A physiological strength exercise model for rats

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Abstract

The lack of good strength exercise models for rodents has been hampering our understanding of skeletal muscle hypertrophy. Although it has been much criticized, most studies use surgical overload models such as synergist ablation to induce hypertrophy. There have been numerous attempts to establish more physiological strength exercise models similar to strength exercise in humans, but the effects have been variable and they often involve stressful conditions.

Consequently, we designed a new exercise cage to induce muscle hypertrophy in rat skeletal muscle. In this model rats voluntarily climb on a demanding substrate in order to obtain food and drink. What mainly sets our model apart from previously attempted strength exercise models is that the animals are kept continuously in the exercise environment throughout the experiment, and are able to move freely and climb on their own volition. We compared the effect of voluntary training with an established overload model; synergist ablation. The animals were divided into four experimental groups: control, overload, trained and trained + overload. The animals' food intake was measured every day, and individual bodyweight was measured once a week throughout the experiment. After 31 days of exercise the *extensor digitorum longus* (EDL) and *tibialis anterio*r (TA) muscle wet weight and fiber cross sectional area were measured.

EDL cross sectional area measurements showed a 102 % increase in the trained compared to the control group. The trained group also had an increase of 74% when compared to the overload group. Also, the trained + overload group had a 123% increase when compared to the control group. Further, comparing the overload group to the trained and the trained + overload group we saw a 74, and a 92% increase in muscle fiber cross sectional area, respectively. Finally, measurements of cross sectional area in the TA muscle showed a 97% increase in the trained group compared to the control group.

These results suggest that we have successfully established a new strength exercise model that has an effect on muscle hypertrophy that surpasses previously established exercise models including surgical overload.

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1 Introduction

1.1 Skeletal muscle hypertrophy

It is well established that skeletal muscles have a remarkable ability to adapt to functional demands. In response to increased mechanical load such as strength exercise or overload, skeletal muscles gain mass by increasing the amount of contractile proteins in individual muscle fibers; a process termed hypertrophy (Russell et al., 2000). In response to disuse, such as denervation or hind limb suspension, the muscles will become smaller due to a decline in muscle fiber protein content; a process termed atrophy (Bodine et al., 2001).

Because muscle mass is a great determinant of muscle strength, research into muscle hypertrophy is of great interest to the general public, including athletes, patients rehabilitating from injury-induced atrophy, and the elderly population struggling with decreased mobility and reduced quality of life due to reduced muscle mass. However, a problem in this field has been to establish animal models that closely mimic human skeletal muscle adaptions. In this thesis, we have investigated a physiological exercise model for skeletal muscle hypertrophy in rat with the future aim of improving the current understanding of cellular and molecular mechanisms regulating muscle mass. In particular, we have developed an exercise cage were the rats have to climb in order to obtain food and drink as a new, non-invasive and voluntary exercise model to induce skeletal muscle hypertrophy.

Current literature suggests that an increase in muscle mass is predominantly caused by hypertrophy of single muscle fibers (Allen et al., 1999; Gollnick et al., 1981; White et al., 2010) although some reports suggest that an increase in muscle fiber number (hyperplasia) can occur in mammals and birds (Alway et al., 1990; Antonio and Gonyea, 1993). Essentially, regulation of muscle fiber size reflects the balance between protein synthesis and degradation. Thus for muscle hypertrophy to occur, the rate of protein synthesis must exceed the rate of protein degradation (Kumar et al., 2009), with the net result being an accumulation of protein and increased muscle fiber area (Kimball et al., 2002).

Exercise induced skeletal muscle hypertrophy is a complex process that involves many signaling modules such as mechanosensors at the muscle fiber membrane and/or contractile and cytoskeletal elements, calcium-mediated signaling, regulation of protein metabolism, activation of stem cells and hormones. To which relative extent these factors contribute to skeletal muscle growth has proven difficult to answer (Adams and Bamman, 2012). It is well known that repeated mechanical load of sufficient magnitude, frequency and duration eventually will lead to muscle hypertrophy. However, muscle hypertrophy is the

accumulation of a complex and poorly understood array of coordinated cellular and signaling processes. Thus, results would be dependent on the model used in studying muscle hypertrophy.

1.1.2 Cellular and molecular mechanisms regulating skeletal muscle hypertrophy

Skeletal muscle fibers are the largest cells in the human body with a diameter up to 50μm, and with lengths up to several decimeters. The muscle fiber has a large cytoplasmic volume and is one of the few syncytia in vertebrates, i.e. each muscle fiber can contain hundreds of nuclei (Bruusgaard et al., 2003). Myonuclei are situated between the myofibrils and the cell membrane in the periphery of the muscle fiber, and each nucleus is believed to regulate the gene product of a finite volume of cytoplasm called the nuclear domain (Cheek, 1985). This concept implies that myonuclear number and cytoplasmic volume have to increase in parallel for the nuclear domain to remain constant (Cheek 1985). Supporting this hypothesis, Bruusgaard et al. (2003) found a correlation between muscle fiber size and the number of myonuclei, suggesting that the number of myonuclei increase with increasing cell volume. Myonuclei are post mitotic, therefor myonuclear addition during muscle hypertrophy is dependent on satellite cell activation (Relaix and Zammit, 2012). Satellite cells are quiescent muscle precursor cells which when activated can proliferate and differentiate into myonuclei (Morgan and Partridge, 2003).

It has generally been believed that addition of myonuclei is a prerequisite for muscle hypertrophy to occur (Adams et al., 2002). Using *in vivo* imaging, Bruusgaard et al. (2010) found that the addition of myonuclei precedes hypertrophy. Mice were overloaded by synergist ablation, and they observed that the addition of nuclei occurred after 6-8 days post surgery, while the increase in cross sectional area occurred after 8-12 days. However, recently McCarthy et al. (2011) showed that satellite cell activation is not necessary for hypertrophy. They demonstrated that satellite cell depleted mice had the same increase in cross sectional area as the control group after two weeks of overload by synergist ablation. This indicates that existing myonuclei, at least until the muscle reaches a certain size, are able to sufficiently increase protein synthesis to cope with the larger myonuclear domains. In conclusion, the study done by Bruusgaard et al. (2010) suggests that an increased number of myonuclei is a major cause of hypertrophy, but it does not exclude the notion that some hypertrophy can occur in the absence of new nuclei in satellite cell depleted muscle fibers as suggested by McCarthy et al. (2011)(Gundersen, 2011).

There are numerous regulatory signaling pathways that have been identified regulating development of load-induced skeletal muscle hypertrophy. For instance, in adult skeletal muscle the mammalian target of rapamycin (mTOR) is currently considered to be essential in the integration of a multitude of upstream signaling pathways which, when activated, results in an increased translational efficiency (Miyazaki et al., 2011). Insulin-growth factor 1 (IGF-1), together with its downstream kinase Akt is one of the most well characterized upstream triggers of mTOR. The IGF-1/P13K/Akt pathway function as a positive regulator of muscle mass. Increased load such as strength training or overload, induce muscle hypertrophy by activating this pathway. IGF-1 activates Akt, which in turn inactivates inhibitors of mTOR. Activation of mTOR increases protein synthesis by two mechanisms: Firstly, mTOR activates its downstream effector, 70-kDa ribosomal protein S6 kinase (p70S6K) which is important for translation of mRNAs during protein synthesis (Kimball, 2002). Secondly, mTOR inactivates the translational repressor 4E-BP1, and thereby activating eIF4E, which in turn allows for activation of the ribosome and protein synthesis (Atherton et al., 2005)(Figure 1).
Mechanical stretch is also thought to activate mTOR through Akt which inactivate the inhibitor Rheb, or through phospholipase D (PLD) and phosphoric acid (PA) in the absence of IGF-1 (Hornberger et al., 2005; Hornberger and Chien, 2006; Miyazaki and Esser, 2009) (Figure 1).

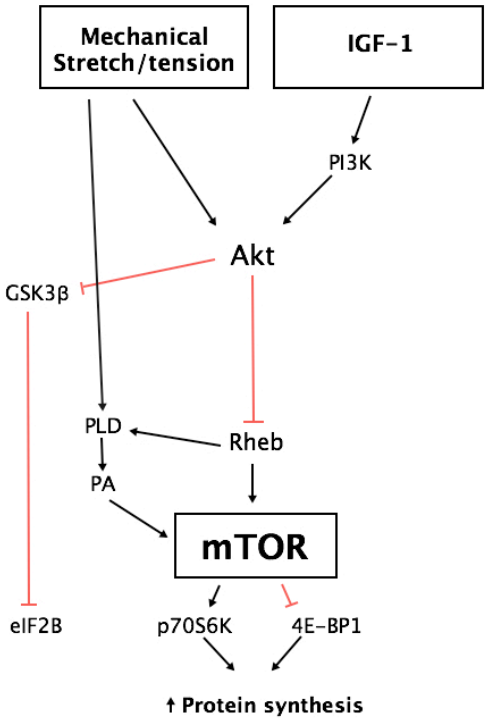


Figure 1. Simplified scheme modified from Miyazaki and Esser (2009) illustrating positive regulators that may contribute to protein synthesis and subsequent hypertrophy in skeletal muscle.

1.2 Experimental models for muscle hypertrophy

Muscle adaptions are specific to the exercise stimulus (Nader and Esser, 2001), and endurance exercise for example during treadmill running leads to physiological and biochemical adaptions such as changes in mitochondrial density, capillary supply, changes in metabolic enzymes, and increased maximal oxygen uptake (Holloszy and Booth, 1976). In contrast resistance exercise leads to physiological adaptions including muscle hypertrophy and increased strength (McDonagh and Davies, 1984).

