Chapter 1

1.1) Introduction

Skeletal muscle comprises about 40-50% of total body weight in humans and rodents (Adibi, 1976). It is of obvious importance for locomotion and is one of the most metabolically active tissues in the body. It is composed of a number of cylindrical muscle fibres, which are bound together by connective tissue and are linked to bones by tendons, located at each end of the muscle. The muscle fibres are further subdivided into thick and thin filaments known as myofibrils. The thick filaments (myosin) and thin filaments (actin) are arranged in a repeating pattern along the length of the myofibril, and each unit of this repeating pattern is known as a sarcomere. Myosin and actin's primary functions are contraction of the muscle fibre, which is required for movement of limbs.

However, during conditions of stress, skeletal muscle, and in particular the actin and myosin molecules, are the primary targets for catabolism, to provide amino acids for fuel production (Tawa *et al.*, 1994).

Also, proteins in skeletal muscle undergo a fairly continuous process of synthesis and degradation which regulates both the overall muscle protein mass and the levels of specific proteins within the muscle (Kettelhut *et al.*, 1988). The overall rates of synthesis and degradation must be balanced because even a small decrease in synthesis or increase in degradation can cause a marked loss in body mass (Tawa *et al.*, 1994). Accelerated proteolysis is the predominant mechanism inducing muscle loss. Increased protein breakdown results in release of amino acids from muscle tissue (Tawa *et al.*, 1994). A large portion of these amino acids are used by the liver for acute phase protein synthesis and gluconeogenesis (Tawa *et al.*, 1994). Other amino acids and in particular glutamine are taken up by enterocytes and cells of the immune system and serve as an important source of energy for these cells (Tawa *et al.*, 1994). Thus, in the early phase of muscle wasting the catabolic response of skeletal muscle may be

beneficial to the organism. However, severe or continuous muscle protein breakdown may lead to muscle wasting, weakness and reduced ambulation. The loss of body mass associated with skeletal muscle wasting may result from a wide variety of pathological conditions such as HIV/AIDS (Spence *et al.*, 1990), sepsis (Voisin *et al.*, 1996), uncontrolled diabetes (Pepato *et al.*, 1996), acidosis (e.g. accompanying chronic renal failure, Mitch *et al.*, 1994), burn injury (Chai *et al.*, 2002), surgical trauma (Mansoor *et al.*, 1996) starvation (Kee *et al.*, 2003), as well as from skeletal muscle disuse (Kandarian *et al.*, 2002). Severe muscle wasting can also result in increased risk for pulmonary implications if respiratory muscles are affected (Argiles *et al.*, 1996).

Atrophy of skeletal muscle may lead to longer hospitalisation and recovery times following injury (Mansoor *et al.*, 1998), illness or surgery. Thus skeletal muscle atrophy negatively affects health care costs and the quality of life, which necessitates the development of procedures to reduce muscle atrophy and/or improve recovery of muscle mass. A number of procedures (as discussed later in section 1.6) have been successful in reducing skeletal muscle atrophy. However, most of the pharmaceutical treatments are expensive. The need therefore exists, especially in a country such as South Africa, to develop cost effective treatment regimens to counteract the negative complications associated with skeletal muscle atrophy.

Inducing atrophy in human subjects is generally not feasible, thus researchers studying atrophy are mostly using rodent models, because skeletal muscle is not hugely different between larger mammals and rodents. Most of the literature review will focus on rodent models. These models are mostly used to mimic real atrophy conditions in humans and to study possible mechanisms of atrophy or recovery methods, which can potentially be used to develop future treatment regimens.

1.2) Role-players in skeletal muscle atrophy

In the next few paragraphs I will review several different role players involved in skeletal muscle wasting. These involve methods for the breakdown of structurally intact muscle proteins released large proteins and smaller peptides.

The lysosomal (cathepsins), calcium dependant (calpains), and the adenosine triphospahte (ATP)-ubiquitin-proteasome pathways are the three known proteolytic systems responsible for the breakdown of the bulk of skeletal muscle protein (Attaix *et al*., 1998). It seems as if these systems work in conjunction with one another to degrade muscle. It is known that the ubiquitin-proteasome system cannot degrade intact myofibrillar proteins (Solomon *et al.*, 1998). Thus degradation of myofibrillar and other sarcomeric proteins by the ubiquitin-proteasome pathway is dependent on their release from the sarcomere. This is accomplished by the calcium-dependant proteolytic system, whereas the lysosomal proteolytic pathway degrades proteins which cannot be recognised by the ATP-ubiquitin-proteasome pathway. Each of these mechanisms will now be discussed in more detail, with particular reference to studies performed in rodent models.

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1.2.1) Calcium dependant degradation

Calcium dependant proteases seem to initiate skeletal muscle degradation by cleaving proteins important in linking components of the sarcomere, such as titin, which anchors myofillaments to the z-disks and maintains sarcomeric proteins' alignment within each individual sarcomere (Huang *et al.*, 1998). The 2 enzymes calpain I and II are responsible for this cleaving process, which disassembles the sarcomere and releases myofibrillar proteins such as myosin and actin, which are degraded by the ubiquitin-proteasome pathway. The calpains are unable to degrade the myofibrillar proteins released. It has been shown that inhibition of the calcium-dependant proteases is able to prevent proteolysis associated with 3 days of hindlimb unloading (Tischler *et al.*, 1990), but is unable to do so after 9 days (Taillandier *et al.*, 1996). A possible reason for the lack of effect of the

inhibitors after 9 days of hindlimb unloading may be that the calcium dependant proteases already had their effect (disassembly of the sarcomere). Therefore inhibition of the calpains would have little effect on muscle proteolysis because the myofibrillar proteins would already have been released to be degraded by the ubiquitin-proteasome pathway. Similar findings have been reported in sepsis-induced atrophy models (Fischer *et al.*, 2001, Hotchkiss *et al.*, 1994). It should be noted that these four studies all used different inhibitors, except for Fischer and Hotchkiss who both used dantrolene. However, the type of inhibitor used in these studies should not make a difference to the overall outcome of proteolysis, because all these inhibitors completely inhibit the calpains (Tischler *et al.*, 1990, Hotchkiss *et al.*, 1994, Taillandier *et al.*, 1996, Fischer *et al.*, 2001).

1.2.2) Lysosomal degradation

The lysosomal enzymes do not seem to degrade myofibrillar proteins. Rather their major role is to degrade endocytosed (Kominami *et al.*, 1987) and membrane proteins, which include receptors, ligands, channels and transporters (Cuervo and Dice, 1998). Cathepsins L, B, D and H are the major lysosomal enzymes involved in proteolysis (Bando *et al.*, 1986, Kominami *et al.*, 1985).

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Although these systems seem to work together and cathepsins and calpains are activated in most catabolic conditions (Taillandier *et al*., 1996, Baracos *et al*., 1995), skeletal muscle protein degradation is largely accomplished by the ubiquitin-proteasome pathway.

1.2.3) Ubiquitin-Proteasome degradation pathway

To fully understand the role of the ubiquitin-proteasome degradation pathway in atrophy it is necessary to understand the complexity of the pathway's subunits. The proteasome complex is located in the cytosol and proteins degraded in this process are first marked for degradation by covalent linkage to the small co-factor, ubiquitin, which is itself a protein. There are two main steps in the proteasome pathway: (i) covalent attachment of the polyubiquitin chain to the

protein substrate (Argiles *et al.*, 1996; Taillandier *et al.*, 2004) and (ii) recognition and degradation of the ubiquitin-tagged proteins by the 26s proteasome (see Fig. 1.1 below for diagrammatic illustration). Next I will briefly describe the processes of ubiquitination and protein degradation.

Ubiquitination

For degradation to occur the substrate must be tagged by at least four ubiquitin molecules. Thus only polyubiquitinated proteins are degraded. Ubiquitination of protein substrates is regulated by at least three different enzymes: ubiquitin activating enzyme (E1), ubiquitin conjugating enzyme (E2) and ubiquitin ligase (E3). It is a complex multiple-step process but in brief occurs as follows. Ubiquitin is firstly activated by the attachment of ATP to E1. Hence, protein breakdown is actually initially an ATP-consuming process. The activated ubiquitin is then transferred to E2, freeing E1 to participate in another activation. Meanwhile E3 recognizes and binds to the specific protein to be degraded and then binds to E2 which is carrying the activated ubiquitin. This large complex is transient plus dynamic and results in the transfer of the ubiquitin from the E2 to the protein substrate with release of both E2 and E3. The process is repeated until a chain of 4 or more ubiquitins are linked to each other and then to the protein substrate. The poly-ubiquitin tagged protein substrate is then transferred to the 26s proteasome complex, where the substrate is internalized and degraded into peptide substrates by the 20s proteasome (Argiles *et al*., 1996).

Degradation by the 20s proteasome

The central part of the 26s proteasome consists of the 20s proteasome, which is responsible for proteolysis. This barrel shaped complex consists of four stacked rings, each ring containing seven subunits. The α-subunits form the outer ring and are non-catalytic and the catalytic β-subunits form the 2 inner rings. Protein substrates enter the 20s proteasome through the outer ring. This is only possible if the proteasome has been activated by proteasome activator (19s) complexes (also called (PA-700), which bind to the 2 α-rings of the 20s proteasome in the

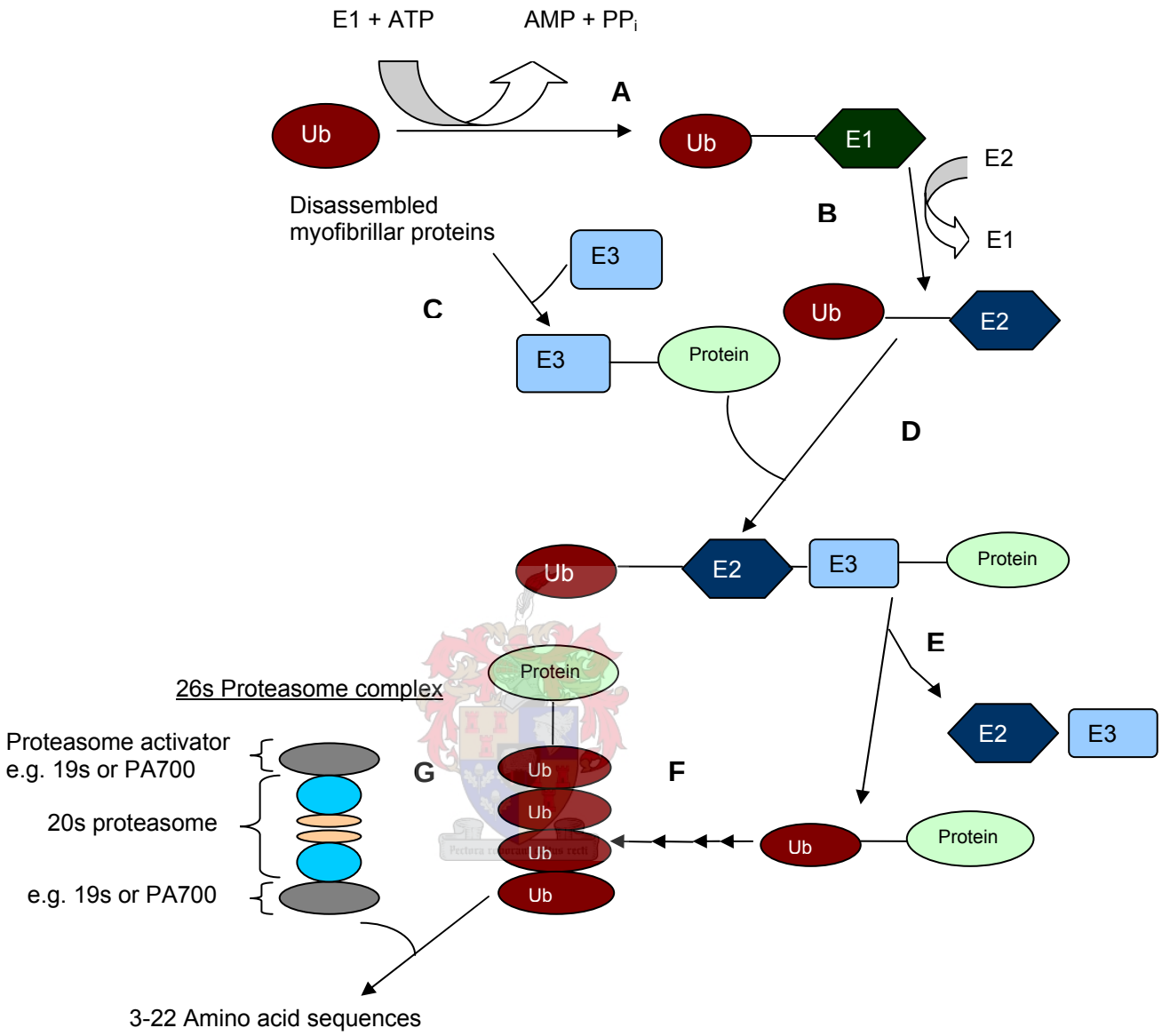


Fig. 1.1 Illustration of the reactions involved in the degradation of skeletal muscle proteins by the ubiquitin-proteasome pathway. (A) Ubiquitin (Ub) is activated by the Ubiquitin-activating enzyme (E1), and ATP is hydrolysed. (B) E1 then transfers Ub to the ubiquitin-conjugating enzyme (E2). (C) The disassembled myofibrillar protein is recognized and bound by a ubiquitin protein ligase (E3). (D) E3 bound to the protein substrate binds to E2 carrying the ubiquitin. (E) E3 transfers ubiquitin from the E2 to the target protein. (F) The process (A – D) is repeated until a polyubiquitin chain is formed. (G) The poly-ubiquitin labelled protein is then transferred to the 26s proteasome complex where it is degraded by the 20s proteasome into peptides of various lengths (Taillandier *et al.*, 2004, Argiles *et al.*, 1996).

presence of ATP to form the 26s proteasome. This is therefore a second ATP-consuming process. The 20s proteasome degrades proteins into peptides of 3-22 amino acids (Taillandier, 2004). A feature of the 20s proteasome is its specificity. It has at least five proteolytic activities, which include chymotryptic-, tryptic-, peptidylglutamyl peptidase-, branched chain amino acid preferring- and small neutral amino acid preferring activities (Orlowski *et al.*, 1993). Each of these enzymes has a preferred sequence of amino acids to target for digestion.

Exogenous peptide substrates have been used in *in vitro* studies to measure the hydrolysing activities of the chymotryptic, tryptic and peptidylglutamyl peptidase activities respectively (Hobler *et al.*, 1999, McCormack *et al.*, 1998). The chymotryptic activity is the most commonly studied, because lactacystin (a specific proteasome blocker) is able to completely block the chymotrypsin-like proteasome activity without inhibiting the activity of other proteasome and non-proteasome proteases. Thus making the chymotrypsin-like proteasome activity a valid method to measure the specific activity of the 20s proteasome.

1.2.4) Comparative assessment

Attaix *et al.* (1998) reviewed the role of the ubiquitin-proteasome pathway in muscle wasting in comparison to the lysosomal and calcium dependent systems. Selective inhibitors of the different proteolytic systems have been used to elucidate the contribution of each system to protein breakdown. Attaix *et al*. (1998) concluded that the ubiquitin-proteasome pathway is activated in every catabolic condition studied in rodents. These included fasting, denervation atrophy, hindlimb unloading, aging, diabetes, acidosis, burn injury, trauma and glucocorticoid and thyroid hormone administration.

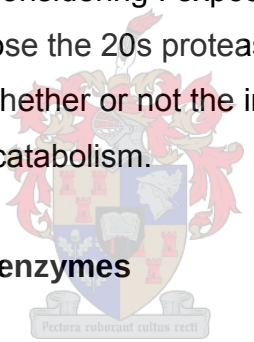
Indeed the pathway is not only activated but is also largely responsible for the increased muscle degradation in these conditions. For example, Baracos *et al.* (1995) implanted rats with Yoshida ascited hepatoma (YAH). Rats implanted with YAH show a rapid and selective loss of muscle protein. To define which proteolytic system contributes to the loss of protein in epitochlearis muscle in this

condition, the harvested muscle was incubated *in vitro* under conditions that modify different proteolytic systems. When muscles were incubated in a calcium free medium, containing sodium dantrolene (which inhibits release of intracellular calcium) it had no effect on protein degradation in the YAH treated rats or controls. Also, inhibition of the lysosomal degradation system with methylamine resulted in a small reduction (12%) of protein degradation. In contrast, when muscle was incubated in the absence of glucose (resulting in ATP depletion) to determine the contribution of the ATP-ubiquitin pathway the enhanced proteolysis in muscles of tumour bearing rats was totally abolished. However, Taillandier *et al.* (1996) reported increased activities of cathepsin B, cathepsins B + L and m-calpain enzymes after 9 days of hindlimb unloading (by 111%, 92% and 180% respectively in rat *m. soleus* muscle). Increased (messenger Ribonucleic acid) mRNA levels of cathepsin B, cathepsins B + L and m-calpain enzymes (by 84%, 220% and 210% respectively) paralleled the increases in the enzymes' activities. But inhibition of the calcium dependent and lysosomal pathways failed to suppress increased protein breakdown in muscle of hindlimb unloaded rats (Taillandier *et al.*, 1996). Thus the authors suggested that increased ubiquitin-dependant proteolysis is the most probable pathway responsible for the muscle wasting observed in muscle of hindlimb unloaded rats. In support of this statement they found increased mRNA levels for various components of the ubiquitin-proteasome degradation pathway. These components included ubiquitin, E2, C2 and C9 subunits (part of the α-subunit rings of the 20s proteasome), which increased by 105 %, 65%, 133% and 61% respectively compared to control animals. Similar studies have been done on muscle of metabolic acidotic (Mitch *et al.*, 1994), burn injured (Fang *et al.*, 1998), denervated (Furuno *et al.*, 1990) and septic (Tiao *et al.*, 1994) animals with the ubiquitin-proteasome pathway always responsible for the majority of skeletal muscle protein degradation to peptides.

After 3-22 amino acid peptides are produced by the 20s proteasome, they undergo further hydrolysis into free amino acids downstream of the proteasome.

It has been shown that extralysosomal-peptidase tripeptidyl-peptidase II (TPP II) degrades peptides released by protein breakdown via the proteasome pathway (Hasselgren *et al.*, 2002). The resultant individual amino acids can be transported out of the muscle and delivered to the liver, or other organs, or they can be further degraded within muscle. For e.g. tyrosine aminotransferase can catabolise tyrosine in muscle, but glutamate is exported after its conversion to glutamine. During normal protein turnover these mechanisms are all active to a certain extent, but are upregulated by disease or stress.

The study done for this thesis is the first to our knowledge, to evaluate proteasome activity in response to an intermittent, mild stress protocol. Given that the 20s proteasome is activated and responsible for protein degradation in most catabolic conditions, and considering I exposed my rats to the stress protocol for 7 and 14 days, I chose the 20s proteasome as my main marker of protein degradation to assess whether or not the intermittent, mild stress-inducing model caused protein catabolism.



1.3) Glucocorticoid inducible enzymes

1.3.1) Glutamine synthetase

Glutamine serves an important role as substrate for respiratory ATP production in selected cell types, renal ammoniagenesis, as gluconeogenic precursor and carrier of nitrogen between tissues. Muscle and lungs are the major storage sites of glutamine, with more than 50% of the total free amino acid pool in skeletal muscle. Glutamine metabolism is largely regulated by glutaminase (catabolises glutamine to glutamate and ammonia) and glutamine synthetase (GS, synthesises glutamine from glutamate and ammonia). Overall utilisation of glutamine increases during a number of catabolic states including sepsis (Elgadi *et al.*, 1998), tumour induction (Chen *et al.*, 1993), glucocorticoid treatment (dexamethasone) (Muhlbacher *et al.*, 1984) and burn injury (Rennie *et al.*, 1986). This is associated with glutamine export by skeletal muscle into the blood circulation (Muhlbacher *et al.*, 1984), which is associated with increased

glutamine consumption by other tissues such as the gut (Newsholme *et al.,* 1986), liver and immune system (Curthoys *et al*., 1995*)*.

However, the proportion of glutamine incorporated in major muscle proteins cannot account for the quantities of free glutamine released from skeletal muscle during catabolic states, suggesting that most of the glutamine originates from glutamate. Glutamine synthetase (GS) catalyzes the ATP-dependant conversion of glutamate and ammonia to form glutamine (Minet *et al.*, 1997). Despite an increase in GS expression and activity in skeletal muscle during conditions of stress, the intracellular glutamine pool becomes depleted, indicating that the maximal glutamine release rate exceeds the maximal rate of synthesis (Jepson *et al.*, 1988). Furthermore the circulating pool of glutamine does not increase indicating accelerated uptake by other organs. This suggests that upregulation of GS expression is a very important response to stress.

The increase in GS expression in skeletal muscle seems to play a key role in the development of atrophy induced by glucocorticoids. Labow *et al.* (1999) injected male Sprague-Dawley rats with methionine sulfoximine (MSO) to deplete muscle glutamine stores. (MSO is an analogue of glutamine, which binds to the GS protein, and inhibits GS activity). They subsequently injected rats with the glucocorticoid analogue Dexamethasone (DEX) to mimic the acute stress response. Quadriceps muscle of rats treated with MSO and DEX in combination or MSO with the vehicle ethanol, resulted in muscle glutamine depletion. However only the MSO/DEX treated rats had a significant increase in GS mRNA. Thus it seems as though muscle glutamine depletion by itself does not stimulate GS mRNA accumulation and that glucocorticoids are necessary for the induction of glutamine synthetase, as shown in previous studies (Falduto *et al.*, 1992; Hickson *et al.*, 1996). However rat studies have shown that glutamine infusion can attenuate corticosterone induced skeletal muscle atrophy (Hickson *et al.*, 1996; Hickson *et al*, 1995). Hickson *et al.* (1996) showed that glutamine infusion in corticosterone treated female Sprague Dawley rats resulted in 200-250% lower

induction of GS activities when compared to saline treated controls in *m. plantaris*, *m. gastrocnemius* and *m. quadriceps* muscles. This was associated with a 74, 91 and 73% prevention of atrophy in the *m.gastrocnemius*, *m.quadriceps* and *m. plantaris*, respectively. Glutamine thus seems to have anti-catabolic effects, possibly by inhibiting the GS activity rather than influencing gene expression. This result was also reported in an earlier study by another research group (Hammarqvist *et al.*, 1989).

In view of the relationship between elevated GS expression and skeletal muscle atrophy, it seems as if increased GS activity could be a key role player downstream from the ubiquitin-proteasome during conditions causing skeletal muscle atrophy. I therefore decided to assess muscle GS activity as an indicator of skeletal muscle degradation.

1.3.2) Tyrosine Aminotransferase (TAT)

TAT is active in most tissues (Chesnokov *et al*., 1990; Nemeth *et al.*, 1977), including skeletal muscle and is a key enzyme in the catabolism of tyrosine (Wurtman and Fernstrom, 1975). Because TAT is found in much higher concentrations in the liver than any other organ and its activity increases several fold in the liver after administration of glucocorticoids (Chesnokov *et al.*, 1990), it is the organ regularly used to examine changes in TAT activity. Its activity can be induced by endogenous glucocorticoids (Chesnokov *et al.*, 1990), insulin (Holten and Kenney, 1967) and glucagons (Haley and Harper, 1982). Furthermore stressors such as immobilisation (Nemeth and Vigas 1975), surgery (Laskowska-Klita and Bong, 1981), starvation (Kato and Saito, 1980) and exposure to chloroform (Stefan, 1976) have been able to induce changes in liver TAT activity in rat models. Assessment of TAT activity in tissues other than liver, is scarce. In a controversial study performed in Prague, Nemeth (1977) subjected male rats to 400 revolutions in drums rotating at a speed of 60 per minute. To illustrate the extreme nature of this experiment I will explain the stress procedure in detail. The drum consisted of two triangular projections attached within. During the

experimental procedure a rat was placed up the side of the drum. When the drum started revolving, the rat fell to the bottom and when it reached the top again was picked up by the following projection. The paws of the rats were taped together to prevent them from attempting in a natural way to break the fall. TAT activity increased in the liver of rats subjected to the stress procedure compared to non-stressed rats. In contrast, no changes of TAT activity were found in the other organs examined, including the *m. quadriceps femoris*. It should be noted though, that this was a very severe and traumatic procedure to induce stress, with the following typical injuries associated with the procedure: broken teeth or being knocked unconscious, bruising of the head, paws and scrotum, haemorrhage into muscle and injuries to the liver, kidneys, lungs and intestines (Bayly, 1952). The combination of severe shock and multiple injuries is not comparable to stress exposure in daily life.

In contrast I will be using a mild procedure to induce stress. They also used an acute (3 days) in contrast to the more chronic (7 and 14 days) stress procedure that I will use. Other than this study (Nemeth, 1977) I was unable to find any other studies in the literature using skeletal muscle to examine changes in TAT activity. However, catabolism of skeletal muscle is associated with increased utilisation of amino acids, including tyrosine as a fuel source (Tawa *et al.*, 1994). Thus because TAT is involved in tyrosine catabolism and its activity is known to be increased in liver during stress, I decided to use it in my study as a possible marker of muscle protein degradation.

1.4) Endocrine response to stress

1.4.1 Corticosterone

The hypothalamic-pituitary adrenal axis is activated during stress. The two primary glucocorticoids released in response to stress are cortisol and corticosterone. Most animals can make either corticosterone or cortisol. Rats secrete mainly corticosterone (Allen-Rowlands *et al.*, 1980), while the primary glucocorticoid secreted by humans (and other primates) is cortisol (Schteingart *et*

al., 1980). Elevations in circulating glucocorticoids are associated with increased protein catabolism (Kayali *et al.*, 1987), decreased protein synthesis and increased amino acid efflux from muscle (Shah *et al.*, 2000).

1.4.2) Testosterone

In contrast, testosterone has been reported to increase protein synthesis and skeletal muscle growth (Danhaive and Rosseau, 1988). This hormone, which is elevated in response to activation of the hypothalamic-pituitary-testicular axis, is suppressed in continuously stressful conditions such as immobilisation (Almeida *et al.*, 1998). Since decreased testosterone levels in response to stress are usually accompanied by increases in corticosteroid levels (Bricout *et al.*, 1999; Cumming *et al.*, 1983), it is difficult to isolate individual effects of either lack of anabolic- or increased catabolic hormones on muscle atrophy.

