University of Veterinary Medicine Vienna

Research Institute of Wildlife Ecology

(Supervision: Dr. Teresa Valencak)

Impact of dietary polyunsaturated fatty acids on *in vitro* force production of skeletal muscles in the European brown hare

MASTER THESIS

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Bernhard Neumayer

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Internal supervisor: Dr. Teresa Valencak, Research Institute of Wildlife Ecology

Reviewer:

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1. Aim of the study

This study tests the hypothesis that polyunsaturated fatty acids (PUFAs) positively correlate with the maximum running speed (MRS) in mammals by supporting the peak force production of skeletal muscles. Fatty acids which contain at least two or more double bonds in their aliphatic chain are considered to be polyunsaturated. Unsaturated fatty acids are lipids which are essential for the mammalian body and cannot be synthesized de novo. There is experimental evidence indicating that a certain ratio of omega-3 and omega-6 fatty acids in membranes significantly relates to MRS in many different mammals (RUF et al., 2006). These fatty received their classification according to the location if the first double bond starting from the methyl end of the lipid. Consequently omega-3 fatty acids have their first double bond at the third carbon atom and omega-6 fatty acids at the 6^{th} carbon atom. Maximum running speed could be a direct result of generally increased muscular performance caused by the high PUFA content within the skeletal muscles of all fast- running animals (RUF et al., 2006). Closely connected to that it was suggested that the enhanced locomotor performance seen in animals with high contents of polyunsaturated fatty acids within the membrane of the sarcoplasmic reticulum may affect the Ca^{2+} ATPase (SERCA) (RUF & ARNOLD, 2008). This in term would influence muscular performance since the SERCA enzyme plays a key role in muscle contraction, as this membrane enzyme is responsible for creating a Ca^{2+} gradient that is necessary for the onset of cross-bridge movement and subsequently muscle contraction. It is likely that transmembrane proteins which pierce the lipid bilayer and therefore are in direct contact with both sides of the compartments are altered by the surrounding membrane lipid composition (LUNDBAEK et al., 2010). Perhaps this is causing alterations in the enzymatic functions of these transmembrane proteins. It has been known that membrane fatty acid composition alters the membrane properties in general and therefore might also influence the activity of associated proteins. A change in the ratio of different membrane lipids, such as an increased incorporation of PUFAs, might influence membrane properties such as thickness and fluidity. Therefore also the conformational structure of the embedded enzymes, including the Ca^{2+} ATPase's might be altered. However, the manner in which the ratio of n-6 to n-3 polyunsaturated fatty acids exactly contributes to the maximum running speed in mammals remains unclear. One way to assess this question, as to whether PUFAs significantly contribute to the maximum running speed in mammals, is to examine whether or not they lead to an enhanced muscular performance in general. My study represents

the first step in trying to answer the question as to whether PUFAs alter the mechanical abilities of striated muscles, such as the peak isometric, isotonic and tetanic force production. This might be correlated to the observed increased maximum running speed of mammals with a high PUFA content. Therefore, in this study (n = 43) European brown hares (*Lepus europaeus*) were chosen to test the above introduced hypothesis. All animals were split into three groups and subjected to three different diets distinct in fatty acid compositions- (see methods for detailed description of the diets). I hypothesize that intake of fatty acid-enriched diets over a period of 8 weeks will induce clear differences in the fatty acid composition of cellular membranes including the membrane of the sarcoplasmic reticulum where the SERCA enzymes are located. Furthermore, I predict that the altered enzymatic function of the SERCAs might lead to a change in peak force performance of the skeletal muscles. Hence, in this study M. extensor digitorum lateralis was chosen for determining the isometric, tetanic and isotonic force production. As will be further explained on page 14 (also see appendix) M. extensor digitorum lateralis meets all the requirements for the quantification of muscular force *in vitro*.

M. extensor digitorum lateralis is located at the lower arm of the thoracic limb, was isolated and maintained by perfusion and oxygenation through a customized system. Furthermore, electrical pulses produced by a generator (Figure 7) induced muscle twitch responses that were detected by a strain gauge (further discussed in materials methods) and recorded digitally. With this technique I could gain insights into the relationship between peak skeletal muscle forces and the PUFA content of muscular membranes. Furthermore, I aimed to test the hypothesis that PUFA's enhance skeletal muscle performance in the mammalian body.

2. Introduction

2.1 Animal model

Lepus europaeus belongs to the family of the *Leporidae* which includes 15 different subspecies. They mainly live in open areas and can be found mostly near agricultural places. An extraordinary feature of *Lepus europaeus* is its capability to reach a maximum running speed of approximately 72 km/h (LUMPKIN and SEIDENSTICKER) which in fact, is four times faster than mammals with a similar body size. It is this remarkably feature that made this mammal most optimal for this study. Additionally, hares have the perseverance to run for approximately 1.6 km until they tire. Although they are capable of extreme locomotor performances, little is known

about the underlying molecular principles that facilitate it. Anatomical constructions alone cannot explain the locomotive performances of hares, since there are strong anatomical similarities seen between hares and rabbits which in turn don't show these features. A possible explanation for the locomotive differences seen in both animals might be the high proportion of polyunsaturated fatty acids that can be found in the European hare compared to other mammals (VALENCAK et al., 2003). The presence of certain PUFAs inside the membrane might influence the number and performance of the Ca^{2+} - ATPase (SERCA) of the sarcoplasmic reticulum. This enzyme plays a key role in muscle contraction. Hence, examining the peak isometric, tetanic and isotonic force production capacity of hare muscles under the influence of different PUFAs could help to understand this phenomenon. Based on these attributes, *Lepus europaeus* was considered an optimal animal model for examining the impact of polyunsaturated fatty acids on different muscle performance parameter as there are plenty of existing data available on muscle fatty acid composition (VALENCAK et al., 2003). It is even known that hares have a preference for certain food plants rich in their omega-6 (linoleic acid) fatty acid content (REICHLIN et al., 2006). The fact that hares are 4 times faster than mammals of similar body size underpins the need to identify reasons for their outstanding running speed. Finally, general body size of the European hare and subsequently general skeletal muscle size makes muscle preparation and handling for maintaining whole muscles *in vitro* easier than with small mammals, such as mice.

Figure 1: Running European hare. *Lepus europaeus* is four times as fast as animals with a similar body size. Source: eckert-rimbach.de

2.2 Mammalian Muscles

Muscle tissue is a well characterized and specialized tissue with the unique ability to contract and generate force. Hence, muscles allow the mammalian body to move and also many organs require the use of muscular contractions in order to fulfill their tasks. The process of contraction is basically the same in all three different kinds of muscular tissues that can be found in the mammalian body, but only skeletal muscles are voluntarily controlled by the central nervous system (MACINTOSH et al., 2005). Striated muscle cells are large syncytial cells composed of multiple cell nuclei located near the cell membrane. Myocytes can reach a length of up to 15 cm

and get 100 µm thick (ALBERTS et al., 2002, LIEBER & BODINE-FOWLER, 1993). The underlying modules of muscle contractions are the sarcomeres, which are composed of two contractile elements: actin and myosin, that are parallel convoluted into each other. These sarcomeres can be seen as the basic unit of the muscle cell and are responsible for the cross-bridge movement. The origin for the onset of the cross-bridge interaction lies inside the sarcoplasmic reticulum which provides a 1000-fold Ca^{2+} that plays a key role in muscle contraction and enables myosin to bind the actin (ALTBERTS et al., 2004). Many sarcomeres in a row form a larger unit called myofibril. The myofibrils are functional units inside the muscle cell surrounded by mitochondria which in turn serves as an ATP supply for the cross-bridge movement. Several myofibrils parallel to each other form the entire muscle cell, also known as muscle fiber. The basis of muscle contraction is given by the contractile machinery which is mainly formed by actin and myosin filaments, along with tropomyosin and troponin C. The interaction of actin and myosin is facilitated by the hydrolysis of ATP, mainly provided by surrounding mitochondria (VECCHINI et al., 1978).

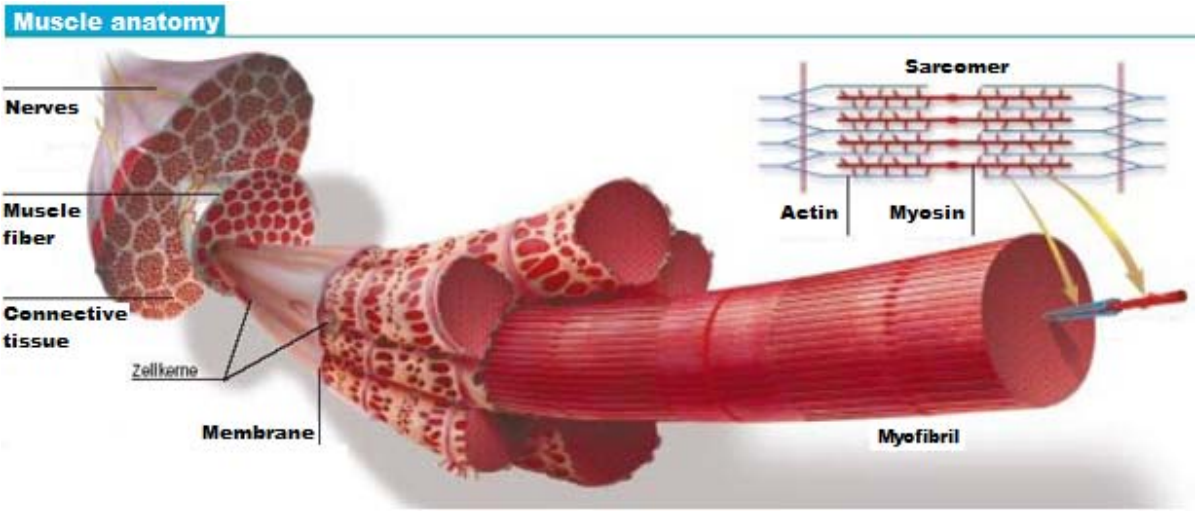


Figure 2: Schematic anatomical structure of skeletal mammalian muscles. The sarcomere is the smallest unit that muscles cells consist of. Many sarcomers in a row form a bigger unit which is called myofibril. Lots of myofibrils parallel to each other form the entire muscle cells.

2. 3 Ca^{2+} ATPase's of the sarcoplasmic reticulum

Onset of muscle contraction is achieved by rapid Ca^{2+} influx into the cytosol along a gradient established by a high number of transmembrane SR Ca^{2+} ATPases (SERCAs) inside the membrane of the sarcoplasmic reticulum. SR Ca^{2+} ATPases are encoded by three different genes that are spliced alternatively in order to form different isoforms. The different SERCA enzymes are classified into three groups according to their location inside the mammalian body. SERCA1a is an isoform which is mainly expressed in fast twitching skeletal muscles, whereas SERCA2a is mainly found in slow twitching muscles, as well as in cardiac tissue. In contrast to SERCA1a and 2a, SERCA3 is only expressed in non-muscular tissue such as the epithelium. All different forms of SERCAs underlie a wide range of direct and indirect modifications which precisely control the enzyme activity. One example of indirect inhibition of SERCA2a in cardiac tissue is the phosphoprotein phospholamban. The dephosphorilated form of phospholamban is an inhibitor of SERCA2a in muscle cells. This regulation is facilitated by a decreased affinity of SERCA2a to Ca^{2+} when phospholamban is bound. Usually SERCAs have a high affinity for Ca^{2+} and clear the cytosol from it under the use of ATP hydrolysis by actively pumping it into the sarcoplasmic reticulum, which establishes a gradient. It is the rapid shift of Ca^{2+} concentrations that allows Ca^{2+} to function as an intracellular second messenger for the onset of muscle contraction. The number of SERCAs is directly correlated with the Ca^{2+} concentration inside the sarcoplasmic reticulum. In other words this means that the better SERCAs function and the higher their number inside the sarcoplasmic membrane the faster and more concentrated gets the Ca^{2+} gradient. A high Ca^{2+} concentration allows a faster signal for the onset of cross bridge interactions and enables the binding of Ca^{2+} with a maximum number of troponin C, an essential step in muscle contraction. On the other hand, a fast shift of Ca^{2+} from the cytosol back to the sarcoplasmic reticulum enables the muscle to reach its relaxation time in a shorter time which means that the next excitation can happen again sooner. SERCAs, which will further be discussed in this study, are possibly subject to manipulations like other membrane proteins as well, by the lipid bilayer in which they are located. Martonosi and his demonstrated that the Ca^{2+}

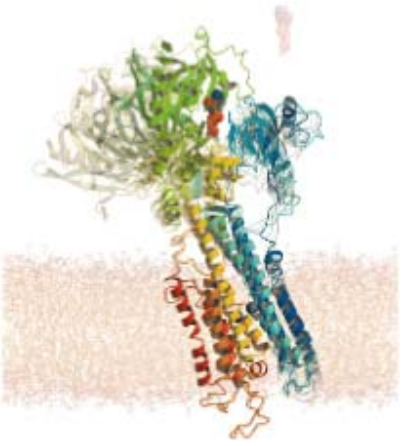


Figure 3: Illustration of the Ca^{2+}-ATPase conformation inside the lipid bilayer. Chikashi Toyoshima worked on revealing the calcium pump structures using crystallography techniques. Source: http://www.pnas.org

ATPases of the sarcoplasmic reticulum require the presence of phospholipids as a treatment with phospholipase C leads to an inhibition of ATPase activity and subsequently inhibition of Ca^{2+} transport (MARTONOSI et al., 1968, DIEHL et al., 1965). Therefore, it is possible that SERCAs are subject to an active manipulation by the surrounding phospholipid bilayer. This is reason enough to conclude that the composition of the lipid bilayer has an impact on the activity of SERCAs, possibly leading to measurable differences in skeletal muscle performances. Furthermore, this mechanism might be the cause of the observed MRS in mammals with a higher PUFA content. Hence, a fast muscle contraction frequency requires optimally functioning SERCAs to keep relaxation time as short as possible.

