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Inactivity and exercise training differentially regulate the abundance of Na^+, K^+-ATPase in human skeletal muscle

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Abstract

Physical inactivity is a global health risk that can be addressed through application of exercise training suitable for an individual's health and age. People's willingness to participate in physical activity is often limited by an initially poor physical capability and early onset of fatigue. One factor associated with muscle fatigue during intense contractions is an inexcitability of skeletal muscle cells, reflecting impaired transmembrane Na^+/K^+ exchange and membrane depolarisation, which are regulated via the transmembranous protein, Na^+,K^+-ATPase (NKA). This short review focuses on the plasticity of NKA in skeletal muscle in humans following periods of altered usage, exploring NKA upregulation with exercise training and downregulation with physical inactivity. In human skeletal muscle, the NKA content quantified by the [^{3}H]ouabain binding site content shows robust, yet tightly constrained upregulation of 8-22% with physical training, across a broad range of exercise training types. Muscle NKA content in humans undergoes extensive downregulation with injury that involves substantial muscular inactivity. Surprisingly, however, no reduction in NKA content was found in the single study which investigated short-term disuse. Despite clear findings that exercise training and injury modulate NKA content, the adaptability of the individual NKA isoforms in muscle (α_{1-3} and β_{1-3}) and of the accessory and regulatory protein FXYD1, are surprisingly inconsistent across studies, for exercise training, as well as for injury/disuse. Potential reasons for this are explored. Finally, we provide suggestions for future studies to provide greater understanding of NKA regulation during exercise training and inactivity in humans.

Linking health, physical activity, muscle excitability and Na^+,K^+-ATPase

Inactivity or disuse causes diverse negative health outcomes, with inactivity recognised as a contributing factor to many cardiovascular and metabolic diseases, as well as declines in mental health (21, 46, 110). Deconditioning (i.e. lack of fitness) is also recognised as a key factor adversely affecting muscular performance in many chronic diseases (16, 60, 94) and in patients receiving organ transplants (114). An important consideration of this is poor physical conditioning and associated *muscle fatigue*, which directly limit muscle function and the capability to perform repeated muscular contractions that are essential to develop or sustain muscle strength and metabolic health, as well as to prevent severity of sarcopenia (1, 115). On the other hand, physical training does improve health, muscle mass and performance in patients with chronic disease and the general population including by attenuating or delaying muscular fatigue and thereby increasing an individual's capacity to perform exercise. This differs to the intent of training in elite athletes, in which optimizing physical training protocols is critical for ensuring maximal performance of skeletal muscle during competition.

Fatigue during muscle contractions is a topic of major debate still after more than a century of study and is likely to involve both central and peripheral components (1, 78, 84). Full discussion of fatigue is beyond the scope of this short review, so here we focus on one important component of fatigue occurring early in the excitation-contraction cycle, membrane excitability. Membrane excitability is linked with the release of K^+ from the contracting cell into the extracellular space with each action potential and is accompanied by an influx of Na^+ from the extracellular space into the muscle cell; repeated action potentials can lead to depolarisation of the membrane leading to inexcitability of the muscle fiber, thus contributing to fatigue (78).

The Na^+,K^+-ATPase (NKA) plays a critical role in the regulation of concentration gradients for K^+ and Na^+ ions and thus, in the maintenance of membrane potential to enable continued propagation of action potentials along the sarcolemma and into the transverse tubular system (18, 78, 101). Understanding the NKA adaptability in muscle to training and downregulation to inactivity are of key interest for muscle NKA, Na^+/K^+ regulation and fatigue. The present review extends earlier reviews which included sections on muscle NKA, but focussed on training and electrolyte regulation (77, 80) on muscle NKA regulation (18) and NKA and its contribution to fatigue at a cellular level (78). Here we explore NKA adaptability in response to increased physical activity through exercise training regimes, as well as downregulation with reduced physical activity, through injury and induced inactivity. The

review focusses on skeletal muscle in humans wherever possible and includes focus on NKA isoforms as well as total content. All biopsies in these studies were taken from the vastus lateralis muscle, unless otherwise stated. In order to fully understand the findings, we also briefly discuss important methodological techniques used to measure NKA in human skeletal muscle and their implications.

NKA in skeletal muscle

The NKA is a heterodimer comprized of an alpha subunit with ten transmembrane segments, and beta subunit, as well as an accessory protein from the FXYD family (18, 87). In human skeletal muscle the exact locations of NKA are not yet determined, whereas in rodent muscle the NKA are predominantly located in the plasma membrane' and within the t-tubules' (56). The α subunit comprizes four isoforms (α_{1-4}), but with only α_{1-3} expressed at the protein level in skeletal muscle; the β subunit comprizes three isoforms (β_{1-3}), with each expressed in skeletal muscle (89). The specific functions of the α isoforms have not yet been clarified in human muscle, but have been assumed to be similar to those identified in skeletal muscle of other species. In rodent skeletal muscle, the α_1 isoform is important for Na^+/K^+ regulation under basal conditions and has also recently been found to have an important intracellular signalling role in skeletal muscle growth, using an α_1-modified murine model (63). The α_2 isoform, also the most abundant α isoform, is primarily responsible for regulating the large Na^+/K^+ fluxes that occur during muscle contractions (42, 44, 75, 98). The role for the α_3 isoform in skeletal muscle remains unclear. The β_1 isoform is highly abundant in skeletal muscle (11) and is critical in NKA integration into the cell membrane (28) and plays a key role in regulating NKA enzymatic activity (64). The β_1 isoform is highly expressed in slow muscle but is near undetectable in fast muscle, where the β_2 isoform is heavily abundant (55, 91, 109). Thus, both the β_1 and β_2 isoforms must make heterodimers with both α_1 and α_2 isoforms to enable NKA activity and the composition of these heterodimers differs between slow and fast muscles in the rodent. The role of the NKA β_3 isoform in skeletal muscle is however, unclear. In human skeletal muscle, fiber-type heterogeneity is an important consideration of muscle performance, thus the expression of NKA isoforms in different fiber-types are of high interest. The α_2 was shown to be more abundant in Type II fibers in two studies (17, 108), conversely two other studies found no difference in the abundance of α_2 in either fiber-types (119, 120) while the β_2 isoform was more abundant in fast than slow twitch fibers (17, 120).