1.4.3) Relationship between corticosterone and testosterone

It has been proposed that the reduction in testosterone in response to stress is due to glucocorticoids. This relationship has been shown in amphibians (Moore and Miller, 1984), reptiles (Tokarz, 1987), birds (Wingfield, 1985), stallions (Colborn *et al.*, 1991) and female rats (de Catanzaro, 1987). However, Retana-Marquez *et al.* (1998) observed that administration of different doses of glucocorticoids to male Wistar rats for 4 or 8 consecutive days failed to decrease plasma testosterone levels. More recently the same authors (Retana-Marquez *et al.*, 2003) exposed male Wistar rats to either immersion in cold water (ICW), immobilisation or electric foot shocks (EFS) and evaluated plasma corticosterone and testosterone on days 1, 5, 10 and 20. Testosterone decreased significantly in the EFS and ICW treated rats on days 1, 10 and 20, whereas corticosterone increased only in rats exposed to ICW. This would suggest that a decrease in testosterone is not necessarily associated with an increase in corticosterone. Further evidence suggests that decreased testosterone is a late response. Almeida *et al.* (1998) exposed pre-pubertal male Wistar rats to immobilisation for 6 hours a day in plastic tubes for 15 (pubertal rats) and 60 days (adult rats)

respectively. Immobilisation caused a significant increase in plasma corticosterone levels after 15 and 60 days with a concomitant decrease in LH and testosterone after 60 days compared to pair-fed controls in young and old rats.

Few studies have explored the significance of the balance between these two hormones in influencing skeletal muscle growth or atrophy. Crowley *et al.* (1996) examined the interactions between corticosterone and testosterone in the regulation of skeletal muscle in male Long Evans rats. Testosterone to corticosterone ratio (T:C) was manipulated for 16 days to evaluate the relative contribution of each hormone to skeletal muscle protein balance. Body mass and skeletal muscle atrophy of the *m.plantaris* muscle was observed in the corticosterone injected rats. Testosterone was ineffective in attenuating the corticosterone-induced atrophy in rats that received both testosterone and corticosterone. This result supports earlier studies (Tsika *et al.*, 1987; Witzmann *et al.,* 1988).

The role of testosterone treatment on muscle mass has also been evaluated in another model of atrophy, namely disuse atrophy where it also did not seem to ameliorate the disuse atrophy. Bricout *et al.* (1999) showed that a combination of hindlimb suspension (HS) and testosterone administration (10 mg/kg body wt) caused a decreased affinity and binding capacity of steroid to steroid receptors in skeletal muscle of male Wistar rats. However, the fast twitch EDL exhibited a higher affinity for steroid than the slow twitch soleus. They suggested that testosterone treatment might be more effective in fast twitch than slow twitch muscle. This would suggest that fibre type should be considered as well when evaluating factors influencing- or markers of muscle wasting. Furthermore the protocol used may influence results. Although endogenous testosterone does not seem to be a useful parameter to investigate, I will review the use of pharmaceutical anabolic steroids as a treatment because my study will include a herbal agent that I will assess as a possible treatment.

1.4.4) Anabolic steroids

In contrast to the findings of Crowley *et al.* (1996) and Bricout *et al.* (1999), synthetic anabolic steroids have been able to ameliorate the atrophy inducing effects of hindlimb suspension (Joumaa *et al.*, 2002), hindlimb cast immobilisation (Harjola *et al*, 2000) and administration of glucocorticoids (Pellegrino *et al.*, 2004) in other studies.

Joumaa *et al.* (2002) used an anabolic androgenic steroid (nandrolone decanoate, 15 mg/kg body wt) weekly for 6 weeks prior to hindlimb suspension, which partially attenuated the hindlimb suspension induced loss in *m. soleus* mass of male Wistar rats. Similarly, clenbuterol (beta-agonist, 30 mg/l treatment in drinking water), another androgenic steroid, has also been able to partially counteract the hindlimb suspension-induced atrophy of male Wistar rats (Pellegrino *et al.*, 2004). Pellegrino *et al.* (2004) showed that clenbuterol (1.5 mg/kg body wt) was not only able to attenuate the atrophy-inducing effects of dexamethasone (5.7 mg/kg body wt) on the *m. soleus*, but actually predominated over the effects of dexamethasone such that the *m. soleus* muscle hypertrophied in C-57 mice treated with both clenbuterol and dexamethasone. Anabolic steroids seem to be quite potent, but have side-effects. Therefore, in human subjects testosterone treatment is often used in lower doses as a replacement therapy and with some success (Urban *et al.*, 1995, Ferrando *et al.*, 2002). In the studies of Urban *et al*. (1995) and Ferrando *et al*. (2002) serum testosterone concentrations were measured in male subjects (60 – 98 years) with total serum testosterone less than 17 nmol/l. Once a week for 6 months the total serum testosterone was measured and adjusted (by testosterone injections) to between 17 and 28 nmol/l to approximate concentrations found in young men.

Testosterone replacement therapy in hypogonadal elderly men decreased muscle protein breakdown (Ferrando *et al.*, 2002), increased protein synthesis (Urban *et al.*, 1995), increased lean body mass (Bhasin *et al.*, 1997; Snyder *et al.*, 2000) and increased muscular strength (Ferrando *et al.*, 2002; Urban *et al.*,

1995). Ferrando *et al*. (2002), Snyder *et al*. (2000) and Urban *et al*. (1995) all injected hypogonadal men (65 years and older) with testosterone (specific concentrations not mentioned) to produce serum concentrations of those of younger men (between 17 and 30 ng/ml), whereas Bhasin *et al*. (1997) injected hypogonadal men (19 – 47 years) with a dose of 100 mg per week for 10 weeks.

Similarly, placebo-controlled studies of testosterone replacement in HIV-infected men have demonstrated an increase in fat free mass in patients who were specifically selected for low testosterone concentrations (Bhasin *et al.*, 1998; 2000; Grinspoon *et al.*, 1998). However, steroid hormone treatment along with anti-retrovirals may have severe side-effects not yet elucidated, and for many patients the cost of an additional treatment is prohibitive. In contrast, herbal treatments are available in markets and are not expensive. The need exists to evaluate these alternative treatments against muscle atrophy related to chronic disease. Therefore it is still relevant to perform further experiments in animal models. Possibly models that do not induce atrophy quite as severe as that seen with castration or hindlimb suspension would be more similar to wasting in slow chronic disease conditions. Some chronic diseases are also associated with increased serum cortisol concentrations. (Reiche *et al.*, 2005, Lindemann *et al.*, 2004), indicating that it is feasible to use stress models.

1.5) Experimental stress models

A variety of experimental conditions, which include glucocorticoid administration (Pellegrino *et al.*, 2003), electrical foot shocks (Marquez *et al.*, 2003), immersion in cold water (Marquez *et al*, 2003), exposure to white noise (Harkin *et al.*, 2002), sepsis (Elgadi *et al.*, 1998), implantation with tumours (Llovera *et al.*, 1996), etc. have been used to elicit atrophy of skeletal muscle and behavioural responses in rodents.

The response to stress in rodents depends on the type of stressor, the severity and the number of successive exposures to the stimulus. In the next section I will

briefly discuss the various stress protocols used previously, followed by a more detailed discussion of the protocols specifically relevant to this thesis.

1.5.1) Systemic disease and or pathology

Conditions such as sepsis (Elgadi *et al.*, 1998) and tumour bearing (Llovera *et al.*, 1996) are characterised by severe weight loss in a short period of time and the short life-span after acquiring the condition. These extreme experimental protocols are not useful to study adaptations to more chronic conditions, or a more physiological range of daily stress.

1.5.2) Disuse

Disuse atrophy occurs when a certain muscle group is not being used, such as in certain experimental conditions or in cases where muscle cannot be used due to injury. Experimental protocols include hindlimb unloading (the hindlimbs are suspended in the air and the rat is only able to move on its forelimbs), casting of one limb (Harjola *et al.*, 2000) and strapping limbs down. Here I will discuss only hindlimb unloading and specifically to indicate findings related to glucocorticoids and fibre type.

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Hindlimb unloading is used to simulate conditions of weightlessness and anti-gravity, such as in space travel, and selectively induces rapid atrophy of slow-twitch fibres (Taillandier *et al.*, 1996). Since many muscles containing slow twitch fibres are continuously active to maintain posture, this atrophy is most likely due to a lack of mechanical strain. However, it is interesting to note that serum glucocorticoids and glucocorticoid receptors in *m. soleus* are also upregulated, indicating that stress may also play a role during simulated weightlessness. Since, it has been shown that the ubiquitin-proteasome pathway is largely responsible for skeletal muscle atrophy during hindlimb unloading (Taillandier *et al.*, 1996), and considering that Combaret *et al.* (2004) showed that the ubiquitin-proteasome pathway is regulated by glucocorticoid treatment, it seems possible that glucocorticoids induce fibre selective atrophy through the ubiquitin-

proteasome pathway. However, exogenous glucocorticoids selectively induce atrophy of fast twitch skeletal fibres (Falduto *et al.*, 1990), whereas endogenous glucocorticoids are not fibre type selective.

1.5.3) Physical stressors

To more clearly separate the role of stress from other atrophy-inducing models, some models have specifically attempted to induce physiological stress. Stressors have included electrical foot shocks (Marquez *et al.*, 2003) and tail flicks (Kallina and Grau, 1995). The latter is induced by exposing the rat tail to heat. Although the rat voluntarily flicks the tail away from the heat, short duration pain is used to evoke stress. The tail is usually exposed to a heat source, such as a plate or water, which is heated to a temperature of 48 - 54º C, whilst the rat is restrained (Hawranko *et al.*, 1999; Kallina and Grau, 1995). Most studies use this stressor to study the pain reactivity (latency of tail flick) of rodents after being exposed to a certain stressful stimulus rather than to study the possible effect on muscle atrophy (Kallina and Grau, 1994, Pinto-Ribeiro *et al.*, 2004). Rats usually display a decreased nociception after repeated exposure to the heat source, thus indicating a stress-induced analgesia. Therefore, short duration exposure to heat can be considered a valid stressor.

1.5.4) Immobilisation

In the context of stress, immobilisation refers to the placement of experimental animals in a small holder for a short duration. They are able to turn themselves, but are unable to carry out any other locomotor activities (Grippo *et al.*, 2002, Harkin *et al.*, 2002). Rodents are able to habituate to this mild form of stress, as shown by Retana-Marquez *et al*. (2003), who reported no change in corticosterone after 15 and 20 days of immobilisation compared to controls. They showed lower corticosterone levels after repeated immobilisation (2 or either 6 hours per day for 20 days) compared to acute immobilisation stress. Similar observations have been reported (Ottenweller *et al.*, 1994) after intermittent restraint for 21 days (Natelson *et al.*, 1998) and after tail shocks to immobilised

rats. A possible explanation for these observations may be that the rat becomes habituated to repeated mild stressors such as immobilisation and tail flick tests. Therefore immobilisation is usually used together with other mild forms of stress such as cage tilt and white noise to induce a more pronounced stress response (Grippo *et al.*, 2002). See Table 1 below for a list of immobilisation studies that measured corticosterone.

1.5.5) Other factors influencing results in rodent models

Soiled bedding and Cage tilt

'Soiled bedding' is a stressor which involves adding water to the bedding of the housing cage of the animal for an hour or more, such that all bedding is soiled. Cage tilt is also used as a stressor and involves tilting the cage (with rodents inside) usually along the vertical axis. These two stressors are usually used as chronic mild stressors together with other mild stressors such as white noise and handling to induce a stress response in rodents (Grippo *et al.*, 2002; Bekris *et al.*, 2005). Grippo *et al.* (2002) exposed male Sprague-Dawley rats to the following stressors in random order: cage tilt, soiled bedding, continuous overnight illumination, paired housing (2 dominant males), restraint in a small cage, and exposure to an empty water bottle following a period of water deprivation, stroboscopic illumination (300 flashes/min) and white noise. They reported an increased resting heart rate, decreased heart rate variability and exaggerated heart rate responses to novel stress. Harkin *et al.* (2002) also reported a significant increase in heart rate and body temperature following repeated exposure to these stressors.

Housing

When conducting research using rodent models a number of factors which could have an impact on the outcome measures should be considered. Firstly it is important to note whether the rodent is housed individually or in a group and whether new rats are introduced or not. Social stress associated with housing a subordinate and dominant animal together, as previously shown, can significantly

Table 1:1 List of studies using immobilization restraint in a confined space that measured serum corticosterone concentrations

Authors and Date	Strain, sex, weight or age at start of study	Protocol	% Change of corticosterone from control group	List of other outcome measures
Almeida *et al*., 1998	Male Wistar 3 months old	6 hr/day for 60 days	57.6% increase	ACTH, Testosterone, LH
Retana-Marquez *et al.*, 2003	Male Wistar 300 – 350 g	2 and 6 hr/day for 14 days	No difference	Body mass, Adrenal mass, Testicular mass
Stamp & Herbert, 1999	Male Lister Hooded 300 – 350 g	60 min/day for 14 days	No difference	Body mass, DHEA, Heart rate
Gadek-Michalska and Bugajski, 2003	Male Wistar, 150 – 160 g	10 min/day for 10 days	100% increase	ACTH
Bhatnagar *et al.*, 2005	Male Sprague Dawley 85 days old	30 min/day for 8 days	50% higher in controls	Body mass, Testosterone

affect stress levels in a study using male mice (Keeney *et al.*, 2001). They showed that repeated social defeat resulted in an increase in core body temperature across 24 hours in subordinate animals and a very large increase in plasma corticosterone levels compared to their respective controls. Also, individually housed rodents tend to show higher behavioural arousal and higher corticosterone levels when compared to those group-housed (Bartolomucci *et al.*, 2003; Sharp *et al.*, 2002).

Secondly, witnessing experimental procedures can also have a stressful effect on animals. Sharp *et al.* (2002) used heart rate (HR) and mean arterial blood pressure (MAP) to determine whether male Sprague-Dawley rats were significantly stressed when present in a room while decapitation or other common experimental procedures were being performed. Small, but significant sustained

increases in HR and MAP occurred in single-housed rats when witnessing decapitation and a routine cage change, whereas the responses in animals housed with one or three cage mates were more transient.

Thus effort should be made to minimise such confounding factors. Ways in which to achieve this include housing rats in groups; performing experimental procedures separate from the animal housing areas and limiting the number of people handling the animals. Furthermore it should be noted that stressors which impact on the home environment of animals (such as soiled bedding) elicit a greater stress response than stressors which have no impact on the home environment of the rat (Harkin *et al.*, 2002).

Strain of rat

Comparisons of results obtained using different strains of rats should also be interpreted with caution. Strains can differ physiologically and in behavioural traits as shown in a study by Schumacher *et al.* (1991) where they compared 18 behaviour patterns in 3 different strains of rats [Spontaneously hypertensive (SHR), Wistar-Kyoto (WK) and Wistar (WI) rats]. Restraint with a jacket and tether produced a decrement in nose poking, quadrant changes (movement in the cage) and grooming. Rearing was also decreased in all strains and was almost totally suppressed in SHR rats. However in similar studies by other groups, SHRs exhibited more locomotor behaviour and rearing and produced more faecal boli in response to new environments (Knardahl *et al.*, 1984; Hard *et al.*, 1985). Both types of response indicate greater stress, even though restraint with a jacket and tether resulted in less movement. SHR also exhibited higher circulating concentrations of adrenalin, nor-adrenalin and corticosterone in response to footshocks (McCarty *et al.*, 1978) and some forms of restraint stress (Kvetnansky *et al.*, 1979). However, their hypertension might confound other physiological parameters.

Time of day and type of stressor

Plasma corticosterone and adrenocorticotropin hormone (ACTH) have a circadian rhythm. In animals such as the rat both hormones exhibit lower levels during the early morning and reach a peak during onset of darkness. An inverse pattern is observed in humans (Horrocks *et al.*, 1990) and the rhesus monkey (Kalin *et al.*, 1985), since primates are awake by day and rodents by night.

Stressful situations induce a rise in corticosterone and ACTH that is dependent on the time of day. ACTH and corticosterone concentrations increase maximally in response to stress at the beginning of the light cycle and the minimum response occurs at lights-off (Bradbury *et al.*, 1991). Retana Marquez *et al.* (2003) applied four different stressors (electrical foot shocks, immobilisation for 2 or 6 hours and immersion in cold water (15º C) for 15 minutes each) to different groups at the start of the light or dark phase. Each of the stressors increased plasma corticosterone levels significantly when it was applied at the start of the light phase, whereas in the dark phase only 2 hours of immobilisation and immersion in cold water induced a significant increase in plasma corticosterone. These results indicate acute effects of the stressors, which were measured within a few hours after the stressors were applied.

With chronic exposure (12 days), electrical foot shocks failed to produce significant changes in the corticosterone levels in either the light or dark phase. This would suggest that the response of hypothalamic-pituitary-adrenal axis (HPA) to stressors depends on the time of the day which the stressor is applied and also on the frequency and duration of the exposure as mentioned previously. Thus we would suggest combining milder stressors such as immobilisation, tail shock and cage tilts in a continuous, unpredictable fashion to prevent habituation.

Muscle fibre type

Rat skeletal muscle consists of both slow and fast twitch fibres. Slow twitch muscle fibres express the type I myosin heavy-chain (MHC) protein, whereas

fast-twitch muscle fibres can be further subdivided into three phenotypes (Type IIA, IIB and IIX) according to the myosin heavy chain protein they express (Polla *et al.*, 1994). Muscle fibres may exhibit a bi-directional fibre type shift with a sequence determined by the transition in MHC expression: I↔IIA↔IIX↔IIB, where transformation of muscle fibres in either direction depends on a number of factors. A number of experiments have been carried out to elucidate the specific fibres affected by stress and potential stress treatments and whether or not any fibre type transition occurred. These included muscle denervation (Windisch *et al*., 1998), foot shocks (Windisch *et al*., 1998), hindlimb suspension (Stevens *et al*., 2004), hypophysectomy (Yamaguchi *et al*., 1996), compensatory overload (Yamaguchi *et al*., 1996), testosterone (Harjola *et al*., 2000) and glucocorticoid treatment (Pellegrino *et al*., 2003). Each of these treatments had a large degree of fibre type selectivity. Therefore, when using a rodent stress model, fibre type specific effects should be investigated.

High concentrations of endogenous glucocorticoids selectively induce atrophy of the superficial regions of fast twitch muscles that contain mainly IIX and IIB (MHC) in white fibres, whereas a smaller degree of atrophy occurs in the deep regions that contain mainly IIA MHC in red fibres (Falduto *et al.*, 1990). However slow-twitch, type I fibres generally do not atrophy from hormone treatment (Falduto *et al.*, 1990). The glucocorticoid dose (e.g. 100 mg/kg body weight cortisone acetate (CA), or 5.7 mg/kg body weight dexamethasone) administered to rats in these studies is comparable with the highest dose used in human therapy for ailments such as asthma. Thus it is difficult to extrapolate these findings to stress studies where endogenous glucocorticoids are upregulated. This result stresses the importance of using a physiologically relevant model where possible, to prevent difficulty in interpretation of results obtained.

There have been contradictory conclusions in the literature on whether or not glucocorticoids can induce a shift in muscle fibre type distribution. Several studies have reported that no change in fibre type distribution occurs (Dekhuijzen

et al., 1992), whilst others reported a shift from type II fibres to type I (Falduto *et al.*, 1990).

Polla *et al*. (1994) were the first to take into account the existence of a third isoform of fast twitch MHC in a rat study investigating corticosteroid-induced myopathy. Monoclonal antibodies were used to identify all four fibre types (Type I, IIA, IIX and IIB) on the basis of their myosin heavy chain composition. Male Wistar rats were given cortisol acetate (CA) for 11 consecutive days and its effects on the distribution and cross-sectional areas of the different fibre types were investigated at the end of this period. EDL and *m. soleus* samples were obtained as examples of predominantly fast and slow muscles respectively. While EDL muscle mass was significantly reduced after CA treatment, the *m. soleus* muscle size seemed unaffected. CA produced a significant reduction of average cross-sectional area in all three fast fibre types of EDL muscle compared with controls. In addition CA resulted in a significantly lower proportion of IIB fibres and a concomitant increase in the percentage of type IIA fibres in EDL muscle. There was a significant reduction in the proportion of type IIA fibres in the soleus muscle. Also in the EDL muscle, the IIX fibres were reduced more than the IIA and there was conversion to type I. Thus this study indicates that exogenous glucocorticoids cause a fibre type transition from IIB in the direction of type I muscle. These results are in general agreement with other studies, which have demonstrated that glucocorticoids selectively induce atrophy of fast twitch fibres (Falduto *et al.*, 1990; Pellegrino *et al.*, 2004).

Chronic low frequency electrical stimulation and hypothyroidism (Kirschbaum *et al*., 1990) are also associated with an upregulation of type I MHC, while hyperthyroidism and muscle denervation induce the opposite transition from the slow to fast phenotype (Devor and White, 1996).

Type II fibres have also been shown to selectively atrophy in experimental models of burn injury (Chai *et al.*, 2002) and sepsis (Tiao *et al.*, 1994). These two

studies both illustrated an increased protein breakdown and upregulated expression of subunits of the ubiquitin-proteasome proteolytic pathway in the predominantly fast twitch EDL muscle but not in the *m. soleus* muscle. The ubiquitin proteasome proteolytic pathway is the major contributor to protein breakdown in most catabolic states, and is known to be upregulated by glucocorticoids (Combaret *et al.*, 2004).

1.6) Recovery

In this section I will review different recognised recovery interventions used after stress-related muscle atrophy. In addition I will discuss a relatively new treatment which is of specific interest to this thesis.

1.6.1) Hormone treatment

The effects of anabolic hormones [such as insulin, growth hormone and insulin-like growth factor-1 (IGF-1)] in experimental conditions of catabolism have been extensively studied.

Insulin stimulates protein synthesis (Prod'Homme *et al*., 2005), and inhibits protein breakdown in most cells and tissues (Heslin *et al*., 2002). Evidence suggests that insulin reduces protein breakdown by inhibition of both the lysosomal and the ubiquitin-proteasome dependant pathways (Kee *et al.*, 2003). However insulin treatment is associated with hypoglycaemia (Carter, 1998) and systemic insulin resistance has been reported in sepsis and injury (Hasselgren *et al.*, 1992). Therefore it cannot be considered for the treatment of patients with muscle wasting conditions.

Recent studies suggest that growth hormone (GH) alone has only modest enhancing effects on anabolism (Weber, 2002; Rennie, 2003). GH supplementation in combination with exercise has been shown to be effective in ameliorating skeletal muscle atrophy accompanying muscle unloading (Allen *et al.*, 1997) and burn injury (Suman *et al.*, 2003). It has also been shown to

increase lean body mass in HIV-infected men after 6 weeks of treatment (Schambelan *et al.*, 1996; Waters *et al.*, 1996). Unfortunately GH administration does have adverse effects such as causing diabetes and glucose intolerance (Blackman *et al.*, 2002) and increasing the risk for cancer. Insulin-like-growth factor 1 (IGF-1) synthesis is also stimulated by GH, but this effect of GH is inhibited in critically ill patients. This makes IGF-1 itself more attractive than GH as a treatment for catabolic diseases. The anti-catabolic effects of IGF-1 are effective in burn injuries (Herndon *et al.*, 1999; Fang *et al.*, 1998). IGF-1 has also been reported to improve protein synthesis in male Sprague Dawley rats with experimentally induced sepsis (Fang *et al.*, 1998). In this model it also decreased ubiquitin and E2 mRNA levels. Together these two lines of evidence support that nett protein catabolism was reduced. However it was unable to prevent the sepsis-induced increase in myofibrillar and total protein breakdown. This suggests that the muscles remained sensitive to sepsis, possibly becoming resistant to the anti-proteolytic effect of IGF-1, which has also been reported in another severe model namely burn injury (Fang *et al.*, 1998).

1.6.2) Pharmacological drug treatment

Other treatment modalities such as glucocorticoid-receptor antagonist RU38486 (Llovera *et al.*, 1996), anti-cytokine [such as pentoxyfilline an inhibitor of tumour necrosis factor alpha (TNF) synthesis and suramin which is an antiprotozoal drug blocking the peripheral action of cytokines such as interleukin-6 (IL-6) and TNF α] (Costelli *et al.*, 2002), and proteasome inhibitor (Fischer *et al*, 2002) treatments have been used to reverse the effects of stress induced atrophy. Whilst all these treatments have been successful, they are expensive and clinical trials need to be performed to establish any adverse effects, as well as to determine the optimal treatment protocol to be used in human subjects. These procedures can take several years to complete. Alternative accessible treatments should also be tested to establish scientifically their mechanisms of action. These include exercise and herbal supplements

1.6.3) Exercise

Exercise has many beneficial effects but for the purpose of this thesis I will focus on its effects on skeletal muscle atrophy (Hickson *et al.*, 1986). Two exercise models (endurance and resistance exercise) have been extensively used in experimental studies to investigate effects thereof on skeletal muscle atrophy.

Endurance exercise

The study of Hickson *et al.* (1981) was the first to determine whether endurance training was able to attenuate the muscle atrophy caused by exogenous glucocorticoid treatment. Male rats were trained for 12 weeks for ~ 110 minutes a day on a treadmill before treatment with exogenous glucocorticoids, which lasted for 11 consecutive days, began. The training regimen inhibited muscle wasting by about 25% and 50% in the *m. gastrocnemius* and *m. plantaris* muscles respectively when compared to sedentary controls also exposed to glucocorticoid treatment. In another study Czerwinski *et al*. (1987) used a slightly shorter running program (90 minutes a day) and simultaneous glucocorticoid treatment for 11 consecutive days. The exercise attenuated muscle atrophy by 25 % in m. *gastrocnemius* and 60 % in *m. plantaris* muscle. Similarly Falduto *et al.* (1992) introduced running of 90 minutes a day after 4 days of cortisol acetate (100 mg/kg body weight) treatment. The exercise inhibited 23% and 36% of the atrophy in the *m. quadriceps* and *m. plantaris* muscles respectively.

In an earlier study these authors also showed that the effect of endurance exercise on reducing atrophy is associated with attenuation of expression of the glucocorticoid inducible gene, GS (Falduto *et al.*, 1989). In this study, female Sprague-Dawley rats were trained on a treadmill for 12-16 weeks (5 days/week) for a duration of 90 min/day, at a speed of 31 m/min with a 10% gradient. For the last 11 consecutive days the animals received a daily injection of either hydrocortisone 21-acetate (100 mg/kg body weight) or an equal volume of vehicle alone. Glucocorticoid treatment resulted in a 2.4 and 3.3-fold increase in GS activity of sedentary and trained rats which were not exercised. Endurance

training of vehicle- and glucocorticoid-treated animals resulted in GS activities that were 57% and 79% lower in plantaris muscle compared to their respective sedentary controls. *M. plantaris* muscle mass was 23% lower in rats treated with the glucocorticoids compared to vehicle treated control. Endurance training attenuated this response to glucocorticoids, so that mean muscle mass of the vehicle treated sedentary control rats was similar to the glucocorticoid-treated trained animals. This study indicates that endurance training may induce its attenuating effects on glucocorticoid mediated skeletal muscle atrophy through GS.

Marone *et al.* (1994) also treated female Sprague-Dawley rats with cortisol acetate at a dose of 100 mg/kg body weight. The training consisted of running on a treadmill at a speed of 28 m/min for 90 min per day, 6 days per week, for 8-10 weeks. Glucocorticoid treated rats exercised every day during the course of the treatment. Exercise prevented 55, 46 and 43% of muscle mass loss in the *m. quadriceps*, *m. plantaris* and *m. gastrocnemius* muscle respectively. These studies suggest that endurance training is able to attenuate the glucocorticoid induced muscle atrophy regardless of whether the initiation of exercise is before, simultaneous with or following glucocorticoid treatment.