For more than a century scientists have attempted to establish a physiological animal model to study skeletal muscle hypertrophy (Baar and Esser, 1999; Chalmers et al., 1992; Denny-Brown, 1960; Gollnick et al., 1981; Gonyea and Ericson, 1976; Gordon et al., 1967; Helander, 1961; Ho et al., 1980; Ishihara et al., 1998; Klitgaard, 1988; Morpurgo, 1879; Roy and Edgerton, 1995; Tamaki et al., 1992; Wong and Booth, 1988). Progressive resistance training has long been used to increase muscle mass and strength both in humans and animal hypertrophy models. Although hypertrophic and functional outcomes of resistance training have been described, the use of human subjects to investigate muscle hypertrophy is substantially limited (Alway et al., 2005). First, the research into molecular mechanisms underlying the skeletal muscle response to strength training necessitates analysis of whole muscles as opposed to small muscle biopsies. Muscle biopsies are generally sufficient for studies of endurance training, but will not be appropriate for studying cellular mechanisms of skeletal muscle enlargement (Alway et al., 2005). For example, when studying muscle fiber type characteristics, results may differ from biopsy to biopsy even within the same muscle, and may not be representative for the muscle as a whole (Elder et al., 1982). Secondly, human subjects require long periods of adaption, and the response to strength training varies between individuals and depends on previous activity level, age, nutrition, gender, motivation and genetic predisposition (Kraemer et al., 2002). The use of animal models allows for a better standardization of the study conditions, and enables the collection of numerous sorts of data. Hence several *in vivo* strength-training models have been developed in attempts to induce hypertrophy in animal skeletal muscle. However, it has proven difficult to develop a satisfactory animal model that can be used to mimic the human strength training condition (Timson, 1990). There are several complicating factors, e.g. that it is difficult to make a laboratory animal perform strength training that can be compared to human strength training. Furthermore, voluntary training in animal models usually requires operant conditioning, involving the use of either positive or negative reinforcements, which could lead to large variability between animals (Wirth et al., 2003).
Thirdly, motivational tools such as reward or deprivation of food have been demonstrated to be ineffective, since the animal will only perform its task until the effort required by the animal

exceeds the desire for food (Timson, 1990). Employing a model using food deprivation as the main stimulus might have a negative effect on muscular hypertrophy either due to lack of motivation to perform the task at hand, or as a result of calorie restriction (Timson, 1990). Surgical methods such as tenotomy (severing the tendon of a synergistic muscle) and synergist ablation (complete or partial removal of a synergistic muscle) have both been demonstrated to be effective in increasing muscle mass, but is criticized as models for human strength training due to the unphysiological conditions of these methods (Taylor and Wilkinson, 1986; Timson, 1990). All in all, there are no satisfactory animal strength training models that are optimized for the study of human strength exercise induced muscle hypertrophy, thus it is important with further developments this field.

1.2.1 Compensatory hypertrophy models

Compensatory hypertrophy entails severing the tendon of a synergistic muscle or complete removal of a synergistic muscle. Forcing the remaining muscle to produce the same tension alone as the whole muscle group combined (Denny-Brown, 1960). Consequently, this will lead to increased functional load, resulting in muscle hypertrophy (Denny-Brown, 1960). To date, two of the most used models of compensatory hypertrophy are tenotomy and synergist ablation (Timson, 1990). Both models elicit a rapid and robust hypertrophic response, but some researchers have found that data collected earlier then 10 days after intervention predominantly result from an initial period of inflammation and edema (Armstrong et al., 1979; Timson, 1990). The tenotomy model generally focuses on the days following surgery, as this is when the greatest increase in muscle wet weight is observed (Lesch et al., 1968; Mackova and Hnik, 1973; Seiden, 1976). The timescale of the tenotomy model is very limited due to rapid reattachment of the severed tendon, and it appears that the increase in muscle wet weight is partly due to edema caused by inflammation from the surgical trauma rather than true functional hypertrophy (Armstrong et al., 1979).
The synergist ablation method is a widely used hypertrophy model, and the response differs from the tenotomy model (Ianuzzo and Chen, 1979). There are two distinct phases of muscle enlargement caused by synergist ablation. The first phase is an immediate inflammatory response in the muscle due to the surgical procedure itself (Armstrong and Ianuzzo, 1977; Armstrong et al., 1979). In the second phase there is a slower response where the muscle starts to respond to the increased functional demand (Ianuzzo and Chen, 1979). The increase in muscle wet weight is larger and appears to be more consistent compared to the tenotomy model where the increase in muscle wet weight appears to be transient (Ianuzzo and Chen, 1979). Likely, this results from avoidance of muscle reattachment that would devoid the overload effect on the remaining synergist (Timson, 1990).

Armstrong et al. (1979) reported a significant increase in the rat *plantaris* muscle wet weight one hour post surgery, and 97% of this resulted from increased water content. After 24 hours, the experimental *plantaris* muscle was ~34% heavier than in the contralateral control, but water accumulation accounted for 91% of the increased muscle weight. The initial inflammatory response subsides two weeks post surgery, and true contractile hypertrophy was at this time measurable (Adams et al., 1999; Gollnick et al., 1981).

The degree of hypertrophy after synergist ablation relies substantially on the activity of the animal (Gollnick et al., 1981; Roy and Edgerton, 1995). Active animals such as mice, may display greater hypertrophy after synergist ablation than larger and less active animals (Roy and Edgerton, 1995). For such animals implementation of a post surgery activity such as running on a treadmill increases muscle hypertrophy (Chalmers et al., 1992; Roy and Edgerton, 1995). One of the earliest exercise-induced hypertrophy models in combination with synergist ablation was presented by Morpurgo (1879). In this study, attempts were made to induce hypertrophy in dogs by surgical removal of the *sartorius* muscle from the contra lateral leg of the dogs, and subsequently exposed them to a training regime over 60 days. After the intervention period the remaining *sartorius* muscle was surgically removed, and a 53%-55% increase in muscle size was demonstrated. Other researchers have employed running as a model for muscle enlargement in other animals such as rats and guinea pigs (Gordon et al., 1967; Helander, 1961; Holmes and Rasch, 1958), but they were all unable to demonstrate a significant hypertrophy. On the other hand Ishihara et al. (1998) demonstrated that endurance activity such as running on a wheel with progressive load gave a hypertrophic response in the *plantaris* muscle of rats.

1.2.2 Exercise-induced hypertrophy models

Electrical stimulation

Wong and Booth (1988) developed a model for resistance training in anesthetized animals. The right foot of the animal was strapped to a metal plate pedal with adhesive tape. A free moving pulley with weights attached to it was fastened to the same bar as the foot pedal (Figure 2).

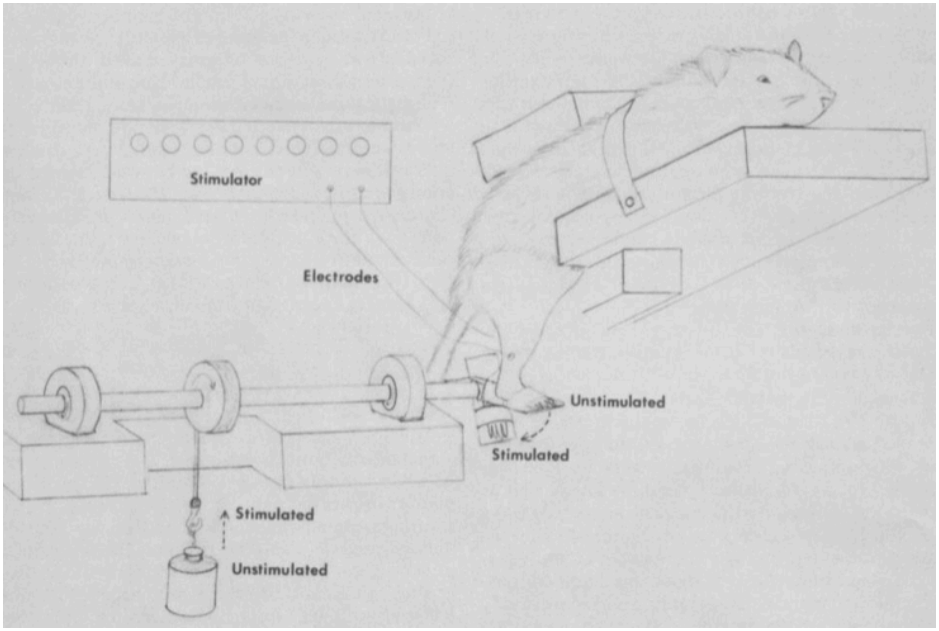


Figure 2. Electrical stimulation, Wong and Booth (1988). Anesthetized animals performing plantar flexions by sub cutaneous electrical stimulation of the plantar flexors, resulting in a muscle wet weight increase of 13-18% compared to control.

Movement of the foot due to plantar flexion resulted in lifting of the weights. The anesthetized rats were secured in a horizontal position onto a platform with the right foot fixed to the pedal. Two electrodes were subcutaneously inserted and positioned bilaterally along the surface of the plantar flexor muscles of the lower hind limb. Muscle contraction was elicited by electrical stimulus of 1 ms pulses at 100 Hz and 15 V with a 2.5 s train duration. The training regimen consisted of six repetitions, four sets over a period of 16 weeks. At the end of the experiment, the rats were able to lift a load between 600 and 1100 grams per repetition, compared to 200-800 grams at the start of the experiment. Muscle wet weight of individual weight-trained plantar flexors (*gastrocnemius* (GAS), *plantaris* (PLA), *soleus* (SOL) and *tibialis anterior* (TA) were 13-18 % greater than in the contralateral non-trained leg ($P<0.05$).

Baar and Esser (1999) demonstrated a significant increase in *extensor digitorum longus* (EDL) and TA muscle wet mass using a modified version of the Wong and Booth (1988) training model. In this experiment they surgically implanted electrodes at the sciatic nerve before its point of trifurcation to ensure contraction of all the muscles in the distal part of the hind limb. The electrodes were run subcutaneously to the base of the neck and secured for subsequent stimulations (Figure 3).