2. 4 Membranes and their impact on proteins

Biological bilayer membranes serve as hydrophobic films which separate the cell from its environment. For a long time the picture of biological membranes was dominated by an un-polar and passive structure with no active molecular biological role. In the past few years however, researchers began to gain inside in the complexity of the physio-chemical properties of cell membranes and their influences on other cellular processes. These lipid bilayers, which enable the cell to maintain compartments, are directly influenced by the composition of the fatty acid dietary uptake (LUNDBAEK et al., 2010) and seem to play an active role for some molecular processes. Membranes are composed of a great variety of phospholipids which vary in length and saturation of their hydrogen bonds and therefore differ in their physical behavior. It is the ratio and presence of these different phospholipids which shape the physio-chemical properties of the membrane and mediate and influence interaction between proteins or directly act on proteins (LUNDBAEK et al., 2010). The composition of phospholipids within the membrane modulates different physical properties such as membrane thickness and dynamic properties, and therefore changes permeability and fluidity of the membrane, also anchoring of membrane proteins. Unfortunately, today it is not fully understood how membranes shape the function of associated proteins such as the CA^{2+} ATPase of the SR. The most convincing model however, is the ratio and composition of fatty acids within the membrane that has an impact on the conformation of embedded proteins and therefore alters their function. It was further demonstrated by Fiehn et al. that different types of plasma membranes within a cell also differ concerning their fatty acid composition in rat skeletal muscles. This implies that the different tasks of different membranes

such as the sarcoplasmic reticulum, mitochondria and the sarcolemma are linked to their fatty acid composition (MEAD et al. 1971). Furthermore the content of fatty acids in membranes reflects dietary fatty acid composition and intake since they are dependent on the availability of circulating fatty acids in the blood stream (SPECTOR et al., 1992). Therefore, dietary driven changes in the membrane fatty acid composition of rat and human muscle membranes can be observed within days after induction of a manipulated diet (HELGE et al. 2012, PAN and STORLIEN, unpublished observations). Since the European hares that were used in this study were subject to a polyunsaturated fatty acid rich diet for about 8 weeks, one can conclude that there was sufficient time to change the fatty acid membrane composition on a molecular level which might possibly result in an impact on muscle performance. Together, these findings clearly indicate that the composition of membranes is of major biological importance on a cellular level und furthermore, can be easily influenced by environmental parameters, such as diet and sport.

2. 5 Muscle Mechanics

Skeletal muscles are able to contract in different ways in order to fulfill their tasks. These different types of contractions mostly happen simultaneously, and can therefore, only seen as isolated processes *in vitro*. Due to the nature of this study, isometric, isotonic and tetanic contractions only, along with basic muscle mechanic principles, will be further discussed below.

2.5.1 Pre-stretching

Understanding the impact of muscle length and therefore, pre-stretching of the muscle is important for correctly conducting the experimental procedure. Pre-stretching of the muscle defines not just the passive force generated by stretching the elastic elements of the muscle cell, but also the maximal active force that can be produced by the muscle. Consequently, the force depends on the degree of muscle pre-stretching at initiation of muscle contraction. The reason for this phenomenon is the length of the sarcomeres. Once the muscle is greatly stretched, the myosin and actin filaments no longer cover each other optimally, subsequently results in a decreased force production. If the muscle is not stretched enough then force production is also minimal because of a shortened sarcomere that does not give the actin and myosin filament enough room to shorten.

2.5.2 Length-tension relationship

The establishment of the length-tension relationship is required for further tests because it provides the researcher with necessary information regarding the peak force production that varies with different muscle length (BLIX 1895, GORDON et al., 1966). The length-tension relationship is done by causing isometric twitch responses at different muscle lengths. Consequently, the peak force and the muscle length are plotted against each other. As it can be seen in figure 4 the capability of the muscles to produce maximum force is decreased in low and long muscle length but is optimal in the intermediate ranges, also where the optimal muscle length can be found. The underlying principle of this phenomenon can be found at the molecular level and is defined by the sarcomere length, which in turn, is defined by the degree of actin- and myosin filament overlaps. Optimal sarcomere length lies around 2µm (LIEBER & BODINE-FOWLER, 1993).

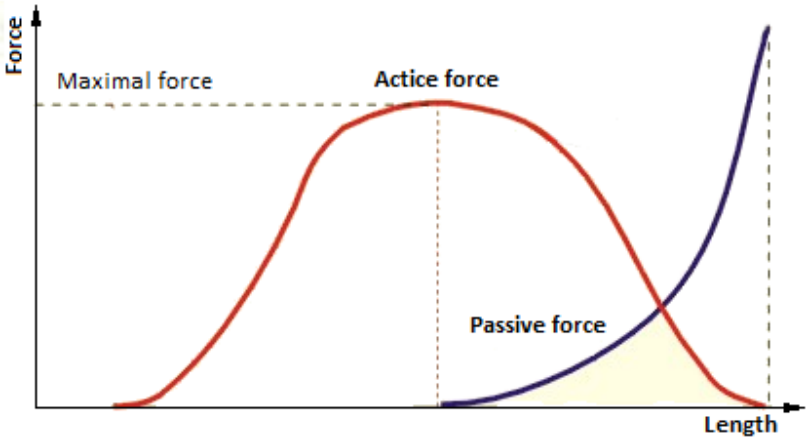


Figure 4: Illustration of the Length-tension relationship. Maximal force production of mammalian muscles correlates with the muscle length. This phenomenon is based on the sarcomeres which length is in an optimal range between 2 – 2.2 µm. Too much or not enough tension on the muscle results in an inadequate cover ratio of actin and myosin filaments. This results in a diminished force production.

2.5.3 Isometric force production

Muscles contract in an isometric manner when they generate force without changing their length at the same time (BLIX, 1895). One example for an isometric muscle contraction is carrying a weight on a constant high. In this scenario the muscle would not change its length but still produce a certain force. To examine the isometric force production the muscle needs to be kept at a constant length while applying electric stimuli. The force that is generated during isometric contractions is dependent upon the muscle length and therefore, may vary. Isometric force production reaches its peak at the optimal muscle length which can be assessed by the length-tension relationship.

2.5.4 Isotonic force production

Muscles contract in an isotonic manner when they change their length with a constant force. Experimentally seen, this can be achieved by allowing a muscle to shorten during contraction while attaching a load to the muscle. Therefore, the muscle lifts up a weight while it shortens during stimulation.

2.5.5 Tetanic force production

Tetanic muscle contractions are caused by the arrival of many action potentials on the neuro-muscular junction. The closely spaced nerve signals initiate the next Ca^{2+} efflux from the SR although the SERCAs are not finished clearing the cytosol from Ca^{2+} that entered the cytosol from the previous nerve impulse. Consequently a high frequency of muscle stimulations causes a permanent depolarization. This results in an overlay of single twitch responses and a permanently contracted muscle.

2.6 Polyunsaturated fatty acids

Unsaturated fatty acids can be divided in two groups: mono and polyunsaturated fatty acids. These lipids are essential to the mammalian body and have to be provided by dietary uptake since mammals lack enzymes that could synthesize them de novo. Thus, PUFA content of cellular membranes can easily be manipulated by diet. In other words this means that PUFA pattern reflects the PUFA content of the diet. PUFAs in general have several different important functions in the mammalian body including immunological regulations (TOMOBE et al., 2000) and hibernation (recently reviewed by RUF and ARNOLD, 2008). PUFA content in muscle cells even depends on seasonal changes in fish (COSSINS et al. 1977), birds (CHAINIER et al., 2000) and hares (VALENCAK et al., 2003). Valencak and colleagues demonstrated that muscular phospholipids are highest in hares with a mean of 67% compared to any mammalian tissue. Furthermore, the high content of omega-6 fatty acids in salmons correlates with their high swimming speed (MCKENZIE et al., 1998). Together these data suggest a strong correlation between membrane PUFA content and muscle performance. However, the role of these lipids in muscular tissue of fast non-hibernating mammals remains unclear and needs to be examined.

3. Material & Methods

3.1 Methods

3.1.1 Ethics statement

Before the start of my studies, all the experiments were discussed and approved by the institutional ethics committee in accordance with good scientific practice guidelines and national legislation (Number 07/08/97/2012). For my study the hares were fed three different diets ad libitum and had their body weights weighed on the day of measurements. They were killed by cervical dislocation through the hands of a very experience person and did not suffer at any time during the experiment. All the animals used in my study were born and raised in the breeding colony at the Research Institute of Wildlife Ecology in Vienna.

3.1.2 Animals and group size

Experiments were performed *in vitro* using *M. extensor digitorum lateralis* (see page 21) from adult European brown hare of both sexes (32 males and 11 females). 43 animals [mass 3586 ± 268 g (mean ± SD)] weighing between 3065 and 4150 g were subject to different dietary compositions and housed separately during the entire study. Some animals (see Table 1) were kept in large compounds whereas other were kept in cages. Hence, it was possible to assess the impact of different housing conditions on the muscular force production. All animals were divided into three dietary groups: a saturated, an omega-6 enriched diet and an omega-3 enriched diet (see below. Food and water was provided ad libitum during the whole period of time. The first group (n=11) received food pellets enriched with 10 % saturated fatty acids (coconut oil, brand name: “Ceres”, Austria), the second group (n=10) received food pellets enriched with omega-3 fatty acids (linseed oil for horses, Raiffeisen, Austria) and the third group was fed with omega-6 enriched food pellets (sunflower oil, “Clever”, Austria). The pellets that provided the basis for all the diets represent a special hare diet mixed to match stomach contents of European hares (ONDERSCHEKA and TATARUCH, 1982) and were produced for the Research Institute by Raiffeisen, Salzburg. Before analyses of the diets and distribution to the animals the dried pellets were weighed and added 10% oil: in the saturated fatty acid group, the coconut oil was melted at 35° for half an hour in a drying oven (see list of equipment on page 28), then weighed

to the nearest g and finally added to the pellets. In the two other groups as oils were liquid at room temperature oils were weighed as were the dried pellets and finally combined. Every 2 hours the pellets and the oil were mixed and completely absorbed after one day (Peter Steiger, personal communication). From previous studies we knew that 10% oil is easily and completely absorbed by the pellets and that the hares accept the oil enriched diets (VALENCAK et al. 2009, VALENCAK and RUF, 2009). All the mixed diets were stored in a refrigerator to prevent the contribution of “autoxidation) of the PUFAs. Prior to distribution the refrigerated pellets to the animals, the diets were taken out and allowed to warm up to room temperature.