Endurance training has also been used as a method to reverse the atrophy induced by immobilisation. Appell (1986) trained female specific pathogen free (SPF)-NMRI mice for 7 days on a treadmill at a speed of 0.3 m/s at an inclination of 5% for 30 minutes per day either prior to or after 7 days of hindlimb plaster cast immobilisation to the right hindlimb. The contralateral limb served as control. The mice which were immobilised first and trained afterwards had a 13% decrease in fibre diameters of their *m. anterior tibialis* muscle compared to exclusively immobilised rats which had a 35% decrease in fibre diameter. The post-immobilisation training however was associated with severe ultrastructural alterations in the muscle. Muscle of mice which were trained before immobilisation had an atrophy of only 5%.

Deschenes *et al.* (1997) found that treadmill running of 6 weeks (5 d/week) at a pace of 24 m/min at a 0% grade after denervation of the *m. soleus* muscle could attenuate the cross sectional fibre area size reductions in denervated *m. soleus* muscle. These findings correlate with those of Delp and Armstrong (1988) who used the same denervation model along with a slower running speed. Endurance training has thus been shown to be capable of at least partially preventing glucocorticoid-mediated muscle atrophy and modifying atrophy induced by other methods. Many of the running protocols used in these studies included an element of resistance since rats/mice exercised against a gradient. As a model, resistance training itself is less frequently used.

Resistance exercise

Resistance training has also been shown to be effective in attenuating the muscle wasting effects of glucocorticoids. Gardiner *et al.* (1980) performed a 6 week study on male Sprague-Dawley rats lifting weights with trunk and hindlimb muscles 4 times per week while simultaneously receiving triamcinolone acetonide (synthetic anti-inflammatory glucocorticoid) at a dose of 1 mg/kg. The training inhibited loss of *m. gastrocnemius* muscle mass by approximately 46% compared to rats receiving glucocorticoid treatment only, whereas the *m. soleus* muscles were similar in size and strength for these two treatment groups. Therefore the resistance protocol did not work because of the predominantly slow fibres in the *m. soleus.*

Resistance exercise (Fluckey *et al.*, 2002; Linderman *et al.*, 1994; Allen *et al.*, 1997) has also been used to study the effects thereof on hindlimb unloading which causes atrophy predominantly in the “anti-gravity” *m. soleus* muscle. Linderman *et al.* (1994) and Allen *et al.* (1997) used similar exercise training protocols in albino and female Sprague-Dawley rats respectively. The exercise involved rats climbing a 1 meter ladder mesh-grid placed at an 85° incline with an additional weight of 50% of body mass attached to the tail. The rats exercised 3 times a day (08:00, 12:00 and 16:00) for 5 days (10 repetitions per day). The

resistance exercise training protocol was unable to attenuate the atrophying effects of hindlimb unloading on the *m. soleus* in the study by Linderman *et al*. (1994). However, the exercise was able to inhibit atrophy (expressed relative to body mass) of the *m. gastrocnemius* and *m. plantaris* muscles, which were similar in relative mass to that of ambulatory control animals, suggesting a response in fast-twitch muscle only. Allen *et al.* (1997) did show an attenuating effect of the exercise on the *m. soleus* muscle wet weight. They however ablated the synergistic *m. plantaris* and *m. gastrocnemius* muscle 7 days before the suspension period. Taking this into account the *m. soleus* was much more overloaded. The overload may have induced the fibre hypertrophy, but it is possible that functional overload induced inflammation and oedema which would have been aggravated in the muscle weakened by the hindlimb unloading, and this could account for the increased muscle wet weight. In a somewhat older study Herbert *et al.* (1988) used the same exercise protocol as that of Linderman *et al.* (1994) and Allen *et al.* (1997) with male Sprague-Dawley rats but with 1 extra session per day and a higher added weight. Rats exercised 4 times a day (when not hindlimb suspended) with a load equal to 75% of their body mass attached to their tail. After 7 days *m. soleus* wet weights were 19% higher in rats that were exercised during the period of hindlimb suspension (HsE), compared to rats which were only hindlimb suspended (HS). Furthermore when *m. soleus* mass was expressed relative to body mass it was similar in controls and HsE group, but 32% lower in HS compared to controls. Thus the frequency and intensity of resistance exercise appears to be important for attenuating *m. soleus* muscle atrophy during hindlimb suspension.

Fluckey *et al.* (2002) used a flywheel resistance exercise apparatus, which has the advantage of rats being able to train whilst being hindlimb suspended. To avoid a brief footshock stimulus (underneath the hindlimbs) the rat had to fully extend its hindlimbs to press an illuminated lever with its nose and subsequently had to flex to return to the resting state. The authors described the exercise as similar to a squat performed by humans with involvement of the hip, knee and

ankle joints. The resistance training protocol consisted of 11 sessions over a 4-week period, with two sets of a maximum of 25 repetitions for each session. Exercised rats had a significantly higher *m. soleus* muscle mass to body mass ratio when compared to animals that were hindlimb suspended alone.

In summary these 5 studies illustrate that resistance exercise is able to attenuate skeletal muscle atrophy in the muscles recruited during a particular exercise protocol. However, the only mechanisms investigated included assessments of glucocorticoids in a few studies and GS activity in a few more.

Effects of exercise on the ubiquitin proteasome pathway

Components of the ubiquitin proteasome pathway have been shown to increase with an initial bout of exercise. Of the various components, ubiquitin itself has been best documented and is upregulated by several types of both acute and chronic exercise. Spontaneous wheel running has been shown by Sandri *et al.* (1995) to increase ubiquitin protein levels of mice. Wakshlag *et al.* (2002) observed an increase in ubiquitin-conjugated proteins and the p31 subunit within muscles of working field dogs during the peak hunting season compared with values measured in the pre-season. Neither of these studies aimed to induce muscle damage and my interpretation of their data is that exercise may increase overall protein turnover rate, influencing both anabolism and catabolism.

In contrast, in human subjects most studies on the activation of the proteasome by exercise have focussed on the types of exercise that are known to induce muscle damage. Thompson and Scordalis (1994) found a 55% increase in free ubiquitin and ubiquitin-conjugated proteins relative to non-exercised control muscles 2 days after a bout of eccentric-isokinetic exercise of human biceps. Willoughby *et al.* (2003) also observed increases in ubiquitin mRNA protein levels of the E2 protein and the 20s proteasome 6-24 hours after eccentric knee extension exercise performed by healthy volunteers.

However, several studies exist that show repetitive exercise can inhibit the ubiquitin-proteasome pathway. In the study previously mentioned by Willoughby *et al.* (2003) where increases were reported in ubiquitin, E2 and the 20s proteasome content 6 and 24 hours after acute knee extension exercise, they also showed that these increases were attenuated after a second exercise bout. Kee *et al.* (2002) found decreases in protein breakdown rate, chymotrypsin-like activity of the proteasome and the rate of ubiquitin dependent casein hydrolysis in treadmill exercised rats (5 consecutive days) compared to muscles of unexercised rats. Jones *et al.* (2004) studied the effects of 6 weeks of exercise rehabilitation after 2 weeks of knee immobilisation and detected decreases in mRNA levels of the 20s proteasome. Thus these studies illustrate that the ubiquitin-proteasome pathway is affected by exercise and that continuous exercise is able to downregulate components of the pathway. Therefore, exercise could be used to modify muscle wasting that accompanies various diseases.

Strength training studies on HIV-infected people have yielded positive effects with regard to muscle mass and body mass gain suggesting a downregulation of wasting although muscle biopsies were not measured (Evans *et al*, 1998). Spence *et al.* (1990) examined the effects of strength training in HIV-infected men who all had experienced and recovered from one episode of *Pneomocystis carinii* pneumonia (PCP) infection, but with no AIDS-defining opportunistic infection. They found an increase in isokinetic power in the exercised group compared with a moderate loss of strength in the non-exercised group. These functional changes were related to mean body mass loss of 1.9 kg in the control group compared with a gain of 1.7 kg in the exercise group and a significant improvement in combined mid-arm and mid-thigh circumference in the training group compared with a decline in the control group. Roubenoff *et al.* (1999) used a more aggressive programme of progressive resistance training in both men and women with HIV. They found a mean increase in body mass of 2.9 kg, of which 2.2 kg was lean mass and 0.7 kg was fat.

However, it is not always easy for persons with diseases such as HIV/AIDS or cancer to do exercise because of physical complications. Also, exercise exerts its effects mainly on the muscle that is recruited by the particular exercise regimen. Thus while exercise remains an important method in deterring skeletal muscle atrophy, other less strenuous procedures should be explored.

Other than exercise three types of treatments have generally been tested to determine whether they could either limit or prevent the catabolic effects of conditions such as sepsis, injury, cancer etc: 1) nutritional, 2) hormonal and 3) pharmacological interventions. I will briefly discuss each of these interventions.

1.6.4) Nutritional supplementation

Protein under-nutrition inhibits the muscle protein degradation (Brodsky *et al.*, 2004; Tawa *et al.*, 1992). Brodsky *et al.* (2004) compared healthy human subjects (9 males and 4 women) fed either the average minimum adult protein requirement (0.71 g/kg fat free mass per day) or a higher protein intake more typical of an American diet according to the authors (1.67 g/kg fat free per day). They illustrated that subjects fed the average minimum adult protein requirement exhibited 68% lower urinary excretion of 3-methylhistidine (indicator of myofibrillar protein breakdown) when compared to adults, who were fed the American style diet. However, there was no difference in the expression of mRNA for proteasome subunits, C2 and C3. Similarly Tawa *et al.* (1992) observed a suppression of skeletal muscle protein breakdown in rats fed a protein deficient diet. They found a 40-60 % reduction in proteolysis rate, measured by tyrosine release rates from skeletal muscle. (Tyrosine is neither synthesized nor degraded by skeletal muscle). However in these two studies, nutrition was not used as a treatment to try to reverse pre-existing muscle wasting.

Studies have used nutrients such as branched chain amino acids (BCAA), glutamine and arginine to determine their effects in catabolic conditions (Cerra *et*

al., 1985; Zanetti *et al.*, 1999). In a series of studies on cellular turnover *in vivo* Mortimore's group (Poso *et al*., 1982; Poso and Mortimore, 1984; Mortimore and Poso, 1986; Lardeux and Mortimore, 1987a; Lardeux *et al*., 1987b; Mortimore *et al*., 1987c), demonstrated that supplementation with amino acids can inhibit protein degradation (Mortimore and Poso, 1986), with leucine having the largest inhibitory effect (Poso *et al*., 1982). More recently Hamel *et al.* (2003) studied the effect of individual amino acids on *in vitro* proteasome activity. Inhibition of the chymotrypsin enzyme activity of the proteasome was seen with leucine, phenylalanine, tryptophan, lysine and arginine, suggesting that the effect reported earlier by Mortimore and colleagues occurs via the inhibition of chymotrypsin-induced proteolysis. However, protein mobilisation in muscle is complex and includes many other elements besides ubiquitin-proteasome activity.

As previously described glutamine has anti-catabolic effects, which may occur via regulation of the glutamine synthetase enzyme. However, it seems as if glutamine supplementation does not always result in an anti-catabolic response in skeletal muscle. Although protein synthesis and whole body nitrogen balance were improved glutamine-enriched solutions were given to patients with moderate trauma (Hammarqvist *et al.*, 1989), in patients with more severe cachexia, the anti-catabolic effect of glutamine was less convincing (Karner *et al.*, 1989).

Thus it seems as if selected amino acids may have an anti-catabolic (decreased protein breakdown), and even anabolic (increased protein synthesis) effects in conditions that cause catabolism. It could be that they exert their anti-catabolic effect through attenuation of proteasome activity. Amino acids are however not so effective when catabolism is severe and ongoing. In a South African context it is also too expensive. Whether or not other nutrients can affect protein turnover/catabolism is also under investigation in some laboratories.

Eicosapentaenoic acid (EPA) supplementation has been shown to attenuate cancer associated weight loss, which may be mediated through its inhibition of the proteasome degradation pathway. Mikhail *et al.* (2003) showed that after supplementing tumour bearing rats with EPA, the subunits (mRNA levels of 14 kilo Dalton ubiquitin-conjugating enzyme and ubiquitin-conjugating enzyme alpha) of the proteasome degradation pathway decreased. Whether it can exert a similar effect on catabolic diseases other than cancer is not known. At present it is unclear what mechanisms are targeted by the various treatments upstream of the proteasome. Disease-induced wasting may result from circulating factors, such as pro-inflammatory cytokines that have intracellular effects in muscle. Therefore nutritional interventions need not focus solely on those with close links to skeletal muscle anabolism, such as the amino acids.

1.6.5) Herbal nutritional supplementation

Recently studies have focused on the effects of natural plant medicine on stress and other chronic conditions (Rai *et al.*, 2003; Punkt *et al.*, 1999). These substances have the benefit of being cheaply produced, although substantive research to evaluate effects is still needed.

Pectora roborant cultus recti

Sutherlandia frutescens (also known as 'cancer bush'), a shrub with attractive flowers, is an indigenous South African herb, which is found mainly in the Western Cape and Karoo. It has been used for many decades by traditional healers to treat a variety of symptoms, which include inflammation, gastro-intestinal tract problems [http://www.sutherlandia.org/Sutherlandia-antioxidant properties.html (July 2005)], cancer [http://www.sutherlandia.org/cancer.html (July 2005)] and stress [http://www.sutherlandia.org/chronic-fatigue-sydrome.html (July 2005)]. Traditionally, the herb is ingested in the form of a tea made from the leaves, which has a bitter taste. Recently it has been used to treat people suffering from AIDS, with reports of attenuation of weight loss and remarkable recovery [van Wyk *et al*., (1997), http://www.sutherlandia.org/aids.html (July 2005)].

Chemical studies have identified that the plant is rich in L-canavaline, gamma-aminobutyric acid (GABA) and pinnitol (Singh *et al.*, 2001) and also contains small amounts of saponins. L-canavaline (a non-protein α-amino acid) is reported to have anti-cancer (Swaffar *et al.*, 1995) and anti-viral properties (Green, 1988). GABA is an inhibitory neurotransmitter and has mood elevating effects (Inagawa *et al.*, 2005), while pinnitol has anti-inflammatory (Cuellar *et al.*, 1997) and anti-diabetic effects (Kim *et al.*, 2005). Although the individual effects of these compounds are known, the combined effect of these as contained within the *Sutherlandia* herb has been scientifically reported only in the last two years.

Tai *et al.* (2004) showed that an ethanol extract of *Sutherlandia* had a concentration dependant anti-proliferative effect on several tumour cell-lines. They also demonstrated that *Sutherlandia* has ant-oxidant activity by reducing free radical cations in culture. Similarly Fernandes *et al.* (2004) showed that a hot water extract of *Sutherlandia* had hydrogen peroxide- as well as superoxide scavenging activities. Thus these novel findings support the anecdotal evidence that *Sutherlandia* has anti-inflammatory capabilities, which is probably the action of pinnitol that was described earlier, although other components are not excluded.

Pectora roborant cultus recti

Smith and Myburgh (2004) showed that a warm water extract of *Sutherlandia* is able to attenuate an immobilisation stress-induced increase in plasma corticosterone levels in rats. In a subsequent *in vitro* study, one of the pathways by which *Sutherlandia* has its effect was elucidated further (Prevoo *et al.*, 2004). Given these positive results, it is of importance to further investigate this herbal remedy as a possible anti-atrophy treatment.

1.7) Aims

1. To evoke a mild stress response in male Wistar rats using an incremental, but intermittent stress protocol.

2. To examine the effect of the stress regimen on parameters of atrophy, which include body mass and skeletal muscle mass. To determine specifically if *m. extensor digitorum longus* and *m. soleus* are affected differently or not.
3. To examine mechanisms possibly contributing to atrophy, specifically serum corticosterone concentrations, activity of the 20s proteasome, glutamine synthetase and tyrosine amino transferase activities.
4. To further investigate using this model whether *Sutherlandia* and/or exercise has any effect on plasma corticosterone and the selected glucocorticoid inducible enzymes TAT and GS, and whether the protein degradation pathway is affected by *Sutherlandia* supplementation.

Chapter 2

2) Materials & Methods

2.1) Experimental Animals

Male Wistar rats from the University of Cape Town breeding facility, weighing 240 – 320 g on arrival were used. Weight matched rats were divided into 6 groups so that each group had rats with a similar range of body mass. The rats were anaesthetised with ether and their ears cut with scissors for marking purposes. Then 1 rat from each of these groups was randomly selected and placed into a cage (50 x 30 x 20 cm) to make up groups of 4 rats per cage. Each cage housed a different experimental group. The housing room was soundproof, maintained on a 12:12 light/dark cycle (with lights on at 07:00) and temperature controlled (27 ºC). Standard rat chow and water were available *ad libitum* throughout the experiments. For three weeks the rats were allowed to acclimate to their new housing facility and recover from the anaesthetics, since the anaesthetic procedure is a stressful event, which could influence the effect the experimental stressors would have on the rat (Hashimoto *et al.*, 1988). Rats were accustomed to handling by weighing them each day before experimental procedures began and also throughout the study. Ethical approval was obtained from the research subcommittee C of the University of Stellenbosch.

2.2) Experimental design

The study consisted of three experiments. The first experiment lasted for one week. It consisted of only two groups, namely an unstressed control group (C) and a stressed group (S) with 6 rats in each. The second experiment lasted for 2 weeks (see Fig. 2.1 for diagrammatic illustration) and consisted of two control and 2 stressed groups, with 6 rats in each group. The control rats were not subjected to any intervention during the first week, but received either placebo (group CP) or *Sutherlandia* (group Csu) injections from the start of the second week daily for the entire week.

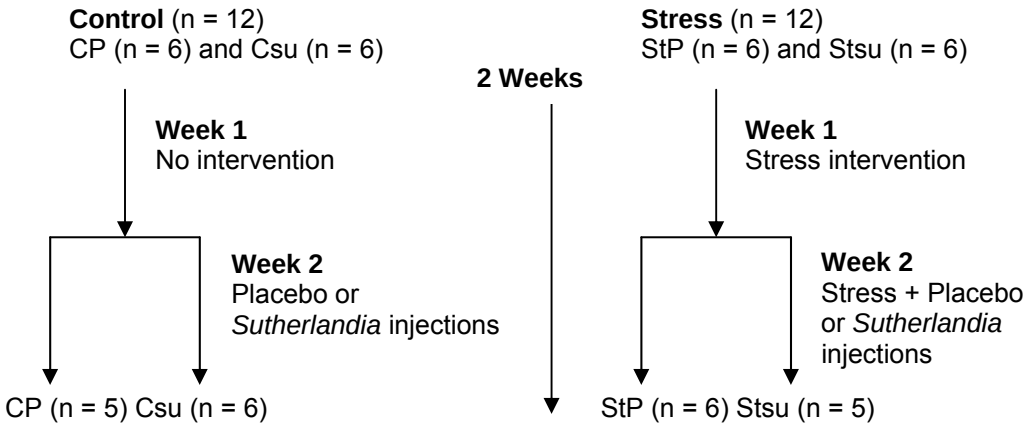

Figure 2.1 A diagrammatic outline of the 2-week experimental design of experiment 2

The 2 stressed groups were subjected to the stress protocol for the entire two weeks (see Table 2.1 for outline of stress protocol) and were injected with either a placebo (group StP) or *Sutherlandia* (group Stsu) solution daily from the start of the second week for the entire week. All the rats survived the entire duration of the study except for 1 rat each from Groups CP and Stsu. Both rats died from causes unrelated to the experiment. One rat had a broken neck (unsure how it happened), reported to me by the animal house caretaker and the other rat was probably fatally wounded during a fight with another rat because it had wounds on its mouth and neck. Dissection of both rats was undertaken by my supervisor to determine possible diseases related to the deaths, but none were detected.

The third experiment (see Fig. 2.2 for diagrammatic illustration) also lasted two weeks. The study consisted of a control (C) and stressed (S) induced group, with 12 rats in each group. Group C were not subjected to any intervention during the first week, while group S was subjected to the stress protocol for the entire two week experiment. Six rats from each group were sacrificed after the first week (Group C and S). The six remaining rats from each group were subjected to the exercise protocol (CE and StE) from the start of the second week for the entire week. Thus experiment 2 lasted two weeks, with all the rats surviving the entire duration of the experiment.

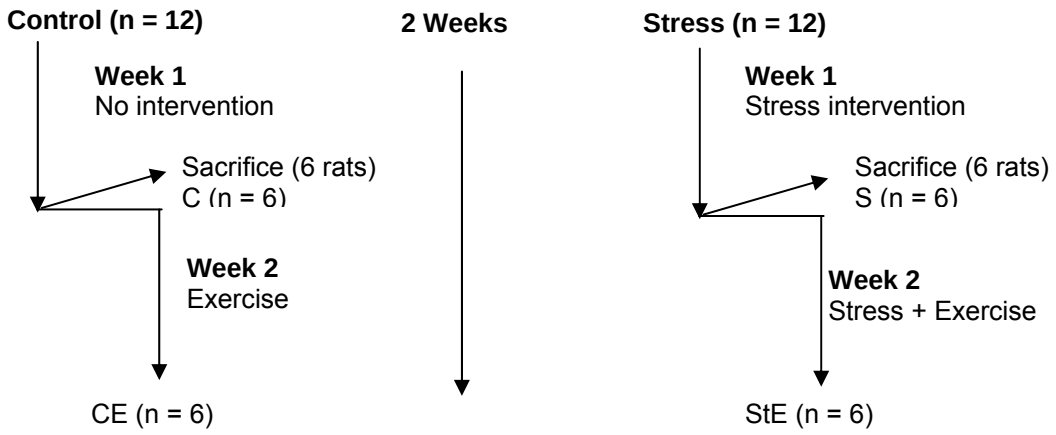

Fig. 2.2 A diagrammatic outline of the 2-week experimental design of experiment 3

2.3) Stress interventions

All rats divided into groups receiving stress, were subjected to the same incremental stress protocol of 2 weeks, which was unpredictable to them. The protocol was shown in a pilot study (see Appendix A) to induce an initial weight loss and to inhibit further weight gain. All stress procedures were carried out in the same room, separate from the housing facility. Four different stressors were used, namely immobilisation, cage tilt, water-soiled bedding and tail flick in response to high temperature exposure (whilst being immobilised). The protocol followed for each stress-inducing intervention is briefly described in the next few paragraphs, and the time-schedule for the entire protocol is given in Table 2.1.

2.3.1) Immobilisation: Rats were immobilised in a Perspex cage that was specially designed for this purpose by our research group (Department Physiological Sciences, Exercise group). Each immobilisation cage consists of 6 different compartments each big enough to house a single rat (dimensions: w x h x l, 6 x 8 x 18 cm). The rat is unable to move freely inside the container. The duration of each immobilisation session was 30 minutes (Almeida *et al*., 1998).

2.3.2) Cage tilt: The objective of this method was to change the body position of the rats whilst they were being immobilised. The immobilisation cage was turned

upside down so that the rat was oriented with its head facing down; this intervention lasted for thirty minutes as well (Harkin *et al.*, 2002).

2.3.3) Water soiled bedding: Another method to induce stress was to pour water (300 ml) into the housing cages. This has been shown previously to induce a stress response in male Wistar rats (Harkin *et al*., 2002). The rats were kept in the watersoiled cages for 1 hour and thereafter returned to their normal living conditions, which is similar to the protocol of Harkin *et al.* (2002).

2.3.4) Tail flick: The rat's tail was placed in a warm waterbath (49º C) until the rat voluntarily flicked it out (Kallina *et al*., 1994). This was done at the start of an immobilisation session whilst the rats were in their immobilisation cages according to the method described in paragraph 2.3.1.

2.4) Recovery interventions

Groups CP (Control placebo), Csu (Control *Sutherlandia*) and CE (Control exercise) received saline injections (0.9% NaCl, 2 x 0.5 ml/day), *Sutherlandia* injections (4 mg/ml, 2 x 0.5ml/day) or exercise (1 x 30 minutes/day) respectively for the duration of week 2 (Table 2.1). Groups StP (Stress placebo), Stsu (Stress *Sutherlandia*) and StE (Stress exercise) were treated exactly the same, but in addition were subjected to the stress stimuli as explained earlier.

2.4.1) Preparation of the *Sutherlandia* supplement

The Sutherlandia herb is traditionally ingested in the form of a tea. Therefore a warm water extract was prepared by adding boiling water to dried Sutherlandia leaves (8 mg/ml) (supplied by PhytoNova, Cape Town, South Africa). This was then infused for 18 hours at room temperature. The extract was then diluted 1:2 in 0.9 % sterile saline to produce an extract of 4 mg/ml, and 0.5 ml of the diluted extract was injected intra-peritoneally twice a day: once after the first stress session and once after the second session of stress each day (see Table 2.1 for time points of injections).

Table 2.1 Outline of the 2-week stress protocol with details of the day to day incremental stress procedures used, as well as time points that the recovery interventions were given

(* Interventions were given at the same time each day as shown on Day 8 for the entire 2nd week)

Day	Time	Procedure
1 – 2	08:00 – 08:30	Immobilisation
3 – 4	08:00 – 08:30	Immobilisation
	12:00 – 12:30	Immobilisation
5 – 6	08:00 – 08:30	Immobilisation
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Immobilisation
7	08:00 – 08:30	Cage tilt
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Cage tilt
8 –14	**Recovery Intervention**	
8	08:00 – 08:30	Soiled bedding
	08:40	* Placebo or *Sutherlandia* injection
	12:00 – 12:30	Immobilisation
	14:00 – 14:30	* Exercise
	15:30 – 16:00	Cage tilt
	16:10	* Placebo or *Sutherlandia* injection
9	08:00 – 08:30	Soiled bedding
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Soiled bedding
10	08:00 – 08:30	Soiled bedding
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Cage tilt
11	08:00 – 08:30	Tail flick + Immobilization
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Cage tilt
12	08:00 – 08:30	Cage tilt
	12:00 – 12:30	Immobilisation
	15:30 – 16:00	Tail flick + immobilisation
13 - 14	08:00 – 08:30	Soiled bedding
	12:00 – 12:30	Tail flick
	15:30 – 16:00	Cage tilt

Sutherlandia is commercially available as 700 mg tablets each containing 300 mg *Sutherlandia* leaf powder. Manufacturers of these tablets recommend a dose of one tablet twice daily (PhytoNova, Cape Town, South Africa). This recommended dose of the commercial product equals 9 mg per kilogram body mass for humans. This corresponds to a daily dose of about 3.4 mg per rat, which was rounded up to 4 mg per rat, to allow for weight gain during the study period.

The rats receiving placebo were injected with 0.5 ml of 0.9 % sterile saline (placebo) twice a day at the same time the *Sutherlandia* injections were administered to the Csu and Stsu groups.

2.4.2) Resistance training intervention

Groups CE and StE were subjected to an incremental weight training protocol. This was achieved by running in a motor driven wheel with the rat loaded with lead weights. Lead was cut into 10 g, 20 g, 50 g and 100 g square pieces. The weights were strapped around the rat's body, as near as possible to the forelimbs, with plaster (for details of how the weights were strapped see Appendix B). Each side of the body carried the same weight.