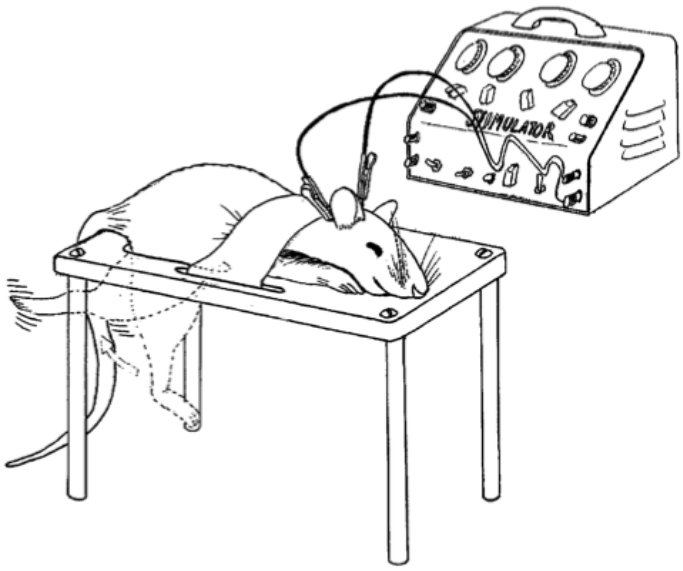


Figure 3. Electrical stimulation, Baar and Esser (1999). Anesthetized rats performing plantar flexion due to electrical stimulation of the sciatic nerve before its point of trifurcation, resulting in a 14% increase in muscle wet weight in the dorsiflexor muscles compared to control.

Another modification to the Wong and Booth (1988) model was that they utilized the fact that the plantar flexors (GAS, SOL and PLA) produce more force than the dorsiflexors (EDL and TA) resulting in a net plantar flexion of the ankle, thus a concentric contraction of the plantar flexors, and an eccentric contraction of the dorsal flexors. This experiment gave a 14% muscle wet weight increase in the EDL and the TA compared to control, but no increase in the GAS, SOL and PLA muscles.

Weight lifting models

Gonyea and Ericson (1976), used operant conditioning to exercise cats in a weight-lifting task. The cats were trained to move a bar a certain distance with their right forelimb to receive a food reward. A minimum of five days was spent conditioning the cats to complete the task prior to the initial experiment. Upon termination of the experiment, 12 muscles in the experimental forelimb and the left contra lateral control were weighed, and a 7-34% increase in muscle weight was reported.

Weight-lifting models for rats with the intention of simulating the squat-exercise in humans have been proposed by numerous researchers (Ho et al., 1980; Klitgaard, 1988; Tamaki et al., 1992; Wirth et al., 2003). In the model suggested by Klitgaard (1988), 19 months old rats were trained to perform a plantar extension in order to obtain a food reward. A weighted lever was placed around the neck of the rat to add resistance to the plantar flexion (Figure 4).

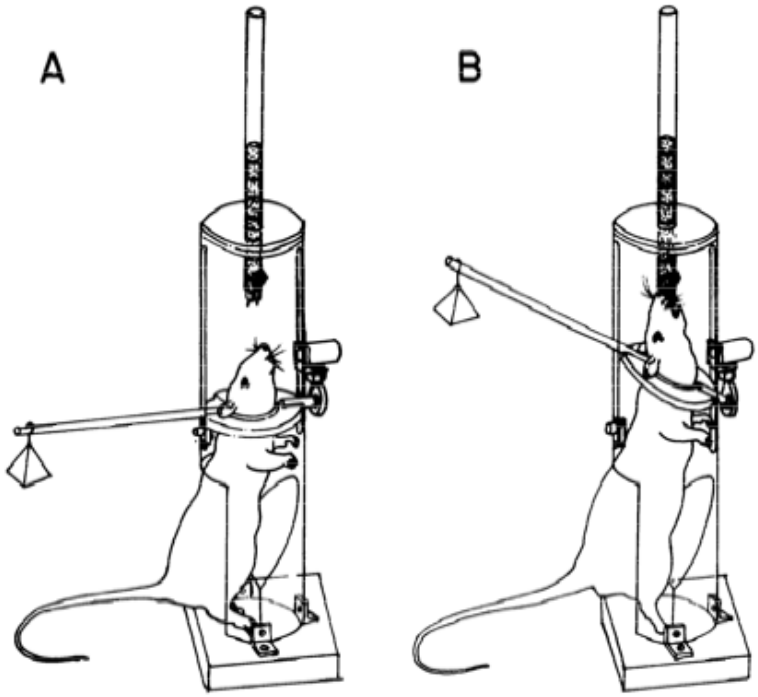


Figure 4. Squat-exercise, Klitgaard (1988). Rats lifting a weighted lever to receive food reward in a Plexiglas tube, resulting in an increse in wet weight of 33% in the *soleus* and 24% in the *plantaris* muscle.

The animals were conditioned for two weeks prior to the experiment. The first day of conditioning the rats were fastened in the Plexiglas tube for 24 hours to motivate adaption to the training model. For motivation to perform the task the animals were deprived of food the day before a training session. At the end of the 36 week experiment the wet weight of the *soleus* (corrected for bodyweight) had increased by 34% (left limb), 31% (right limb)and 24%

increase in both the left and right *plantaris* muscle compared to control animals, respectively. Also mean body weight decreased from 509 g to 418 grams during the experiment. This experiment was conducted on old rats, and by the end of the experiment they were 29 months old. The muscle weight of the 19 month old rats were not measured, therefore it is not possible to determine whether the results are en effect of actual muscle hypertrophy or an effect of exercise decreasing age-related atrophy (sarcopenia)(Klitgaard, 1988; Timson, 1990).

In another squat-exercise study, Ho et al. (1980) placed a belt-like chain with weights attached around the animals abdomen. The rats were conditioned to respond to visual light stimulus by standing upright on their hind limbs to grasp a steel bar projecting from the top of a chamber. If the rat did not respond to the light stimulus within a specified time, an electrical current was applied through the grid floor of the chamber. The rats were trained 4 days per week for 8 weeks and consisted of 16 successful lifts per day. This training regime resulted in a 21% muscle wet weight increase in the *adductor longus* muscle, but no increase in the *rectus femoris* muscle.

Tamaki et al. (1992) described a squat-training model were the torso of rats was fitted with a canvas jacket keeping them in an upright position. The canvas jacket was attached to a wooden arm holding weights (Figure 5).

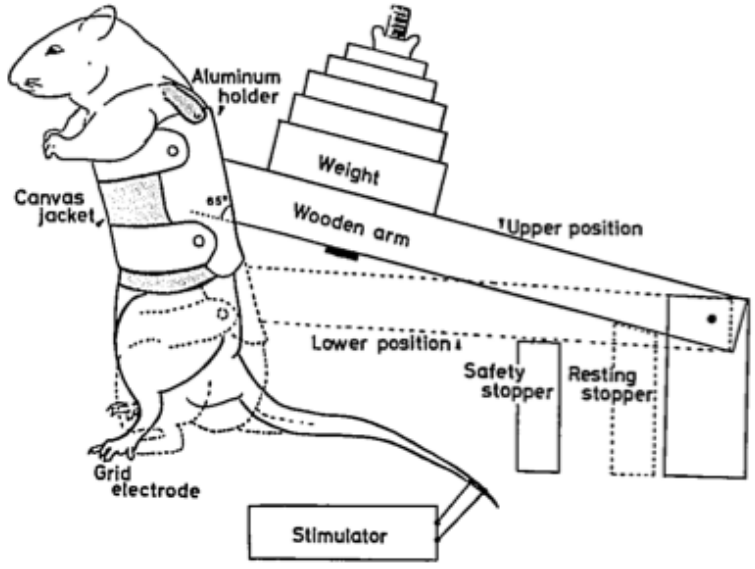


Figure 5. Squat-exercise. Tamaki et al. (1992). Rats performing hind limb extension stimulated by electric shock to the tail resulting in a muscle wet weight increase of 21% in the *adductor longus* muscle.

An electrical shock was given to the tail of the rat through a surface electrode resulting in that the rats repeatedly extended their hind limbs in a squat-like manner. The rats performed

15 sets of 15 repetitions per set, four days a week for 12 weeks, with a load of 65-75% of 1 repetition maximum (RM). The squat-training group was compared to a sprint-training group following the same training schedule. The squat-training group showed a 31.4% weight increase in the *plantaris* muscle and a 17.9% increase in the *gastrocnemius* muscle compared to sedentary controls, but the EDL and *soleus* muscles were not significantly enlarged.

Climbing models

Models with animals climbing with progressive load have been much used for studies on muscle enlargement (Duncan et al., 1998; Hornberger and Farrar, 2004; Scheffer et al., 2012; Yarasheski et al., 1990). Yarasheski et al. (1990) developed a model were rats were forced to climb 40 cm upwards, on a 90° incline for a food reward after a restricted diet aiming at investigating the effects of resistance training on skeletal muscle (Figure 6).

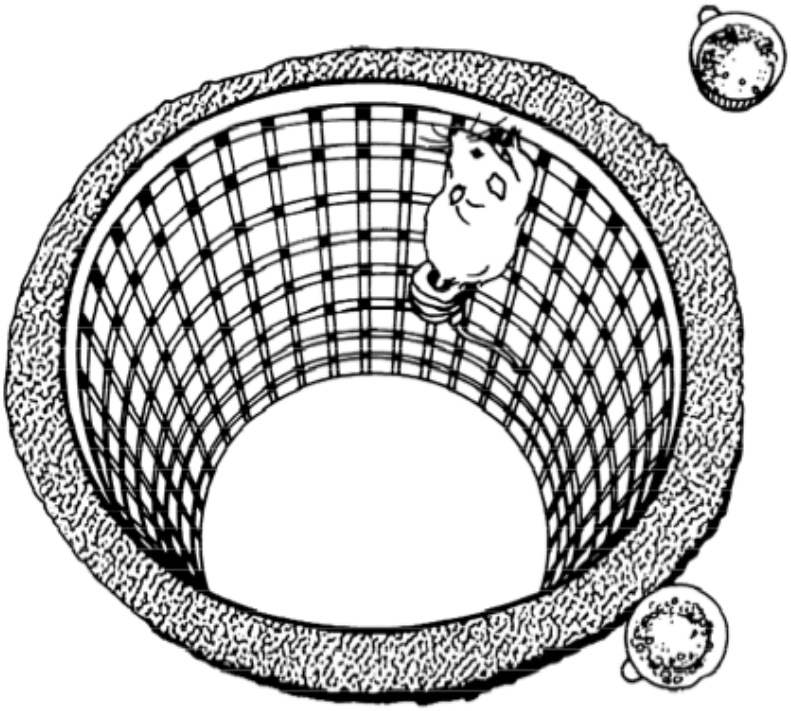


Figure 6. Climbing model, Yarasheski et al. (1990). Rats climbing with led coil attached to the tail in order to receive food reward after restricted access to food. This resulted in a muscle wet weight increase of 7.8% in the *rectus femoris* muscle, and muscle dry weight measurements displayed a 9% increase.