Phospholemman (FXYD1) is the main isoform of the FXYD family expressed in skeletal muscle, where it mainly associates with the NKA α_1 and α_2 isoforms (29, 99, 100); a further isoform, FXYD5, is also expressed in skeletal muscle (13, 72). FXYD1 binds to the α subunits in an unphosphorylated state and reduces α subunit Na^+ affinity (26), whereas when FXYD1 is phosphorylated, Na^+ affinity is increased (10). FXYD1 acts as a main substrate for protein kinase A and C phosphorylation in skeletal muscle (30), and it appears that FXYD1 is necessary for maximal activation of the NKA (100). FXYD1 is not expressed in a fiber type specific manner (108) but does undergo fiber-type specific phosphorylation after brief and intense acute exercise bouts (108). FXYD5 upregulation has also been shown to be responsible for increasing NKA activity (72), but nothing is known regarding its possible fiber-type specificity. Further information regarding the activation of the NKA acutely can be found in an excellent recent review (97).

Measurement of NKA in Human Skeletal Muscle - Methodological Considerations.

Outcome measures.

1. *[^{3}H]ouabain binding site content*

The [^{3}H]ouabain binding site content technique provides an absolute measurement of the NKA in molar units (pmol.g wet wt^{-1} muscle). Readers are referred elsewhere to detailed discussion of the [^{3}H]ouabain binding site content methodology and its significance (18, 19). In brief, the [^{3}H]ouabain binds stoichiometrically to the α subunit of the NKA, thereby allowing quantification of the content of these subunits, with the specific α isoform detected dependent on the differing affinity to ouabain of α isoforms in some species, and the concentration of ouabain used. The NKA are located in both the sarcolemma and the transverse tubules in muscle (18). The [^{3}H]ouabain binding site content in rat soleus muscles was identical when using either cut muscle pieces or intact muscles, thus this method ensures quantification of all NKA in sarcolemmal and transverse tubular membranes, at least for NKA that incorporate the α_2 isoform (79). Similar analyses have not been conducted on human muscles, since muscle biopsies contain cut pieces only. Due to the high affinity of ouabain binding to all α isoforms in human muscle (93, 111), the [^{3}H]ouabain binding site content can also be referred to as the total NKA content in human skeletal muscle. In rat muscle the α_1 isoform makes up approximately 20% of the NKA α subunits; however the α_1 has a lower affinity to cardiac glycosides which doesn't allow for the α_1 to be detected using the standard [^{3}H]ouabain binding site content technique (42). Thus, in rodent muscles, the standard [^{3}H]ouabain binding site technique detects all α_2 but not α_1

isoforms and is not a full quantification of total content. Regardless, the α_2 is believed to be the major isoform in skeletal muscle (44). Thus research in rodents which showed e.g. increases in [^{3}H]ouabain binding site content with training, represent a gain in the NKA α_2 isoform protein (58), whereas e.g. increases in human muscle with training, would primarily reflect increases in α_2, but could also include changes in the α_1 or α_3 isoforms (82). A limitation of the standard [^{3}H]ouabain binding site content technique for studying adaptability in human skeletal muscle is that it cannot differentiate between binding to the three α isoforms, although using different concentrations of ouabain have been used for this purpose in muscle in some other species (61). A second limitation of the [^{3}H]ouabain binding site content technique is the slow incubation time for [^{3}H]ouabain to the muscle NKA, typically around ~2 h to saturate all sarcolemmal and t-tubular membranes, which means that impacts of processes changing within muscle on a more rapid time frame on NKA including hormonal changes, nutritional supplementation and acute exercise on e.g. translocation might not be detected (97). However, this latter limitation is not relevant to interpretation of the total NKA content in muscle, in particular with training or inactivity interventions, as biopsies are generally taken under resting conditions before and after a medium-long term intervention. Hence this long in-vitro incubation time for NKA content measurements will not affect training induced changes in resting skeletal muscle.