Rats were familiarised to running in the wheel for 7 days (for details of the familiarisation phase see Appendix C). After the familiarisation phase they were allowed to rest for a week before commencing with the actual exercise intervention, which lasted for 7 days (from day 7 to 14, see Table 2.1).

On the first day of the exercise intervention, rats carried 10% percent of their body weight whilst running at 15 m/min (Yarasheski *et al.,* 1990 and Harrison *et al.*, 2002). This speed was kept constant throughout the protocol while the weights were incrementally increased by 10% each day until 40% of body mass was reached (day 4), after which it was kept stable for the last 3 days. Exercise

intervention was carried out $1^{1}/_{2}$ hour after the second stress session of each day (Table 2.1).

2.5) Sample collection

Initiation of the intervention protocols was staggered, so that no more than 6 rats were sacrificed on one day. All sacrificing took place between 09:00 and 11:00 to prevent diurnal changes in hormone levels from becoming a confounding factor, which might mask small inter-group differences. Rats were decapitated within 3 minutes after removal from housing cages. Blood was collected by exsanguinations directly from the ascending aorta, through a heparinised funnel into 10 ml serum separator (SST) tubes (Vacutainer, Beckton Dickinson). Blood was allowed to clot at room temperature for 10 minutes, after which it was placed on ice. Clotted blood samples were then centrifuged at 0 °C for 10 minutes at 3000 rpm, serum aliquoted into eppendorf tubes and stored at – 40 °C until subsequent analysis.

Whole muscle samples were collected from the *m. extensor digitorum longus* (hereafter EDL) and *m. soleus* (hereafter simply soleus) of both hind legs. All muscle dissections were performed within 15 minutes after sacrifice. All samples were snap frozen in liquid nitrogen (within 1 minute of dissection) and then stored at –80 °C until subsequent analysis.

2.6) Analysis

Snap-frozen muscle samples were analysed for glutamine synthetase activity, 20s proteasome activity, and tyrosine aminotransferase activity, all of which were expressed relative to muscle protein concentration. Serum samples were analysed for total serum corticosterone concentrations.

Methods used for the above analyses are described below in paragraph 2.6.1 to 2.6.5

2.6.1) 20s Proteasome activity determination assay

Proteasome activity was determined using an assay kit supplied by (Chemicon International, catalogue no. APT280, Temecula, USA). The kit provides a means for assaying proteasome activity that recognizes the peptide substrate Leucine-Leucine-Valine-Tyrosine (LLVY, Meng *et al*., 1999). The assay is based on detection of the fluorophore 7-amino-4-methylcoumarin (AMC) after cleavage from the labeled substrate LLVY-AMC. The free AMC fluorescence can be quantified using a 380/460 nm filter set in a fluorometer (FLX800 Bio-Tek Instruments, Inc., Vermont, USA)

I will now describe the laboratory procedure used to extract the 20s proteasome enzyme from muscle tissue and the assay used to determine 20s proteasome activity.

Extraction of 20s Proteasome

Extraction was done on the EDL and soleus muscles from the right hind leg.

I. Muscle tissue was weighed (wet weight), thawed and cut finely in a weighing boat.
II. It was placed in a film canister (For the volume of the aggregate used a film canister was the appropriate size for the homogenization procedure with an Ultra turrax) and ice-cold buffer (pH 7.5) containing 50 mM Tris-HCl and 20% glycerol was added (buffer-tissue ratio, 8:1, v: w; typically 2ml buffer: 250 mg tissue).
III. Homogenisation was by means of a mechanical method (Ultra Turrax, Janke & Kunkel, KG) at a speed of 8000 repetitions per minute for 3 cycles of 10 seconds each.
IV. Homogenate was transferred to eppendorf tubes.
V. The homogenate was then centrifuged at 2 000 g for 10 minutes.
VI. The supernatant was further centrifuged at 10 000 g for 30 minutes.
VII. The subsequent supernatant (2 aliquots per sample) was then stored at -80º C until subsequent analyses.

Note: Extraction was done on *4 samples at a time*. *All steps were done on ice except for centrifugation and homogenization with Ultra-Turrax. Homogenization of muscle with Ultra-Turrax was not longer than 10 seconds at a time.*

The kit components include:

- A 10x assay buffer which consists of a 1.5 ml vial containing 250 mM 4-(2-hydroxyethyl)-1-piperazine athenesulfonic acid (HEPES), (pH 7.5), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Nonidet P-40 (NP-40), and 0.01% sodium dodecyl sulphate (SDS) (w/v);
- A 500 µg peptide substrate (Suc-LLVY-AMC),
- One vial containing 9.4 µg lactacystin (20S proteasome inhibitor) and
- A vial containing 2.2 µg AMC standard.

Generating an AMC standard curve

I. AMC was reconstituted with 800 µl dimethyl sulfoxide (DMSO) (giving a 15.625 µM stock solution).
II. Four 200 µl aliquots were made in 1.5 ml opaque Eppendorfs and 3 were stored at –20 °C until subsequent use.
III. The remaining aliquot was used to prepare a dilution series (in duplicate) in 1.5 ml opaque eppendorfs.
IV. The reconstituted AMC was diluted (1:2) with 1x Assay buffer to yield AMC standard in the concentration range of 0.121 µM –15.625 µM.
V. 100 µl of each dilution were added (in triplicate) to wells in a 96 well fluorometer plate.
VI. 100 µl of Assay buffer and 50% DMSO + Assay buffer were each included as blanks.
VII. Fluorescence was read using a 380/460 nm filter set.

Note: - AMC is light sensitive, explaining the use of opaque eppendorfs.

- *Readings were done in duplicate.*
- *A background reading of the fluorometer plate was done at least 30 min before the actual assay.*

Determination of Activity

I. Samples and proteasome substrate were thawed on ice.
II. Proteasome substrate stock solution was diluted 1:20 with 1x assay buffer in 1.5 ml opaque eppendorfs.
III. Assay mixtures (see Table 2.2 below) were prepared in 1.5 ml opaque eppendorf tubes.
IV. Assay buffer and DMSO were included as blanks.
V. The total volume from each sample (see Table 2.2) was added to a well in a 96 well fluorometer plate.
VI. Samples (covered with wrapping foil) were incubated for 2 hours at 37ºC.
VII. Fluorescence emission was read at 460 nm after excitation at 380 nm.

An additional experiment was done to validate whether 20s proteasome is present in the extraction sample.

I. Lactacystin was reconstituted with 20 µl DMSO giving a 1.25 mM solution
II. This was divided into 4 aliquots and stored at – 4 ºC until subsequent analysis.
III. 5 µl was added to the test sample (see Table 2.2).

Table 2.2 Example of actual Assay mixtures used to determine 20s Proteasome activity

	Assay Buffer	Extraction buffer	Proteasome extract	Lactacystin	d. H_2O	Substrate	Total volume
Buffer blank	10 µl	10 µl	-	-	80 µl	-	100 µl
Substrate blank	10 µl	10 µl	-	-	70 µl	10 µl	100 µl
Test sample	10 µl	7 µl	3 µl	-	70 µl	10 µl	100 µl
Test sample + lactacystin	10 µl	10 µl	3 µl	5 µl	62 µl	10 µl	100 µl

N.B The addition of lactacystin yielded an 85 % inhibition of the 20s proteasome activity. This result validated that the 20s proteasome was present in the muscle sample extract and was responsible for the substrate degradation in the assay mixture.

Note: - We used opaque Eppendorfs since the proteasome substrate is light sensitive

- Readings were done in duplicate

- A background reading of the fluorometer plate was done at least 30 min before the actual assay.

The fluorescence value was used to determine 20s proteasome enzyme activity (see Appendix D (i) for example of 20s proteasome enzyme activity calculation), which was expressed relative to muscle protein concentration (determined via Bradford assay method, see 2.6.4 for description of method).

2.6.2) Glutamine synthetase (GS) activity assay

Glutamine synthetase (GS) activity was measured by a colorimetric method (Minet *et al,* 1997). In short, in the physiological state GS catalyses the formation of L-Glutamine from L-Glutamate and NH_4^+. When replacing NH_4^+ with

hydroxylamine, y-glutamyl-hydroxamate is formed, which gives a characteristic color reaction after addition of ferric chloride.

I will now describe the laboratory procedure used to prepare the muscle tissues for analysis and the assay used to determine GS activity.

GS extraction

Extraction was done on the EDL and soleus muscle from the left hind leg

I. Muscle tissue was weighed (wet weight), thawed and cut finely in a weighing boat on ice.
II. It was placed in a film canister (For the volume of the aggregate used a film canister was the appropriate size for the homogenization procedure with an Ultra turrax) and 50 mM imidazole (pH 6.8) extraction medium was added (buffer-tissue, 8:1, v: w, typically 2ml buffer: 250 mg tissue).
III. Homogenisation was by means of a mechanical method (Ultra Turrax, Janke & Kunkel, KG) at a speed of 8000 repetitions per minute for 3 cycles of 10 seconds each.
IV. Homogenate was transferred to 1.5 ml reaction vials (eppendorfs, 2 aliquots per sample).
V. The homogenate was centrifuged at 4 500 g for 15 minutes at 4 ºC.
VI. The supernatant (2 aliquots) was stored at 0 ºC until subsequent analyses for enzyme activity within 1 week.

Note: Only 4 samples were homogenised at a time. *All steps were done on ice except for centrifugation and homogenisation with Ultra-Turrax. Homogenisation with Ultra-Turrax was not longer than 10 seconds at a time, before sample was placed back on ice.*

Preparations of stock solutions

The following stock solutions were made:

- 250 mM Imidazole chloride (pH 6.8),
- 50 mM L-Glutamine,
- 25 mM Hydroxylamine (pH 6.8),
- 25 mM sodium arsenate, and
- 2 mM $MnCl_2$,

All solutions were prepared in distilled water except for glutamine, which was dissolved in imidazole chloride (pH 6.8). The Imidazole chloride and hydroxylamine solutions were adjusted to pH 6.8 with potassium hydroxide. All the solutions were stored at +4º C except for glutamine, which was stored at -20º C. Reagents are stable for up to a month after storage (Minet *et al*., 1997).

Assay of enzyme activity

- The above-mentioned stock solutions were added (see table below for specific volumes) to 3 reaction vials to yield final concentrations (see table below) of the reagents in the mixture.

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Table 2.3 Example of reagent mixture of GS enzyme assay

Reagent	Volume (µl)	Concentration (mM)	pH
Imidazole-chloride	200	50	6.8
L-Glutamine	100	50	_
Hydroxylamine	200	25	6.8
Sodium arsenate	100	25	_
$MnCl_2$	200	2	_
ADP	100	0.16	_
Supernatant sample	100	_	_

Next is a brief description of the assay procedure used to determine GS enzyme activity.

I. Sample and adenosine diphosphate (ADP) were added last (in this order) to the reaction mixture (see Table 2.3) just before the whole mixture was placed into the 37º C waterbath.
II. The mixture was then incubated in a waterbath at 37º C for 20 minutes.
III. The reaction was stopped by adding 2 ml of a solution containing 2.42% $FeCl_3$, 1.45% trichloro-acetic acid (TCA) and 1.82% hydrochloric acid (HCl).
IV. It was then mixed (shaking the film canister) and 1 ml transferred to 1.5 ml reaction vials (eppendorfs).
V. This was then centrifuged at 4 500 g for 5 minutes to remove insoluble material.

The absorbance of the supernatant was determined spectrophotometrically (Varian, Cary 50, Chicago, USA) at 540 nm.
Values were corrected by a blank containing the total incubation mixture except for the homogenate supernatant and ADP. All determinations were performed in duplicate.

Note: - Sample extract was thawed beforehand
- Worked on ice
- Eight samples were assayed at a time (in duplicate)

The absorbence values were used to determine GS enzyme activity (see Appendix D (ii) for example of GS enzyme activity calculation). GS activity was expressed relative to muscle protein concentration (determined via Bradford assay method, see 2.6.4 for description of method).

2.6.3) Tyrosine aminotransferase (TAT) activity assay

TAT catalyzes the first reaction in the pathway by which tyrosine is initially converted to 4-hydroxyphenylpyruvate (ρHPP) and finally degraded to acetoacetate and fumarate.

The assay is based on the conversion of ρHPP to ρ-hydroxybenzaldehyde (ρHBA) after addition of KOH, which can be measured spectrophotometrically (Hayashi *et al.,* 1967).

Tissue Extraction

- Extraction of 20s proteasome and TAT were done on the same tissue with the same extraction method as described earlier (see 2.6.1, extraction of 20s proteasome).

Stock solutions of the following reagents were prepared:

- 0.007 M L -tyrosine in 0.125 M KH_2PO_4 buffer (pH 7.6),
- 0.5 M α-Ketoglutarate (αKG), adjusted to pH 7.0 by the addition of 10 M KOH,
- 0.005 M Pyridoxal-5′ phosphate (PLP) adjusted to pH 6.5 by the addition of 10 M Potassium hydroxide (KOH), and
- 10 M Potassium hydroxide.

All solutions were prepared in distilled water except for tyrosine, which was prepared in potassium dihydrogen phosphate (KH_2PO_4).

TAT Activity Determination

The above-mentioned stock solutions were added (in the indicated volumes below) to 1.5 ml eppendorf tubes to yield final concentrations (as indicated in brackets below) of the reagents in the mixture.

I. 160 µl of Tyrosine (7 mM) in 125 mM KH_2PO_4,
II. 4 µl of α−ketoglutarate (500 mM),
III. 2 µl of pyridoxal 5'-phosphate (5 mM) and

IV. 20 µl of tissue sample

(A blank was prepared by addition of 14 µl of 10 M KOH to the reaction mixture excluding the tissue sample).

I. The blank samples and reaction mixtures were incubated at 37 ºC for 10, 20, and 30 minutes respectively.
II. The reaction was stopped by adding 70 µl of 10 M KOH.
III. The conversion of ρHPP to ρHBA was allowed (30 minutes) at room temperature.
IV. The reaction mixtures were transferred to 96 well plates.
V. The endpoint absorbance was read at 330 nm with an Enzyme-Linked Immunoserbent Assay (ELISA) plate reader (ELX800, Biotek Instruments, Inc., Vermont, USA).

One unit of enzyme was defined as the quantity that catalyzes the formation of 1 µmol of ρHPP per minute at 37ºC.

Note: - Sample extract was thawed beforehand
- Worked on ice
- Eight samples were assayed at a time
- All samples were done in duplicate

The change in absorbence per minute was used to determine TAT enzyme activity (see Appendix D (iii) for example of TAT enzyme activity calculation), which were expressed relative to muscle protein concentration (determined via Bradford assay method, see description of method later).

2.6.4) Protein concentration determination

The Bradford assay was used to determine the protein concentration of each of the final supernatants of the muscle samples which were used to determine the

specific enzyme activities previously described. The Bradford assay was done on the same day that the specific enzyme activities were determined.

Table 2.4 Reagents used in the Bradford assay

Reagents	Final Concentration
Coommassie G250 powder	0.02%
95% (v/v) Ethanol (EtOH	4.75%
Distilled water	
Phosphoric acid (H_3PO_4; 85% M/V)	8.75%
Whatman no. 1 filter paper	
Bovine Serum Albumin (BSA) crystals	
100 mM Phosphate Buffer pH 7.4	

Preparation of Bradford reagent

I. Coommassie Brilliant Blue G250 (0.1 g) was weighed and 34 ml 95% ethanol added.
II. 50 ml 85% (m/v) phosphoric acid (H_3PO_4) was then slowly added and stirred with a glass rod.
III. The above mixture was added to 100 ml distilled water (dist.H_2O) in a 500 ml volumetric flask. Dist.H_2O was added to up to the 500 ml mark.
IV. It was then filtered through filter paper (x3),
V. and stored at 4°C in a brown glass bottle.

Generating a BSA standard curve

I. Bovine Serum Albumin (BSA, 5 mg) was weighed and 10 ml of 100 mM phosphate buffer added (pH 7.4) to get 0.5 mg/ml BSA.
II. This was stored at 2 °C.

III. A dilution series (see Table 2.5 below) of the 0.5 mg/ml BSA was prepared in test tubes.

Table 2:5 Mixtures used to generate a BSA standard curve

Tube no	0.5mg/ml BSA (µl)	Phosphate buffer (µl)	[BSA] (mg/ml)
0 (Blank)	0	60	0
1	12	48	0.1
2	24	36	0.2
3	36	24	0.3
4	48	12	0.4
5	60	0	0.5

I. 3 ml of Bradford reagent was added to each of the above tubes
II. After 5 minutes each of the reaction mixtures were decanted into 3 ml cuvettes and read at 595 nm on spectrophotometer (Varian, Cary 50, Chicago, USA)

Sample concentration determination

I. Sample (5 µl) was added to 55 µl of 100 mM Phosphate buffer (pH 7.4) in test tubes.
II. 3 ml of Bradford reagent were added and immediately vortexed.
III. It was then incubated for 5 minutes at room temperature. (The mixture is stable for 1 hour).
IV. It was then decanted into 3 ml cuvettes and the absorption immediately read at 595 nm.

Note: - Sample extract was thawed beforehand.
- Worked on ice.
- Eight samples were assayed at a time.
- All samples were done in duplicate.

2.6.5) Hormone analysis

Serum corticosterone concentration was determined using an enzyme immunoassay kit (IDS limited. Catalogue no. AC-14F1, Lot: 52895, Fountain Hills, USA). The assay is based on the principle of competitive binding. The microtiter plates are coated with a polyclonal corticosterone antibody. The corticosterone in the test sample competes with enzyme (horseradish peroxidase) labeled corticosterone for binding to the antibody coated wells. A colour reaction is formed after addition of tetramethylbenzidine (TMB) substrate, which binds to the horseradish peroxidase. The amount of bound peroxidase is inversely proportional to the corticosterone concentration in the sample. Thus the intensity of the colour developed is inversely proportional to the concentration of corticosterone in the sample. The colour reaction was quantified with an ELISA plate reader (ELX800, Biotek Instruments, Inc., Vermont, USA) at 450 nm. The percentage binding of each standard was calculated (Percentage binding = mean absorbance of each standard/mean absorbance of '0' standard x 100). A standard curve was then constructed in an Excel worksheet (Microsoft Office 2003) by plotting the percent binding (on x-axis) obtained from each standard against the concentration (y-axis). An exponential curve was gained. The concentration of corticosterone in each sample was obtained by multiplying the value read from the curve with the dilution factor. The kit does not give an exact concentration range, however as a guide they show the concentration range of 16 normal rat plasma samples from 23 - 363 ng/ml.

I thank dr. Martin Kidd for assisting with the calculation of the hormone concentrations.

2.6.6) Statistical analysis

All data were entered into a Statistica (version 7) worksheet. Box plots were plotted in Statistica, while all other graphs were constructed in Excel (Microsoft Office 2003). All data are expressed as means ± SD. Comparisons between Group C and S were performed using the non-parametric t-test. All other analysis

was made using analysis of variance (ANOVA). If statistically significant differences were found by ANOVA, comparisons between groups were further made by Bonferroni *post hoc* analysis. Results were considered significant when $P < 0.05$. A qualified statistician (dr. M. Kidd) assisted the candidate with all statistical analyses.

Chapter 3

3) Results

In this chapter I will present the data in order to answer the questions set out in my aims section.

3.1) Effect of stress exposure alone for 1 week

This section presents the comparison of results obtained for rats sacrificed after 1 week (groups C and S). There was no difference in body mass at the start of the study between group C and S (C: 562 ± 91 g; S: 575 ± 44 g; P = 0.75). At the end of the 1 week protocol, there was an effect of stress on body mass, with the S group losing 47 ± 15 g (8 %) of body mass (Fig. 3.1.1), whereas the body mass of group C was steady over the 1 week during which C received no intervention.

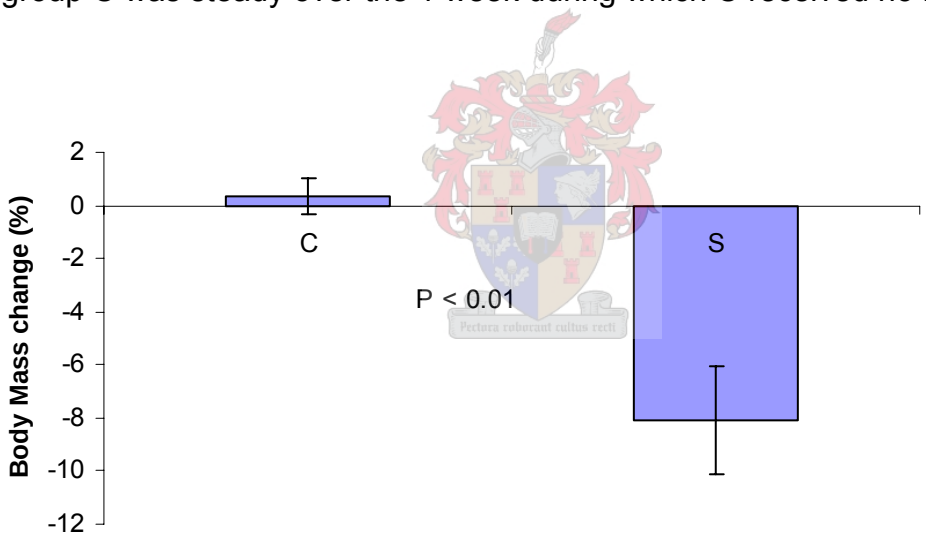

Fig. 3.1.1 Effect of stress on body mass expressed as percentage change. Control (n = 6; C) and stressed rats (n = 6; S) sacrificed after 1 week. Data presented as means ± standard deviation (SD), statistical analysis was performed using the non-parametric t-test.

Whole muscles were weighed, but also did not differ between groups for either muscle (soleus mass: C = 251 ± 27 mg, S = 261 ± 17 mg, EDL mass: C = 231 ± 32 mg, S = 222 ± 20 mg). Because of the inter-individual differences in body mass between the rats within each group, I analysed these data in two more ways. Due to non-significant correlations between body mass and muscle mass

(soleus: r = 0.30, EDL: r = 0.06), I will not report muscle mass covarying for body mass. Instead I calculated the soleus mass to body mass ratio. This derived parameter also indicated that there was a range in muscle masses within each group. There was no significant difference in the soleus mass to body mass ratio between groups C (0.45 ± 0.06 mg/g) and S (0.49 ± 0.03 mg/g) or EDL mass to body mass ratio (C: 0.42 ± 0.09 vs. S: 0.42 ± 0.03 mg/g).

Despite no difference in the gross measurements described above, measures of proteasome activity did differ. There was a significant difference in proteasome activity in the soleus muscle between groups C and S ($P < 0.05$, Fig. 3.1.2 A), as well as in the EDL muscle ($P < 0.05$, Fig. 3.1.2 B). The relative increase in mean proteasome activity was 24.8% for the soleus and 39.2% for the EDL.

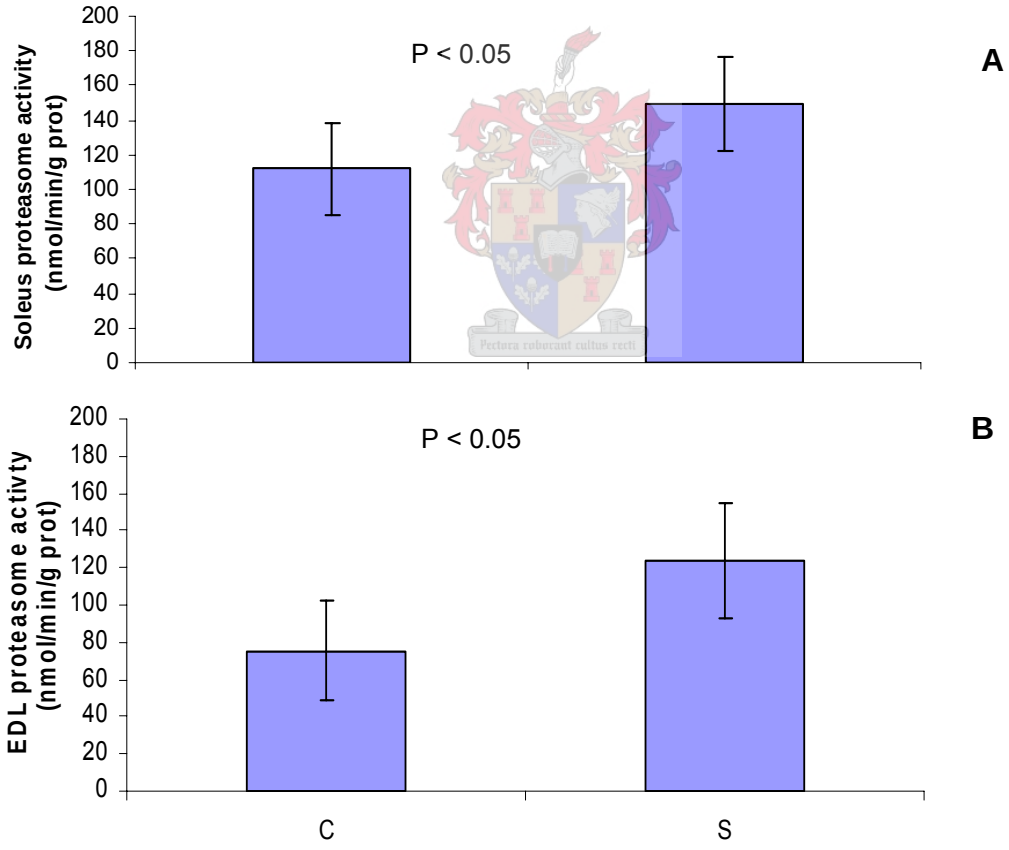

Fig. 3.1.2 Effect of stress on proteasome activity in (A) soleus and (B) EDL muscle of rats sacrificed after 1 week (day 1 to 7). For abbreviations and statistical analysis see legend to Fig. 3.1.1.

In contrast to the proteasome activity described above, there was no significant difference in GS activity in either the soleus (Group C: 869 ± 200 nmol/min/g protein and Group S: 954 ± 58 nmol/min/g protein) or EDL muscle (Group C: 1181 ± 518 nmol/min/g protein and Group S: 1058 ± 98 nmol/min/g protein) between groups C and S. Although there seems to be a relative increase in soleus GS between S vs. C, the SD in group C was quite high. There was also no significant effect of stress on TAT activity in either the soleus (Group C: 8.74 ± 2.07 µmol/min/g protein and Group S: 8.68 ± 2.77 µmol/min/g protein) or EDL muscle (Group C: 9.20 ± 2.67 µmol/min/g prot and Group S: 8.73 ± 2.97 µmol/min/g prot) in both groups C and S.

Stress also had no significant effect on serum corticosterone concentration (C: 55.3 ± 57.9 ng/ml vs. S: 45.0 ± 49.6 ng/ml). There was one outlier in each group (see Fig. 3.1.3).