3.1.3 Customized tissue bath and force measurement system

The system I present here was entirely customized because of the size and cost barriers associated with commercially available whole organ bath's systems and force transducing systems. To my knowledge, there is no single commercially system available that could deal with the size and the maximum force production of *Lepus europaeus* muscles at the same time. Despite these issues, my system enabled me to run my study in a straightforward manner quantifying isomeric, tetanic and isotonic muscle forces and it was compiled at a very attractive price at the same time. In case of interest, I’m happy to share my experiences to everyone interested and I can be contacted on email any time.

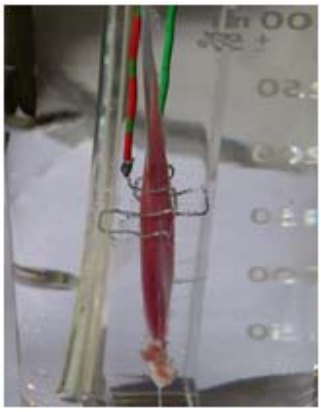


Figure 5: M. extensor digitorum lateralis mounted vertically inside the experimental chamber. The proximal tendon is facing the bottom of the glass. Muscle is surrounded by bubbled Krebs - Henseleit Buffer and flanking horizontally both platinum electrodes.

Figure 6: Both force transducers connected to a Plexiglas. Front transducer is immobilized and hence used for isometric measurements. The other transducer was moveable and therefore used for isotonic measurements.

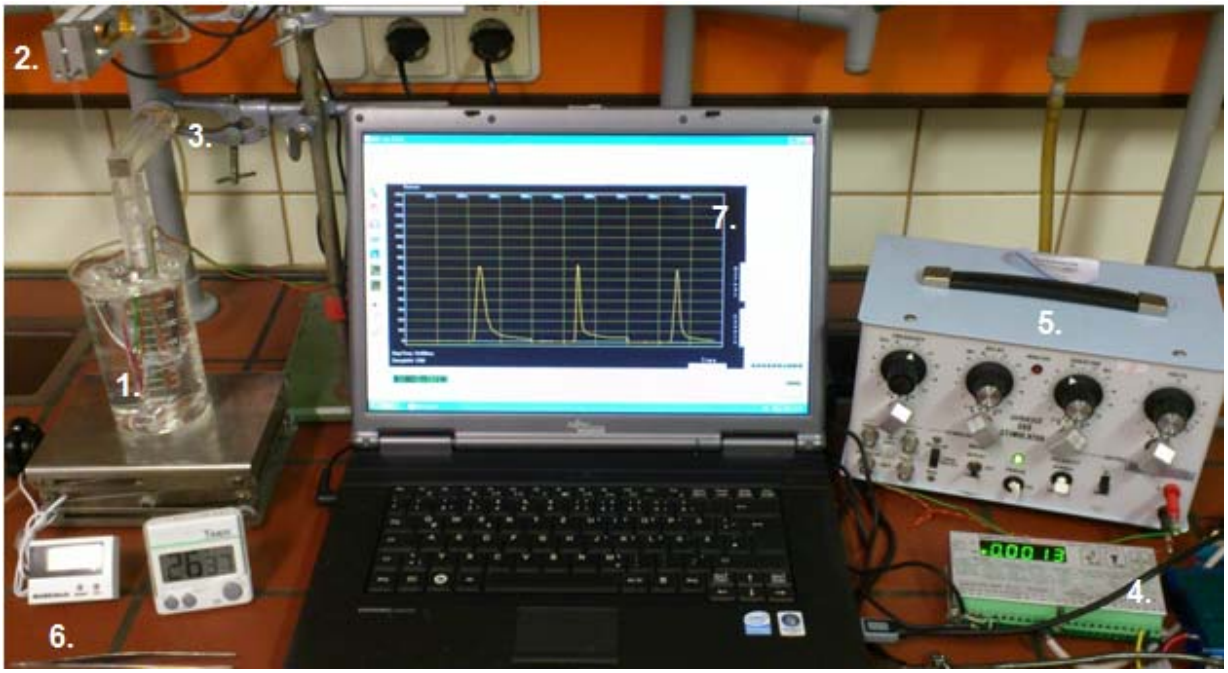


Figure 7: Overview of all system components. 1. Muscle containing chamber, 2. Isometric & isotonic force transducer, 3. Muscle holder, 4. Signal enhancer, 5. Pulse generator, 6. Thermometer, 7. Software.

3.1.4 Rabbits

Before using the first hares for this study, I optimized the experimental setting and the parameters by using 14 rabbits to gain first insides concerning the overall feasibility of the planned study. Hence, these first data served as a basis for the experiments mentioned in this study. First thoracic and pelvic limbs of the rabbits were carefully dissected to find ideal muscles candidates for force measurements *in vitro*. As mentioned in the Material and Methods section and further discussed in the Appendix, muscle size is limited due to several reasons. This is especially an issue when working with "big" animals such as rabbits or hares because of the increased muscle size. Because of the nature of this study it was originally planned to use skeletal muscles of the hind limb since they play a major role in hare locomotor performances. However, no muscle could be found on the hind limb that fulfilled the requirements for small size and a superficial anatomical location for rapid muscle sampling. Consequently, *M. extensor digitorum lateralis* that is located on the lower arm of the thoracic limb was chosen because of its correct size and anatomical properties (see Material and Methods). Once the most ideal muscle candidate for this study was found, rapid preparation if the was practiced to keep muscle sampling time as short as

possible (for the reasons why see appendix). Thus, muscle sampling could be performed in a few minutes (<5 min.) and it furthermore it could be to demonstrated that the muscles could still contract while their maintenance *in vitro*, the determination of the most optimal electrical impulse began. This was done by changing electrical parameters such as voltage amplitude, frequency, and pulse duration and also by using varying muscle length. All this provided first insights in the possible range for the correct pulse parameters in hares. Hence, the search for the ideal stimulation parameters in hares was kept to a minimum which contributed positively to the viability of the muscle.

3.1.5 Experimental procedure protocol

After the animals of all three dietary groups received their specific fatty acid diet for about 8 weeks they were killed and the *Musculus extensor digitorum lateralis* was isolated and exposed to electrical stimulation. Optimal stimulation parameters were assessed prior initiation of the study and applied to all animals throughout all measurements. Hence, all sampled muscles received the same electrical impulse. Two of the 43 hare sampled muscles used in this study did not show any response to electrical stimulation, which was probably due to mistakes made during the muscle sampling process. Hence, muscle sampling technique resulted in 95.4 % success rate. For statistical analysis the mean of the 3 strongest measured muscle contractions (see example below) was calculated using the highest value that occurred in each peak. At the same time only the highest value produced by the highest peak per experiment was used for the same statistical calculations in parallel. This was done to counteract a possible contribution of muscular fatigue.

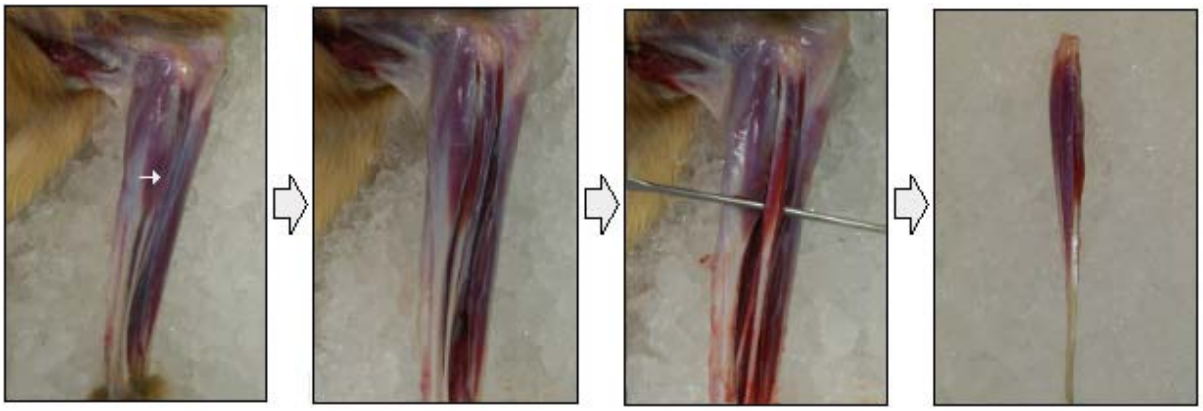
Maintenance of isolated muscles *in vitro* and furthermore electrical stimulation followed by force measurements is a convenient method that has been used by researches over decades (VAN DER HEIJDEN et al., 1999; BARCLAY et al., 2010; BARTON & LYNCH, 2011). The skeletal muscle was maintained in an experimental chamber containing “Krebs-Henseleit-Buffer” (see page 29 for exact composition), mimicking physiological conditions such as osmotic strength. All experiments were undertaken at room temperature. After preparing the muscle from the animal body, the tendons were connected on either sides with non-elastic strings (see list of equipment) and the proximal end was attached to an immobilized Plexiglas and the distal tendon was attached to a force transducer (Sömer GmbH, Germany, see figure 7). Hence, the muscle was

mounted vertically in with 100% oxygen bubbled Krebs-Henseleit Butter solution and allowed to acclimatize for 10 minutes before applying any electrical stimuli. The whole procedure of isolation and preparation of the muscle was accomplished within 5 minutes after the death of the animal was confirmed. Contraction of the muscle was achieved via electrical stimulation using an impulse generator (Grass Technologies, USA) that was kindly provided by the department of neurophysiology of the University of Vienna. Two platinum electrodes (Neolab, Germany) were needed to deliver the electrical impulse to the muscular tissue. The electrodes were bent multiple times to form and mimic plates with a subsequently increased surface for establishing a twitch response. The electrodes were placed horizontally next to the muscle to ensure tight contact between the electrodes and the muscle surface. Hence, the muscle was placed in the center of both electrodes. First of all, I determined the optimum muscle length, which served as a basis for further experiments. This was achieved by continuously adjusting the muscle length after each single twitch response with the focus on the maximal peak twitch force which was displayed on the computer screen by varying amplitude sizes. Optimal length was achieved when signal amplitude and hence force output was maximal. In accordance to other studies, I allowed each muscle to rest for 60 s before the next pulse was applied (BARTON & LYNCH, 2011). Resting time intervals are important to keep the intracellular nutrition and oxygen levels in balance since each twitch consumes oxygen and nutrition. The waiting intervals minimized the contribution of fatigue to the experiment. Once I defined the optimal muscle length (by choosing the muscle length that correlated with the highest signal), I established the frequency-force relationship. I did this by stimulating the muscle at increasing frequencies starting from 10 to 250 Hz. Electrical pulse duration was set to be 200 ms during this experiment. As shown in figure 14, force production was highest using a frequency of 120 Hz. The range of frequencies was only performed once and the optimal frequency was kept throughout all experiments. Next pulse duration was assessed by increasing the pulse duration from 5 to 200ms with regard of maximal force output (see figure 13 on page 25). The highest muscular force was achieved with pulse durations of 90 – 100 ms. Finally, I determined the optimal pulse amplitude by stimulating the muscle with increasing voltages. In this case highest force production was highest when the generators voltage output was set maximal. Consequently, I am unable to conclude that 100 V correlate with the highest possible force production of *M. extensor digitorum lateralis* (see discussion). Once muscle length, frequency force relationship, optimal voltage amplitude and pulse duration was determined, all optimal force production parameters were available and kept

throughout all experiments. Then it was possible to assess the maximum isometric, isotonic and tetanic force production of *Musculus extensor digitorum lateralis in vitro*. The measurements were completed within 1 h after death of the animal.