2. *NKA isoform proteins*

Western blotting is commonly used to investigate possible changes in NKA isoform abundance and phosphorylation with inactivity and training. The immunoblot technique should detect all NKA protein for the specific isoform probed, regardless of their membrane location, or incorporation into a functional NKA dimer. Thus the technique would also be expected to detect any isoform proteins present. In contrast the [^{3}H]ouabain binding site technique detects functional NKA dimers, being locked into a conformation by vanadate to facilitate ouabain binding. That these different techniques are detecting some differences is suggested by the considerably different responses in percentage terms to training (see later). Thus whilst immunoblotting allows investigation of relative changes in abundance (e.g. with training), this does not allow quantification with molar units (19). Different analytical techniques are used which should be considered when evaluating differences in findings between research groups. Some studies used a fractionated muscle lysate for western blotting analyses (9, 106, 107), whilst others employed whole homogenate as the preparation of the sample (6, 88, 89, 96, 118, 120). Readers are referred to two excellent methods papers regarding western

blotting for more detail on these issues (76, 90).This difference in sample preparation may have an effect on the yield of the isoform retrieved (76, 90). An additional issue, which is not well documented within the literature, is the heating of a sample over 60°C, which can lead to aggregation of integral membrane proteins interpretations may be inaccurate compared to studies where no heating was employed. As a semi-quantitative technique, western blotting probably has greater variability in the magnitude of change compared to quantitative techniques such as [^{3}H]ouabain binding site content. The typical error of western blotting for NKA isoforms was recently reported to be 10-30% (17). Thus, western blotting may not have the sensitivity to detect small changes in NKA isoforms. Another issue with western blotting is that potential adaptations may not have been detected due to proteins being measured in a mixed-muscle homogenate sample, rather than in individual muscle fibers. It is possible that some studies failed to detect actual changes in NKA isoform proteins that occurred in one fibre type only, by not measuring NKA isoforms at the single fiber level. To overcome this, researchers have begun isolating segments of single fibers from human muscle biopsies and performing western blots. So far, single fiber analyses have been utilized to investigate effects of both training and inactivity (96, 118, 120), these changes are described in more detail within the inactivity and training sections of this manuscript.

Other methodological considerations

1. Intervention differences

Many training studies that measured isoform abundance adaptations used trained populations, such as elite cyclists, football players, as well as recreationally active participants. The varying athletic status is likely to be important, as an earlier cross sectional analysis indicated that well-trained participants had a higher abundance of NKA isoforms, relative to recreationally active participants (88). Therefore, the level of stimulus required to increase the abundance of NKA isoforms in muscle may be greater in athletes compared with recreationally active and non-trained individuals. The experimental design also varies tremendously, making it difficult to make direct comparisons between studies, or to investigate any association between upregulation of NKA isoforms with any training modality or duration. Some studies also utilized High-Intensity Interval Training (HIT), speed endurance training (SET), or sprint training (ST) in replacement of regular training (51), or to supplement training (6, 8, 41), whilst others combined multiple training modalities within the same

study (106, 117). This makes their findings more difficult to compare with studies that used SET, ST, repeat sprint exercise (RSE) or HIT as the sole training modality (86, 92, 118, 120).

Finally, the sample size studied is important and insufficient statistical power may limit the capacity to detect changes in NKA isoform abundances in a number of training studies. The typical sample size for studies ranged between 8-15 participants, however in instances where n=15 were studied, these were often divided into two different groups (51, 86). A challenge of this research is finding sufficient numbers of volunteers willing to undergo invasive procedures on multiple occasions, which explains why sample sizes are often limited. This issue of small sample size and lower power is especially prevalent in invasive training studies with humans, nonetheless, future studies should embark on larger scale, simple training interventions to minimise potential effects of insufficient statistical power. This could be achieved by multicentre trials across institutions to recruit increased numbers of participants thus generating larger data sets.

Disuse effects on muscle NKA content

Both injury and disuse models have been used to study the broad effects of inactivity within skeletal muscle (12). Common disuse models include bed rest (54) with studies extending for as long as 119 days (65), or immobilisation, which typically involves a cast placed around a limb to prevent dynamic muscle contractions and movement. A less constrictive approach is that of Unilateral Lower Limb Suspension (ULLS), in which participants wear one shoe with an extended sole (~10 cm) and walk with the assistance of crutches, causing one leg to become unloaded (105), with the contralateral leg acting as a control leg (96). A model used in athletes is reduced muscular usage or detraining that occurs with cessation of training, often after completion of a competitive season (106). The literature examining the effects of injury or inactivity on NKA in human skeletal muscle is currently sparse, being limited to only six studies, likely due to the extremely difficult nature of these studies, which combined with invasive measurements involve major disruption to a participant's daily life. Thus future studies are still required to understand the effects of inactivity on muscle NKA. In lieu of these challenges surrounding human volunteers, different models of human inactivity including astronauts, as well as a multicentre approach, should be used to investigate effects on muscle NKA. Here we have reviewed findings of the current studies which have investigated inactivity and NKA in human skeletal muscle.