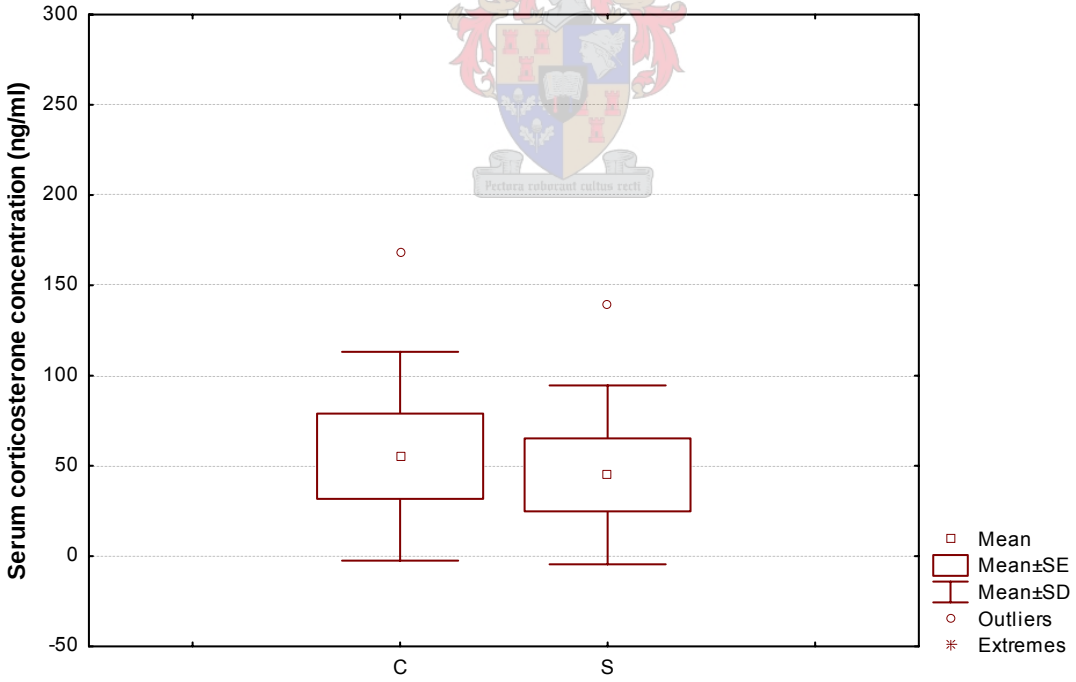

Fig. 3.1.3 Box plot analysis of serum corticosterone concentration

3.2) Was *Sutherlandia* supplementation able to reverse the effects of stress despite continuation of the stress intervention?

To answer this question, four groups were compared to assess the effect of two interventions namely Stress (St) or *Sutherlandia* (Su). Similar to the first section one group was subjected to stress, whereas one was not; however to control for the possible effect of injecting Su both these groups were injected with a placebo (CP and StP). Two corresponding groups received Su (Csu, Stsu).

Body mass of the four groups at sacrifice was: CP = 546 ± 78 g, StP = 521 ± 34 g, Csu = 575 ± 41 g and Stsu = 482 ± 41 g. I also determined how body mass in these rats had changed over the days. There was a significant main effect of stress on body mass, with the absolute body mass change from day 1 to 14 ($P < 0.05$), day 1 to 7 ($P < 0.01$) and day 7 to 14 ($P < 0.05$) significantly different in StP and Stsu compared to groups receiving no stress. Similarly there was a significant effect of stress on percentage change in body mass from day 1 to 14 (Fig. 3.2.1 A, $P < 0.01$). Bonferroni *post hoc* analysis revealed that the difference between CP and StP was a trend, whereas Stsu clearly lost significantly more body mass than Csu ($P < 0.01$).

Stress caused a significant reduction in absolute body mass in the first week (from day 1 to 7) of the experimental protocol (Fig. 3.2.2) in CP vs. StP ($P < 0.0001$), CP vs. Stsu ($P < 0.001$), Csu vs. StP ($P < 0.001$) and Csu vs. Stsu ($P < 0.001$).

However there was no significant difference in absolute body mass change from day 7 to 14 between any of the groups.

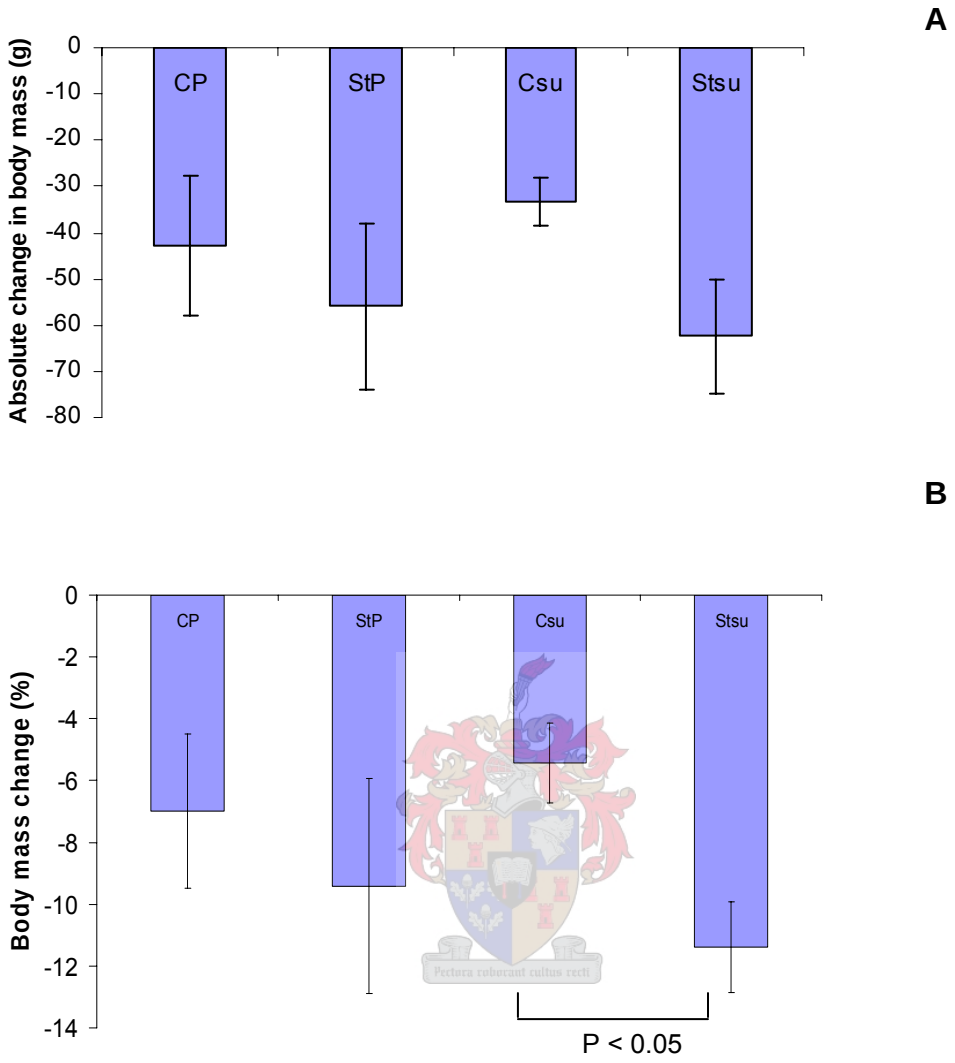

Fig. 3.2.1 (A) Absolute change and (B) percentage change in body mass over 2 week study duration (day 1 to 14) of control rats which received either placebo (CP) or *Sutherlandia* injections (Csu) or stressed rats which received either placebo (StP) or *Sutherlandia* injections (Stsu). Statistical analysis was performed using a factorial ANOVA with Bonferroni *post hoc* test: No interaction effect; group differences indicated.

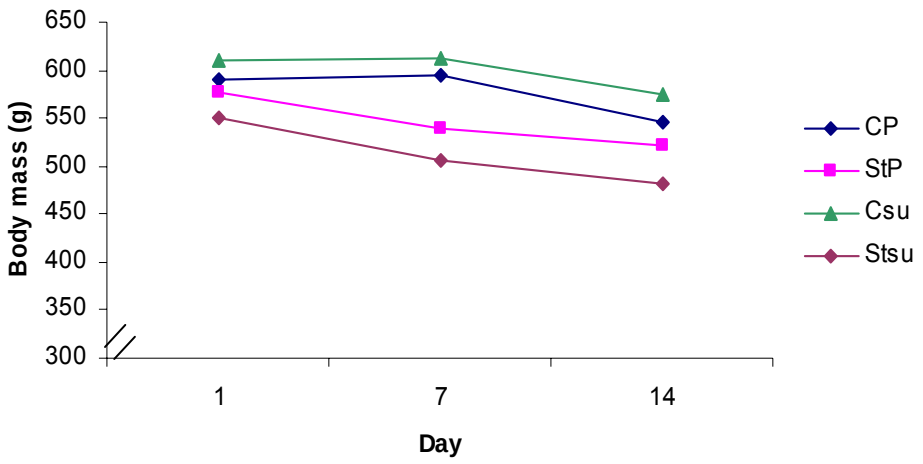

Fig. 3.2.2 Body mass change from the start (day 1) of the study to the day of sacrifice (day 14)

Actual soleus mass was: CP = 277 ± 15 mg, StP = 239 ± 8 mg, Csu = 273 ± 10 mg and Stsu = 248 ± 20 mg. Actual EDL mass was: CP = 261 ± 12 mg, StP = 209 ± 7 mg, Csu = 260 ± 16 mg and Stsu = 239 ± 14 mg. Stress was unable to induce a significant effect on soleus muscle mass to body mass ratio (Fig. 3.2.3 A).

However, EDL to body mass ratio was significantly less in StP vs. CP (Fig. 3.2.3 B, $P < 0.05$) and *Sutherlandia* treatment was able to significantly attenuate the stress induced loss in EDL muscle mass in Stsu vs. StP (Fig. 3.2.3 B, $P < 0.05$). The inserted graph in Fig. 3.2.3 (B) illustrates the interaction between the different groups with $P < 0.05$. Stressed placebo (StP) rats lost EDL muscle mass compared to control placebo rats (CP) as a result of stress but the effect of stress on the EDL muscle was inhibited when stressed rats received *Sutherlandia* treatment.

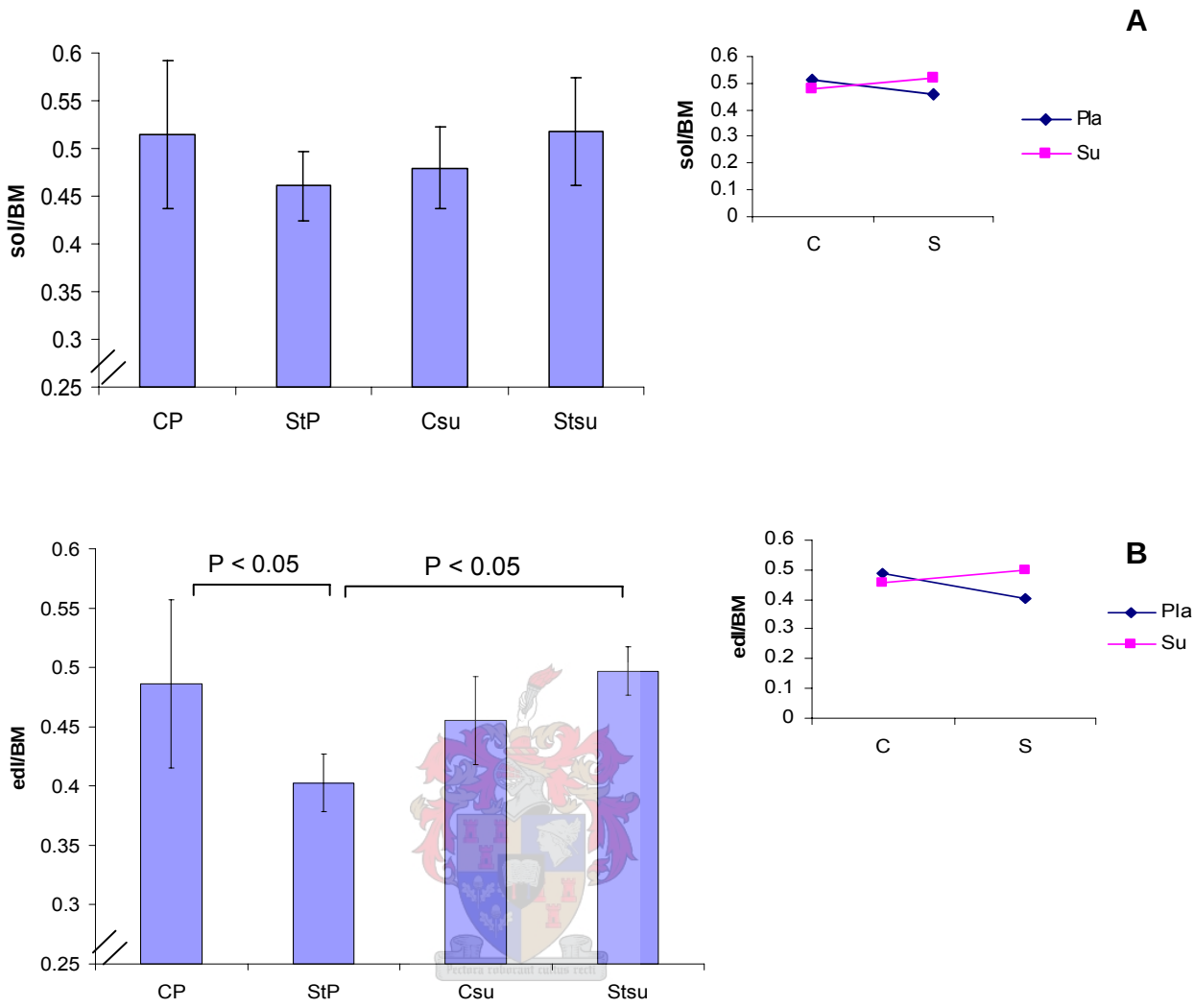

Fig. 3.2.3 Inter-group differences in (A) soleus to body mass (sol/BM) ratio and (B) EDL to body mass (EDL/BM) ratio. Statistical significance of Bonferroni *post hoc* analysis indicated in the bar graphs, while the inserted graphs are used to indicate interaction between groups (C and S) and treatment (*Sutherlandia* and Placebo). For group abbreviations, see legend to Fig. 3.2.1.

There was also a significant main effect of stress on proteasome activity in the soleus ($P < 0.05$). Bonferroni *post hoc* analysis revealed that proteasome activity in soleus muscle was significantly higher in StP vs. CP (Fig. 3.2.4 A, $P < 0.01$), StP vs. Csu ($P < 0.05$) and StP vs. Ssu ($P < 0.05$). Similarly the interaction graph (insert in Fig. 3.2.4 A, $P < 0.05$) indicates that stress caused a significant increase in proteasome activity in the soleus muscle of stressed placebo (StP) rats compared to control placebo (CP) rats but the effect of stress on proteasome

activity was inhibited when stressed rats received *Sutherlandia* treatment (Stsu). However, since Csu differed from CP, one could also interpret the results to mean that there was not an additive effect of stress and *Sutherlandia* in group Stsu.

The proteasome activity in EDL was increased by stress. However the increase in proteasome activity as a result of stress was significantly attenuated when treated with *Sutherlandia*, indicated by a significant difference between these two groups (Fig. 3.2.4 B, Stsu vs. StP, $P < 0.05$). $P < 0.05$ for the interaction effect of *Sutherlandia* and stress on EDL proteasome activity (Inserted graph in Fig. 3.2.4 B)

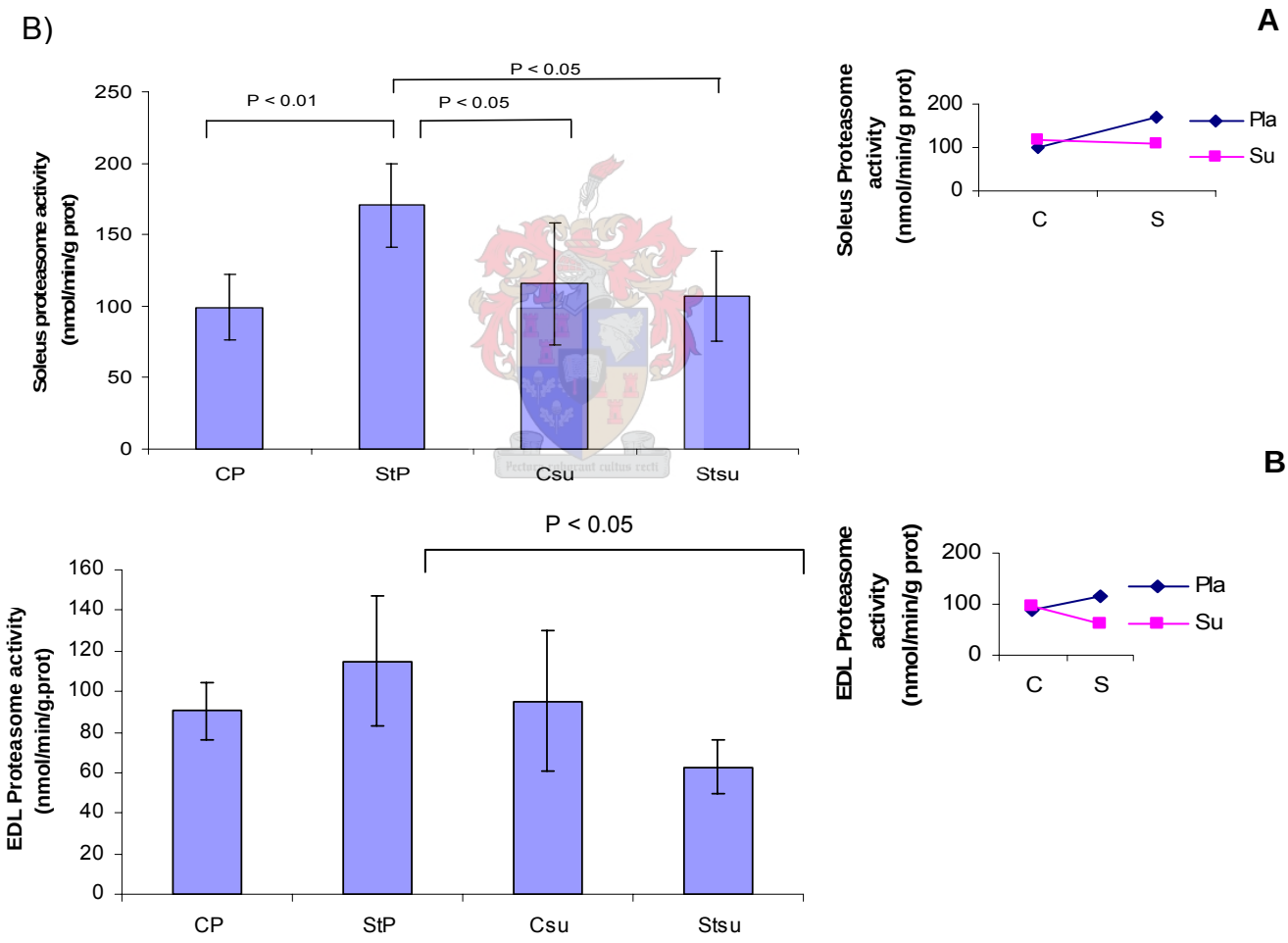

Fig. 3.2.4 Inter-group differences in (A) soleus proteasome and (B) EDL proteasome activity measured by Bonferroni *post hoc* analysis (bar graphs). Inserted graphs indicate interaction between *Sutherlandia* and placebo treatment in (A) soleus proteasome and (B) EDL proteasome activity of control and stress rats. For group abbreviations, see legend to Fig. 3.2.1.

Stress also had a significant main effect on GS activity in the soleus ($P < 0.01$) and EDL ($P < 0.01$). The GS activity in soleus muscle was significantly higher in StP vs. CP (Fig. 3.2.5 A, $P < 0.01$) and StP vs. Csu ($P < 0.01$). *Sutherlandia* treatment was unable to attenuate the stress-induced increase in GS activity (Csu vs. Stsu), (Fig. 3.2.5 A), although the level of significance was reduced to $P < 0.05$. Similarly GS activity in EDL was significantly greater in StP vs. CP (Fig. 3.2.5 B, $P < 0.01$) and StP vs. Csu ($P < 0.01$), with *Sutherlandia* treatment unable to significantly attenuate the stress induced increase in GS activity (Fig. 3.2.5 B).

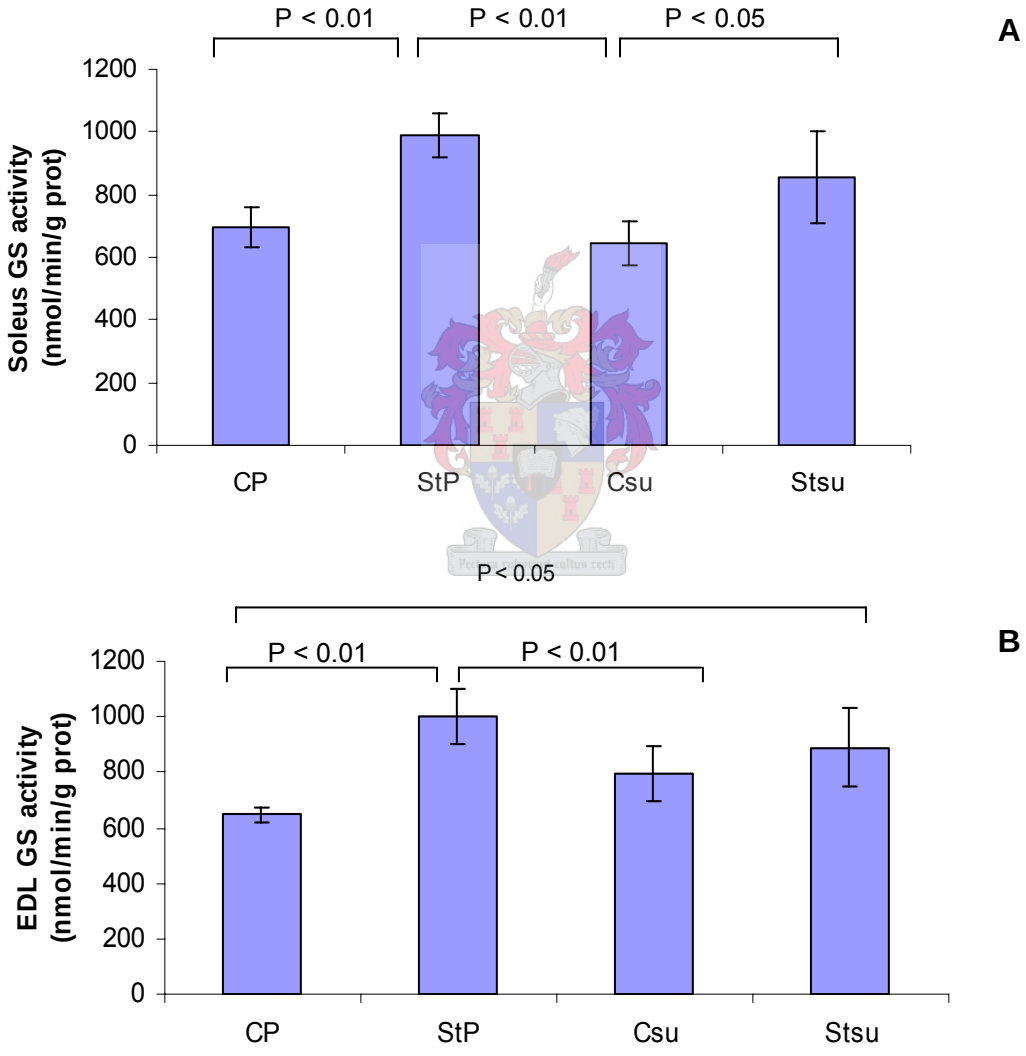

Fig. 3.2.5 GS activity in (A) soleus and (B) EDL muscle of control rats which received either placebo (CP) or *Sutherlandia* injections (Csu), or stress rats which received either placebo (StP) or *Sutherlandia* injections (Stsu). For statistical analysis refer to legend to Fig. 3.2.1

Stress had no significant effect on TAT activities in either the soleus (Fig. 3.2.6) or EDL. Similarly *Sutherlandia* administration was unable to induce any significant effect on TAT activities in either the soleus or EDL muscle. However there was an interaction effect of TAT activity in the soleus muscle between the different groups (Fig. 3.2.6 insert). The interaction graph in Fig. 3.2.6 illustrates that TAT activity increased in soleus of stressed rats compared to stress but the effect of stress on TAT activity was inhibited when stressed rats received *Sutherlandia* treatment (Stsu).

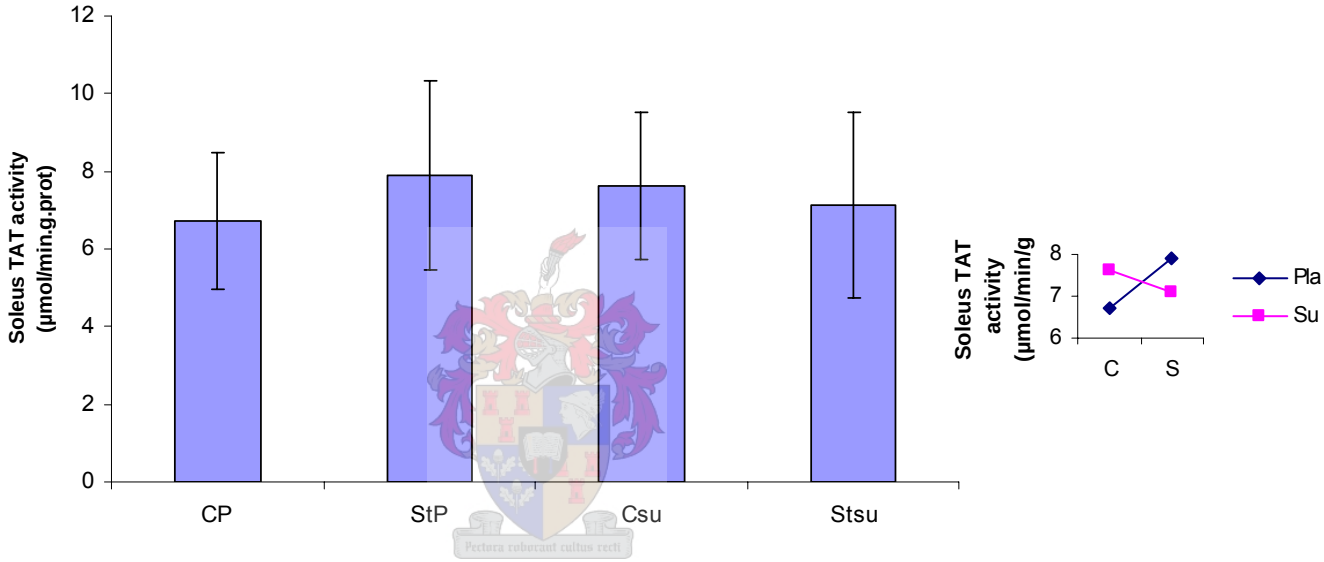

Fig. 3.2.6 No inter-group differences in soleus TAT activity (Bonferroni *post hoc* analysis), while the inserted graph indicates interaction between *Sutherlandia* and placebo treatment on soleus TAT activity of control and stress rats.