3.1.6 Muscle preparation

Prior to all measurements the hares used in my study were killed by cervical dislocation and placed immediately on ice during the entire muscle isolation process. Muscle sampling was initiated approximately 30 seconds after death was confirmed by checking that respiration has ceased as well as reflexes stopped. Chilling down the muscles on ice should decrease the cellular demand for nutrition and oxygen during the preparation procedure. Also, it kept any lytic process to a minimum and prolonged the time-window for the following experimental procedures and contributed positively to the maintenance of the muscle. The *M. extensor digitorum lateralis* that is located on the lateral side of the lower arm of the thoracic limb (see page 21) was chosen for this experiment because of its optimal size regarding critical diffusion distances and its anatomical location for rapid sampling of the muscle. It was always tried to maintain the connection between muscle and circular system as long as possible before removing the muscle to keep the optimal conditions for the cells. Muscles of the front limb were exposed by skinning the leg from distal to proximal by using a scalpel and a scissor. Next, muscle fascia on the cranial and caudal end of the muscle were removed (see figure 8 B) and the muscle was sampled by most-, carefully moving a metal stick from distal to proximal along the bone and therefore gently disrupting the connection of muscle and bone (see figure 8 C). Tendons were kept intact on both sides. Finally, the connection of the distal tendon to the bone was interrupted using the scalpel. The maintenance of the muscle integrity is the most important aspect of this study and of major importance (BARTON & LYNCH, 2011). Hence, during muscle preparation the muscle was periodically washed with cooled Krebs-Henseleit Butter solution and furthermore, direct touching of the muscle, stretching, tearing, pulling, as well as allowing it to dry out, was prevented as much as possible. The entire muscle preparation was performed in less than 5 minutes starting from the first initial cut till the final transfer of the muscle into bubbled Krebs-Henseleit Buffer.



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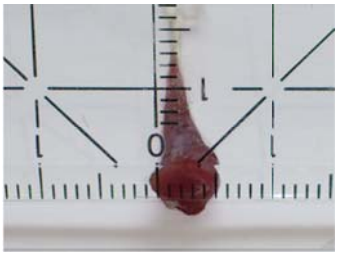
All isolated muscles were immediately fixed vertically inside the test chamber and allowed to rest for 10 minutes before initiation of measurements. To perfect the muscle sampling technique, sixteen rabbits were used before initialization of the experiment.



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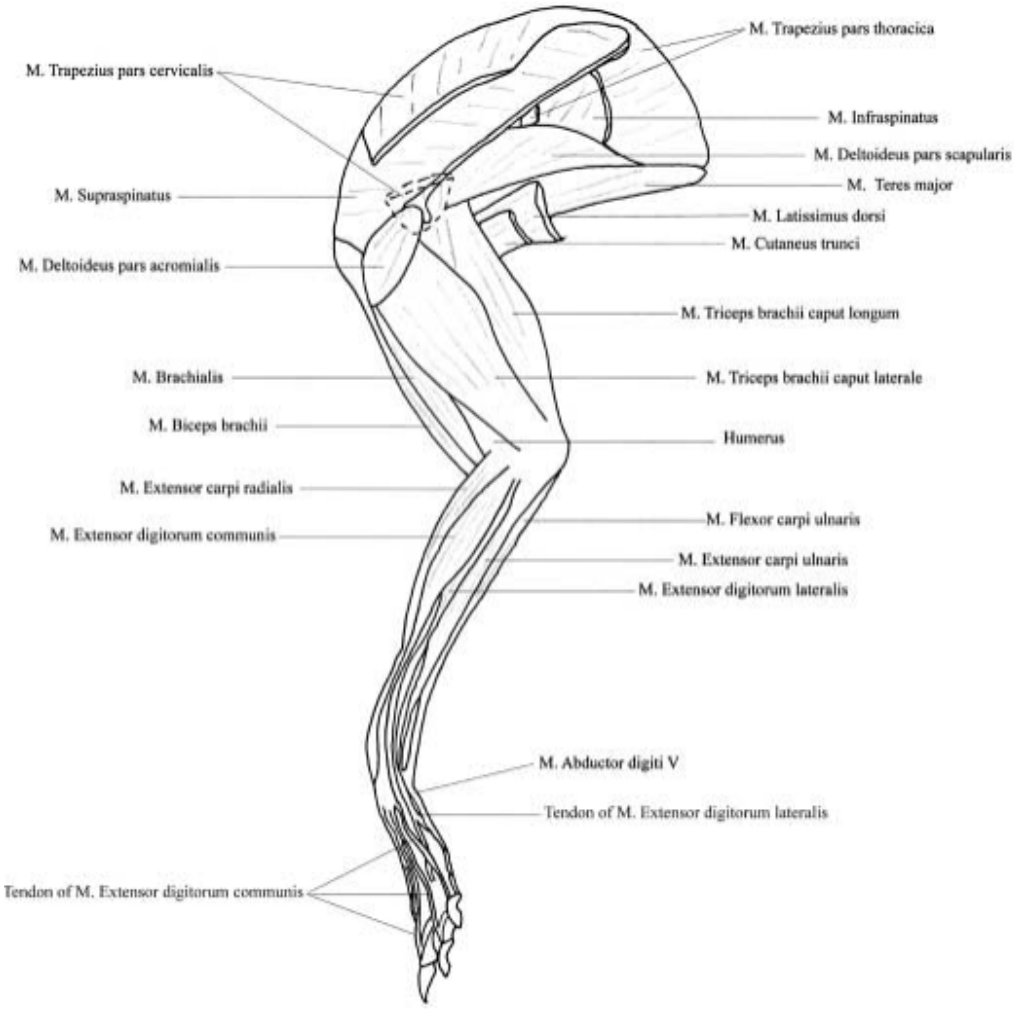

Figure 11: Scheme of anatomical location of EDL in the thoracic limb in hares published by Wiliams et al., 2007).

3.1.7 Maintenance of mammalian tissue in vitro

For this experiment physiological Krebs-Henseleit Buffer was chosen to be the optimal medium for maintaining the viability of the isolated muscles *in vitro*. Furthermore, the muscles were bubbled with 100% O_2 in a 1 l bottle (Air Liquide, Austria) during all experiments. Bubbling allows the solution to saturate with gas (oxygen) and increases the gas availability for the cells. The high oxygen content compensates for the lack of oxygen carrying proteins such as hemoglobin (BARTON et al. 2011). Furthermore, the components of the Krebs Henseleit Buffer (see page 29) support altogether muscle viability by controlling osmotic and ionic strength, as well as metabolic demands of the cells and an extracellular pH of 7.40. Furthermore, bubbling

with oxygen helped to ensure a continuous flow of medium inside the chamber. The solution volume of the experimental chamber was kept at a constant volume of 300 ml.

3.1.8 Stimulation of isolated muscles in vitro

As a first parameter for creating optimal twitch responses, the optimal voltage amplitude was examined. This was achieved by increasing the amplitude from 10 to 100V with the focus on the maximal force production of the muscle. Next the optimal impulse duration was assessed by applying stimuli ranging from 5 to 200ms. After optimal amplitude and duration of the pulse were established the optimal muscle length was measured by increasing the muscle stretching force. Finally, the frequency that results in the strongest tetanic force production was determined by increasing the pulse frequency from 10 to 200Hz. Isotonic contraction was facilitated by allowing the muscle to shorten when applying electrical stimuli. Thus, the isotonic force transducer was allowed to move up and down along a turning moment. During isotonic measurements 125 g were attached to the muscle. This weight was chosen after evaluating maximal force production under the influence of different attached weights (data not shown). Once all best parameters given the *in vitro* conditions were established in order to ensure optimal stimulation conditions, they were kept constant throughout the experiment for all the measurement's and applied on each muscle five times for isometric and isotonic stimulations and three times for tetanic stimulations. As recommended by (MELLORS & BARCLAY, 2001, BAXI et al., 2012) two platinum electrodes which are resistant to oxidation were placed horizontally next to the muscle and were allowed to directly touch the tissue in the middle of the muscle. This leads to an establishment of an electrical field that flanks the muscle. The created field stimulates the muscle and the muscle responds with a twitch to the electrical stimuli. This twitch is assessed by a force transducer and recorded digitally on a computer. Contraction of isolated muscles in vitro requires a certain resting time between each single twitch response in order to prevent the contribution of fatigue and subsequently decreased force production to the experiment. Isometric and isotonic twitch response experiments were done 5 times, whereas tetanic experiments were done in triplets. After all experiments were successfully performed, muscles were weighted and finally a sample of each muscle was frozen and stored in liquid nitrogen and used later for studies of SERCA content.

3.2 Statistical tests and analysis

After data sampling was completed, all data were transferred into an excel sheet (Microsoft office 2010). This file contained 143 columns with up to a few thousand data points per column. Summary statistics were done by computing means and standard errors of the mean. Before parametric tests such as ANOVA models were applied, I tested for normality. The force production [N] (see chapter below for strategy) was log transformed and then used as dependent variable in an analysis of variance model (ANOVA) with sex, cage and weight as independent factors. As we used 41 different individual hares, the data obtained were independent from each other so we did not include any random factor. Statistical analyses were computed in S-Plus 6.1 for Windows (Insightful Corporation, Seattle, USA). Fore measurement graphs illustrating isometric, isotonic and tetanic force measurements and show means +/- SD.

3.3 Force Measurements

Output signals of both force transducers were enhanced by a DASIII enhancer before entering a personal computer and visualization using the DOP 2.6 Software which was also provided by Sömer Messtechnik GmbH (Germany). The transducers were set to maximum of 600 measurements per second. Hence, measurements were continuously taken every 1.6 ms. This resulted in a few thousand values per experiment. The force transducers used in this study are commonly available strain gauges which are widely used for any electrical purposes. The gauge changes its electrical resistance when subject to any deformation. This results in a signal that is proportional to the applied force. This signal in term can be subsequently converted in weight and force. The First optimal pulse parameters were assessed by examining different electrical stimulation patterns to achieve maximal muscle performance including peak force production (mentioned above). Once optimal values were assessed they were kept throughout the entire experiment. Hence, all muscles were subject to the same electrical impulse.

3.4.1 Determining optimal pulse amplitude

As a first parameter for creating optimal twitch responses, the optimal voltage amplitude was examined. This was achieved by increasing the amplitude from 10 to 100V with the focus on the maximal force production of the muscle. Figure 18 shows the digitalized signal production of an isometric force transducer connected to a stimulated *M. extensor digitorum lateralis in vitro*. Each peak represents a single stimulation of the muscle with increasing voltage amplitudes every 60 seconds. As can be seen in figure 12, the highest force production was observed when stimulating the muscle with 100 V. Thus, all further experiments were conducted using

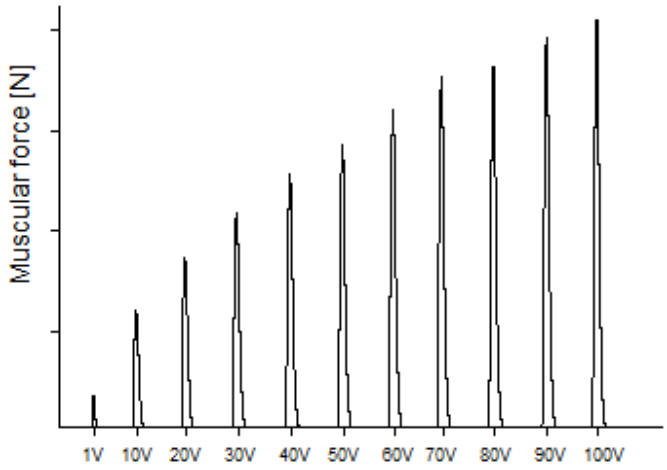

Figure 12: *M. extensor digitorum lateralis* exposed to increasing voltage amplitudes. Stimulation started with 1 Volt and ended with 100 V because of technical limitations. Each peak on the X axis represents one muscular twitch response. The highest force production was measurable with the highest voltage amplitude. It was not possible to show that force production decreased or increased with higher voltages.

amplitudes of 100 V. However, due to technical limitations it was not possible to show that possible force production of EDL was highest when applying 100 V because, higher amplitudes could not be created. Thus, I cannot conclude that 100 V caused the highest possible force production because I could not apply any higher voltages to the muscle. Even when stimulation amplitudes were suboptimal, all muscles were exposed to the same impulse and I assume that my theory of dietary impacts on the skeletal muscle force production was not influenced by this. However, it's unfortunately not possible to conclude that the measured muscular force production is the highest possible that could be provoked *in vitro*.

3.4.2 Determining ideal pulse duration

The optimal impulse duration was assessed by applying electrical stimuli ranging from 5 to 200 ms. As can be concluded from figure 13, the highest force production of EDL could be achieved with an electrical impulse duration of 90 ms. Lower or higher impulse durations resulted in a diminished force production compared to the one observed around 90 ms. Thus, all further experiments were conducted using a signal duration of 90 ms.