Led coil-weights were secured around the base of the tail of the rat, and the weight was increased by 30 g every 3rd day. The rats completed 20 successful climbs per day, five days a week for eight weeks. The *biceps brachii* and the *brachialis* muscle wet weight were measured, but there were no significant differences from the sedentary control group, however they found a small increase (7.8%) in the *rectus femoris* muscle wet weight, and

muscle dry weight measurements showed a 9% increase, respectively. Climbing is predominantly a hind limb activity, which requires the activation of both the upper end the lower muscle compartments of the rats hind limbs (Duncan et al., 1998). Therefor analyzing for limb muscles such as biceps brachii and the brachialis will not be productive when using a climbing model to study muscle hypertrophy.

Duncan et al. (1998) attempted to induce muscle hypertrophy by modifying the model developed by Yarasheski et al. (1990). Briefly, modifications involved analyzing different muscles and increasing the intervention period from 8 to 24 weeks. The rats repeated four bouts of 12-15 repetitions each, four days a week with increasing loads. The experiment resulted in a significant muscle wet weight increase in the EDL and the *soleus muscle* in the trained compared to the control ($P<0.05$). Cross sectional area measurements showed a significant increase in the EDL, the *soleus*, the *plantaris* and the *rectus femoris* muscle ($P<0.05$).

Hornberger and Farrar (2004) developed a model wherein rats were trained to climb a 1.1 meter vertical (80° incline) ladder with weights attached to their tails (Figure 7). The rats were trained every three days for a period of eight weeks, and each training session consisted of 4-9 climbs, and additional weight was added based on previous performance. They found a 23 % increase of the *flexor halluces longus* (FHL) muscle weight. Additionally, a 24% increase in both total and myofibrillar protein content in the FHL were found.

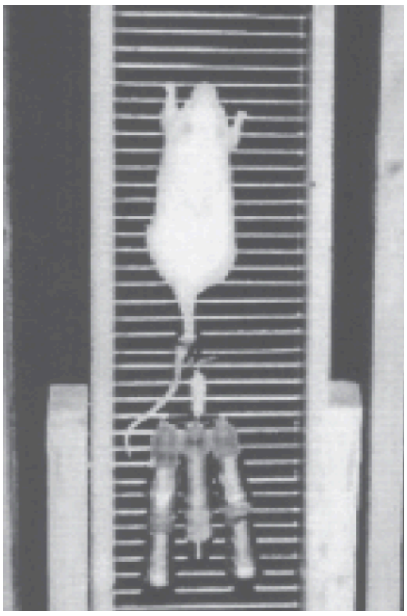


Figure 7. Climbing model, Hornberger and Farrar (2004). Rats climbing with increasing weights attached to the tail, resulting in a 23% increase in muscle weight in the *flexor hallucis longus* compared to control.

An important consideration to be made when choosing an appropriate model to study muscle hypertrophy is that the results are probably model-specific. It is important to consider what model is best fit in terms of which aspects of muscle hypertrophy the researcher is investigating (Adams and Bamman, 2012). For example, synergist ablation may be an effective model in terms of studying cellular signaling pathways leading to acute skeletal muscle adaptions (Miyazaki and Esser, 2009), but for studies on long term adaptions the researcher would be advised to choose a more physiological exercise model since the increase in muscle mass during surgical ablation seems to reach a plateau 4-5 weeks following surgery (Baldwin et al., 1977; Ianuzzo and Chen, 1979). The models highlighted in this thesis involve the use of either surgical manipulation, non-voluntary electrical stimulation under anesthesia or the utilization of positive or negative rewards to stimulate the animal to perform the exercise. Thus, when choosing an appropriate model factors such as: magnitude of muscle enlargement, degree of food restriction acquired for motivation, negative reward (i.e. pain) and time spent executing the experiment should be considered. A summary of the aforementioned models is displayed in Table 1.

Table 1. Comparison of selected previously established exercise models for rats. RF: *Rectus femoris*; AL: *Adductor longus*; SOL: *soleus*; PLA: *Plantaris*; GAS: *Gastroknemius*; TA: *Tibialis anterior*; EDL: *Extensor digitorum longus*; FHL: *Flexor hallusis longus;* n.s: Non significant. # Significant increase in CSA in EDL-fibers with low SDH-activity, (P<0.05); ## Significant increase in SOL-fibers with high SDH-activity (succinate dehydrogenase), (P<0.05); ## Significant increase in SOL-fibers with intermediate SDH-activity (P<0,01).

	Training model	**Motivational tool**	**Animal age (start of experiment)**	**Duration (weeks)**	**Muscle wet weight increase compared to control**	**Fiber CSA Increase compared to control**
Wong and Booth, 1988	Electrical stimulation	Anesthesia	-	16	GAS, 18% PLA, 18% SOL, 13% TA, 16%	-
Baar and Esser, 1999	Electrical stimulation	Anesthesia	-	6	EDL ,14% TA, 14%	-
Klitgaard, 1988	Squat-training	Food restriction	19 months	36	SOL, 33% PLA, 24%	-
Ho et al., 1980	Squat-training	Electrical shock	84 days	8	RF, n.s. AL, 21%	n.s
Tamaki et al.,1992	Squat-training	Electrical shock	4 weeks	12	PLA 31% GAS 19%	-
Yarasheski et al., 1990	Climbing	Food restriction	2 weeks	8	RF, 8%	20% in type IIb-fibers, 13% in type IIa-fibers
Duncan et al., 1998	Climbing	-	3 weeks	26	EDL, 12% SOL, 16%	EDL, 21% # SOL, 18%## SOL, 59%###
Hornberger and Farrar, 2004	Climbing	Electric shock	3 months	8	FHL, 23%	-

1.3 Aims of study

Develop a physiological strength exercise model for skeletal muscle hypertrophy in rats. Specific aims of the study were to:

- Develop a new non-invasive animal strength exercise model to induce muscle hypertrophy.
- Investigate the effect on muscle enlargement from synergist ablation compared to non-invasive continuous and voluntary training.
- Study the effect of combining synergist ablation with post surgery exercise.

2 Materials and methods

2.1 Experimental cages

Synergist ablation has been the preferred method for studying muscle hypertrophy in several research groups, including our own (Bruusgaard et al., 2010; Egner et al., 2013; Gollnick et al., 1981; Miyazaki et al., 2011). This method has been successful in mice-studies, but the results have been inconsistent in rats (unpublished results). This is likely due to the space limitations of standard rat cages in animal facilities, where the rats remain somewhat sedate during the experiments. In this study we wanted to test a new, non-invasive and voluntary exercise model. We developed a training cage were the rats had to climb in order to obtain food and drink. The training cages were made of Plexiglas (31×52×46cm), with a removable wire mesh frame (29×49×43cm). The food tray was placed in the lid of the cage (4,5×11×7,5cm), and the water bottle placed 30 cm above the cage floor. The animal facility standard polycarbonate cages (35×55×19cm) were used as control cages (Figure 8).
32 male Sprague Dawley rats were randomly distributed into 4 experimental groups, with 8 rats in each group, and 4 rats in each cage.

- Control
- Overload
- Trained
- Trained + overload

The control and overload groups were placed in standard cages, while the trained and the trained + overload groups were placed in the exercise cages (Figure 8). After one week of habituation bilateral synergist ablation surgery was preformed on the overload groups.

In order to, as subjectively as possible, monitor climbing frequency the animals were monitored by a 24 -hour infrared motion sensor web camera (D-link, DCS-2132L). Food was given *ad libitum* throughout the experiment, and food intake was measured every weekday to study any differences between the experimental groups. Also, body weight was measured for each rat once a week to monitor differences between individuals during the 31-day experiment.

A)

B)

Figure 8. Exercise cage and standard cage. A) Picture illustrates the execise cage displayed to the left, and the standard control cage is displayed to the right. **B)** Exercise cage picture illustrating a rat from the trained group climbing.

2.2 Animal experiments

2.2.1 Animals

All animal experiments were performed at the Department of Biosciences (IBV) at the University of Oslo, Norway, with approval from The Norwegian Animal Research Committee (FDU). Male Sprague Dawley-rats (200-400 g) were used, and the treatment and housing of the animals were in accordance with the criteria set by EU and FDU. All experiments were carried out by personnel certified to perform experiments on live animals (FELASA, class C). The rats were housed on a reversed 12 hour light/dark cycle. Light intensity in light phase was >100 lux. The temperature in the housing quarter was kept at 21±1°C, with a humidity level of 55±10%, and a ventilation rate of 5-20 times per hour.

2.2.2 Surgical procedures

Prior to all invasive experiments the rats were anaesthetized with Isoflurane (Baxter, Oslo, Norway) mixed with regular air at a flow of around 5-6 L/min. Anesthesia was induced by placing the rat in an induction chamber with 5% isoflurane. To confirm deep anesthesia the *metatarsus* region was pinched to ensure absence of retraction reflex. The rats were then transferred to a mask with isoflurane concentration between 2.5-3%. Changes in respiration frequency and frequent pinching of the metatarsus region were used to regulate anesthesia dosage.

The animals received subcutaneous injections of Temgesic (buprenorfin, 0,04 mg/kg) for analgesia post surgery.

2.2.3 Synergist ablation

The anaesthetized animals were placed on a heated platform to ensure stable body temperature throughout surgery. The rat hind limb was then shaved, and commercially available hair removal cream was applied (Boots), and then washed with 70% ethanol. Finally, the leg was fixed to a surgical platform. Subsequently, an incision was made lateral to the *tibia* to surgically expose the *tibialis anterior* (TA) muscle. The *tibialis* tendon was identified, and cut. Approximately 75% of the TA was removed to overload the synergist muscle, EDL (Figure 9). During surgery, the open wound was continuously kept wet by applying ringer-acetate solution (131323, B. Broun Petzold). Upon termination of surgery, the incision was then closed with sutures (Softsilk, S-1172, 12 mm, Syneture), and analgesia (Temgesic) was administered prior to awakening to reduce post-operative pain.