Serum corticosterone (Fig. 3.2.7) concentrations had a high level of inter-individual variation with some statistically identified outliers and there was no significant difference between any of the groups.

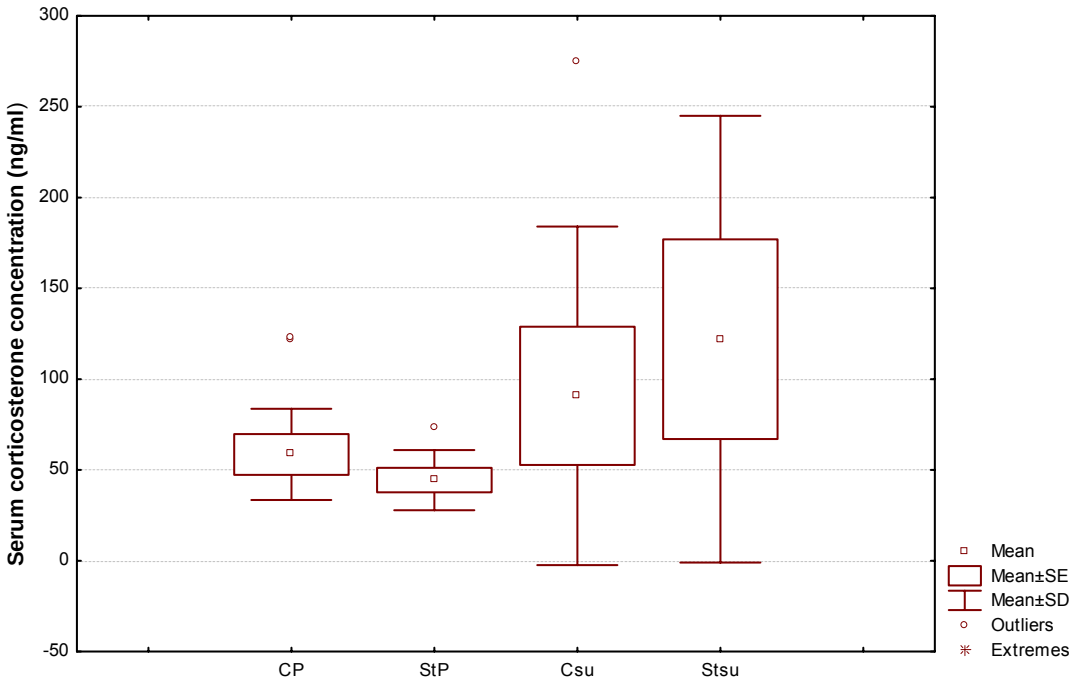

Fig. 3.2.7 Box plot analysis of serum corticosterone concentration

3.3) Was exercise able to reverse the effect of stress despite the continuation of stress?

To answer this question, we compared the results of 4 groups: C and S sacrificed after 1 week and CE and StE which were treated exactly the same as C and S in week 1, but had exercise added in week 2. See Fig. 3.3.1 for absolute body mass change from the start (day 1) of the study to the day of sacrifice (day 7 and 14). Bonferroni *post-hoc* analysis indicated that exercise was unable to reverse the stress induced loss in body mass (Fig. 3.3.2). Since the fat mass component of body mass could possibly account for body mass loss induced by exercise, this does not imply that parameters used as markers of muscle breakdown would be elevated. The exercise protocol significantly attenuated the stress-induced increase in EDL proteasome activity (Fig 3.3.3 A) in StE vs. S. However, there was no significant difference between any of the groups for all other parameters (see Table 3.1).

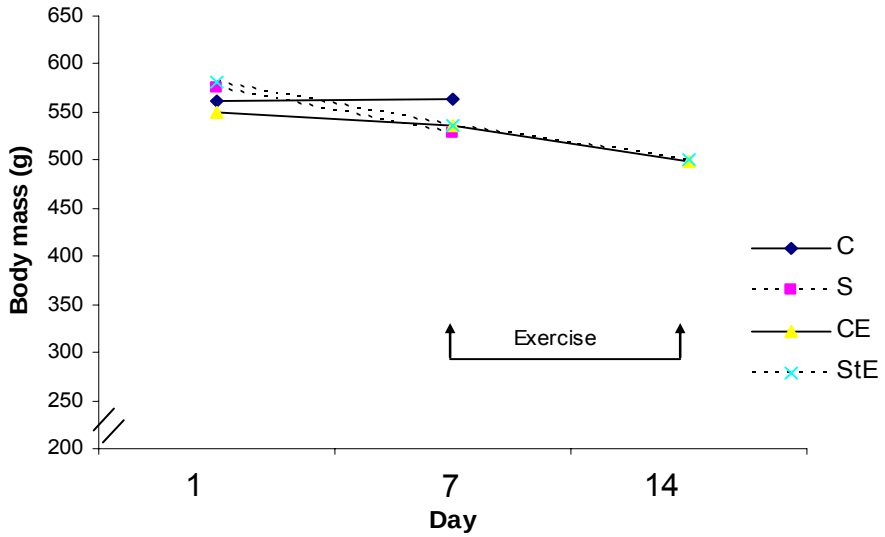

Fig. 3.3.1 Body mass change from the start (day 1) of the study to the day of sacrifice (day 7 and 14)

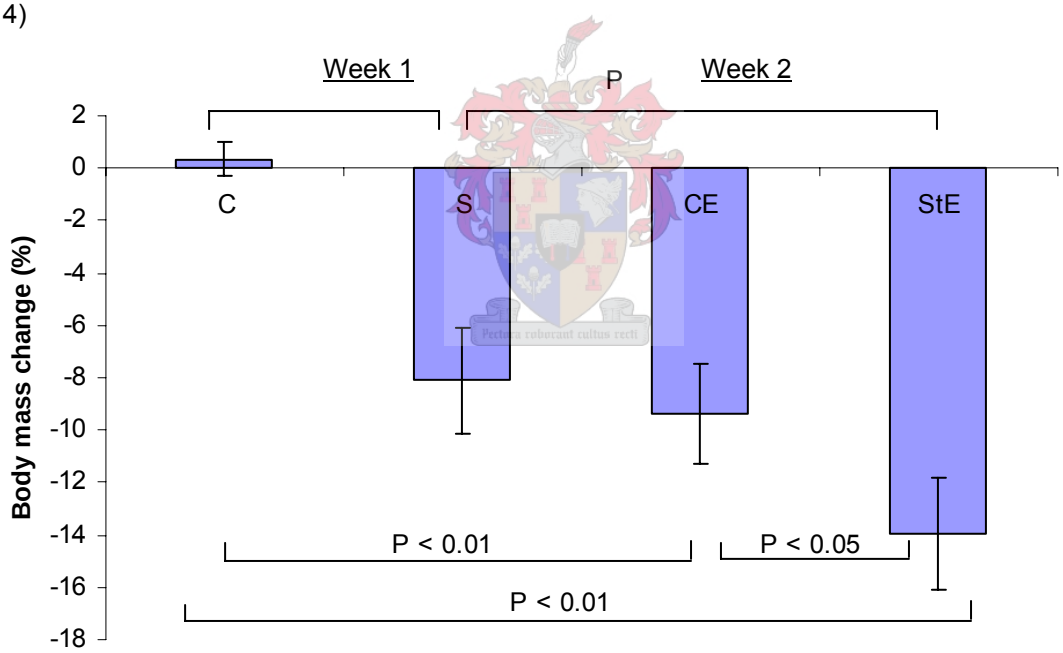

Fig. 3.3.2 Comparison of percentage body mass change between control (C) and stress rats (S) sacrificed after 1 week and exercise rats (CE and StE) participating in the 2 week study protocol.

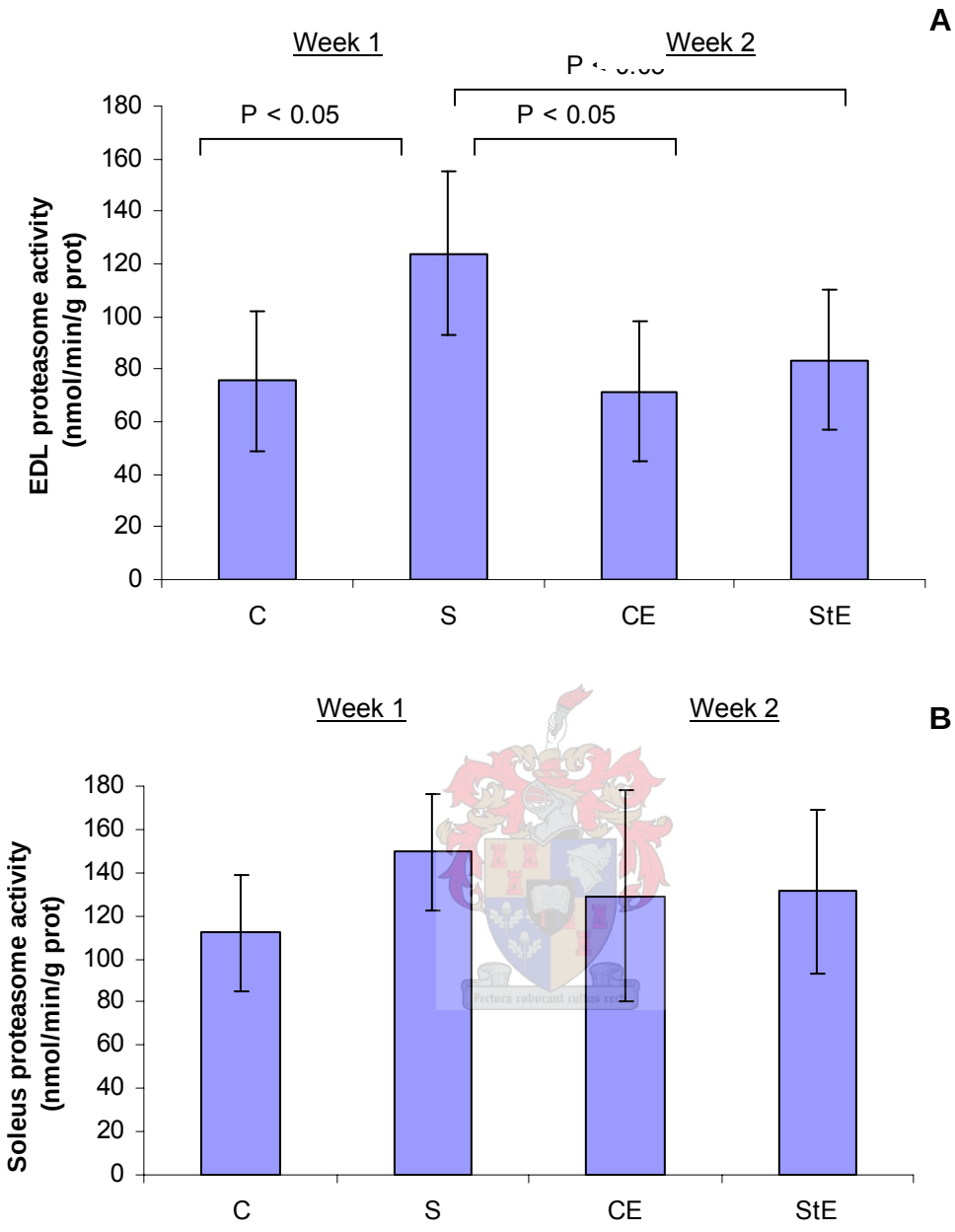

Fig. 3.3.3 Comparison of proteasome activity in (A) EDL and (B) soleus muscle between control (C) and stress rats (S) sacrificed after 1 week and exercise rats (CE and StE) participating in the 2 week study protocol.

Table 3.1: Results of non-significant data for Groups C, S, CE and SE

	Group C	Group S	Group CE	Group SE
Soleus mass (g)	251 ± 27	261 ± 17	242 ± 14	252 ± 26
EDL mass	231 ± 32	222 ± 20	220± 13	214 ± 24
Sol/BM (mg/g)	0.45 ± 0.06	0.49 ± 0.03	0.50 ± 0.06	0.49 ± 0.03
EDL/BM (mg/g)	0.42 ± 0.09	0.42 ± 0.04	0.44 ± 0.03	0.42 ± 0.03
Sol. proteasome activity (nmol/min/g prot)	112 ± 27	149 ± 27	129 ± 49	131 ± 38
Soleus GS activity (nmol/min/g prot)	869 ± 200	954 ± 58	1021 ± 105	999 ± 183
EDL GS activity (nmol/min/g prot)	1181 ± 518	1058 ± 98	1019 ± 65	989 ± 165
Soleus TAT activity (µmol/min/g prot)	8.74 ± 2.07	8.68 ± 2.77	8.83 ± 1.16	8.93 ± 2.75
EDL TAT activity (µmol/min/g prot)	9.20 ± 2.66	8.73 ± 2.97	8.86 ± 2.65	6.96 ± 2.519
Corticosterone (ng/ml)	55.4 ± 57.9	45.0 ± 49.6	72.9 ± 47.5	87.4 ± 80.3

Data presented as means ± SD

Pectora roborant cultus recti

Chapter 4

4) Discussion

In the next sub-sections I will attempt to elucidate with possible explanations the findings obtained in the results section. Although not the only findings that I will discuss the main findings for experiment 1 were:

i) 1 week of stress exposure decreased body mass and increased the proteasome activity of both predominantly fast and slow twitch muscles.

ii) Proteasome activity was more responsive to 1 week of stress than GS or TAT activities.

4.1) One week of stress exposure

In the next sub-section I will discuss the effects of the 1 week stress protocol on each of the parameters measured.

4.1.1) Body and muscle masses

Our stress intervention caused a significant reduction in body mass after 1 week in Group S (Fig. 3.1.1), whereas no significant change was measured in the absolute or relative muscle weights of the soleus and EDL. Thus the stressed rats (Group S) lost body mass, which was not accounted for by any significant decrease of muscle mass. This was not an expected result, since body mass loss in response to stress is often associated with concomitant atrophy of muscle mass (Hickson *et al*., 1996, Elgadi *et al*., 1998).

However my study did differ from these and there are some possible explanations for my results:

i) *Not strong or sustained enough stress stimulus*

It is probable that the psychological stress intervention used in my study was not severe enough, or sustained for long enough, to affect skeletal muscle mass within one week.

Compared to continuous stress interventions such as sepsis (Voisin *et al.*, 1996), hindlimb immobilization (Taillandier *et al.*, 1996) and glucocorticoid administration (Hickson *et al*., 1996), our psychological stress model included only 30 minutes of immobilisation stress exposure on Day 1, increasing to 3 x 30 minutes of 2 different types of stress exposure (immobilisation and cage tilt) on day 7. The intermittent nature of the stress may have had a milder effect than the 24 hours of continuous exposure used by the other studies mentioned above. Although there are other studies in the literature using intermittent stress models (Grippo *et al.*, 2002, Kallina and Grau, 1995), to my knowledge none of them measured both body mass and mass of individual muscles.

It is still necessary for me to propose an explanation for the loss of body mass. As opposed to e.g. hindlimb suspension (Taillandier *et al*., 1996) which specifically targets the peripheral limb, the response to psychological stress is systemic, including endocrine and cytokine responses that can influence any type of tissue (Grippo *et al*., 2005). I propose that a systemic stress response was intermittently present and that it may have influenced the fat deposits. There is evidence from human subjects that acute exposure to psychological stress markedly increases the lipolytic activity in adipose tissue (Hagstrom-Toft *et al.*, 1993, Wennlund *et al*., 1994). Therefore, in my study catabolism of fat may have spared the muscle tissue by a significant amount, despite upregulation of muscle proteasome activity (for further discussion of proteasome results see section 4.1.2).

ii*) Larger muscle groups*

Another possible explanation is that larger muscle groups could have been affected by stress to a greater extent, instead of the soleus and EDL muscles which I investigated. For example, Hickson *et al*. (1996) did show decreased muscle mass in gastrocnemius and quadriceps muscle groups. However Mitch *et al*. (1994) did show decreased muscle mass in another small muscle, the

epitrochlearis muscle, within 1 week of imposing acidosis. Again this protocol is much more severe than mine.

iii) *Group size*

In this part of my study there were 6 rats in each group (C and S; n = 6 each). It is possible that with more rats, I could have shown a decrease in EDL mass, because a small change of -3.9% was observed. Other studies have shown that fast twitch muscles are more susceptible to catabolic stimuli (Hobler *et al.*, 1999, Hickson *et al*., 1996).

4.1.2) 20s Proteasome activity

Despite no significant effect on muscle mass, there was an effect of stress on both the soleus and EDL, since the 20s proteasome activity was significantly increased in both muscles in Group S vs. C (Fig. 3.1.2 A & B). This finding might seem to be in opposition to the maintenance of muscle mass, since it could be expected that activation of the 20s proteasome should cause skeletal muscle degradation.

Possible reasons for increase in 20s proteasome activity without reduction in muscle mass, are:

i) *Intact sarcomere*

Although the 20s proteasome was activated, the myofibrillar proteins were still intact as part of the sarcomere, since it is well known that the 20s proteasome is unable to degrade intact myofibrillar proteins (Solomon *et al.*, 1998). Thus although the 20s proteasome was activated, there could have been no substrate to degrade. Although it goes against most results to suggest that the proteasome was activated without the calcium-dependant proteases (such as calpain, which releases the myofibrillar proteins from the sarcomere (Huang *et al.*, 1998), the response of the calpains to psychological stress of a mild nature have not been investigated. If this is true then it would indicate that the ubiquitin proteasome

pathway is not regulated secondary to increased amounts of substrate made available to it. Unfortunately we did not measure activity of any proteases from the calcium-dependant pathway, which would have elucidated the above theory.

ii) *Unprocessed protein substrate*

Another possible reason is that the 20s proteasome is not the rate limiting step in the ubiquitin-proteasome pathway. Components such as ubiquitin or E2 might not have been increased by my protocol. Thus even if the myofibrillar proteins were released from the sarcomere, the activated 20s proteasome would not have been able to degrade the unprocessed protein substrate.

iii) *Responsiveness*

In studies where the animals are sacrificed, it is possible to measure e.g. proteasome activity only at 1 time point. However, the overall response of the tissue is influenced by the whole time course (including speed of response and half life of response). Studies with more severe stress (e.g. sepsis) have shown elevated proteasome activity within 16 hours (Hobler *et al*., 1999) and the elevation was 1.5 times that of control. Mine is the first study that I know of to indicate a moderate (+ 25% and + 39% for soleus and EDL respectively) proteasome response to mild intermittent psychological stress. The ubiquitin-proteasome pathway has been shown to be regulated by glucocorticoid treatment (Combaret *et al.*, 2004), therefore it would be logical to assume that corticosterone would be elevated in S.

4.1.3) Serum glucocorticoid (GC) concentration

There were no significant differences in the serum glucocorticoid concentrations (Fig 3.1.3) between C and S. This might indicate that our stress intervention was unable to activate the hypothalamic-pituitary-adrenal (HPA)-axis significantly and that proteasome activity upregulation was in response to a different signal. Although this is difficult to explain, there are several possible explanations:

i) *Failure to activate HPA axis*

Glucocorticoids are known to decrease the rate of protein synthesis (Shah *et al.*, 2000) and increase the rate of protein degradation (Kayali *et al.,* 1987). However, glucocorticoids are not required for all forms of atrophy. Previous research has shown that although disuse atrophy (hindlimb unloading, and denervation) is associated with an increase in serum glucocorticoids, RU38486 (a glucocorticoid antagonist that blocks the glucocorticoid receptor of skeletal muscle) treatment does not block atrophy (Konagaya *et al*., 1987; Tischler, 1994). Specifically, Konagaya *et al.* (1987) demonstrated that GC-blocking was unable to attenuate the denervation-induced atrophy and, similarly Tischler (1994) indicated that GC-blocking had no attenuating effect on hindlimb unloading induced atrophy. My psychological stress model included immobilisation, which could have affected the proteasome without affecting GC concentrations. although this was also not continuous such as in the other models.

ii) *Intermittent protocol*

Although our stress protocol was unable to induce a significant increase in serum corticosterone concentration, that was evident on the day of sacrifice, this is not an indication that the S rats were not exposed to higher GCs over the 7 days. This is quite possible since the protocol was not continuous such as in the models mentioned earlier, and acute responses may have subsided.

iii) *Habituation*

Another possible reason for no difference in serum corticosterone concentration on the day of sacrifice is that the corticosterone response of Group S had already habituated to the stress intervention toward the end of the week. This is supported by previous studies, which have used a mild restraint stress (Ottenweller *et al.*, 1987, Pitman *et al.*, 1988). The restraint stress used in these studies was also unable to alter serum corticosterone concentrations when compared to untreated controls. Again, this explanation might indicate that the 20s proteasome was activated by a regulator other than corticosterone examined

in the present study. Other possible regulators include cytokines such as TNFα, which transiently increase in serum in response to stress (Grippo *et al*., 2005) or catecholamines. I found no literature on a possible link between catecholamines and the muscle proteasome. However I found one study indicating that the increased activity of the proteasome in a rat model of biliary cirrhosis was associated with increased muscle TNFα (Lin *et al*., 2005).

iv) *Small sample size and large variation*.
Possible differences in serum corticosterone concentration between Group C and S could have been masked by the small sample size and the large inter-individual differences in both groups.

4.1.4) GC-inducible enzymes

GS and TAT enzyme activity were also unaffected by the 1-week stress protocol in Group S vs. C. Since both enzymes are glucocorticoid-inducible (Minet *et al*., 1997, Chesnokov *et al*., 1990), this result correlates well with the glucocorticoid results of the present study, which showed no difference between C and S. Also, the 1-week stress intervention was unable to induce skeletal muscle degradation, which would have caused amino acid release into the circulation. Again, these data suggest that GS and TAT are not controlled similarly to the proteasome.

4.1.5) Summary of findings for experiment 1

Thus it seems as if the 1-week stress intervention was unable to induce atrophy of the skeletal muscle under investigation, which is likely due to the lack of significant response of serum corticosterone concentration, GS and TAT activities. However the 20s proteasome activity increased in Group S compared to C in both the soleus and EDL, without a concomitant skeletal muscle atrophy. I conclude that upregulation of the proteasome may precede conclusive evidence of atrophy. An escalation of the stress intervention might have been able to cause skeletal muscle degradation. We will attempt to answer this question in the next section.

4.2) Stress exposure for two weeks

To determine whether or not *Sutherlandia* supplementation (Su) was able to reverse the effects of stress, despite continuation of the stress intervention, required that I have another control group, namely that of Su without stress. Because Su was administered intra-peritoneally it became necessary to also administer placebo to the C group. Group CP spent week 1 without intervention and week 2 with a placebo injection twice a day. Group Csu also spent week 1 without intervention and week 2 with Su injection twice each day.

The main findings for experiment 2 were:

i) a significant decrease in EDL mass to body mass ratio in StP vs. CP, which was attenuated by Su treatment in Stsu. The same interaction effect was seen in the soleus to body mass ratio, although the *post hoc* tests did not reach significance.

ii) a significant increase in soleus proteasome activity in StP vs. CP, which was attenuated by Su treatment in Stsu vs. StP. Similarly Stsu had significantly lower EDL proteasome activity than StP.

iii) Body mass itself did not follow the same trends

In the following sections I will first discuss the results of the control groups and the effect of stress, and later I will discuss the effect of Su.

4.2.1) Body mass

All groups lost body mass, including both of the control groups (CP and Csu). Some possible causes are presented below.

(i) *Inflammation*

CP and Csu rats only started losing weight after initiation of the injections (Fig. 3.2.1). This could indicate the presence of an inflammatory response to the injections. However, Smith and Myburgh (2004) injected their rats over a 28 day period with the same placebo and *Sutherlandia* solutions as in the current study. They did not find any alterations in resting serum IL-6 levels in response to either

the placebo or *Sutherlandia* injections, even though their *Sutherlandia* solutions were also unsterilised. Inflammation therefore seems an unlikely cause of the observed loss in body mass. However, it is possible that rats in the study by Smith and Myburgh (2004) adapted over time in their longer protocol, leading to no increased cytokines measured. In my study there is also some evidence against inflammation as a possible reason for weight loss. In the present study the placebo solution was sterilised and the *Sutherlandia* solution was not and the rats in both control groups lost the same amount of body mass. I observed that the placebo-injected non-stressed rats (CP) lost ~7% of body mass (Fig. 3.2.1 B) over 2 weeks, that all was lost in the second week, and that this was similar to the stress-related 8% loss of body mass in group S discussed previously. This might indicate that the CP rats were actually similarly psychologically stressed by the injections. This is a more likely explanation than the possibility that the injections (from the start of the second week, for 1 week) caused an inflammatory response. Unfortunately no cytokine concentrations were determined in this study, so we are unable to make a conclusion in this regard.

ii) *Handling*.

Another possible explanation is that the handling procedure needed for injection of the rats may have been stressful in addition to the injection itself. For the injection procedure, one person gripped the rat around the scapulae with one hand and kept the hindlimbs apart with the other hand, while another person administered the injections. It has been shown that handling of animals functions as a stressor, which may stimulate an increase in circulating glucocorticoids (Holst *et al.*, 1998). Acute increases in response to stress results in serum corticosterone concentrations of approximately 260 ng/ml, whereas chronically unstressed rats typically have ~20 ng/ml *vs*. ~80 ng/ml in chronically stressed rats without influence of acute stress (Smith and Myburgh, 2004, Retana Marquez *et al*., 2003).

However, there was no difference in serum corticosterone concentrations between any of the 4 groups and concentrations were similar in CP and StP compared with the rats sacrificed after the first week. Potential differences between groups might have been masked by the large inter-individual differences and the outliers. It must be concluded that corticosterone concentrations reflected their chronic condition, whereas some rats had an acute stress response just before sacrifice.

iii) *Stress*

Although all experimental groups lost body mass over the course of the 2-week study, the placebo-supplemented stressed rats (StP) and *Sutherlandia*-treated stressed rats (Stsu) lost significantly more mass (main effect of stress). This is in accordance with other studies reporting stress-induced weight loss over the same time course (Marti *et al*., 1993, Marquez *et al*., 2004).

Body mass was reduced by 8.1% in stressed rats (S) over 1 week and 9.4% in placebo-supplemented stressed (StP) rats (Fig. 3.2.1 B) after 2 weeks of stress. This is considerably less than the loss of ~ 40% reported in studies which used more severe stressors such as glucocorticoid treatment (Combaret *et al.*, 2004) and cancer (Costelli *et al.*, 2002), and only slightly less than more severe immobilisation procedures (strapping of limbs, fibre cast of hindlimbs) which caused ~ 15% loss of body mass (Marti *et al.,* 1993). Studies using a similar immobilisation technique (confinement in Plexiglas tube) reported stable body masses during the course of the stress procedure which lasted 20 days (Retana-Marquez *et al.*, 2003). My result was therefore not unexpected, since rats can habituate quickly to the mild confinement stress alone. Although my study used multiple stressors in an intermittent unpredictable fashion and this was sufficient to cause a main effect, *post hoc* significance was lacking.