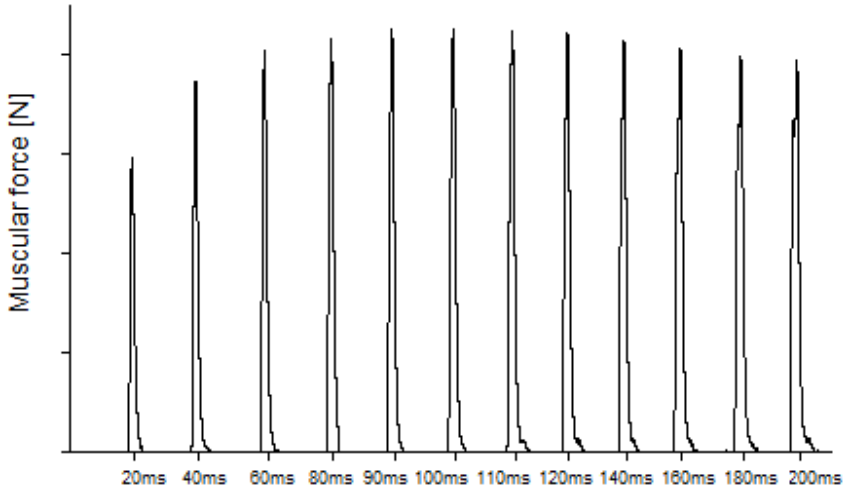

Figure 13: *M. extensor digitorum lateralis* exposed to increasing signal durations. Stimulation started with 20 ms and ended with 200ms. Each peak on the X axis represents one muscular twitch response. The highest force production was measurable using a stimulation duration of 90ms.

3.4.3 Determining correct impulse frequency

The most optimal impulse frequency was determined by applying stimuli with increasing frequencies and a signal duration of 200 ms to keep the signal length as long as possible because this ensured that tetanic contraction could reach its plateau. As can be concluded from figure 14 the highest measurable force production correlated with an stimulation frequency of 120 Hz. Any other frequency did not correlate with a similar force production. Thus, all further experiments were conducted using a signal frequency of 120 Hz. Further it can be clearly seen that increasing frequencies lead to an increased fusion of each single contraction which flattens the tetanic plateau.

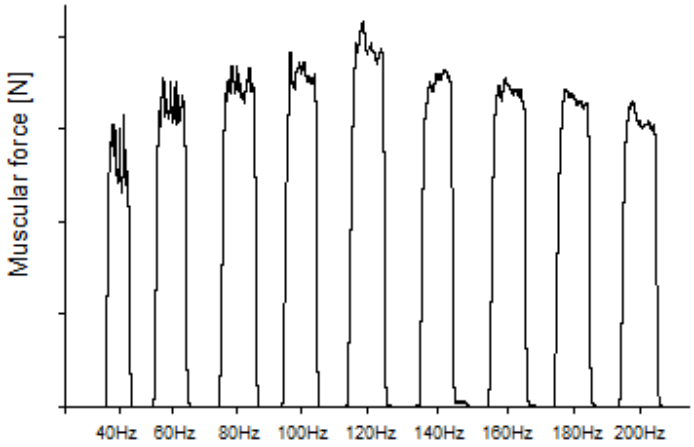

Figure 14: *M. extensor digitorum lateralis* exposed to increasing signal frequencies. Stimulation started with 40 Hz and ended with 200 Hz. Each peak on the X axis represents one muscular twitch response. The highest force production was measurable using a stimulation frequency of 120 Hz.

3.4.4 Determining optimal muscle length

After optimal amplitude and duration of the impulse were established the optimal muscle length was measured by increasing the muscle stretching force (Figure 14). This was done by keeping the electrical stimulation parameters constant but only changing the muscle length itself indirectly by varying the muscle stretching force. Since the muscle was fixed vertically, a change in the high of the force transducer changed the force that was pulling the muscle up. Consequently this force changed the length of the muscle. I concluded from this that keeping the muscular length constant by indirectly controlling the muscle lengthening force is a much more accurate compared to measure the muscle length directly. Hence, this method allowed the precise control of the passive stretching of the muscle (previously discussed) compared to assessing the muscle length since it's difficult to say where the exact borderline between tendon and muscle cells lies. As can be seen in figure 15, the force output seen in *M. extensor digitorum lateralis* is highest when applied a passive stretching force of 0.223 Newton. Thus, this indirect parameter for the most optimal muscle length for this *in vitro* experiment was kept for all further muscle samplings.

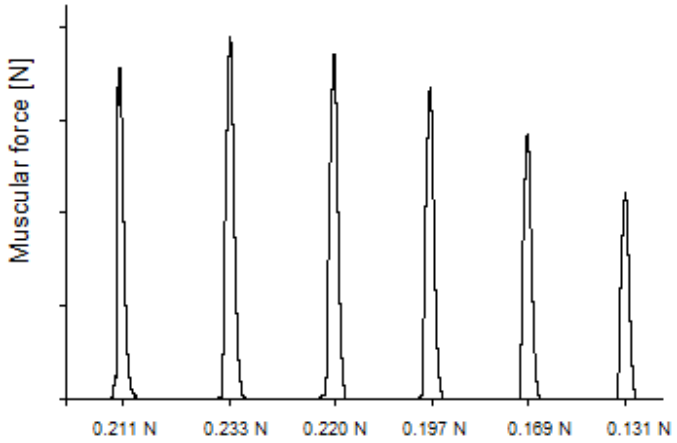

Figure 15: *M. extensor digitorum lateralis* exposed to different passive muscle stretching forces. Muscle stimulation was done using varying muscle lengths. Each peak on the X axis represents one muscular twitch response. The highest force production was measurable using a passive stretching force of 0.223 Newton.

4. Materials

4. 1 Equipment	Manufacturers
Force transducer (Plattform Wägezelle 1004)	Soemer Messtechnik GmbH
Signal enhancer (DAS III)	Soemer Messtechnik GmbH
Test and Analysis Software DOP	Delta Electronics, Inc.
Software drivers	Delta Electronics, Inc.
USB – RS485 (COM port) Adapter	Conrad Electronic GmbH Co KG
Wires	Conrad Electronic GmbH Co KG
Electric soldering iron	Conrad Electronic GmbH Co KG
Platinum electrodes	neoLab ©
Plexiglas	Acryplex GmbH Wien®
Thermometer	Conrad Electronic GmbH Co KG
Strings	Klos Heinrich KG
Oxygen	Air Liquide Austria GmbH
50 ml centrifuge Tubes with Printed Graduations and flat Caps	VWR
Pulse Generator	Grass SD9 Stimulator, USA
Scissors	Bayer Austria GesmbH
Scalpel	Bayer Austria GesmbH
Forceps	Bayer Austria GesmbH
Coconut oil	Ceres, Austria
Linseed oil	Raiffeisen Warenbetriebe, Salzburg, Austria
Sunflower oil	Clever, Austria
Food pellets	Raiffeisen e-force GmbH, Austria
Retainer	Bochem Instrumente GmbH
Scale	Mettler AT200

4.2 Krebs-Henseleit Buffer

Double distilled water was used to produce the Krebs-Henseleit Buffer (BARTON et al. 2008). Fresh solution was made daily and each ingredient was allowed to dissolve completely before adding the next. After all ingredients dissolved completely the pH value was adjusted to 7.35 using concentrated HCl. Adjusting the pH was done at room temperature while the solution was permanently bubbled with 100 % oxygen to ensure the saturation of the solution with oxygen before the onset of the experiment.

Composition in mM:

NaCl	117.2	(6.89g/L)
KCl	4.74	(0.35g/L)
$MgSO_4$	1.2	(0.295g/L)
$CaCl_2$	1.25	(0.133g/L)
$NaHCO_3$	25	(2.1g/L)
KH_2PO_4	1.2	(0.163g/L)
D-Glucose	11	(1.9g/L)

5. Results

5.0.1 Example for repeated isometric and tetanic stimulation of the same muscle:

Both figures shown below represent the same muscle which was stimulated five times each for isometric (Figure 16), isotonic (data not shown) measurements and three times for tetanic contractions (Figure 17) due to increased fatigue when using tetanic stimuli. The peak values of each contraction were used for further statistical calculations. All sampled muscles were subject to the measurements shown below.

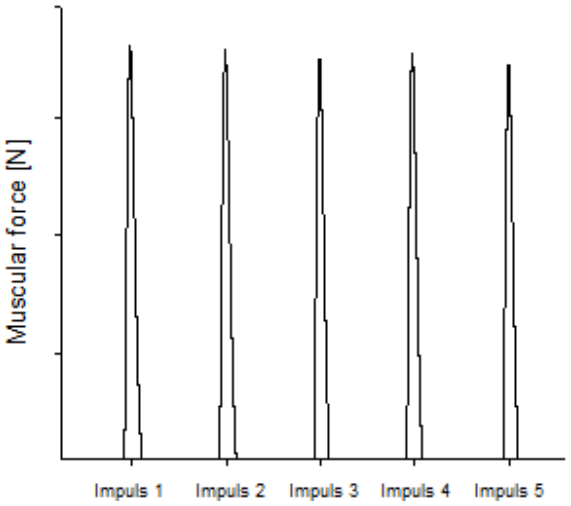

Figure 16: Example for assessing isometric muscular force in hares. The figure shows isometric force exhibited by EDL. Stimulated was repeated five times.

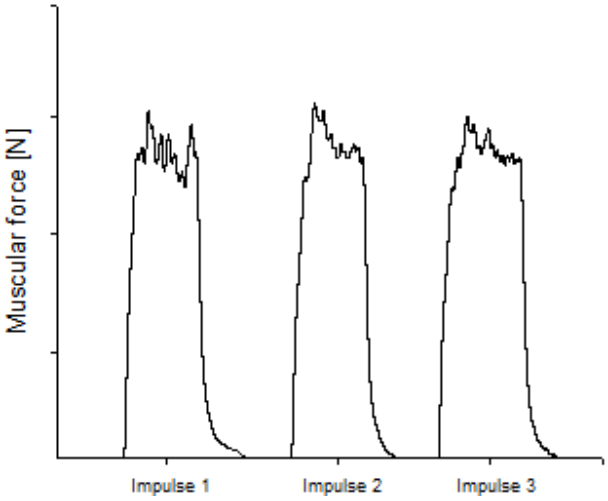

Figure 17: Example for assessing tetanic muscular force in hares. The figure shows the force exhibited by tetanic stimulation of EDL. The muscle received three times the exact same electrical stimulation.

5.1 Influence of diet on muscular force production

Statistical evaluation of the gained data did not reveal any significant link between the uptake of fatty acid enriched food and the maximum force production of *M. extensor digitorum lateralis in vitro* ($F_{1,x} = 0.4709$; $P = 0.6586$). However, as can be seen in Figs 18 - 23 we saw a tendency that omega-3 fatty acid enriched food has an adverse impact on *in vitro* force production of isolated EDL in all three types of force production (Figure 18 - 23). In contrast to omega-6, animals fed with omega-3 and saturated fatty acids seemed to exhibit a higher force production capacity (Fig 18 - 23). This impression is manifest in all groups (Figures 18 – 20 (mean values) and figures 21 - 23 (peak values)). The corresponding data further reveals that omega-6 fatty acids increased

isometric force production of EDL *in vitro*. Additionally the omega-3 fed group showed the highest variation in all 3 different muscle contraction parameters (Figure 18 – 23, table 1). In contrast to this the variation was lowest in the omega-3 group and intermediate in the animal group fed with saturated fatty enriched food pellets (Figure 18 – 23, table 1).

5.2 Gender driven impacts on EDL force production

Surprisingly the underlying data revealed that hares exhibit strong gender driven differences concerning their *in vitro* force production capacity of EDL ($F_{1,x} = 5.55$; $P = 0.0242$; Figure 24). Female hares were capable of a significantly higher isometric, tetanic and isotonic force production compared to males (see figure 24 for isometric measurements). This effect was independent of age or weight ($F = 1.339$, $P > 0.05$). Females from the saturated fatty acid group showed the highest isometric force production capacity among all groups and the highest variability was seen again in the omega-3 fatty acid group regardless of the animal's sex. Additionally female force production patterns reflected the patterns mentioned previously. Force production in males was always higher in the omega-6 group (Figure 24). This effect was highest during isometric muscle contractions (Figure 24).