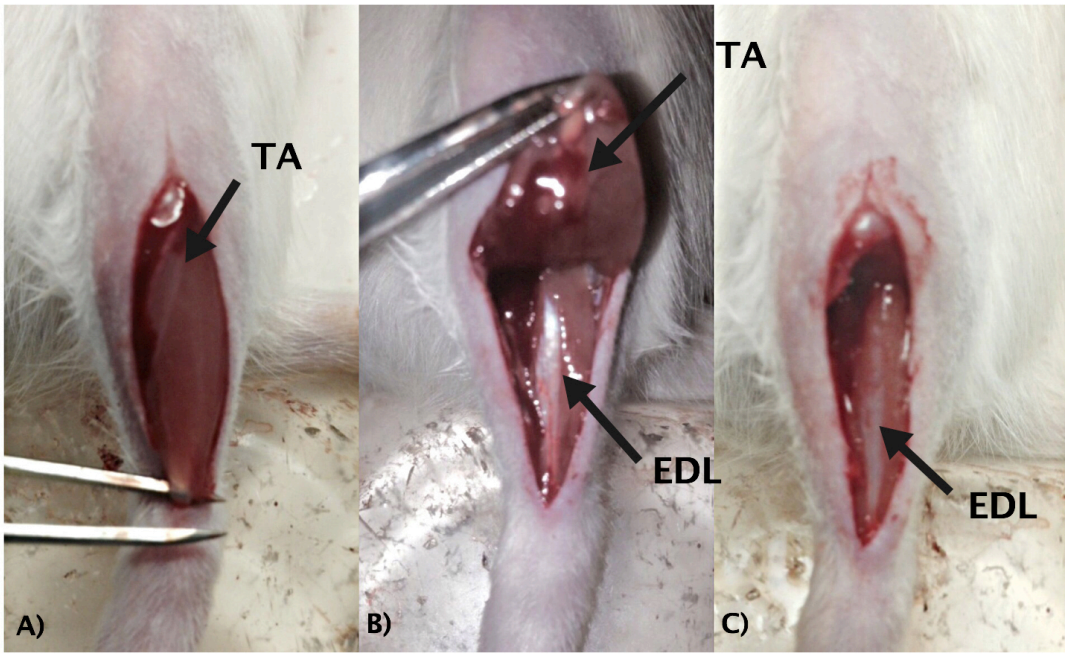


Figure 9. Synergist ablation procedure. A) Identification of the distal TA tendon. **B)** The distal tendon is then cut and 2/3 of the TA is then removed to induce overload of the EDL muscle. **C)** The EDL muscle after removal of the TA.

2.3 Histology

2.3.1 Removal of muscles

31 days after surgical ablation the animals were placed under anesthesia and the remaining synergist EDL and the TA were removed for downstream analysis. In the overload and trained + overload groups, only the EDL was removed due to previous removal of the TA, while in the control and trained groups both the TA and the EDL muscle were removed for analysis. In order to investigate a correlation between muscle wet weight and Increase in muscle fiber size, the muscles were weighed immediately after removal, and frozen.

2.3.2 Snap-freezing of muscles

To ensure rapid freezing, the muscle was slightly stretched and pinned up on a platform as close to the *in situ* muscle length as possible. The muscle was subsequently dropped in a beaker containing 2-Methylbutane (Sigma-Aldrich) cooled to -160°C immerged in liquid nitrogen. The muscles were then transferred to cryotubes (microtube 2ml, PP, Sarstedt), and stored at -80°C until cryosectioning.

2.3.3 Cryosectioning

The muscles were sectioned into 10 μm thick cross sections using a cryostat (CM1950, Leica Biosystems). During sectioning each sample held a temperature of -21°C, and serial sections were placed on superfrost plus glass slides (Thermo Fisher Scientific, Oslo, Norway). Sections were allowed to dry at room temperature before storage at -80°C until subsequent immunohistochemistry procedures.

2.3.4 Staining for laminin

Laminin is a ubiquitously expressed non-collagenous glycoprotein in connective tissues, a major constituent of the basement membrane of muscle fibers. Muscle sections were stained using a polyclonal primary antibody against laminin (L9393, Sigma). Secondary antibody conjugated to FITC fluorochrome (F9887, Sigma) was used to visualize binding of the primary antibody to the basement membrane of the muscle fibers. Illuminating FITC with blue light (488nm), allowed for visualization using a fluorescent microscope (Figure 10).

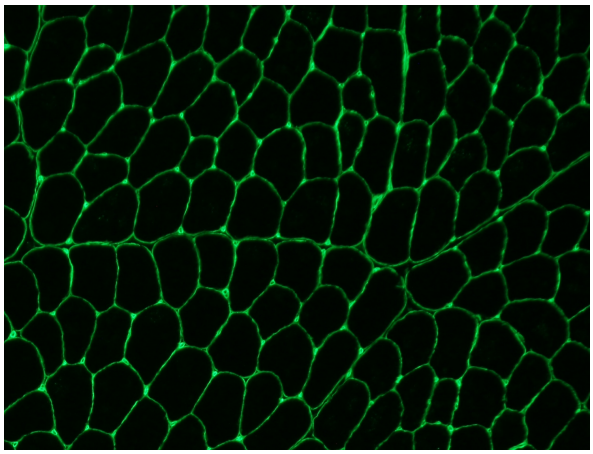


Figure 10. *Tibialis anterior* cross section micrograph of cryo section stained with antibody against laminin (green).

2.3.5 Fluorescent microscopy

Sections stained with fluorophore-conjugated secondary antibodies were photographed in a dark room using a single-lens camera (Canon 60D) connected to a microscope (BX50W1, Olympus). The sections were photographed with a water immersion lense at 10X magnification.

2.3.6 Measurements of cross sectional area

The image-files were blinded by assigning random file names to prevent bias, and then imported to Photoshop CS6 (Adobe Systems, San Jose, CA, USA) for processing. Systematic uniform random selection of muscle fibres was done, and the selected muscle fibres were then measured by tracing the cell membrane (figure 8), and calculating a pixel value. Conversion to μm^2 was performed on the basis of a micrometer-scale image using the same microscope settings.

2.4 Statistical procedures

For statistical comparison of food intake and body weight measurements, a Friedman test with a Dunn´s multiple comparison post test was performed. For statistical comparison of the EDL cross sectional area measurements and muscle weight, a Kruskal-Wallis one-way analyses with a Dunn´s multiple comparison post test was used. For statistical comparison of the TA cross sectional area measurement and muscle weight, a Mann-whitney test was done. The significance level was set to 0.05.

3 Results

3.1 Food intake and Web camera monitoring

Web camera monitoring of the animals showed a high climbing frequency in the training cages during the dark cycle (awake cycle of the animals). The web camera had a motion sensor that was activated by movement in the cages. We observed that the animals had a high climbing frequency even when they were not obtaining food or drink, and seemed to be positively affected by the environment of the exercise cages. The mean food intake in each group was measured every week-day during the experiment, and subsequent analyses showed that when comparing the mean food intake for all the groups during the 4 weeks of the experiment, there were no significant differences between the groups. However, when comparing each group per week the results show that in week 2, post surgery, the overload group had a 6% higher food intake than the control group (P<0,05; Figure 11).

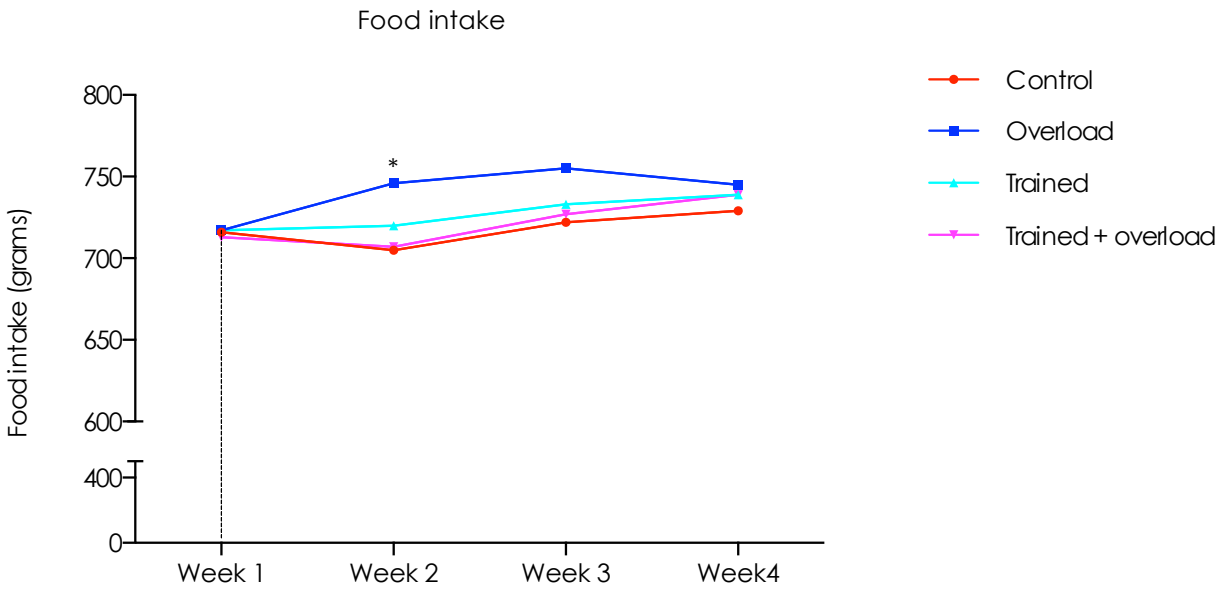


Figure 11. Food intake measurements. The stippled line marks the day of surgical intervention. Each dot represents the collected food intake of each group during one week. * Statistically different from control group (P<0.05).