Different types of injury that induce severe localised inactivity have been found to decrease muscle NKA content, including shoulder impingement syndrome, anterior cruciate ligament injury (using a contralateral limb as a control), paraplegia and partial spinal injury compared to ambulant, age-matched controls (13, 22, 67, 95). The muscle NKA content was reduced with these injuries, with declines ranging from 20-23% in patients with ruptured anterior cruciate ligament (n=6, mean age 25 years, 5-50 weeks post-injury) (95), 27% in patients with shoulder impingement syndrome (n=6, mean age 44 years, at least 11-77 months post-injury) (67), 34% in paraplegia patients (n=6, mean age 32 years, 1-19 years post-injury) compared to the deltoid of the same patients (22); and as much as a 45% decline in chronic cervical spinal injury patients (n=6, mean age 44 years, injured for multiple years) compared to controls (13). However, for all of these studies, it is possible that in addition to enforced muscular inactivity, effects consequent to the injury per se, or medical treatment may also have had impact on muscle NKA. Thus a preferred approach is to investigate muscle unloading per se in otherwise healthy individuals, but to date only a single study has investigated the impacts of voluntary unloading on NKA (96). A surprising finding was that 23-days of ULLS failed to cause any decrease in NKA content, despite substantial impairment of muscle mass and function, including exercise performance (96). One interpretation of the lack of NKA downregulation after ULLS compared to the marked reductions in muscle NKA content with injury, is that differences may in part be attributed to the short time frame of the ULLS intervention. In animal models, where lifespans are much shorter, short-term inactivity induced substantial reductions in muscle [^{3}H]ouabain binding site content when expressed relative to muscle wet weight; falling by 20% in soleus muscle after 1 week limb casting in rats (58), by 23-25% in gastrocnemius muscle and 18-19% in the plantaris muscle, after 2-3 weeks partial immobilisation using a prosthesis in guinea pigs, (66) and by 39% after 9 weeks limb casting in sheep (52). Substantial recovery in muscle [^{3}H]ouabain binding site content in sheep muscle occurred after subsequent 9 weeks of remobilisation (52). Immobilisation in young rats (5 days old) for 7 days reduced the normal gain that occurred at that age in [^{3}H]ouabain binding in soleus muscles by 33% (112). Partial immobilisation for 3 weeks also allowed eventual recovery of [^{3}H]ouabain binding site content (66). Inactivity subsequent to training also reduced the muscle [^{3}H]ouabain binding site content; 6 weeks of swim training induced ~41% and ~46% upregulation in soleus and extensor digitorum longus muscles, respectively, whereas 3 weeks of subsequent rest reduced NKA by ~34% and ~26%, respectively (58). Therefore the results from these inactivity

studies in animals suggest that either a longer duration or greater severity of unloading may be required to depress NKA content in human skeletal muscle and the balance between mRNA mediated synthesis and degradation rates of NKA proteins. Other factors concomitant with injury, such as enhanced local inflammation (69, 70) and changes to neurotrophic factors (103) may also exert effects additive to those of disuse per se, but these are untested in relation to NKA expression

Disuse effects on muscle NKA Isoform abundances

Only three studies have investigated the effects of injury and inactivity on muscle NKA isoform abundances in humans. Patients with chronic cervical spinal injury (n=6, mean age 44 years, injured for multiple years) had 75%, 52% and 38% lower NKA α_1, α_2 and β_1 abundances in the vastus lateralis muscle, respectively, compared to healthy controls (13). Interestingly, those patients who were able to perform daily activities despite partial cervical spinal injury (n=6, mean age 49 years) actually exhibited no differences in NKA isoform abundances in the paralysed vastus lateralis muscle (13). Following 3 weeks of muscular disuse induced by ULLS in healthy young adults, there were no changes in the α_1 or α_2 isoform abundances, whether measured in either whole muscle homogenates or in single muscle fibers (96). However, after ULLS, the β_1 isoform protein abundance was lower in Type II fibers (40%) and was also restored following resistance training; no changes were detected in homogenates (96). NKA heterodimers with a β_1 isoform have been suggested to support higher NKA activity by having a greater affinity for Na^+ than the α/β_2 heterodimer (64); thus a loss of β_1 may imply a reduced number of functional NKA heterodimers present in Type II fibres of skeletal muscle after ULLS. The functional effects of possible reduction in β isoforms are not clear, as skeletal muscle is thought to have an excess abundance of β compared to α subunits (64). Similarly, no changes in the α_1, α_2 or β_1 isoform abundances were found after a less severe inactivity model, comprising cessation of training for two weeks following the end of a soccer season and with isoforms measured in fractionated lysates (106). These studies strongly suggest, consistent with findings in NKA content, that reductions in muscle NKA isoforms are only induced by a severe lack of physical activity over a prolonged period. This conclusion is surprising given the large and rapid reductions in NKA isoforms evidenced in animal models. In rat muscle, the marked reductions in [^{3}H]ouabain binding site content with one week inactivity represent mainly a reduction in the NKA α_2 isoform protein (58), due to its high affinity to ouabain (18) and as the dominant α isoform expressed in muscle (42, 44). Changes in NKA α_2 isoforms are also highly complex and time-dependent. Hindlimb suspension in rats reduced