5.3 Impact of housing conditions on muscular force production

Hares kept in bigger compounds exhibited a stronger *in vitro* force production capacity of EDL compared to animals which were kept in cages ($F_{1,x} = 2.943$; $P = 0.0248$, Figure 25). Females showed again an increased muscular force production capacity compared to males, regardless of their housing conditions. This effect was again, independent of the animal's age or weight.

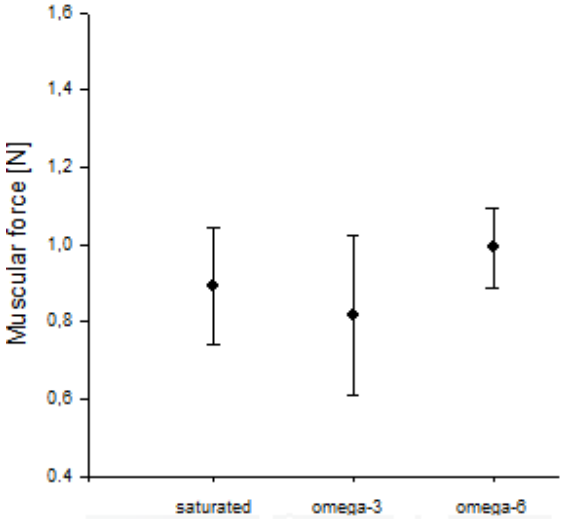

Figure 18: Isometric force production using the mean of the three strongest muscle contractions observed during one experiment. Strongest muscular force seen in animal group fed with omega-6 enriched food. Highest variation can be observed in the omega-3 group.

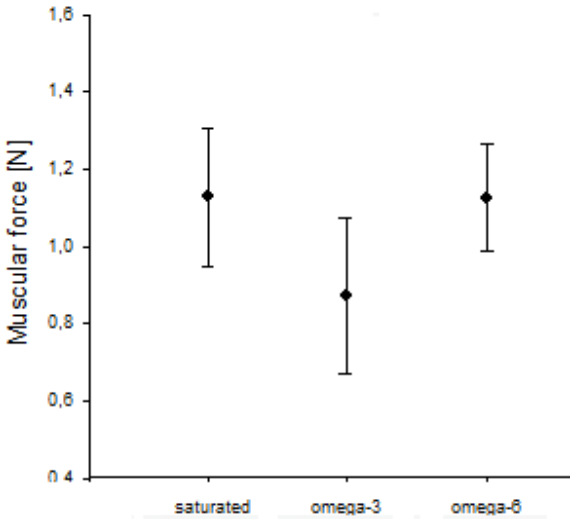

Figure 19: Tetanic force production using the mean of the three strongest muscle contractions observed during one experiment. Strongest muscular force seen in animal group fed with saturated fatty acid enriched food.

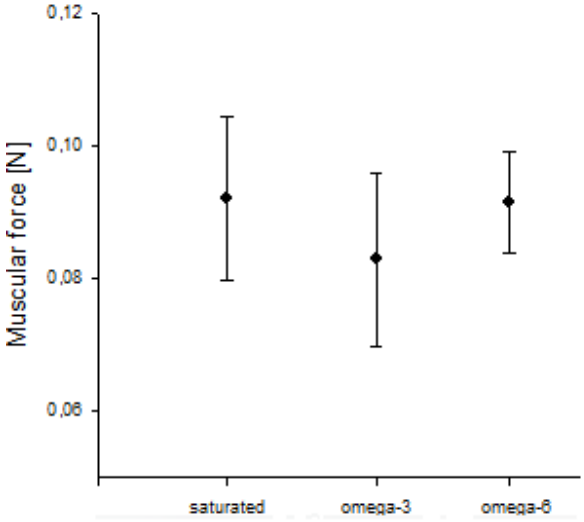

Figure 20: Isotonic force production of EDL using the mean of the three strongest muscle contractions observed during one experiment. Strongest muscular force seen in animal group fed with saturated fatty acid enriched food. Highest variation can be observed in the omega-3 group.

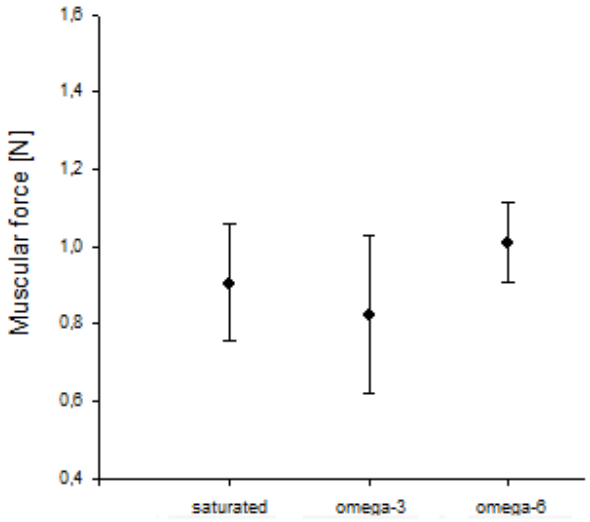

Figure 21: Isometric force production of EDL using the highest peak values observed during all muscle contractions done per experiment. The strongest muscular force was seen in the experimental group fed with omega-6 fatty acid enriched food.

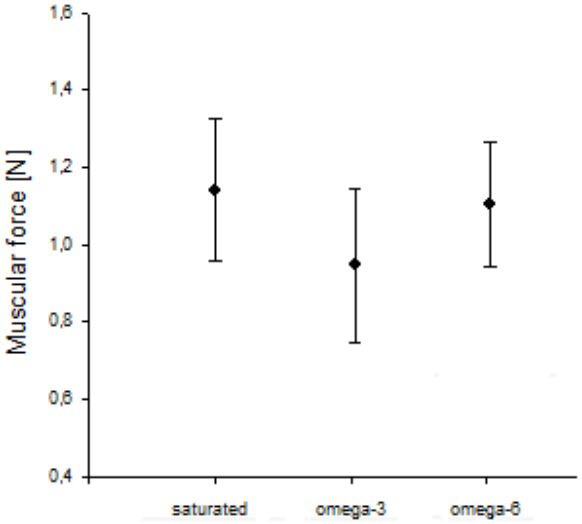

Figure 22: Tetanic force production capacity of EDL using the highest peak values observed during all muscle contractions done per experiment. Strongest muscular force can be observed in animal group fed with saturated fatty acid enriched food.

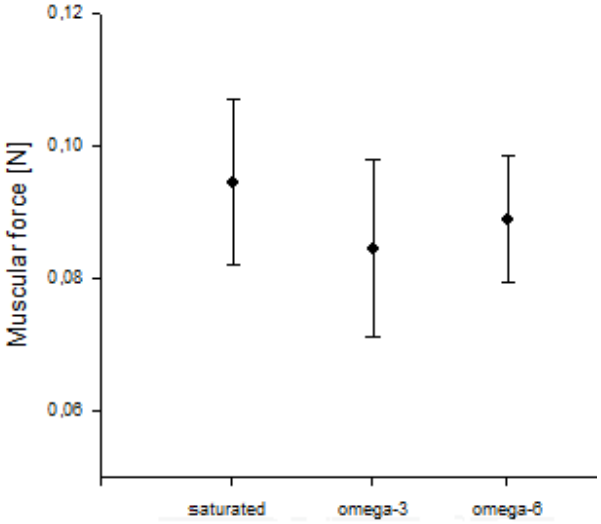

Figure 23: Isotonic force production capacity of EDL using the highest peak values observed during all muscle contractions done per experiment. Strongest muscular force can be observed in animal group fed with saturated fatty acid enriched food.

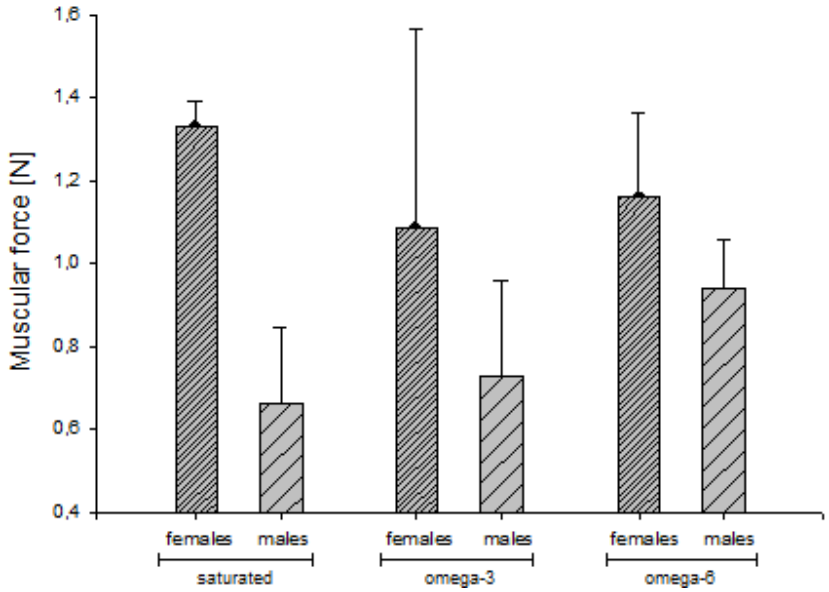

Figure 24: Isometric force production differences between both sexes according to their specific fatty acid enriched diet. Females show a significantly increased isometric, tetanic and isotonic force production compared to males of the same group. Females from to the saturated fatty acid group showed the highest isometric force production capacity.

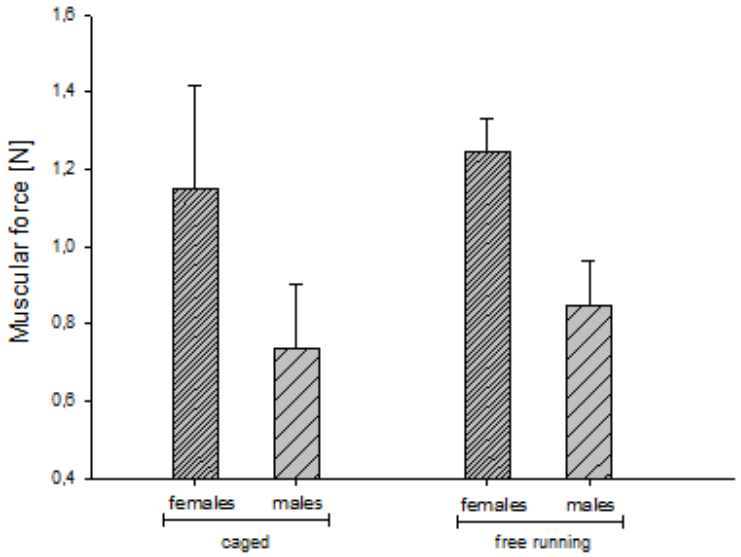

Figure 25: Effect of housing condition on isometric force production. Hares kept in bigger compounds exhibited a stronger *in vitro* force production capacity of EDL compared to animals kept in cages. Females showed an increased muscular force production capacity compared to males, regardless of their housing conditions. This effect was again, independent of the animal's age or weight.

6. Discussion

This study aimed to test the hypothesis that PUFA's enhance skeletal muscle performance in the mammalian body and to gain insights into the relationship between peak skeletal muscle forces and the PUFA content of muscular membranes. Hence, the cellular fatty acid membrane composition was manipulated by feeding different fatty acid diets since there is experimental evidence indicating that a certain ratio of omega-3 and omega-6 fatty acids in membranes significantly relates to MRS in many different mammals (RUF et al. 2006). Maximum running speed could be a direct result of generally increased muscular performance caused by the high PUFA content within the skeletal muscles of all fast- running animals (RUF et al. 2006). Closely connected to that it was suggested that the enhanced locomotor performance seen in animals with high contents of polyunsaturated fatty acids within the membrane of the sarcoplasmic reticulum may affect the Ca^{2+} ATPase (SERCA) (RUF et al. 2006). This in term would influence muscular performance since the SERCA enzyme plays a key role in muscle contraction, as this membrane enzyme is responsible for creating a Ca^{2+} gradient that is necessary for the onset of cross-bridge movement and subsequently muscle contraction. It is likely that transmembrane proteins are altered by the surrounding membrane lipid composition (LUNDBAEK et al., 2010). Perhaps this is causing alterations in the enzymatic functions of these transmembrane proteins including the Ca^{2+} ATPase's. This might be correlated to the observed increased maximum running speed of mammals with a high PUFA content. M. extensor digitorum lateralis was chosen for determining the isometric, tetanic and isotonic force production *in vitro*. This muscle was maintained by perfusion and oxygenation through a customized system: *in vitro*. Furthermore, electrical pulses produced by a generator induced muscle twitch responses that were detected by a strain gauge and recorded digitally.