3.2 Body weight

Body weight was measured at the end of each week to monitor weight gain in individual rats within, as well as between the experimental groups. Body weight analysis show that all the experimental groups had a significant body weight increase throughout the experiment ($P<0.01$). Furthermore, there were no significant differences when comparing the control to the overload group, or when comparing the trained to the trained + overload group, respectively. However, The control and the overload group had a higher mean body weight throughout the experiment compared to the trained and the trained + overload group ($P<0.05$; Figure 12). Measurements of individual body weight showed that all the rats had the same weight gain as the control group throughout the experiment (data not shown).

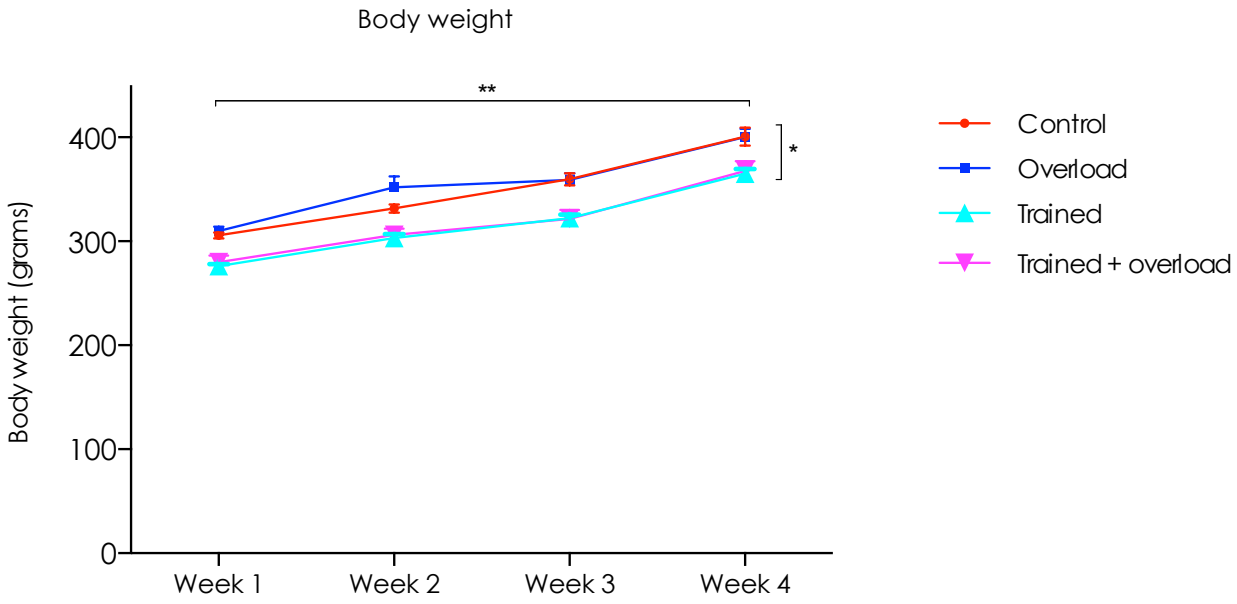
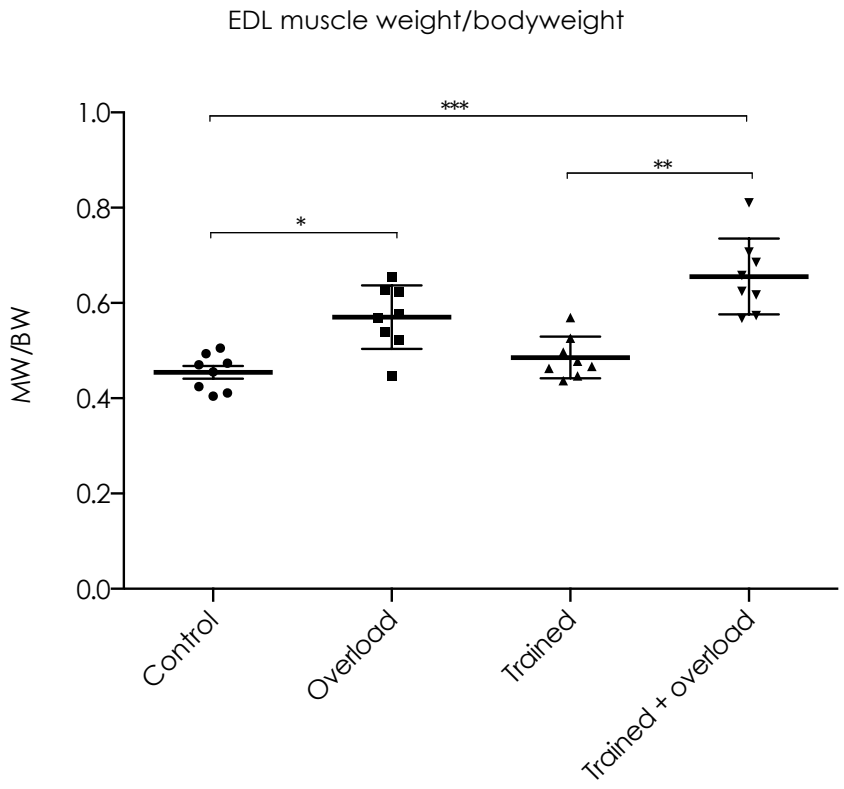


Figure 12. Body weight measurements. Each dot represents the mean body weight of all the rats in each group (n= 8) at four time points during the experiment. Mean with SEM is shown. ** Statistically significant weight increase in all groups during the experiment ($P<0.01$). * Control and trained group statistically different from overload and trained + overload group ($P<0.05$).

3.3 Muscle weight

Muscle wet weight was measured, and the results showed an increase in muscle weight in both overload groups compared to the control and the trained group. There was a 25% increase in muscle weight comparing the control group to the overload group ($P<0.05$) Comparing the control and the trained + overload we saw a 44% increase ($P<0.001$), and the trained + overload group had a 35% increase in muscle wet weight compared to the trained group ($P<0.01$). When comparing the control to the trained group, overload compared to the trained group, as well as the overload compared to trained + overload, no statistical differences in muscle wet weight were observed (Figure 13; Table 1). The muscle wet weight measurements of the TA showed a 13% increase in the trained group compared to the control group ($P<0.05$; Figure 13; Table 2).

A)



B)

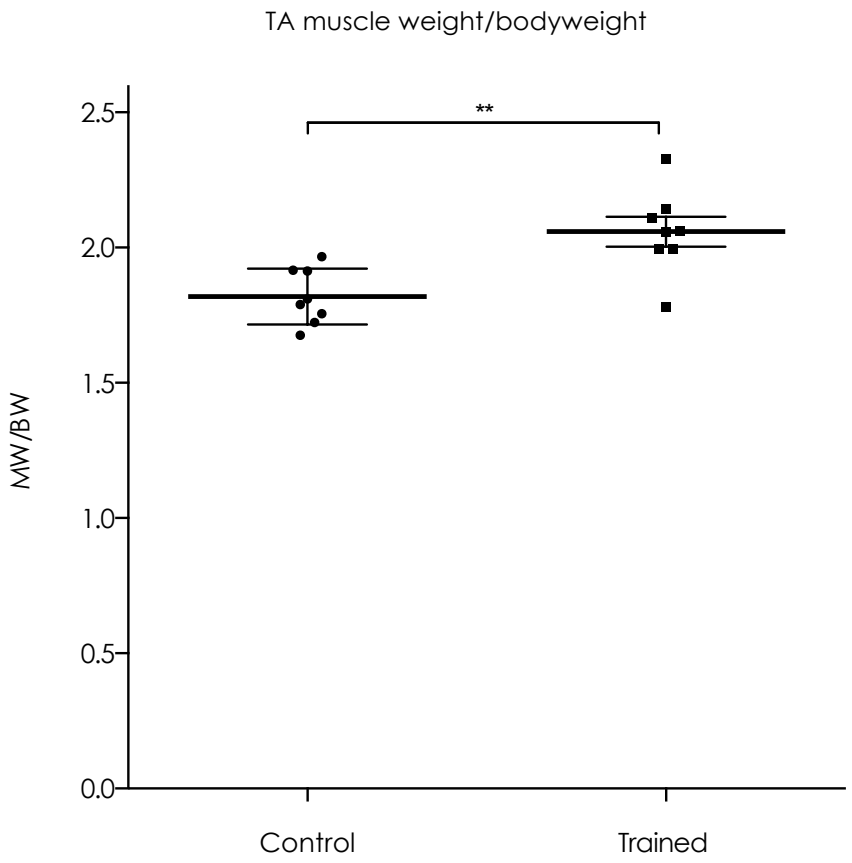
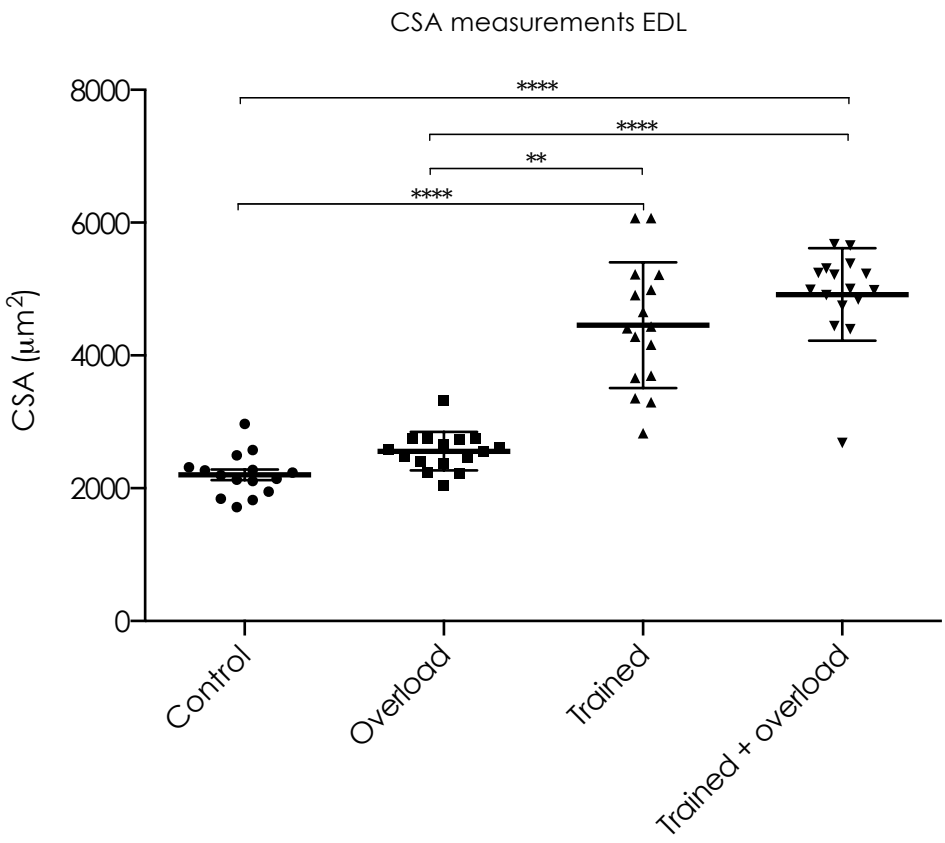


Figure 13. Effect of training cage on muscle wet weight in the EDL and TA. A) Each dot represents the weight of merged left and right EDL muscle corrected for bodyweight (n=8). Mean with SEM is shown. * significantl difference between control and overload group (P<0.05). ** Significant difference between Trained and trained + overload (P<=0.01). *** Significant difference between control and trained + overload (P<0,001). **B)** Each dot represents the weight of the merged left and right TA muscle corrected for bodyweight (n=8). Mean with SEM is shown. **Statistical differences between groups (p<0.01). For additional information see Table 2.