A follow-up study using greater sample size is required before a final conclusion can be made.

4.2.2) Skeletal muscle mass

Stress

The relative mass (relative to body mass) of the EDL muscles were significantly reduced in StP vs. CP (Fig. 3.2.3 A & B) but not of the soleus muscle. This is in accordance with other studies, which have shown that fast twitch muscle is more sensitive to catabolism than slow twitch muscle in a variety of muscle wasting conditions, including burn injury (Chai *et al.*, 2002) and sepsis (Hobler *et al.*, 1999). As discussed earlier our stress procedure may not have been strong enough or sustained enough to cause skeletal muscle degradation after 1 week in Group S vs. C. However, the finding of reduced EDL muscle mass to body mass ratio in StP vs. CP suggests that the longer the intermittent stress protocol is applied, the more effective it is in inducing skeletal muscle atrophy. Again as discussed earlier an initial systemic response may have targeted fat mass degradation (as shown by a decrease in body mass without a concomitant decrease in skeletal muscle mass after 1 week of stress intervention). However continuation of the stress response was sufficient to result in greater body mass reduction as well as skeletal muscle mass degradation.

Degradation of skeletal muscle mass and/or fat mass during conditions of stress, when there is a high energy requirement, is a natural physiological response, which ultimately results in fuel production for ‘fight’ or ‘flight’. Thus my stress protocol had a more prominent atrophy effect after 2 weeks of stress than after 1 week. This is in contrast to other immobilisation studies which have shown a more marked atrophy effect during the first week (Booth, 1982; Appell, 1986). However, protocols such as fibre casts are continuously applied, whereas the rats in our study were stressed only for certain periods in the day and could then return to normal activity.

It was unexpected that this stress protocol would affect only the EDL muscle, since we used immobilisation each day as part of the stress procedure. Muscles consisting of pre-dominantly slow-twitch skeletal muscle, such as soleus are

vulnerable to disuse atrophy, such as denervation (Lu *et al.*, 1997), hindlimb suspension (Bricout *et al.*, 1999) and fibre casting (Harjola *et al.*, 2000). These procedures however have a localized effect reducing all signalling (such as Mechanical growth factor) in response to mechanical strain (Goldspink, 2005). My intermittent psychological stress model has a very short local effect, but a longer systemic effect. The slight protection of the soleus muscle may have been due to mechanical strain still being applied in the immobilisation chamber. Since this is the first study to my knowledge, which has evaluated the effects of an intermittent stress protocol on muscle mass itself, I present a finding which further substantiates previous studies that fast-twitch skeletal muscle is more susceptible to general stress than slow-twitch skeletal muscle.

Sutherlandia

There was no significant difference between CP and Csu in either the EDL or soleus muscle (Fig. 3.2.3 A & B). This suggests that Su treatment in non-stressed rats had no effect on skeletal muscle mass. *Sutherlandia* treatment was unable to alter the stress-induced loss in body mass in Group Stsu vs. StP (Fig. 3.2.1 A & B). However, *Sutherlandia* treatment was able to significantly attenuate the stress-induced loss of relative EDL weight in Group Stsu vs. StP (Fig. 3.2.3 B), whereas there was no significant effect of *Sutherlandia* treatment on relative soleus muscle in the same groups (Fig. 3.2.3 A). Thus Stsu rats lost body mass, which cannot be accounted for by loss of lean body mass. Assuming that the loss in body mass after *Sutherlandia* treatment is due to a decrease in fat mass, it could be that *Sutherlandia* mobilizes fatty deposits, which can be used by organs such as the liver and skeletal muscle for fuel production. This would alleviate the need for skeletal muscle degradation to provide amino acids for fuel production. Further trials are needed though to elucidate this possible mechanism. If further trials substantiate this hypothesis, it would make *Sutherlandia* an attractive treatment for diseases such as HIV/AIDS which is characterized by increases in abdominal fat deposits for patients on anti-retroviral treatment (Mulligan *et al*., 2001) and a loss of skeletal muscle mass (Breitkreutz *et al*., 2001).

4.2.3) 20s Proteasome activity

Control rats (CP & Csu)

Many rodent studies of the effects of stress or other catabolic states on the proteasome investigated upregulation of gene expression (Combaret *et al*., 2004, Kee *et al*., 2003, Tiao *et al*., 1994, Wakshlag *et al*., 2002) rather than the proteasome activity itself. I found only three studies that measured 20s proteasome activity in rat muscle homogenates using methods similar to my own (Hobler *et al*., 1999, Kee *et al*., 2002, Yimlamai *et al*., 2005). However the mean proteasome activity values of control rats in the studies of Hobler *et al*. (1999) and Kee *et al*. (2002) were 20 and 3 times higher in predominantly fast-twitch muscle compared to my own proteasome activity values in control rats. The reason for these differences may be because of the different strain and age of rats used in these two studies. Hobler *et al*. (1999) used very young male Sprague Dawley rats, weighing 40 -60 g, whereas Kee *et al*. (2002) also used male Sprague Dawley rats which were a bit older weighing ~ 200 g. My own male Wistar rats were older than the rats used in both these studies (8 months at start of study) weighing between 450 and 700 g. Also Hobler *et al*. (1999) used an endpoint measurement to measure fluorescence, whereas Kee *et al*. (2002) and I continuously monitored the fluorescence over a 1 hour time period. Thus background fluorescence could have contributed to the much higher 20s proteasome activity values measured in the study of Hobler *et al*., (1999).

Nevertheless my values correspond well with those of Yimlamai *et al*., (2005), with activities in control soleus of ~150 nmol/min/g prot vs. mine of ~ 100 nmol/min/g prot; and activities in control tibialis anterior of ~ 60 nmol/min/g prot vs. mine in EDL of ~ 90 nmol/min/g prot. The only real explanation for these small differences between the two studies could be the gender and the age of the rats (Yimlamai *et al*., 2005: female Sprague Dawley rats; ~200 g). The difficulty in comparing rodent models measuring 20s proteasome activity will be discussed in detail later after I discuss my own study results.

Sutherlandia administration to control rats (Csu) did not have a significant effect on 20s proteasome activity in either the soleus or EDL muscle when compared to placebo-supplemented control rats (CP). Csu rats did have a large inter-individual variation (Fig. 3.2.4 A & B) in 20s proteasome activity in both muscles, which might indicate that Su treatment did have an effect on 20s proteasome activity in some of the non-stressed control animals, especially in EDL. It can't be concluded with the evidence available if these variations were due to Su itself, or the administration method.

Stress

Our results indicate that the 2-week intermittent mild stress protocol used in our study was able to significantly increase the chymotrypsin-like activity of the 20s proteasome in the soleus but there was only a trend in the EDL muscle (Fig. 3.2.4 A & B). Again a possible significant difference in the EDL muscle might have been masked by the small sample size and the large inter-individual difference of group StP in the EDL muscle.

This is in contrast however to other studies, which have shown that fast twitch muscle is more sensitive to catabolism than slow twitch muscle in a variety of muscle wasting conditions, including burn injury (Chai *et al*, 2002) and sepsis (Hobler *et al.*, 1999).

Fibre type

Although fibre type seemed to affect baseline proteasome activity (C) within soleus 1.5 times higher than EDL, this difference was not statistically significant (ANOVA, experiment 1, group C and S, muscle EDL and soleus). The proteasome activity response to stress was also not fibre type specific in my study or others. It is activated in a specific muscle in response to the muscle wasting condition being used, as shown in studies using hindlimb unloading (Taillandier *et al.*, 1996) or sciatic nerve denervation (Medina *et al.,* 1995), where the proteasome activity was increased in the soleus muscle; or sepsis (Hobler *et*

al., 1999) and burn injury (Chai *et al.*, 2002), where the proteasome was increased in predominantly fast-twitch skeletal muscle. Most of these studies did not assay very different muscle types.

I did not find a significant increase in 20s proteasome activity in the EDL muscle after the 2-week intermittent mild stress protocol, but with more rats it is likely it would have been significant.

Muscle mass and proteasome activity

Although soleus to body mass ratio was not significantly decreased by stress, the soleus proteasome activity was significantly increased, with the opposite in terms of statistical significance occurring in the EDL. Significant differences for both parameters in both groups in response to stress could have been masked by the small sample sizes and large inter-individual differences.

Sutherlandia treatment

Sutherlandia treatment significantly attenuated the increased 20s proteasome activity of Stsu rats in both the soleus and EDL (Fig. 3.2.4 A & B) muscle when compared to StP. *Sutherlandia* treatment decreased the 20s proteasome activity in response to the stress stimuli in the soleus of Stsu rats by ~ 37% (Fig. 3.2.4 A) to similar values as that of placebo-supplemented controls (CP) whereas it was decreased by ~ 46% to slightly below placebo-supplemented control values in the EDL muscle (Fig. 3.2.4 B). This might indicate that the predominantly fast-twitch EDL muscle was slightly more responsive to *Sutherlandia* treatment than the predominantly slow-twitch soleus.

Three possible reasons for the attenuating effect of *Su* on stress-induced increase in 20s proteasome activity:

i) *Effect of corticosterone on 20s proteasome*

Smith and Myburgh (2004) showed that *Sutherlandia* is able to attenuate the immobilisation stress-induced increase in serum corticosterone concentrations in rats. An effect on the adrenal gland was supported in a subsequent *in vitro* study by Prevoo *et al.* (2004). Furthermore Combaret *et al.* (2004) showed that the ubiquitin-proteasome pathway is regulated by glucocorticoid treatment. Thus *Sutherlandia* could have had its attenuating effect in this study on the 20s proteasome activity of Stsu vs. StP via a decreased corticosterone response. However the present study was unable to show a decrease in serum corticosterone concentration in response to *Sutherlandia* treatment, so this explanation is not conclusive

ii) *Mood elevating properties of Sutherlandia*

GABA is present in *Sutherlandia* (Brummerhoff, 1969) and has mood elevating effects. Thus it could have indirectly contributed to the decreased 20s proteasome activity via improved recovery between the intermittent bouts of stress and thus an overall decreased GC release. This would not be shown by the single corticosterone sample taken at sacrifice in my study.

iii) *Anti-catabolic properties of Sutherlandia*

Pinitol, another substance present in *Sutherlandia* (Brummerhoff, 1969) has been shown to have actions similar to insulin in diabetic conditions (Kim *et al.*, 2005), whereas insulin has well known anti-catabolic effects in muscle. Insulin has been shown to downregulate components of the ubiquitin-proteasome pathway in cultured myoblasts (Wing and Banville., 1994), starved rats (Kee *et al.*, 2003) and in incubated muscle of non-disease rats (Larbaud *et al.*, 2001). Thus insulin may have its anti-catabolic effects at least in part via the inhibition of the ubiquitin-proteasome pathway, and pinitol may have similar inhibiting actions on the ubiquitin-proteasome pathway, whether given as part of a *Sutherlandia* herbal extract or alone.

Comparison with literature

To compare my 20s proteasome results to those reported in the literature, I have taken into account 5 factors:

i) This is the first study to our knowledge, to evaluate proteasome activity in response to an intermittent mild stress protocol, which can be regarded as a psychological stress experience, as opposed to the physical stressors used in the majority of studies, such as sepsis and cancer, to induce skeletal muscle atrophy.

ii) Most studies report changes of proteasome activity in either a predominantly fast twitch skeletal muscle or predominantly slow twitch skeletal muscle, making it difficult to compare the proteasome activity response between the 2 muscle fibre types in the same study.

iii) The stress protocol in my study was elicited for a longer period of time than most other studies reporting changes in the 20s proteasome activity.

iv) A lot of studies report changes in the components of the 26s pathway without evaluating changes in proteasome activity; whilst it has been shown that components of the ubiquitin-proteasome pathway do not strictly correlate with rates of proteolysis (Kee *et al.*, 2003; Combaret *et al.*, 2004).

v) The proteasome activity assay used to evaluate its activity in muscle extracts, as in the present study, uses an artificial proteasome substrate and does not necessarily reflect the *in vivo* activity of the 20s proteasome. The hydrolysis of this substrate does not require ubiquitinylation and processing of the multi-ubiquitinated peptide substrate through the 26s proteasome complex before it is degraded by the 20s proteasome. Nevertheless Kee *et al.* (2001, 2002) have shown that degradation of casein by isolated 20s proteasome, which needs prior ubiquitinylation, correlates well with 20s proteasome activity evaluated on an artificial proteasome substrate.

The results of this study support the indigenous knowledge that *Sutherlandia* has anti-catabolic properties (http://www.sutherlandia.org/cancer.html), which

possibly acts via the inhibition of the proteasome pathway. Future studies should elucidate whether *Sutherlandia* treatment is able to have similar positive effects on more severe muscle wasting models, such as cancer cachexia.

4.2.4) Corticosterone and stress

At the end of the 2-week intermittent mild stress protocol there was no significant difference in plasma corticosterone concentrations between any of the groups. Possible reasons for this finding.

i) *Habituation.*

At first it might seem that the most probable reason is that the rats habituated to the stress procedure. This is supported by the fact that StP rats had similar serum corticosterone concentrations to CP rats (Fig. 3.2.7). However Group S sacrificed after 1 week had similar serum corticosterone concentrations to StP rats sacrificed after 2 weeks. This argues against a habituating effect the longer the rats were exposed to the stress protocol.

I designed my stress protocol with the specific goal of preventing habituation, exposing rats to different types of stress in an intermittent unpredictable fashion. In a pilot study using the same protocol we observed a loss of body mass after exposure to a novel stressor, but the decrease in body mass progressively declined over the days with continued exposure to the same stressor. Thus I designed the study so that the rats were not exposed to the same stressor for more than 2 days. Almeida *et al*. (1998) demonstrated elevation in corticosterone from ~99 ng/ml (control) to ~232 ng/ml with 60 days of intermittent immobilisation stress. Both my CP and StP rats were closer to ~50 ng/ml indicating a resistance to stress, or a low stress response in StP that resolved between exposures.

ii) *Large inter-individual differences*

Differences between control and stress-induced corticosterone responses in rats sacrificed after week 1 or in those sacrificed after week 2, might have been

masked by the outliers and the inter-individual differences in serum corticosterone concentrations (Fig. 3.2.7). Marquez *et al.* (2004) showed that rats subjected to an initial immobilisation session (1 hour per day for 13 days by strapping of limbs to wooden boards) resulted in individual differences in the corticosterone response, which was reduced after repeated exposure to the stressor. Thus it could be that the rats in the present study showed real individual differences in corticosterone response after the initial stress sessions, which was reduced after 14 days of stress when the SD was smaller than in those subjected only to 7 days of the stress protocol.

iii) *Mild nature of the stressors*

My results are in agreement with previous studies, which also used a mild restraint stress (Ottenweller *et al.*, 1987, Pitman *et al.*, 1988). The restraint stress used in these studies was also unable to alter serum corticosterone concentrations (at the time of sacrifice) when compared to untreated controls. However I used an even milder form of stress than these studies, which used protocols such as tail shocks whilst in restraint chambers (light-tight, sound attenuated, individually ventilated chambers) (Ottenweller *et al.*, 1994) and strapping of limbs (Marquez *et al.*, 2004).

4.2.5) Corticosterone and Su treatment

Interestingly there were large inter-individual differences in corticosterone concentration in response to *Sutherlandia* treatment in both the *Sutherlandia* treated stress rats and the *Sutherlandia* treated control rats (Fig. 3.2.7). This might indicate that the *Sutherlandia* herb caused an inflammatory response, since it was neither sterilized nor filtrated before administration. These results are in contrast to those of Smith and Myburgh (2004), who reported an attenuation of the immobilization-induced increase in serum corticosterone concentration in response to *Sutherlandia* treatment. However they subjected their rats to 28 days of immobilisation, while simultaneously administering *Sutherlandia*. Thus the rats in the present study might not have been subjected long enough (7 day treatment

from day 7 to 14) to the *Sutherlandia* treatment to get used to the handling and i.p. injections. Also the chronic stress response might not have developed sufficiently yet to become more significant than acute effects. Therefore we suggest that future studies evaluating the corticosterone response to *Sutherlandia* treatment, use a longer treatment model of more than a week, or to administer it by oral methods.

4.2.6) Glutamine synthetase activity

StP vs. CP

The stress procedure was able to increase the GS activity in both the soleus and EDL muscle (Fig. 3.2.5 A & B) of StP when compared to CP. This is the first study known to the authors that has evaluated GS activity in response to a mild-intermittent stress protocol. Thus we report a novel finding that a chronic mild psychological stress is able to alter the GS activity in skeletal muscle.

However, the activity values of GS in the stressed rats (StP) are lower than those generally reported in the literature in response to other stressors (Minet *et al*., 1997, Hickson *et a*l., 1996, Chen *et al*., 1993).

There are several possible reasons why the GS is lower in my study:

i) *Model not severe enough*

Most studies evaluating GS activity in skeletal muscle have used a synthetic glucocorticoid analogue (e.g. Dexamethasone) to induce skeletal muscle degradation. Dexamethasone increases GS activity by more than 2.5 fold (Minet *et al*., 1997) and effects are far greater in the fast-twitch fibres, whereas the increase in response to stress (StP vs. CP) in my study amounted to a ~ 1.4 to 1.5 fold increase in both muscles. Thus our stress inducing model may not have been severe enough to cause an increase in GS activity to similar values as those reported in the literature (Minet *et al*., 1997, Chen *et al*., 1993).

Nevertheless I show that stress sets up metabolic pathways for catabolism of protein.

ii) *Habituation*

The intervention protocol used in our study is applied for a longer period of time than most other studies reporting changes in the GS activity (Hickson *et al*., 1996, Chen *et al*., 1993), which may have resulted in habituation of the GS response to the stressful stimuli. Since GS is well known to be glucocorticoid inducible, and as mentioned previously serum corticosterone concentration is gradually decreased over time (in days) after exposure to an initial novel stressor, it could be that the GS activity already decreased in response to decreased serum corticosterone concentrations, even over only 7 days.

iii) *Possible confounding factor*

A possible confounding factor is the fact that appetite could have been altered in response to the stressful stimuli, which would have influenced the GS activity. Food intake was not measured in the current study, thus a reduction in food intake by the stressed animals is a possibility. Previous studies have shown that restraint-induced stress reduces food intake (Ricart-Jane *et al*., 2002, Torres *et al*., 2002)

GS activity and Sutherlandia

As with the parameters previously discussed, there was again no significant difference between CP and Csu. This suggests that Su treatment to unstressed control rats had no effect on the muscle parameters measured, which is further confirmed by the TAT results (described later).

Considering that GS activity did not differ significantly between Stsu vs. StP, one might conclude that *Sutherlandia* treatment did not significantly change the GS activity in the 2nd week of the 2-week mild intermittent stress protocol in either the soleus or EDL muscle (Fig. 3.2.5). However there was a slight decrease in average GS activity (~ 10 – 15 %, not significant) in both the soleus and EDL in

Stsu vs. StP (1001 nmol/min/g prot. vs. 890 nmol/min/g prot, Fig. 3.2.5). If I consider that the StP group differed significantly from the CP group to the $P < 0.01$ level for both muscles, but the Stsu differed from CP by only $P < 0.05$ in EDL and not significantly in soleus, I can actually conclude that there was a small treatment effect.

I have three possible explanations for this smaller effect of Su on GS activity, especially given the fact that the proteasome activity was affected by Su:

i) *Su treatment not applied long enough to reverse the effect of stress*
GS is well known to be glucocorticoid-inducible, thus if *Sutherlandia* has an effect on GS, it is likely that it acts via reducing corticosterone. *Sutherlandia* treatment has been shown in the study of Smith and Myburgh (2004) to decrease the immobilisation-induced increase in corticosterone, if administered in conjunction with stress for 4 weeks. My study differed from theirs in three ways. Firstly Su was introduced only after the first week of stress. Secondly it was administered for only 1 week. Thirdly I used an incremental stress protocol, whereas they only used immobilisation (restraint in a Plexiglas cylinder) as a stress procedure. Thus I conclude that Su treatment in my study may not have been applied for a long enough period to have any significant effect on GS activity, or the dose was not enough to deal with the added stressors.

Furthermore mild stress procedures (such as the one used in the present study) may need a milder treatment procedure. Some studies administer supplements using mini-osmotic pumps (Hashimoto *et al*., 2002) or oral lavarge techniques (Miguel *et al*., 2005) instead of injections.

ii) *Mild stress*
The corticosterone and subsequent GS response to the stress protocol of my study was much less than other studies. Therefore it would be more difficult to show an effect of the *Sutherlandia* treatment.

iii) *Inflammation*

Another possibility is that administration of the *Sutherlandia* herb may have caused a mild inflammatory response, since it was neither sterilized nor filtrated before administration. This would have resulted in an increased corticosterone response with a possible concomitant increase in GS activity in Csu and Stsu. This is unlikely though since there was no increase in serum corticosterone concentration or GS activity of either the soleus or EDL in *Sutherlandia* treated unstressed rats (Csu) compared to placebo-supplemented control rats (CP).

iv) *GS not exclusively glucocorticoid dependant*

Other factors outside corticosterone itself can also affect GS activity. Glutamine supplementation decreases glucocorticoid-induced increases in muscle GS activity (Hickson *et al.,* 1996). Also, IGF-I is known to decrease glutamine release in skeletal muscle (Parry-Billings *et al.,* 1993), to inhibit glucocorticoid-induced glutamine synthetase activity in cultured L6 rat skeletal muscle cells (Kimura *et al*., 2001). It is possible that *Sutherlandia* might affect GS activity through a mechanism related to glutamine availability.

Comparing Fig. 3.2.3 and Fig. 3.2.5 one might speculate that the GS response to stress was more evident than actual loss in muscle mass. The upregulation of the enzyme might indicate that longer exposure would have resulted in more muscle atrophy. A follow-up study, evaluating the effect of *Sutherlandia* treatment on exogenous glucocorticoid-induced skeletal muscle atrophy should clearly elucidate whether *Sutherlandia* has any effects on GS activity in skeletal muscle. This study should assess circulating glutamine concentration. I also propose that further studies using stress-induced skeletal muscle atrophy to evaluate GS activity response should report changes in skeletal muscles consisting of both predominantly fast-twitch and slow-twitch skeletal muscle.

4.2.7) Tyrosine aminotransferase (TAT) activity

In the literature TAT activity has been measured almost exclusively in the liver, because of the high metabolic activity of this tissue. TAT activity has been shown to have diurnal variations (Kato and Saito, 1979), reaching a peak during the dark period and being at its lowest during the early part of the light period in rats fed *ad libitum*. Furthermore a maximal TAT activity is found after feeding time (Fuller and Snoddy, 1968). In the present study we sacrificed our rats during the early part of the light phase and their feeding time was during the dark phase, although enough food was supplied for *ad libitum* feeding.

As is the case for GS, TAT is well known to be glucocorticoid-inducible (Chesnokov *et al*., 1990). Although Csu did not exhibit lower TAT activity than CP. *Sutherlandia* treatment has been shown to decrease the immobilisation-induced increase in corticosterone (Smith and Myburgh, 2004). No effect of Su in unstressed rats may be because muscle TAT activity is not very responsive especially compared to proteasome activity which gave consistent and explainable results. There have also been studies that have not been able to induce an increase in liver TAT activity even in response to an increased secretion of corticosterone into blood (Geller *et al.*, 1964).

TAT activity after stress.

Other studies have shown that liver TAT activity reaches a peak 6-10 hours after the initiation of the stressor and declines thereafter to basal values within 6 hours (Chesnokov *et al.*, 1990, Lasbowska-Klita and Bong, 1979). I sacrificed my rats the day after the last stress procedure, with the stress procedure ending at 16:00 and sacrificing of the first rat taking place at 09:00 of the next day before new food was provided. Thus considering these factors, it is conceivable that we sacrificed our rats at a time when the diurnal TAT activity was at its lowest levels. This could have masked differences in TAT activity between the different groups. Indeed when considering *post hoc* analysis, the 2-week intermittent mild stress

protocol was unable to induce any significant changes of TAT activity in either the soleus (Fig. 3.2.6) or EDL.

Considering the literature which has reported changes in TAT activity in response to stress only in the liver, the current findings might have been expected. However there was a small increase (~ 15% though not significant) in the TAT activity of StP rats compared to CP rats in the soleus muscle. Also the main ANOVA indicated a significant interaction effect indicating that the groups behaved differently. According to the interaction effect (Fig. 3.2.6), TAT activity in the soleus muscle differed between the different groups and TAT activity increased in soleus muscle of StP rats compared to CP rats as a result of stress but the effect of stress on TAT activity was inhibited when stressed rats received *Sutherlandia* treatment.

This was not the case in EDL, which seems to indicate that TAT activity is more responsive in the predominantly slow-twitch soleus muscle rather than the predominantly fast-twitch EDL muscle. Still, *post hoc* significance in soleus and any significance in EDL were lacking. Possible reasons include:

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i) *Small sample size.*
A significant effect might have been masked by the small sample size (n = 6). It is surprising that the TAT activity responded to the stress stimuli in the soleus muscle rather than the EDL, since TAT is glucocorticoid inducible (Chesnokov *et al.,* 1993) and because glucocorticoids affect the fast-twitch fibres rather than the slow-twitch fibres (Falduto *et al.,* 1992).

ii) *Intermittent stress*
Our skeletal muscle TAT activity values are a bit higher though than those reported in the literature for tissue other than the liver (Chesnokov *et al.,* 1990, Nemeth *et al.,* 1977). This might indicate that all 4 groups experienced some stress. However, the effect of stress on TAT activity must be enough to overcome

other influences if significances are to be found. Chesnokov *et al.* (1990) showed that protein availability may regulate TAT activity during the de-induction period (when TAT activity decreases to basal values). They observed a maximal increase in liver TAT activity after a single dose of hydrocortisone treatment, but a second injection was unable to increase TAT activity during the de-induction period. This indicates that the decreased TAT activity is not because of a loss of glucocorticoid concentration. They then injected rats with cycloheximide (a protein synthesis inhibitor) during the de-induction period, which resulted in a rapid decline in TAT activity. Thus TAT activity is not solely dependant on serum glucocorticoid concentration, but can also be affected by protein availability. This could be a possible reason for the lack of a really significant response of TAT activity observed in StP. There could have been a lack of protein catabolism at the time of measurement (day 14) so that TAT enzyme activity was not much different in StP vs. CP in either muscle.

iii) *Time of day of sacrifice*

We chose not to sacrifice our rats immediately after the last stress session because we wanted to eliminate any acute effects of stress. Furthermore chronic stress seems to elevate morning (lights-on) serum corticosterone concentrations but is unable to alter the evening (lights-off) corticosterone response (Ottenweller *et al.*, 1994, Retana-Marqeuz *et al.*, 2003). Therefore we chose to sacrifice our rats during their resting period (early part of light phase), when the serum corticosterone was at its lowest to eliminate confounding factors, such as playing or fighting, which would have affected both the plasma corticosterone and testosterone concentrations. However for future studies it would be best to evaluate TAT activity changes 6-8 hours after the stress procedure to prevent de-induction.

iv) *Small sample size*

The small sample size might have masked a more significant attenuating effect of *Sutherlandia* treatment on the stress-induced increase in soleus TAT activity.