the electrogenic activity of the α_2 isoform protein, measured via ouabain-suppressible activity. Surprisingly, the reduction in electrogenic α_2 activity was accompanied with an initial doubling in α_2 protein abundance after 24 h and with a ~50% elevation still remaining at 72 hours post-intervention, β subunit protein abundances were unfortunately not reported (61). This indicates that the reduction in α_2 electrogenic activity was due to a decline in NKA enzymatic activity per se; interestingly, no changes were found in the same measures for the α_1 isoform in the soleus muscle (61). These changes were subsequently demonstrated in a time frame as short as 12 h post hindlimb suspension (62). These changes in NKA may also be responsive to changes in plasma [K^+], with hypokalaemia having a profound impact on NKA content and specific isoform abundance, with particular effects on α_2 as seen in studies with rodents. When rats were placed on K^+ deficient diets over a period of 1-4 weeks, the α_2 showed a progressive decline and disappeared after 3 weeks (48). It has been suggested that decreased [K^+] may be important in suppressing mRNA to protein translation, at least for the α_2 isoform (7). Conversely, hyperkalaemia typically induces increases in NKA content, as increased K^+ clearance is required; in rats this was observed within 7 days of a high K^+ diet (15). The link between voluntary inactivity and plasma [K^+] changes in humans are not known, however, after 23 days of ULLS plasma [K^+] at rest was not altered (96). Thus, in short-term inactivity studies investigating muscle NKA content or isoform abundances, any alterations are less likely to be changes in plasma [K^+], at least in healthy populations. Hence, the time course of these changes and the underlying mechanisms in human muscle of considerable interest for future studies to explore.

Muscle FXYD following inactivity

Despite its emerging importance in regulating NKA activity (10), few studies have investigated the regulation of FXYD with disuse in human skeletal muscle. Cervical injury patients had 52% lower muscle FXYD1 content compared to healthy controls, with no difference in phosphorylation at $FXYD1^{ser63}$ and $FXYD1^{ser68}$ (13). The amount of basal and phosphorylated FXYD1 in the cervical spinal injury patients capable of ambulation (i.e. able to perform some movements) were not different from the controls (13). There was also an increase of the FXYD5 in the spinal injury patients (13). These few studies indicate that injury and physical inactivity clearly can regulate the abundance of the FXYD1 and 5 proteins. In addition, these findings in cervical injury patients indicate that reductions in the FXYD1 due to inactivity may not be related to the abundance of phosphorylation of FXYD1. It is possible that the unchanged phosphorylation of FXYD1 and increases in FXYD5 compensated for the

dramatic decline in α_1, α_2 and β_1 isoforms and total amount of FXYD1 in these patients, thereby assisting in maintenance of functional NKA. Thus the abundance of the FXYD1 and 5 proteins may regulate the catalytic activity of the NKA despite declines in isoform abundance associated with inactivity.

The effects of disuse on the abundance of FXYD1 in skeletal muscle has not been extensively studied in healthy humans. Following two weeks of cessation of training in soccer players, there was no change in the abundance of FXYD1, however, there was a decrease in the phosphorylation of $FXYD1^{ser68}$ by 19% and 18% at 72 h and 2 weeks after training cessation, respectively (106). Given the training status of these participants, it is likely that FXYD1 proteins were already elevated by training; this is likely to be a typical post-training reduction rather than a true disuse effect.

Effect of exercise training on muscle NKA

Classification of modalities of physical training.

The first investigation into adaptability of muscle NKA with longitudinal exercise training was conducted nearly three decades ago (57). Since then numerous studies have investigated exercise training effects on muscle NKA content, NKA isoforms using a broad range of training modalities, which especially for high intensity training, have adapted over time and thus require definition. For the purpose of comparison of training effects on NKA in this review, exercise training modalities have been classified into three broad categories, defined as Endurance Training (ET), High Intensity Training (HIT) and Resistance Training (RT), as described in Table 1. Each of these exercise types will likely recruit a differing proportion of both Type I and Type II fibres; Type I fibres are more heavily recruited during submaximal endurance exercise, whereas during high intensity exercise, Type II fibres are recruited in additional to Type I (24). Thus the implementation of these exercises may influence NKA isoform contribution to exercise. ET is defined as training that comprizes exercise bouts performed at an intensity between 50-80% of an individual's maximum oxygen consumption (VO_{2max}) and typically sustained for a prolonged period, therefore having a heavy reliance on aerobic metabolic pathways. High Intensity Training (HIT) is defined as training utilising repeated, short duration, intense exercise bouts, interspersed with passive or active recovery periods, requiring a heavy contribution from anaerobic metabolism. HIT typically comprizes 4-10 bouts, of 10 s to 4 min duration, completed at work rates ≥ 90% VO_{2peak}, or with longer ~4 min bouts ≥ 80% VO_2 peak (31, 32, 50, 82). HIT can therefore be further classified into several sub-types of training, including Aerobic

High Intensity Training (AHIT), Speed Endurance Training (SET), Sprint/Speed Training (ST) and Repeat Sprint Exercise (RSE). Aerobic High Intensity Training (AHIT) is defined as repeated bouts of exercise between 1-5 minutes ≥ 80% VO_2 peak (6, 9) or HR max (33, 106) the recovery time is between 1:0.5 up to 1:2 work rest ratio.

Speed Endurance Training comprizes repeated 10-40 s sprint bouts of near-maximal intensity, with a 1:5 work rest ratio (50), this type of training has also previously been termed sprint training (43, 82), but for consistency we will refer to this type of training as SET. Speed training (ST) comprizes 2-10 s maximal exercise, with recovery periods up to 1:10 work rest ratio (50). Repeat-sprint exercise (RSE) comprizes multiple (4-6) high-intensity bursts, each lasting between 2-6 s, interspersed by a brief recovery period (102, 104) and are typically used to be comparable with efforts produced during intermittent team sports, such as soccer, rugby, Australian football and hockey (4, 53, 113).