3.4 Measurement of cross sectional area

CSA measurements of the EDL muscle showed that the trained group had a 102% increase in muscle fiber CSA when compared to the control group (P<0.0001). Comparing the trained + overload group to the control group there was a 123% increase in CSA (P<0.0001). Also, there was a 92% increase between the control overload group and the trained overload group (P<0.001) Finally, when comparing the trained control group to the control overload group, we saw an increase of 74% in the trained control group (P<0.01; Figure 14; Figure 15; Table 2). CSA measurements of the TA muscle also showed a 97% increase in the trained group compared to control (P<0.001; Figure 14; Figure 16; Table 2).

A)



B)

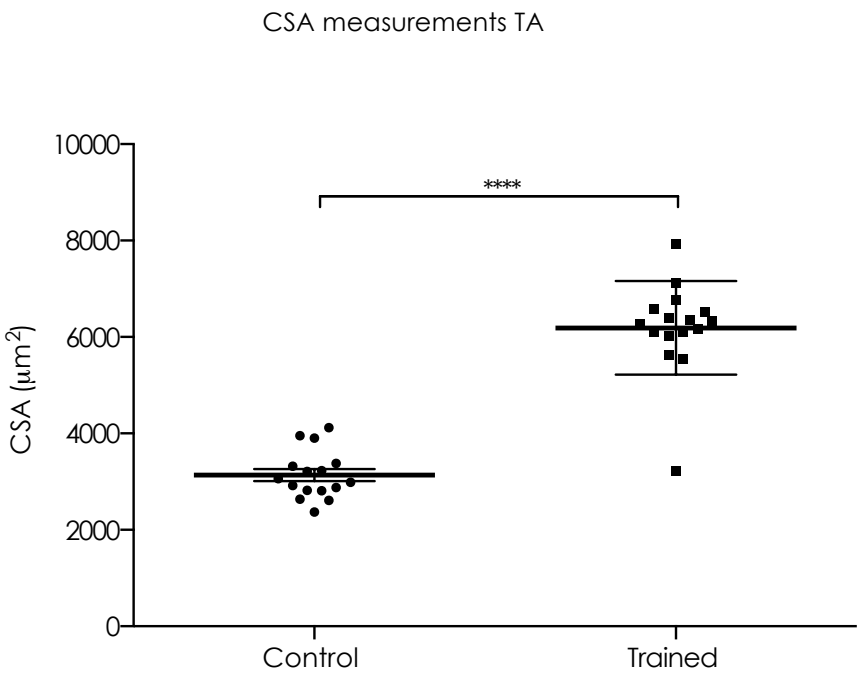


Figure 14. **Figure shows cross sectional area measurements (CSA) of the EDL and the TA**. **A)** Each dot represents one EDL muscle in each group (n=16). Each muscle CSA mean is calculated from n=50 fiber measurements. Mean with SEM is shown. ****Statistical differences between groups (P<0.0001). ** Statistical difference between overload and trained group. (P<0.01). **B)** Each dot represents one TA muscle in each group (n=16). Each muscle CSA mean is calculated from n=50 fiber measurements. Mean with SEM is shown. **** Statistical differences between groups (P<0.0001). For additional information see Figure 15, 16 and Table 2.

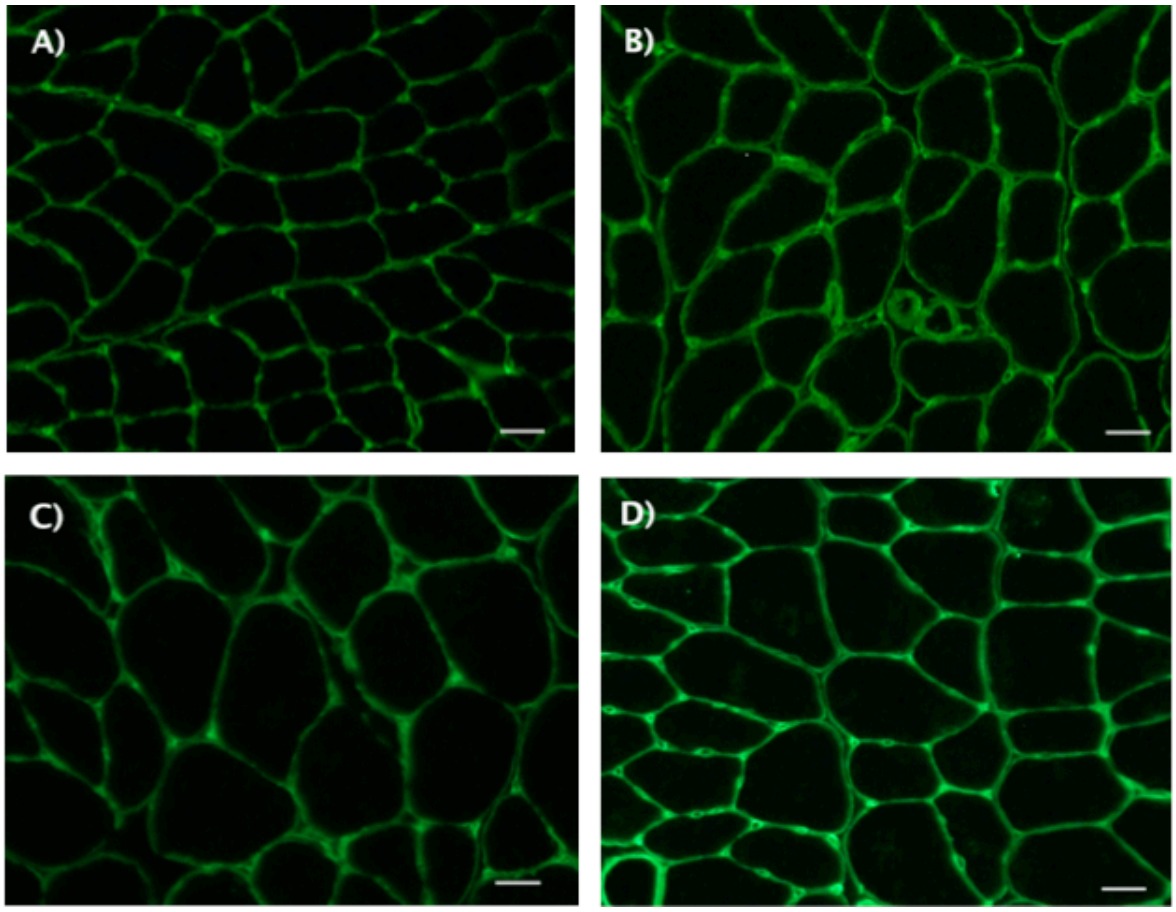


Figure 15. Representative micrographs of EDL cross section stained with antibodies against laminin (green). A) Control. **B)** Overload. **C)** Trained. **D)** Trained + overload. Scale bar = 30μm

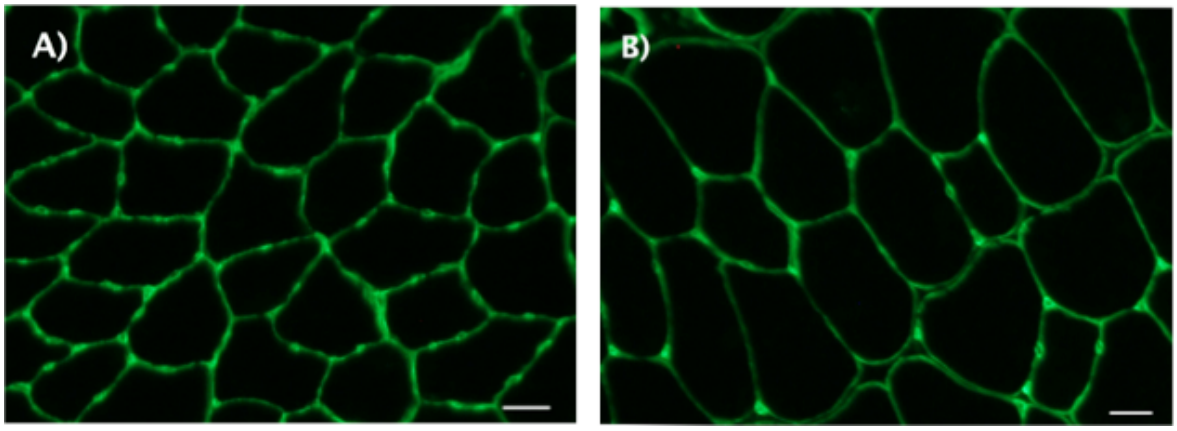


Figure 16. Representative micrographs of TA cross section stained with antibodies against laminin (green).A) Control. **B)** Trained. Scale bar = 30μm.

