~Dog, data = RPEAHSHR)
# p value: p-value = 0.0114 AHR to SHR
```