Working on isolated muscles allows researchers to gain insight in simple biological processes without any systemic influences of the animal's body on the muscle performance. Additionally, it is by far easier to control several different parameters *in vitro* such as solution, pH, temperature, oxygenation and perfusion of the organ of interest. Thus, its often the method of choice when examining the influence of certain parameters on muscular performance. A major disadvantage, however, of using such *in vitro* methods is the task to sustain the tissue outside the body and keeping it stable and functional during all necessary measurements. Thus, attention has to be

spent to all kind of environmental parameters such as temperature, dissection procedure (duration and mechanic forces), ionic strength and pH of the used solution which would result in alteration of the tissue function. Maintaining mammalian tissues *in vitro* such as skeletal muscles is dependent on many factors, including time, surgical dissection, temperature and adequate muscle perfusion with appropriate oxygen and nutrition supply and last but not least, the use of accurate force recording equipment and impulse generators. The key aspect for successfully maintaining whole mammalian muscles *in vitro* and subsequently, accurate measurements, is the choice for the right muscle bathing solution. The muscle surrounding physiological solution should be similar to that of the blood plasma of the species used to optimally maintain cell viability.
Consequently, a system is required that allows the maintenance of tissues e.g. skeletal muscles and guarantees its optimal performance *in vitro*. Experimental design of this study involved the maintenance of an isolated skeletal muscle (*M. extensor digitorum lateralis*) of the European brown hare through a customized system *in vitro* to determine its isotonic, isometric and tetanic force production ability. This required an experimental chamber in which the muscle was continuously profused and oxygenated to achieve proper substrate delivery during the entire experiment. Furthermore, a stimulation device to electrically stimulate the isolated muscle, an isometric and isotonic force transducer as well as digital recording equipment was needed. Furthermore, due to the lack of a fully functioning central nervous system *in vitro,* a system was required that emulates neural functions using electrical pulses in order to induce muscle contractions. It was important to precisely control many different electrical pulse parameters such as the voltage amplitude, duration and frequency in order not to damage the muscle cells. Once all muscles were subject to electrical stimulation the gained data were analyzed with S-Plus.

6.1 Influence of diet on muscular force production

Unexpectedly, evaluation of the data did not reveal a significant link between the uptake of the special diet (increased contents of either omega-3, omega-6 or saturated fatty acids) and the force production of EDL. However opposed to the omega-6 and saturated fatty acid group, animals fed with omega-3 enriched food showed adverse effects concerning the maximum force generation of *M. extensor digitorum lateralis in vitro*. This effect was seen in all three different kind of force productions measured (Figure 18 - 23). In contrast to omega-3, animals fed with omega-3 and saturated fatty acids seemed to exhibit a higher force production capacity. This tendency can be

seen in all groups figure 18 – 20 (mean values) and figure 21 - 23 (peak values). The corresponding data also demonstrate that omega-3 fatty acids are favorable for isometric force production of EDL *in vitro*. There are various reasons why the hypnotized link between PUFAs and a higher skeletal muscle force production capacity could not be confirmed. One reason might be the choice of muscle that was greatly limited because of the choice of animal. It might be possible that there are selective links between defined skeletal muscles like those who greatly participate during running and those who play a minor role in locomotor performances. Even though it might be hardly probable and it already has been proven that PUFA content does not differ between peripheral and central skeletal muscles (VALENCAK et al., 2003). However such a mechanism cannot be entirely excluded. Another explanation might be the special food used in this study. As mentioned in the material and method section the pellets that provided the basis for all the diets in this study mixed to match stomach contents of European hares. Thus, its plausible to conclude that this special food already included enough omega-3 and omega-6 fatty acids. This would mean that the manipulated food could not cause a strong impact since the original composition was already counteracting insufficient supply of the mentioned fatty acids. Hence, a diminished effect would be the consequence.

6.2 Gender specific differences

Evaluation of the data revealed that hares exhibit strong gender driven differences ($P = 0.0242$) concerning their *in vitro* force production capacity of EDL (Figure 24). Females are capable of a significantly higher isometric, tetanic and isotonic force production compared to males. This effect was independent of age or weight. Therefore, it seems that the higher force production ability seen in female hares are due to other sex specific mechanisms. One possible explanation for this observed effect might be the increased fatigue resistance seen in female human muscles (GORE, 2007). Females often have less muscle mass compared to males which subsequently correlates with a lower metabolic demand for oxygen (GORE, 2007). However, no attention was spent to muscle weights during this study. I conclude from this that it is possible that female muscles could handle better the (compared to physiological conditions) low *in vitro* oxygenation levels within the bubbled Krebs Henseleit solution (see discussion). Thus, an increased force production capacity would be the result.

6.3 Influence of housing conditions on muscular force production

Furthermore, I could demonstrate that hares kept in bigger compounds revealed a stronger *in vitro* force production capacity of EDL (independent of age or weight) compared to animals which were kept in cages (Figure 25). It seems plausible that animals which were able of exercising more developed a higher muscle mass and or had increased muscular strength compared to animal which could not run and therefore had diminished muscular force.

7. Summary

This study tested whether polyunsaturated fatty acids (PUFAs) positively correlate with the maximum running speed (MRS) in mammals since high PUFA contents can be found within the skeletal muscles of all fast- running animals. Closely connected to that it was suggested that the enhanced locomotor performance seen in animals with high contents of polyunsaturated fatty acids within the membrane of the sarcoplasmic reticulum may affect the Ca^{2+} ATPase (SERCA) and consequently muscle performance. Thus, in this study (n = 43) European brown hares (*Lepus europaeus*) were chosen to test the above introduced hypothesis. All animals were split in three groups and subjected to three different diets distinct in fatty acid compositions (rich in omega-3, omega-6 or saturated fatty acids). After 8 weeks of receiving this diet, hares were killed and the *Musculus extensor digitorum lateralis* was isolated and maintained in vitro. All sampled muscles were subject to different electrical stimulations in order to examine their isometric, tetanic and isotonic force production capacity. The groups did not differ significantly in the above mentioned force production parameters. Furthermore it could be shown that hares exhibit strong gender driven differences concerning the *in vitro* force production of *Musculus extensor digitorum lateralis.* Females muscles were capable of higher force productions compared to males. Furthermore, it could be demonstrated that the type of housing condition significantly affects the muscular force production.

8. Zusammenfassung

Fettsäuren erfüllen verschiedenste wichtige Aufgaben im Säugetierorganismus. Durch ihre Inkorporation in zelluläre Membranen beeinflussen sind sie nicht nur an immunologischen Prozessen und Winterschlaf beteiligt sondern sie beeinflussen auch die Eigenschaften dieser Membranen wie Dicke und Fluidität. Auf Grund dessen beeinflussen sie auch indirekt funktionelle Eigenschaften von Transmembranenzymen wie die Ca^{2+} ATPasen des sarkoplmatischen Retikulums in dem der direkte Kontakt dieser Moleküle die Konfirmation dieser Proteine beeinflusst. Dies resultiert in einer veränderten Enzymaktivität. Mehrfach ungesättigte Fettsäuren sind für den Säugetierorganismus essentiell, da sie nicht de novo synthetisiert werden können. Damit bestimmt die Zusammensetzung und Verfügbarkeit von Fettsäuren in der Nahrung auch indirekt die Zusammensetzung der zellulären Membranen und beeinflusst damit die Funktion von Enzymen die ebenfalls in diesen Membranen in kooperiert sind. Die Zusammensetzung von mehrfach ungesättigten Fettsäuren in Membranen korreliert auch mit saisonalen Änderungen (VALENCAK et al., 2003). Dr. Teresa Valencak konnte auch bereits zeigen, dass Feldhasen einen überaus großen Anteil von mehr als 65% an mehrfach ungesättigten Fettsäuren (Polyunsaturated Fatty Acids-PUFAs) in ihren Laufmuskeln besitzen. Aus diesem Grund wird ein direkter Zusammenhang zwischen den Fettsäurezusammensetzungen von Feldhasen und ihrer außerordentlichen Laufgeschwindigkeiten geschlossen. Tatsächlich konnte von Fr. Dr. Valencak gezeigt werden, dass der Anteil an n- 6 mehrfach ungesättigten Fettsäuren mit der maximalen Laufgeschwindigkeit eines Säugetieres in signifikantem Zusammenhang steht (Ruf et al., 2006). Ein Enzym, die Calzium-Magnesium- ATPase im sarkoplasmatischen Retikulum (SERCA), ist für den Transfer von Calzium aus dem Zytosol verantwortlich und wurde für die gefundenen Effekte verantwortlich gemacht. Die Aktivität dieses Enzyms ist stark temperaturabhängig und wird vom n-6 zu n-3 Fettsäuren-Verhältnis gesteuert (zusammengefasst in Ruf und Arnold, 2008). n-3 bzw. n-6 Fettsäuren gehören zu den mehrfach ungesättigten Fettsäuren und unterscheiden sich im Vorkommen der ersten Doppelbindung in der Kohlenwasserstoffkette sowie in ihrer physiologischen Wirkung.

Parallel zu den Arbeiten zur Membranzusammensetzung des Feldhasen konnte von Fr. Dr. Valencak festgestellt werden, dass Feldhasen sehr selektiv fettreiche Gräser und Kräuter aus ihrer Umgebung fressen (Reichlin et al. 2006). Da das Fettsäuremuster im Gewebe neben der artspezifischen, regulierten Komponente auch von der aufgenommenen Nahrung stark beeinflusst wird

und die Feldhasen speziell Karotten und z.B. Klatschmohn fressen, schlug sie vor, dass spezielle Fettsäuren aus der Nahrung den Feldhasen schneller machen. Mehrfach ungesättigte Fettsäuren bzw. speziell das n-3 und n-6- Verhältnis im Gewebe stehen also in Kontext mit der Laufgeschwindigkeit und beeinflussen den Kalzium- Stoffwechsel in der Muskelzelle. Diese Studie versuchte nun ihre Wirkungen auf die mechanischen Eigenschaften im Muskel aus bzw. auf die Muskelkraft herauszufinden. Diese Frage soll mein Forschungsvorhaben beantworten und der Feldhase stellt ein sehr aussagekräftiges Modelltier dar. Wir haben für den Versuch 43 Feldhasen, davon 11 Weibchen und 32 Männchen vorgesehen. Die Tiere werden in 3 Gruppen aufgeteilt werden: eine Kontrollgruppe auf Hasenfutterpellets die mit 10% gesättigten Fettsäuren (Kokosfett) angereichert wird (11Tiere), eine experimentelle Gruppe die speziell mit n-3 Fettsäuren gefüttert werden wird (Leinsamenöl, 10 Tiere) und eine dritte Gruppe denen n-6 Fettsäuren (Sonnenblumenöl) vorgelegt werden wird (11 Feldhasen). Der Energiegehalt und auch die genaue Fettsäurenzusammensetzung aller Futtermittel wird von uns bei jeder neuen Futtermischung bzw. Anreicherung mit den Ölen und Fetten durch chemische Analysen ermittelt. Das Pelletfutter selbst wird für das Forschungsinstitut für Wildtiere und Ökologie speziell von Raiffeisen Österreich in Salzburg seit gut 20 Jahren hergestellt und dessen Zusammensetzung wurde anhand von ausgedehnten Mageninhaltsanalysen von bei der Jagd erlegten Feldhasen erarbeitet. Eine artgerechte und den Vorlieben des Hasen nahe kommender Futterzusammensetzung war daher gewährleistet. Nach der Aufteilung der Feldhasen auf 3 Gruppen bzw. entsprechend viele Gehege werden den Tieren 3 Futtersorten ad libitum vorgelegt werden. Das Futter wurde bis zum Verfüttern kühl gelagert werden (um Autoxidationen zu verhindern) und täglich je nach Bedarf nachgefüttert werden. Nach einem Zeitraum von 6-8 Wochen wurden die Feldhasen durch zervikale Dislokation mit einem Eisenknüppel durch die erfahrene Hand eines Tierpflegers getötet. Sofort nach Eintreten des Todes wurde begonnen den *M. extensor digitorum lateralis* entfernt und mit Krebs-Henseleit Lösung in vitro am Leben erhalten. Der Muskel wurde vertikal an einer Wägezelle (Sömer, Deutschland) die selbst bei geringstem Zug eine Spannung erzeugt aufgehängt und elektrisch gereizt wodurch dieser kontrahierte. Die entstandene Kontraktion wurde durch ein digitales Signal in Echtzeit auf dem Computer dargestellt. Auf diese Weise wurden die mechanischen Eigenschaften des Muskels beim Europäischen Feldhasen und der intramuskulären Verfügbarkeit ungesättigter n-3 bzw. n-6 Fettsäuren untersucht. Aufgrund der uns bereits vorliegenden Daten zum Fettsäuremuster der Laufmuskulatur sowie aufgrund ihrer besonders herausragenden maximalen Laufgeschwindigkeit ist der europäische Feldhase (*Lepus europaeus*) das ide-