In summary our results should encourage future studies to evaluate changes in TAT activity of predominantly slow-twitch skeletal muscle, because it seems that TAT activity is responsive to stress in predominantly slow-twitch muscle. Similarly *Sutherlandia* seems to have an effect on TAT activity (although not significant) in the predominantly-slow twitch soleus muscle rather than the predominantly fast-twitch EDL muscle.

4.3) Was one week of exercise able to reverse the effect of stress for two weeks.

Relating to this question I will offer possible explanations to elucidate the findings obtained in the results section.

The main findings for experiment 3 were that:
i) exercise caused a significant percentage reduction in body mass in CE vs. C and in StE vs. S without a concomitant decrease in either of the skeletal muscles masses measured.
ii) exercise was able to significantly attenuate the stress induced increase in 20s proteasome activity in the EDL muscle.

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4.3.1) Exercise, body and muscle mass

Exercise stressed rats (StE) were exposed to stress for days 1-7, and stress plus exercise for days 7-14. My hypothesis was that exercise would be able to reduce or reverse the effect of prior stress on atrophy, despite continued exposure to stress. This effect could theoretically be due to decreased stress perceptions, or due to a localised effect of muscle contraction. I designed my protocol favouring the latter possibility. Therefore I used added weight to enhance the mechanical effect of exercise. I hypothesised that this exercise protocol would induce hypertrophy in fast-twitch muscles of CE and reverse atrophy in StE. StE lost significantly more body mass (- 14%) than rats (Group S) sacrificed after 1 week of stress (- 8%). This cannot be accounted for by the longer duration of stress alone, since StP rats (stressed for 2 weeks) lost just over 9.4% of body mass.

Despite greater loss in body mass in StE, skeletal muscle masses were similar across groups. Thus the exercise intervention (CE and StE) was able to induce a significant loss in body mass, which is not explained by loss of skeletal muscle mass. On the other hand, in the control exercise group, no hypertrophy took place. Possible explanations are that the exercise protocol induced fat mass loss, and therefore had a more systemic effect rather than local effect.

i) *Systemic effect of exercise*

A previous study also in male rats, exercised the rats on a treadmill at higher speed for 38 min/day or lower speed for 60 min/day (Pacheco-Sanchez *et al*., 1994). The rats exercising at higher intensities lost significant carcass fat compared with sedentary controls after 8 weeks on the protocol while the rats exercising at a low intensity had a less marked result. Voluntary running over 12 weeks also reduces fat mass in rats (Nara *et al*., 1999). Both these studies lasted much longer than mine. Tremblay *et al*. (1985) reviewed the literature on exercise-induced fat loss in humans and indicated that longer programmes are more successful, but that it is more effective in dieting.

The combination of exercise and stress has not been studied in a design similar to mine. However, fat cells from rats previously trained for 13 weeks on a treadmill had a higher adrenaline-sensitive lipase activity than untrained animals (Askew *et al*., 1975). This indicates an interaction between the two interventions in my study was possible. Fat is usually mobilized in response to endurance exercise (Tremblay *et al*., 1985), but energy expenditure must have been higher than energy intake to induce loss of fat even with my protocol.

ii) *Local effect of exercise*

My exercise protocol consisted of running in a motor driven wheel at a speed of 15 m/min for 30 minutes each day for 7 consecutive days, whilst carrying weights. The rats ran at the same speed every day, while the weights were incrementally increased from 10% (of body mass) on day 1 to 40% on day 4, whereafter it was kept constant for the remaining 3 days of the protocol.

The resistance exercise protocol did not affect the “predominantly fast-twitch EDL” muscle, despite the fact that it is well known that resistance exercise induces hypertrophy of predominantly-fast twitch skeletal muscle (Wilmore and Costill, 1994). The rats may not have been subjected long enough to the exercise protocol (from Day 7 to 14) to induce skeletal muscle hypertrophy which can take ten or more weeks (Wilmore and Costill, 1994). In another study where exercise was used as a treatment after immobilisation (3 weeks), 8 weeks of high-intensity treadmill exercise almost fully restored muscle fibre size from the atrophy condition (loss of 30%, Kannus *et al*., 1998). In this respect, they showed that high-intensity exercise was more beneficial than low-intensity exercise and that free cage activity was not effective.

The possibility exists that my exercise model was unable to recruit the muscle under investigation. However it does seem as if the EDL muscle was recruited in response to our exercise model, since the 20s proteasome activity was attenuated (~ 33%) in StE rats compared to stress rats (S, Fig. 3.3.3). This was my major second finding for this part of my study.

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4.3.2) 20s Proteasome activity

The resistance exercise protocol significantly attenuated the stress-induced increase of the 20s proteasome activity in the EDL muscle of Group StE vs. S (Fig. 3.3.3). A previous study has shown that the components of the ubiquitin-proteasome pathway are actually increased with an initial bout of exercise but are downregulated quickly with continued bouts of exercise (Willoughby *et al.,* 2003). Thus it is possible that the 20s proteasome activity was increased in my study after the initiation (Day 7) of the exercise protocol, but was downregulated after continued bouts of exercise (Day 8-14). In the EDL, this downregulation was sufficient to return the proteasome activity to normal levels. The effect of stress on proteasome activity was less in soleus than EDL, so that the effect of exercise that I found was not the same as in EDL. In contrast the exercise soleus muscle had quite a large variation in proteasome activity. I conclude that the exercise

protocol might have caused some damage in this muscle not previously used to any overload, because my overload protocol was quite severe (40% of body mass in added weight).

In summary my resistance exercise protocol was probably able to recruit the predominantly fast-twitch fibres located in the EDL muscle and the predominantly slow-twitch muscle fibres located in the soleus.

Corticosterone

Similar to earlier discussions of the corticosterone response, there was no significant difference of serum corticosterone concentrations in either exercise group (CE and StE) compared to stress (S) or control rats (C) sacrificed after 1 week (Table 3.1). A possible reason is that:

i) *The Exercise protocol too severe*

The exercise protocol might have been too severe to have an attenuating effect on the corticosterone response induced by the stress procedures. This could be the case since the serum corticosterone concentration was increased (although not significant, which is possibly due to large-interindividual variations) in the exercised rats (CE = 72.9 ± 47.5 and StE = 87.4 ± 80.3) compared to C (55.4 ± 57.9 ng/ml) and S (45.0 ± 49.57 ng/ml) rats. Thus the exercise could have been experienced as an added stressor by some of the rats. Taking this into account, the mechanical effect on inhibiting the proteasome activity in these groups is even more important.

4.3.3) GS and TAT activities

When considering the effect of stress on GS and TAT activities in the first section, there was no significant difference between groups C and S. Because the same groups were used as control for the CE and StE, an effect of exercise in reducing the effect of stress on these two enzymes was not really possible to detect, because they were not elevated in the first place. Since none of the

results for these two glucocorticoid inducible enzymes were significant, they are difficult to discuss. Therefore, I will just mention a relevant article I found.

It has been shown that endurance exercise is able do decrease the endogenous glucocorticoid-induced increase in GS activity (Falduto *et al.*, 1989). I used a resistance exercise model, which was quite severe as my exercise model. As discussed earlier the serum corticosterone concentration was increased (although not significantly) in the exercised rats (CE and StE) compared to C and S rats. However the increased corticosterone response in both exercise groups was not correlated with GS activity in either muscle. This might indirectly indicate that the catabolic stimulus of stress was counteracted by the positive effect of exercise on skeletal muscle protein balance.

4.3.4) Conclusions

Taking into account the result of all three experiments, I conclude that 1 week exposure to my mild-incremental-intermittent stress procedure was not able to induce skeletal muscle atrophy, despite decreasing body mass. I propose that the observed loss in body mass might be as a result of fatty deposit mobilisation in response to the stress procedure. Furthermore none of GS, TAT or corticosterone was significantly increased in response to the 1 week stress procedure, which might explain why skeletal muscle mass was not reduced. However the 20s proteasome activity was significantly increased in the EDL and showed a trend towards an increase in the soleus, which suggests that upregulation of the 20s proteasome may precede conclusive evidence of skeletal muscle atrophy.

In contrast to the 1 week stress exposure, the 2 week stress exposure was able to significantly reduce the relative EDL muscle mass, whereas it was only a trend in the soleus. I conclude that an escalation of the stress procedure was able to induce skeletal muscle degradation. Furthermore the proteasome activity was increased in the soleus with only a trend in the EDL, the GS activity was

increased in both muscles and TAT showed a trend in the soleus muscle in response to the 2 week stress exposure. I conclude that a larger sample size might have shown more clear elevations in all enzymes in both muscles..

I further conclude that *Sutherlandia* treatment was able to attenuate the relative skeletal muscle atrophy induced by stress in both muscle, although it was only a trend in soleus. It might have its anti-catabolic effects through regulation of the 20s proteasome, since the 20s proteasome activity was attenuated in response to stress in the soleus muscle, while it showed a trend in the EDL. However Su treatment was unable to affect the serum glucocorticoids or the glucocorticoid-inducible enzymes GS and TAT. Therefore the effect on the proteasome activity is likely through a different mediator.

I conclude that exercise spared skeletal muscles mass loss in response to stress in the EDL muscle, which probably indicated that the EDL and not soleus muscle was recruited in response to my exercise procedure. Exercise might have its anti-catabolic effect through reduction of the 20s proteasome activity, since the stress-induced increase in the 20s proteasome activity was attenuated by exercise.

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Chapter 5

5) Future research

My mild-intermittent-incremental stress procedure was able to reduce body mass and not skeletal muscle mass after 1 week, whereas both body mass and skeletal muscle mass was reduced after 2 weeks. I propose that future studies use a mild-intermittent-incremental stress procedure of at least two weeks in duration, when examining its effects on skeletal muscle parameters. I also propose that future studies using a mild-intermittent-incremental stress procedure measure serum fatty acid concentration to assess the effect of psychological stress on fat deposit mobilisation. Since corticosterone was unaffected by stress in my study, although the stress procedure was able to reduce body mass after the 1 and 2 weeks of stress and also skeletal muscle mass after 2 weeks of stress, I propose that mediators other than glucocorticoids might be involved. Therefore future studies using a similar model should asses serum cytokine concentration and catecholamines.

However, I can't conclude that glucocorticoids were not involved because my serum corticosterone concentrations had large inter-individual variation in all groups. This indicates that some rats experienced acute effects of stress and others experienced a more chronic stress effect at the time of sacrifice. Admittedly it is my impression that the rats which were sacrificed first on a particular day had lower corticosterone concentrations than those sacrificed later; indicating that the rats sacrificed later experienced acute effects of stress. When examining chronic effects of stress on serum corticosterone response I propose, although difficult, that as few as possible rats be sacrificed on a particular day to eliminate possible acute effects of stress, walking in and out of their housing room to take a rat out of its living cage possibly stresses the remaining rats, even though the sacrifice was done in a separate room 3 rooms away from the housing room.

After introducing the injection procedure at the start of the second week the control rats (CP and Csu) started losing body mass, indicating that the injection procedure was stressful for the rats. Either the injections or the handling of the rats caused stress. I propose that future studies administer the treatment orally (Hashimoto *et al*., 2002, Miguel *et al*., 2005). This is not that easy with *Sutherlandia* because it is a very bitter herb. In my pilot study I fed agar cubes to the rats containing *Sutherlandia*. I used a separator (designed for the dimensions of each cage) to isolate each rat (4 in a cage) from each other inside the living cage. An agar cube was placed inside each isolated space (for an individual rat), but the rats did not eat the cube quickly enough, which was required considering all the procedures the rats needed to do in a particular day. Furthermore isolating the rats can be considered as an added confounding stressor.

I propose that similar future studies use a larger sample size than my own (n = 6 per group), since a number of parameters showed a trend towards significance, without actually being significant. This might reduce the effect of variations that are brought about by a student handling the experimental animals. It is necessary to learn the procedures first, but it takes a long time to gain experience.

Younger rats may be less stressed by handling. Our facility was not breeding animals at the time of my study, so I did not handle the rats from a young age. I suggest that rats bred for stress studies need to be handled more than rats to be used for other kinds of research from the beginning. Also, male rats seemed to be temperamental. Possibly female rats should be used in stress studies, despite the complications of the oestrogen cycle. Also social domination would have less influence.

Despite the problems I encountered, *Sutherlandia* was able to attenuate the stress-induced atrophy on EDL mass and the stress-induced increase in proteasome activity in both muscles in my study. I propose that studies using *Sutherlandia* as a treatment intervention, be continued. As mentioned earlier I

also propose administering the Su orally to eliminate the confounding stressors of injection and handling. A pair-fed control group could also be added in future studies to asses the effect of Su on food intake.

Although Su had no effect on GS activity, I propose that a follow-up study, evaluate the effect of *Sutherlandia* treatment on exogenous glucocorticoid stress induced skeletal muscle atrophy to positively elucidate whether *Sutherlandia* has any effects on GS activity in skeletal muscle. This study should also evaluate muscle glutamine concentration in response to Su treatment, since glutamine availability can also influence GS activity. The use of a mild dose of exogenous glucocorticoids would prevent some of the difficulties experienced with rats habituating to the stress protocol.

Few, if any other studies have looked at muscle TAT activity. I encourage future studies to evaluate the effect of stress on muscle TAT activity. Also they should use predominantly slow-twitch muscle since my study indicated that it is more responsive in the soleus than EDL in response to stress. This might have been an effect in my particular study, but at least both muscle types should be investigated.

Similar to Su treatment I propose that resistance exercise should be used for a longer duration than in my own study (7 days) to asses its effects on skeletal muscle parameters, since the anabolic effect of resistance exercise might take longer than 7 days to become evident. A minimum of 6-8 weeks, unless the atrophy was very severe first. Then 3 weeks may be sufficient. However, to maintain stress for at least 3 weeks is difficult because rodents get used to it. Exercise was administered in the light cycle. Because I wanted to increase resistance, the exercise was stressful. Possibly future studies could use a less stressful resistance exercise protocol such as ladder climbing (Linderman *et al*., 1994) in the dark phase.

In my literature review and my study, I did not consider signalling pathways or specific gene promoter regions of interest. But for more extensive studies, like PhD work, this would be essential. For example future studies might want to asses the effect of Su on muscle specific genes such as muscle-specific RING finger 1 (Murf1) and rat muscle atrophy F-box (MAF*bx)*. Bodine *et al*. (2001) have shown that Murf1 and MAF*bx* knock-out mice lose significantly less muscle mass after muscle denervation than denervation controls. However this is only one example, the number of signalling proteins and genes involved is vast and would require a really comprehensive literature study before an experiment is decided on.

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Appendix

A) Incremental stress protocol

A pilot study was undertaken to acquire a stress protocol that would cause a constant weight loss in a rat model throughout a 2-week protocol. In a previous study (unpublished) from our laboratory we demonstrated that an immobilisation model causes an initial weight loss but is unable to cause a further loss in body mass. Thus a new model was required for sustained stress, in which we had to continually subject the rats to a novel stressor before they could get used to one particular stressor.

On days 1, 2 and 3 rats were weighed and immediately immobilised (see Paragraph 2.3) for one 30 minute session. The rats lost body mass after the first 2 days of immobilisation but it stabilised after the 3rd day. On day 4 an extra immobilisation session (thus 2 x 30 minutes of immobilisation) was added and again the rats lost weight after two days and stabilised after day 3. A third immobilisation session was added and the trend repeated itself.

However, the body mass loss in response to 3 immobilisation sessions were less marked. We then further subjected the rats to novel stressors in addition to the 3 immobilisation sessions. The trend repeated itself each time a novel stressor (for list of stressors see Paragraph 2.3) was added, although the weight loss response became less marked each time. We decided to use an incremental stress procedure in the actual study (see Table 2.1), adding an extra immobilisation session every 3rd day until the rats were subjected to 3 immobilisation sessions per day. Thereafter a novel stressor was introduced to the rats after every 3rd day.

B) Strapping of the weights

Using rats not involved in the experiment we initially tied the weights onto specially made leather jackets and strapped these around the rats with Velcro or

laces. However it turned out to be too complex. Firstly jackets had to be made for the different sizes of each rat. This was impractical since the rats either grew bigger or lost weight during the experimental protocol depending on which procedure (see Table 2.1) they were subjected to. Secondly fitting the rats with the jacket proved difficult, as 3 persons were required to do it. One person was needed to fit the forelimbs into the jacket, one to hold the rat and another to strap the jacket over the scapulae of the rat. The rats also seemed to slip out of the jacket quite easily and strapping the jacket to tightly inhibited them from moving freely. In addition this procedure seemed too stressful for the rat. Since the rats were subjected to an incremental stress protocol with the exercise as a possible treatment, this additional novel stressor could be a confounding factor.

Thus we had to find a way of strapping the weights with something that fitted more easily around the body, which would also be more difficult for the rat to slip out of.

We decided on fabric plaster (Elastoplast), which fits easily around the scapulae. The plaster was cut into strips, long enough to fit around the scapulae and wide enough for the weights to fit into. The weights were covered inside 2 plaster strips and a third strip was used to strap the plaster-covered weights over the scapulae.

For this procedure, two people were needed to strap the weights; one for holding the hind limbs and one to strap the weights as near as possible to the forelimbs. The rats attempted to push out of the strips using their hindlimbs, thus placing the weights nearer to the forelimbs, which made it more difficult to slip out of. Strapping of the CE and StE rats took less than 1 minute each. After strapping a rat it was placed in its individual running wheel, with commencement of the exercise protocol within 6 minutes.

C) Familiarisation with the motor driven wheel

After strapping, rats had to be familiarised with running in the motor driven wheel. On the first 2 days rats ran at 8 m/min without any strapping. On the third day they were strapped with weights 10% of their body weight and again ran at 8 m/min. Only 3 of the 6 rats were able to complete 30 minutes while running at 8 m/min. The 3 rats which could not complete the exercise were removed from the wheels as soon as they were unable to keep running. It took a further 2 days for all the rats to be able to complete the entire exercise session. The next day, the speed of the wheel was increased to 15 m/min (the actual speed they were required to run at). It took another 4 days or all the rats to be able to complete the running exercise while strapped with 40% of their body weight. They were allowed a further 2 days to familiarise to the running conditions while strapped. The rats were allowed to rest for 2 weeks before commencement of the study.

D) Calculations of enzyme activities

i) *Calculation of 20s proteasome activity*

The tables below (Table 4.1– 4.3) show examples of raw values obtained for samples, AMC standards and protein concentrations, which were used to determine 20s proteasome activity.

Table 4.1 Example of absorbency values obtained for BSA standard concentrations

BSA standards (mg/ml)	Absorbance (in duplicate)		Average
0	0	0	0
0.2	0.3763	0.3562	0.36625
0.4	0.4256	0.3969	0.41125
0.6	0.6643	0.6114	0.6378
0.8	0.7367	0.6925	0.7146
1	0.7943	0.7902	0.79225
Slope [(Abs/(mg/ml)]	**0.75**	**0.7392**	**0.7475**

Determination of protein concentration (Bradford)

E.g. Average raw absorbance value obtained for sample = 0.477 absorbency unit (Abs)

Protein concentration = Average absorbance value / Average slope of BSA standard X Dilution factor

= 0.477~~Abs~~ / 0.7475 ~~Abs~~/ (mg/ml) X 12

= 7.657 mg/ml

Or 7.66 g/l

Table 4.2 Example of raw fluorescence values (of actual sample) read every 5 minutes for 1 hour (Fl/min = fluorescence units per minute)

Time (min)	**Raw fluorescence units (RFU) (done in duplicate)**	
0	2232	2224
5	2309	2291
10	2362	2340
15	2412	2392
20	2464	2436
25	2513	2483
30	2558	2530
35	2599	2572
40	2644	2613
45	2677	2659
50	2717	2705
55	2753	2745
60	2785	2794
Slope (Fl/min)	**9.0**	**9.2**

- RFU values obtained for Blanks were 5 and 6 (done in duplicate) for Assay buffer and 15 and 12 (done in duplicate) for 50% DMSO + Assay buffer respectively.
- The graph drawn for RFU against time showed a linear trend for all samples except for 1 (which were omitted), with a Pearson correlation of higher than 0.90 for all samples.

Table 4.3 Example of RFU obtained for AMC standard concentrations

(Fl/µM = Fluorescence units per concentration of AMC standard)

AMC concentration(µM)	RFU (in duplicate)		Average
15.62	3403	3624	3624
7.81	1805	2046	1925
3.90	855	1076	965
1.95	454	662	558
0.97	227	493	360
0.48	137	262	199
0.24	61	105	83
0.12	34	81	57
0.06	23	44	33
Slope (Fl/µM)	**218**	**228**	**230**

- RFU values obtained for Blanks were 5 (for both duplicates) and 8 (for both duplicates) for Assay buffer and for 50% DMSO + Assay buffer respectively.
- The graph drawn for AMC concentration against RFU showed a linear trend with a Pearson correlation of 0.99.

20s proteasome activity = slope of fluorescence per minute of sample (Fl/min) x slope of fluorescence per concentration of AMC standard (Fl/ µ mol/l) x protein concentration (g/l).

$$= \frac{9.001\ \text{Fl}}{\text{min}} \times \frac{230\ \text{Fl}}{\mu\ \text{mol/l}} \times \frac{7.675\ \text{g}}{\text{l}}$$

$$= \frac{9.001\ \text{Fl}}{\text{min}} \times \frac{230\ \cancel{\text{Fl}}}{\cancel{\mu\ \text{mol/l}}} \times \frac{7.675\ \cancel{\text{g}}}{\text{l}} \times \frac{\cancel{\mu\ \text{mol/l}} \times \text{l}}{230\ \cancel{\text{Fl}} \quad 7.675\ \cancel{\text{g}}}$$

$$= \frac{9.001\ \cancel{\text{Fl}}}{\text{min}} \times \frac{\mu\ \text{mol} \times \text{l}}{230\ \cancel{\text{Fl}} \times \text{l} \quad 7.675\ \cancel{\text{g}}}$$

= 0.005 µ mol/min/g. protein x dilution factor

= 0.005 µ mol/min/g. protein x 100/3

= 0.16 µmol/min/g. protein

= 160 nmol/min/g. prot

ii) *Calculation of GS activity*

Example of raw absorbency values read at 540 nm on spectrophotometer (of an actual muscle sample):

- read at time 0 = 0.089
- 20 minutes after incubation = 0.101

Slope = 0.0006 abs/min

Molar absorption coefficient (ε) for GS = 1004 $M^{-1}.cm^{-1}$ or 1004 l/mol. cm^{-1}

The Beer-Lambert law was used to obtain GS activity

Beer-Lambert law ⟶ A = ε x C x l, where

A = slope of absorbence (Abs/min), C = concentration of GS in solution (mol/l) and l = pathlength of the cuvette in which the sample is contained (cm)

$$\text{Thus} \quad \frac{0.0059\ (1)}{(\text{min})} = \frac{1004\ (\text{l}) \times C \times \text{l}\ (\text{cm})}{(\text{mol. cm})}$$

$$\text{Thus C} = \frac{(1)\ 0.059}{(\text{min})} \times \frac{1\ (\text{mol})}{1004\ (\text{l})} \div \text{Protein concentration}$$

Divide C by protein concentration to express GS activity to protein concentration (see D (i) for determination of protein concentration:

$$\text{GS activity} = \frac{(1)\ 0.0006}{(\text{min})} \times \frac{1\ (\text{mol})}{1004\ (\text{l})} \div \frac{(\text{l})}{7.66\ (\text{g})}$$

= 78 µ mol/min/g prot

Correct for dilutions made:

GS activity = 78 µ mol/min/g prot x 10 (dilution factor)

= 780 nmol/min/g prot

iii) **Calculation of TAT activity**

The tables below (Table 4.4 – 4.7) show examples of raw values obtained for samples, HBA standards and protein concentrations, which were used to determine TAT activity.

Table 4.4 Example of raw absorbency units (of actual sample) read every 10 minutes for 30 minutes (Abs/min = Absorbency unit per minute)

Time	**Absorbance (done in duplicate)**	
10	0.836	0.838
20	1.122	1.138
30	1.509	1.437
Slope (Abs/min)	**0.034**	**0.030**

- Absorbency values obtained for Blanks were 0.112 and 0.103 (done in duplicate).
- The graph drawn for absorbency against time showed a linear trend for all samples, with a Pearson correlation of higher than 0.90 for all samples.

4.5 Example of absorbency units obtained for HBA standard concentrations (Abs/ µM = Absorbency unit per concentration of AMC standard)

HBA concentration (µM)	**Absorbance (in triplicate)**			**Average**
100	0.697	0.694	0.698	0.696
90	0.627	0.632	0.636	0.632
70	0.505	0.504	0.506	0.505
50	0.392	0.392	0.395	0.393
30	0.281	0.272	0.274	0.276
20	0.213	0.211	0.215	0.213
10	0.159	0.16	0.155	0.158
Slope (Abs/µM)	0.0059	0.0059	0.0060	0.0060

- Absorbency units obtained for Blanks were 0.097 (for both duplicates).

- The graph drawn for AMC concentration against absorbency values showed a linear trend with a Pearson correlation of 0.966.

Average slope of absorbance per minute of sample = 0.034
Average slope of absorbance per concentration of AMC standard x protein concentration = 0.0060

Table 4.6 Example of absorbency values obtained for BSA standard concentrations

BSA standards (mg/ml)	**Absorbance (in duplicate)**		**Average**
0	0	0	0
0.2	0.1254	0.1211	0.12325
0.4	0.2348	0.2321	0.23345
0.6	0.3672	0.3785	0.3728
0.8	0.501	0.5111	0.5061
1	0.602	0.6203	0.61115
Slope [(Abs/(mg/ml)]	**0.610**	**0.620**	**0.620**

Protein concentration = 6.27 [See Section D (i) for calculation of protein concentration]

TAT activity = slope of absorbance per minute of sample (Abs/min) x slope of absorbance per concentration (Abs/ μ mol) of AMC standard x protein concentration (g/l)

$$= \frac{0.034\ \text{Abs}}{\text{min}} \times \frac{0.0060\ \text{Abs}}{\mu\ \text{mol/l}} \times \frac{6.27\ \text{g}}{\text{l}}$$

$$= \frac{0.034\ \text{Abs}}{\text{min}} \times \frac{0.0060\ \cancel{\text{Abs}}}{\cancel{\mu\ \text{mol/l}}} \times \frac{6.27\ \cancel{\text{g}}}{\text{l}} \times \frac{\cancel{\mu\ \text{mol/l}}}{0.0060\ \cancel{\text{Abs}}} \times \frac{\text{l}}{6.27\ \cancel{\text{g}}}$$

$$= \frac{0.034\ \cancel{\text{Abs}}}{\text{min}} \times \frac{\mu\ \text{mol}}{0.0060\ \cancel{\text{Abs}} \times \cancel{\text{l}}} \times \frac{\text{l}}{6.27\ \cancel{\text{g}}}$$

= 0.9038 µ mol/min/g prot x dilution factor

= 0.9038 µ mol/min/g prot x 15

= 13.56 µ mol/min/g prot