Resistance Training (RT) is classically defined as moving limbs/ or body segments against various resistances including machines, dumbbells, body weight and cables and is utilized to improve muscle strength and power and to promote muscular hypertrophy. The performance benefits of ET, HIT and RT have been well described elsewhere (31, 33, 50, 85) and hence are not covered here.

Adaptations in muscle NKA content with endurance and high intensity training

The findings of studies investigating training effects on muscle NKA content are indicated in Table 2. In order to summarise this literature, we searched for studies involving humans which had investigated muscle [^{3}H]ouabain binding and/or muscle NKA isoforms with training or inactivity. No studies were excluded and those that measured but failed to detect any upregulation with training were also cited. The studies are broadly consistent, with 8-25% increases in NKA content elicited with training, in 10 out of 12 studies published to date. Furthermore, and importantly, these increases appear to be regardless of the type of training utilised, or the population studied. Only two of these studies did not detect an increase in NKA content; in the first neither the training modality nor fitness status of participants were detailed (57), whilst in the more recent study, the participants were already well-trained cyclists ($VO_{2\ peak}$ 4.9 L.min^{-1}) (6). Thus, it is possible that the training stimulus used was sub-optimal or that the muscle NKA content may already have been elevated before the training intervention (88). Nonetheless, upregulation of NKA content in muscle is clearly a consistent finding. To compare findings from the various studies, the 90% Confidence Interval (90%CI) was calculated utilising each of the percentage increases in NKA content, reported p values and sample size (47).

370 Where the precise p value was not presented, but rather reported as $p<0.05$, we took a conservative
371 approach, using a p-value of 0.049 for consistency across analysis. The study by (57) was not
372 included as insufficient data were reported. The objective was to identify whether there were any
373 apparent differences in adaptation with different training modes. The data reveals firstly that NKA
374 content was consistently increased with training, between 8-22%, regardless of training modality,
375 whether studied in healthy young or older adults, or in Type I diabetics (Figure 1). Furthermore, the
376 percentage increase in NKA content was not related to either the mean training intensity or
377 cumulative training time (Figure 2). An important additional finding was that the training duration did
378 not affect the gain in muscle NKA content. An increase in NKA content was found after only one week
379 of ET (39) and participants undertaking ET exhibited a 22% increase in NKA content after 3 weeks,
380 but with no further increase after 12 weeks (34). Thus, the mean gain in NKA content did not exceed
381 ~25%, even when training exceeded 3 months. Elderly also displayed a similar muscle NKA content
382 upregulation with training, with an 11% increase after 12 weeks of HIT (118). An early cross sectional
383 study demonstrated that older adults who had been active for over 10 years had higher muscle NKA
384 content compared to sedentary older adults, which ranged between 30-40% depending on the type of
385 training, including swimming (30%), running (32%) and RT (40%) (59). It is of interest to compare
386 these findings in human muscles, to those with chronically stimulated muscles in animal models. Low
387 frequency stimulation of extensor digitorum longus muscle in rabbits rapidly increased the [^{3}H]ouabain
388 binding site content by ~41% after only 3 days, 86% after 10 days, then plateaued, with no further
389 increase after 50 days (37). Even larger increases were found in a subsequent study, where a gain in
390 [^{3}H]ouabain binding site content of 60% occurred after 6 days and by 107% after 20 days chronic low
391 frequency stimulation (45). Apparently, there are clear differences between species in the magnitude
392 and rate of adaptation of muscle NKA. Within humans, the importance of the NKA increasing during
393 training has obvious implications for maintaining membrane potential and K^+ clearance during
394 exercise, for improvement of exercise performance (78, 82). But the time course of adaptability in
395 NKA in human skeletal muscle content are needed to understand why and when NKA adaptation
396 reaches a plateau. For example in the studies listed in Table 2, the maximum NKA content increase
397 was ~25%. There are several possible speculations on what may be limiting increases in NKA content
398 with training. A consideration might be that the stimulus of training isn't eliciting the same 'new
399 stimulus' and thus over time, the NKA pool is able to better cope with the demands of the training

session, the physiological challenge is decreased with a lesser requirement for synthesis of new NKA. Secondly, it might be dangerous to synthesize NKA beyond a particular threshold within a given individual. As muscle makes up 40% human body mass its role in clearing increases of K^+ is extremely important. Thus more NKA in muscle would enable greater K^+ clearance and thereby better performance; however this also has large potential effects on post-exercise plasma [K^+]. Hypokalaemia is commonly reported within the first 5-10 minutes of recovery from an acute bout of intensive exercise, in particular exercise utilizing a large muscle mass (e.g. rowing, sprint cycling) (2, 3, 71); this is likely due to an highly activated NKA. Hypokalaemia has important adverse implications for cardiac muscle (71) with a recent study showing the post-exercise hypokalaemia was associated with impaired cardiac hysteresis measured via electrocardiogram (3). This lowered K^+ post exercise therefore has implications for cardiac arrhythmias and sudden cardiac death after exercise (3). Thus a training plateau of the increase in NKA content may be a protective mechanism, however more research is required to determine the time point or physiological point where this plateau is reached.

Adaptations in NKA content with resistance training.