ale Modelltier für das Experiment. Es konnte schließlich gezeigt werden, dass es keinen signifikanten Zusammenhang zwischen der Kraftentwicklung des *M. extensor digitorum lateralis* und der gefütterten Fettsäure Diät gibt. Allerdings zeigte sich eine Tendenz, dass Muskeln die aus der gesättigten sowie der omega-6 Gruppe stammten eine höhere Kraftentwicklung gegenüber den omega-3 gefütterten Tieren aufweisen. Des Weiteren wurde ein signifikanter Zusammenhang zwischen Geschlecht und Kraftentwicklung gefunden, wobei Weibchen eine größere Kraftentwicklung aufwiesen. Dieser Mechanismus könnte allerdings mit der höheren hypoxie Toleranz von weiblichen Muskeln gegenüber Männlichen korrelieren. Abschließend konnte demonstriert werden, dass die Art der Tierhaltung einen Einfluss auf die Muskelkraft hat denn Tiere die in Bodenhaltung gehalten wurden zeigten eine höhere Fähigkeit durch Kraftentwicklung als Tiere der Käfighaltung. Diese Studie stellt den ersten Schritt zur Ergründung des Einflusses mehrfach ungesättigter Fettsäuren auf die mechanischen Eigenschaften der Laufmuskulatur des Feldhasen dar.

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11. Appendix

Table 1:

id	PUFA diet	sex	weight	caged	isometric-	tetanic-	isotonic- force
1	omega-6	♀	3385 g	-	1.880 N	2.032 N	0.126 N
2	omega-6	♀	3300 g	-	0.701 N	0.725 N	0.055 N
3	omega-6	♂	3685 g	-	NA	0.078 N	NA
4	omega-6	♂	4055 g	-	0.135 N	0.181 N	0.028 N
5	omega-6	♂	3700 g	-	1.864 N	1.444 N	0.106 N
6	omega-6	♂	3335 g	-	0.429 N	1.063 N	0.075 N
7	omega-6	♂	3065 g	-	0.904 N	1.009 N	0.091 N
8	omega-6	♂	3175 g	-	1.161 N	1.125 N	0.119 N
9	omega-6	♂	3680 g	-	0.923 N	0.456 N	0.041 N

10	omega-6	♂	3405 g	-	0.107 N	0.182 N	0.016 N
11	omega-6	♂	3285 g	-	1.438 N	NA	0.129 N
12	omega-6	♀	3610 g	-	0.206 N	NA	NA
13	omega-6	♀	3725 g	-	1.880 N	2.032 N	0.126 N
14	omega-3	♀	3610 g	-	0.701 N	0.725 N	0.055 N
15	omega-3	♂	3585 g	+	1.176 N	1.360 N	0.112 N
16	omega-3	♂	3275 g	+	0.818 N	0.974 N	0.110 N
17	omega-3	♂	3775 g	+	0.866 N	0.931 N	0.078 N
18	omega-3	♂	3790 g	+	0.801 N	0.878 N	0.085 N
19	omega-3	♂	3125 g	-	1.867 N	1.856 N	0.145 N
20	omega-3	♂	3520 g	-	0.207 N	0.372 N	0.062 N
21	omega-3	♀	3700 g	-	1.014 N	NA	0.075 N
22	omega-3	♀	3333 g	-	1.047 N	NA	NA
23	omega-3	♂	3770 g	-	1.269 N	1.731 N	0.123 N
24	omega-3	♂	3375 g	-	1.299 N	1.518 N	0.090 N
25	omega-3	♀	3780 g	-	0.840 N	0.718 N	0.112 N
26	omega-3	♀	3570 g	-	1.276 N	1.109 N	0.112 N
27	saturated	♂	3900 g	+	0.199 N	NA	NA
28	saturated	♂	3590 g	+	0.086 N	0.164 N	0.026 N
29	saturated	♀	3700 g	+	1.432 N	1.656 N	0.122 N
30	saturated	♂	3155 g	+	1.076 N	1.821 N	0.086 N
31	saturated	♂	4025 g	+	0.919 N	0.985 N	0.101 N
32	saturated	♂	4065 g	+	0.656 N	1.237 N	0.123 N
33	saturated	♂	3640 g	+	NA	0.409 N	0.014 N
34	saturated	♂	3815 g	+	1.326 N	1.381 N	0.118 N
35	saturated	♂	3360 g	+	NA	0.971 N	NA
36	saturated	♂	3360 g	+	0.316 N	0.082 N	0.059 N
37	saturated	♀	4150 g	-	1.356 N	1.681 N	0.125 N
38	saturated	♀	3775 g	+	1.292 N	1.563 N	0.113 N
39	saturated	♀	3730 g	+	1.148 N	1.615 N	0.121 N
40	saturated	♂	3592 g	+	0.086 N	0.164 N	0.026 N
41	saturated	♂	3700 g	+	1.432 N	1.656 N	0.122 N

11.1 Methodological considerations

Despite the possibility to study physiological reactions under controlled conditions, one has to face limitations as well when dealing with the presented in vitro method. Concerning the chosen animal model in this study the biggest disadvantage is the limitation in size of skeletal muscles. The reason why only small muscles are suitable for their maintenance in vitro is because of the limited diffusion distance of oxygen and other nutrition. However, smaller animal models such as mice hamper dissection procedure and muscle handling. The maximal diffusion distance for oxygen for example ends at ~ 2 mm. Thus, the bigger the chosen muscle the more likely it will be that a hypoxic core inside the muscle center will arise. This subsequently leads in an impairment of muscle function and force production. Besides large diffusion distances, tissues in vitro face other difficulties such as a missing arterial PO_2 pressure and lack of blood flow. However, one possibility to counteract these problems is the choice of a lower temperature since hypothermic tissues are more resistant to limited oxygen supply.

11.1.1 Limitation in size because of critical diffusion distances

Maintenance of whole isolated muscles in vitro is a critical process that requires precise control of many critical parameters such as oxygen since muscles are most sensitive to ischemia. Oxygen and nutrition supply is vital to dissected, isolated muscles and their availability inside the cells determines cellular stability and how long the tissue can be maintained and functions ex vivo. Since dissected muscles are no longer connected to the vascular system, oxygen as well as nutrition supply is dependent on passive diffusion through the muscular tissue. Especially the inner core of the muscle has to face a limited availability of oxygen and nutrition's because of the long diffusion distance from the bathing solution. The depth in which oxygen is able to penetrate the muscle was assessed to be a maximum of 4 mm for muscular resting metabolism (VAN DER HEIJDEN et al., 1999). Hence, the bigger the muscle the more poor gets its oxygen and nutrition supply by diffusion. Consequently the bigger the muscle size the more likely it will be that hypoxic areas within the center of the muscle will evolve. This impairs muscle function, decreases resistance to fatigue, shortens muscle tissue stability and subsequently diminishes force production. Since the main aim of this study was to evaluate the influence of omega-3 and omega-6 fatty acids on contraction force of isolated rabbit muscles, it is obvious, that those

muscles which contribute mainly in the hind-limb movement were most attractive. The sizes of most of these muscles however are in conflict with their possible maintenance outside the body duo to the limitation of oxygen and nutrition supply. Although it was demonstrated by Heijden et al. that trimming bigger muscles (which reduces the cross-sectional area) is a possible way of counteracting diffusion distance limitations, only whole muscles were used in this study to exclude impairment's in force production that would result from trimming procedures and are hard to control. Therefore, it is impossible to maintain whole main hind limb muscles of the European hare in vitro with the here used methods. Consequently M. Soleus was chosen since its contribution in the hind-limb movement and its acceptable size.

11.1.2 Temperature

Solution temperature is affecting numerous parameters such as stability, kinetic parameters and force production of the muscle. The closer the temperature to the physiological value of 37°C the higher becomes the metabolic demand of the cells. This is a big concern regarding the use of in vitro methods. Therefore, the temperature was set to be relatively low (25°C) compared to normal physiological values of rodents to increase ischemic tolerance of the tissue. Temperatures below 20°C were not used since there is evidence that mammalian muscles undergo a decrease in muscle excitation during tetanic contractions under low temperatures (STEIN et al., 1982). A decreased temperature ensures prolonged muscle viability by delaying energy shortage and therefore contributes positively to the experiment (VAN DER HEIJDEN et al., 1999). The advantages of using lowered temperatures compared to the physiological one are slowed down cellular processes with a subsequently decreased demand of oxygenation and nutrition. During the entire preparation process the muscles were periodically cooled with Krebs-Henseleit solution to decrease the metabolic demand of the muscle. It has been demonstrated that tissue oxygen consumption can be decreased by the factor of 2.0 – 2.5 per 10°C reduction in temperature (GOLDBERG et al., 2010). Since the core body temperature of most rodents lies around 37° one might assume that the optimal temperature for maintaining mammalian skeletal muscles in vitro lies around that physiological value. Interestingly (GOLDBER et al., 2010) found that the maximum mechanical efficiency of isolated mouse muscle fibers is little affected by temperature between 20 and 30°C. Based on this finding and other data as well as an SOP written for measuring isometric forces in isolated mouse muscles in vitro (BARTON & LYNCH, 2011) the

optimal temperature for this study was set to be 25°C and maintained during the entire experiment. This prolongs the viability of the isolated mammalian muscles *in vitro* with little interfering the maximum mechanical efficiency output of the muscle. Solution temperature was kept at room temperature. Controlling of the bath temperature was ensured by using a digital thermometer that was placed inside to the muscle containing chamber.

11.1.3 Oxygenation

Diffusion distance is the biggest limiting factor concerning muscle size for almost all possible muscle candidates of *Lepus europaeus*. Since muscles are sensitive to ischemia (SAPEGA et al., 1988) lack of oxygen supply leads immediately to impairment in muscle function. Heijden et al. and others found out that the maximal penetrable depth of tissue by oxygen lies around 1.8 - 4 mm. Such a diffusion distance would still provide the muscle with sufficient oxygen for its resting metabolism (VAN DER HEIJDEN et al., 1999) without the formation of necrotic tissue. Consequently the maximal diameter of any used skeletal muscle is limited with maximal 8 mm. However this is in conflict with any bigger animal model. Based on this M. Soleus was one of the few remaining possible skeletal muscles of the hind-limb with the right size and right anatomical location for rapid dissection to keep the stress for the tissue at a minimum. Besides diffusion distance, temperature is also a limiting factor when it comes to oxygen supply. As mentioned above, increasing temperatures increase the oxygen demand of tissues which would not result in an adequate oxygen supply. Hence, short diffusion distances and low temperatures contribute positively to this experiment (MELLORS & BARCLAY 2001, BARTON & LYNCH, 2011).