Table 2. Values from statistical analysis of muscle wet weight/bodyweight and CSA measurements (in μm^2).

	Control	Overload	Trained	Trained + overload
EDL muscle wet weight/body weight	n=8	n=8	n=8	n=8
Mean	0.455	0.5701	0.4858	0.6555
SEM	0.01333	0.02360	0.01557	0.02815
TA muscle wet weight/body weight	n=8	n=8	n=8	n=8
Mean	1.819		2.059	
SEM	0.03662		0.05480	
EDL CSA	n=15	n=16	n=16	n=16
Mean	2203	2559	4454	4919
SEM	82.53	73.20	236.9	174.8
TA CSA	n=16	n=16	n=16	n=16
Mean	3138		6191	
SEM	125.7		242.0	

4 Discussion

4.1 Our exercise cage method induces hypertrophy that surpasses previous strength exercise models.

The hypertrophic response to our exercise cages exceeds that of previously described strength exercise models (Baar and Esser, 1999; Duncan et al., 1998; Ho et al., 1980; Hornberger and Farrar, 2004; Klitgaard, 1988; Tamaki et al., 1992; Wong and Booth, 1988; Yarasheski et al., 1990) with a doubling of CSA. Previous models have been ranging from13-59 %. In models similar to ours, Yarasheski et al. (1990) obtained a maximum increase of 20% and Duncan et al. (1998) a maximum increase of 59% in CSA. A reason for this discrepancy could be that in their model the rats climbed only 20-50 times a day four or five times a week, whereas in our experiment the rats climbed regularly and at their own volition, seven days a week. Yarasheski et al. (1990) also used food restriction as a motivational tool, while in our model the rats had free access. Our results also show that adding the aspect of exercise to surgical intervention gave a much greater hypertrophy effect than synergist ablation alone. Furthermore, there is apparently no need for surgical intervention as the trained group showed a 74% CSA increase in the EDL, and a 97% increase in the TA muscle compared to the control group. This indicates that the training cage may replace synergist ablation, and function as a new and more physiological exercise model when studying muscle hypertrophy in rat skeletal muscle.

Muscle wet weight measurements did not reflect the CSA measurements. Although muscle wet weight as a measure of hypertrophy has been used by many researchers (Baar and Esser, 1999; Duncan et al., 1998; Ho et al., 1980; Hornberger and Farrar, 2004; Klitgaard, 1988; Tamaki et al., 1992; Wong and Booth, 1988; Yarasheski et al., 1990), the accuracy of this measurement it is questionable. For example, when comparing groups that has underwent surgical intervention to control groups one has to take in to account the possibility that edema and inflammation is contributing to the weight increase (Armstrong et al., 1979; Snow, 1990). Furthermore when comparing trained animals to sedate control groups one should contemplate the idea that sedate animals might have a higher muscle fat content than active animals. Another consideration to be made is that the EDL muscle is a small muscle and the amount of blood, connective tissue, and how much of the tendon is dissected out will have a larger impact on the differences in weight, compared to the TA, which is a significantly larger muscle. Measuring muscle dry weight to correct for possible edema and inflammation is likely to give more accurate results in regards to hypertrophy, however, in this study we were not able to measure dry weight due to the subsequent histological analysis.

4.2 The method is simpler than most established models.

Our exercise cage is a relatively simple model, and minimal initial conditioning was needed as the exercise cages were used as standard housing, and the animals appeared to adapt to the new environment within hours of transfer from the standard animal facility cages. Furthermore, there was no need for time consuming training sessions throughout the experiment, as the animals were climbing voluntarily. Most of the previously described exercise models (Baar and Esser, 1999; Ho et al., 1980; Hornberger and Farrar, 2004; Klitgaard, 1988; Tamaki et al., 1992; Wong and Booth, 1988; Yarasheski et al., 1990) are labor intensive for the researcher, and can go on for months, with the hypertrophy response being minimal compared to the response obtained with our exercise cage.

4.3 The method resembles natural exercise

Deprivation of food as motivation for exercise as used by Klitgaard (1988) and Yarasheski et al. (1990) could lead to caloric restriction and a lack of motivation to perform with maximum effort. This can interfere with results and might not be comparable to natural exercise conditions (Timson, 1990). Further, Li and Goldberg (1976) showed a decrease in protein synthesis and RNA content after one day, and an increase in protein catabolism after two days in food deprived rats. Although the animals in our experiment had to climb to obtain food, climbing activity was not restricted to feeding, and web camera monitoring of the animals revealed a high climbing frequency in the training cages during the dark cycle (awake cycle of the animals). The animals were climbing continuously even when they were not obtaining food or drink, and seemed to be positively affected by the environment of the exercise cages. Moreover, all the groups had approximately the same overall food intake, which indicates that despite having to climb to reach their food, the rats in the training cages displayed no reduction in caloric-intake. However there was a significant, but transient increase in food intake in the synergist ablated animals the week after surgery compared to the control and the trained group which might be due to the need for additional nutrition post surgery. The animals had a mean food intake of approximately 30-35 grams a day, which is higher than the recommended intake of 5g/100g for laboratory rats (Krinke, 2000). Weekly weighing showed that all the individual animals had a normal weight gain compared to control throughout the experiment. The animals in the training groups had a lower mean weight, but this is likely due to their increased activity level, which leads to a higher muscle to fat ratio (Slentz et al., 2004). The trained animals activity level suggest that they would have a

significantly higher metabolism, and observations during surgery suggested that the untrained rats had a much higher level of subcutaneous and visceral fat.

Furthermore there was no need for the use of negative motivators such as electrical shock or restraint (Ho et al., 1980; Hornberger and Farrar, 2004; Tamaki et al., 1992) to force the animal to perform the exercise. Also, strapping weights to the rats tail as done by (Duncan et al., 1998; Hornberger and Farrar, 2004; Yarasheski et al., 1990) may cause degloving injury, and is most likely painful for the rats. These factors may cause systemic stress including hormonal responses that could interfere with, or alter the extent of muscle hypertrophy (Alway et al., 2005). Our exercise cages allows for natural, continuous and voluntary exercise as the animals were allowed to move freely and did not necessitate exposure to any major stress factors such as food restriction, electrical shock or subjecting the animals to the unknown effects of repeated anesthesia during the experiment (Baar and Esser, 1999; Duncan et al., 1998; Ho et al., 1980; Hornberger and Farrar, 2004; Klitgaard, 1988; Tamaki et al., 1992; Wong and Booth, 1988; Yarasheski et al., 1990). Prolonged enhanced levels of stress-induced steroid hormones such as cortisol (corticosterone in rats) have a catabolic effect on skeletal muscle (Hasselgren, 1999), and will decrease protein synthesis, and increase protein breakdown (Hanaoka et al., 2012). Therefor, high stress levels will most likely interfere with the results, and should be minimized to obtain credible outcomes.

4.4 Future directions and applications

Analyses of the rats behavior by using data from the web camera monitoring should be quantified. We are also planning to perform fiber type analyses, to look at alterations in fiber type distribution as a consequence of the cage exercise. Further, assessing stress levels by measuring the cortisol levels in the trained rats and comparing them to the surgically overloaded rats to further validate our model as a stress-free and natural exercise model would be favorable.

Our group is currently working on a new theory of muscle memory (Egner et al., 2013). So far these studies have been performed under artificial conditions such as administration of high doses of testosterone in female animals and overload with surgical ablation. Thus, it is important to test the concept of muscle memory under more physiological conditions that can be related to natural exercise such as the exercise cage described here. With this method, repeated cycles of training, detraining and retraining could be performed under physiological conditions, further validating the muscle memory mechanism.

Finally, Our exercise model could be used in molecular studies since most studies done on molecular adaptions to increased load, is done by using unphysiological models such as

surgical overload. These models might trigger factors such as stress, inflammation and edema, which could alter the results and give an inaccurate representation of underlying molecular mechanisms during skeletal muscle hypertrophy.

4.5 Conclusions

- We have established a new physiological strength exercise model, which is efficient in inducing skeletal muscle hypertrophy.
- Our Exercise cage model elicits a more pronounced effect on muscle hypertrophy than synergist ablation surgery alone.
- Adding the aspect of exercise to synergist ablation has an additional effect on skeletal muscle hypertrophy.

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6 Appendix

6.1 List of abbreviations

ALD	Anterior lattisimus dorsi
Akt	Protein kinase B
CSA	Cross sectional area
EDL	Extensor digitorum longus
eIF4E	Eucaryotic translation initiation factor 4E
FHL	Flexor halluces longus
IGF-1	Insulin growth factor 1
GAS	Gastroknemius
mTOR	Mammalian target of rapamycin
PI3K	Phospoinositide 3-kinase
PLA	Plantaris
p70S6K	70-kDA ribosomal protein S6 kinase
RF	Rectus femoris
SDH	Succinate dehydrogenase
SOL	Soleus
TA	Tibialis anterior
4E-BP1	4E-binding protein 1

6.2 Histochemistry

6.2.1 10X PBS (phosphate buffered saline) solution

Solution:	Amount:
NaCl	80.0 g
KCl	2.0 g
$Na_2HPO_4 \times 2H_2O$	14.4 g
KH_4PO_4	2.0 g

- Dissolve salts in 800 ml of dH_2O
- Adjust volume to 1.1 l, and then adjust pH to 6.8

6.2.2 Staining for laminin

- Remove sections from -80° freezer and let the sections thaw for 30 min before removing the sorrounding foil.
- Apply anti laminin primary antibody produced in rabbit (L-9393, Sigma) in a 1:100 dilution in 1% BSA in PBS. Incubate at 4°C overnight.
- Wash sections 3 × 5 min in 1× PBS (pH 7.2)
- Apply anti-rabbit IgG FITC-conjugated secondary antibody (F9887, Sigma) on primary antibodies in a 1:200 dilution in 1% BSA in PBS. Incubate in room temperature for 1 hour in a dark moist chamber
- Wash sections 3 × 5 min in 1× PBS (pH 7.2)

		Consentrations	Incubation
Primary antibody	L9393	1:100	Overnight, 4°C
Secondary antibody	F9887	1:200	1 hour RT