Three studies have examined the effects of RT on skeletal muscle NKA content. In one study, participants performed RT for 12 weeks, comprising 3 sets of 6-8 repetitions of each of leg press, squat and leg extension exercises, finding that muscle NKA content was unchanged after 4 weeks (34), increased by 16% after 7 weeks, but then remained constant until 12 weeks (34). In another study, well-trained cross country skiers undertook RT comprising five series of four heavy full squat lifts, with a focus on eccentric contractions, completed either once, twice or three times per week, for 3 months (83). They found that NKA content was not significantly increased in the athletes that undertook RT only once a week, but was increased when athletes trained twice and three times per week by 15% in the pooled results (83). In the third study, the effects of 4 weeks RT on muscle NKA content were examined in six healthy participants, with RT undertaken immediately following a 23-day period of ULLS. Interestingly, RT had no effect on the NKA content, despite gains in both muscle mass and strength (96). Regardless, an unchanged NKA content in the context of an overall increased muscle mass would in fact suggest an increased NKA synthesis commensurate with the increased muscle protein content, but detailed studies are required to verify this.

Adaptations in muscle NKA content with exercise training in hypoxia

Whilst almost all exercise training studies reported an increased muscle NKA content, undertaking training with hypoxic exposure actually induced the opposite effect of reducing NKA content, at least for ET. Participants who performed ET in normoxia exhibited a 14% increase in muscle NKA content after ET, whereas a group that trained under hypoxic conditions over 8 weeks had a decline in muscle NKA content by 14% (35). This decline was similar to the 14% reduction found after a 21-day expedition to 6,194 m in recreationally active people (36). Practically, this implies that training in hypoxia per se may not be beneficial for enhancing muscle performance. Mechanistically, this may be due to reactive oxygen species (ROS) which are generated during exercise, ROS generation is amplified when training in hypoxia (74) and ROS may inhibit NKA activity during exercise (81) and thus muscle cellular responses to chronic hypoxia may prematurely impair NKA activity and excitability during training. From a training perspective, the quality and capacity of each training session would then be compromised, with athletes' therefore not reaching required training load and reflective in a lack of NKA responses (5). Regarding chronic hypoxic exposure that caused a reduction in NKA content, although it was hypothesised exposure to hypoxia may result in greater protein breakdown and thus a loss of NKA was seen after 21 days reaching 6,194 m (36), there is little evidence to directly support this explanation. An alternative approach to training in hypoxia, that allows athletes to receive the beneficial adaptions of altitude exposure has been termed Live-High, Train-Low (LHTL) (68). When well-trained endurance athletes continued their normal training whilst undertaking 23 consecutive nights of hypoxic exposure, no change in muscle NKA content occurred (5) thereby intermittent exposure to hypoxia may be more beneficial to NKA and allows athletes are able to train at appropriate intensities while obtaining haematological benefit (27, 49).

Muscle NKA isoform adaptability to training

Over the past decade, there has been considerable interest in determining the malleability of NKA isoforms in human skeletal muscle with training. These studies show highly variable responsiveness of specific NKA isoforms to various training modalities (Table 2). The percent change ±90% CI (47) for most commonly measured NKA isoforms, α_1, α_2 and β_1 with training is presented in Figure 3. Only around one-half of the studies published to date reported increases in these isoforms, with increases found for α_1 in 6 of 13 studies, for α_2 in 6 of 13 studies and for β_1 isoforms in 7 of 13 studies. It is surprising that less than one-half of studies utilizing western blotting detected an increase in the α_2

isoform with training. There is no apparent consistency regarding the upregulation of any isoform with a particular type of training. This may be due to methodological considerations, as outlined on page's 5-7. It is also unclear whether any particular training modality consistently increased one isoform more than another. Only three of these 13 studies detected an increase for each of the $\alpha_{1,}$ α_2 and β_1 isoforms (9, 20, 38). One study utilizing high-intensity single leg cycling reported an increase in both the α_1 and α_2 isoforms (92); while SET (running) increased in both the α_2 and β_1 isoforms (86); two studies which incorporated either regular football (soccer) training or repeated small sided soccer drills (8x2 min) in conjunction with SET running training found increases in α_2 (8, 106); another study found sprint training (running) exclusively increased α_1 (51); while a combination of mixed RT and SET training found an increase only in β_1 (117).

NKA isoform measurements within single muscle fibers

There have been a handful of studies conducted within single fibers to elucidate how the NKA works during exercise and adapts to training. The first study examined acute exercise responses primarily focusing on FXYD1 phosphorylation (108). Following a 5-min bout of intense exercise, corresponding to ~95% of maximal oxygen uptake on a cycle ergometer, there was an increase in phosphorylation of $FXYD1^{ser68}$ in Type II fibers and increased unspecified FXYD1 phosphorylation in both Type I and II fibers (108). Following 4 weeks of RSE training, which comprised 3 sets of 5 x 4 s sprints performed on a non-motorised treadmill, there was a 42% increase in β_1 isoform protein abundance in Type II fibers, with no changes found for other isoforms (120). A 12-week training protocol comprising four 4-min bouts at 95% peak heart rate, performed 3 times per week in adults aged over 65 years, showed a 30%-increase in α_2 in Type II fibers with no other isoforms being upregulated (118). In adults aged between 18-35 years, six weeks of High-Intensity Training (HIT) comprising 4x 30s sprints, with 4 minutes recovery between sprints, induced increases in the α_1 and β_3 isoforms in both Type I and II fibers, β_1 in Type II fibers, and decreases in FXYD1 in Type I fibers (17).

Despite a lack of consistency around training and isoform upregulation, one observation is the studies that found increases in α_2 utilized training that comprized either exercise of high intensity, ranging between 90-150%of VO_2 max or running speed (8, 9, 86, 92, 106), or of high volume, with training sessions lasting between 60-120 minutes (9, 38). One RT study also found increases in the α_2 isoform (20), which might relate to the repeated highly intense contractions performed in RT. It is likely that

intense quadriceps contractions during these high-intensity or high-volume running and cycle training studies (5, 6, 71, 76, 90), as well as during RT (14) also indicate heavy recruitment of the vastus lateralis muscle, hence accounting for the consistent elevations in the NKA α_2 isoform in the vastus lateralis, thus explaining why different modes of exercise training induced similar outcomes for α_2.

Muscle FXYD1 and training

Ten days of training which incorporated both ET at ~75% $VO_{2\ peak}$ for 45-90 min and AHIT (comprising 6x5 min intervals at 90-100% $VO_{2\ peak}$), had no effect on total FXYD1 content or phosphorylation at Ser^{63}, Ser^{68} or Thr^{69}, despite upregulation of each of the NKA α_1, α_2 and β_1 isoforms (9). In contrast, after 2 weeks combined SET and AHIT, FXYD1 phosphorylation on site Ser^{68} relative total FXYD1 was increased by 27% (106). Similarly, in well trained endurance cyclists, subsequent to a reduction in training volume by ~70% and then replaced with SET and AHIT, there was a 30% increase in FXYD1 protein abundance and an increase in non-specific FXYD1 phosphorylation, suggested to be attained through phosphorylation at Ser^{68} (107). An interesting observation is when there was a heavy ET component during 10 d of one-legged cycling training, there were no changes in FXYD1 phosphorylation on sites Ser^{63}, Ser^{68} or Thr^{69} or the total FXYD1 abundance (9). Conversely, when intermittent intense exercise training was predominantly used, both FXYD1 abundance and phosphorylation were increased (106, 107). Together this suggests a higher intensity of training may be required to induce FXYD1 phosphorylation adaptations.

Association between muscle NKA, performance and fatigue

The increases of α_2/β_1 isoforms in skeletal muscle with training reported in a number of studies may have considerable implications for NKA activity and exercise performance, but it is important to acknowledge that these changes have not been consistently reported. The fact that the [^{3}H]ouabain binding sites are increased suggests that the α_2 isoform at least should also be elevated and points to methodological reasons underpinning the inconsistent findings. Both α_2 and β_1 isoforms are believed to be the major isoforms employed during muscle contractions/exercise (64, 98). The α_2 isoform abundance was correlated to high-intensity running during soccer. Importantly, the α_2 and β_1 isoforms are each expressed in Type I versus II muscle fibers with no fibre-type dominance being reported (119, 120). This suggests that both isoforms can exert an effect on the whole muscle, rather than being constrained to a dominant effect in one fiber-type only, as is the case for other enzymes and proteins that are expressed specifically in one fiber-type only in skeletal muscle. The use of co-

518 immunoprecipitation of α, β and γ subunit isoforms would be particularly valuable in identifying fiber-
519 type specific heterodimers. The same could be said for improvements in the NKA α_1 isoform, which
520 was observed to adapt as often as the α_2 isoform, but just as inconsistently, and which also showed
521 the largest reported increase in any isoform, of up to ~80% (9) (Figure 3). Given we do not yet know
522 the relative composition or respective roles of the α subunit isoforms in human skeletal muscle, it is
523 possible that adaptations in the α_1 may play an equally important role as those for α_2, since
524 improvements in performance and K^+ regulation were also seen with increases only in α_1 (51). The α_2
525 key role is to regulate Na^+/K^+ gradients during contractions, and thus it would be expected to be
526 increased in most training studies. However, this review demonstrates that this is not always the case.
527 In training protocols utilising short bouts of only a few seconds duration, the rise in interstitial [K^+] and
528 intracellular [Na^+] may not be as pronounced, in particular, intercellular Na^+ is a potentially important
529 regulator which may trigger the synthesis of new NKA as demonstrated in myotubes (14, 116). Thus if
530 these sprints are too short, there might be insufficient stimulus for complete α_2 activation and or α_2
531 synthesis. The lack of consistency among training studies and the mechanistic research conducted
532 thus far makes speculation difficult. For these reasons, it should not be a surprise that both α_1 and α_2
533 display large adaptability to longer periods of both intense (9, 92) and long endurance exercise (9,
534 39). It is likely that FXYD1 also plays an important role in skeletal muscle function, since a reduction
535 in phosphorylation of FXYD1^{ser68} were associated with declines in physical tests related to team sport
536 performance, namely a repeat sprint test and Yo-Yo IR2 performance, (106, 107).

537 ***Conclusions and perspectives***

538 Exercise training has been demonstrated to robustly increase NKA content with most training types,
539 however individual isoform responses are much more varied. More studies need to be undertaken to
540 determine which isoforms are changed with various types of training inclusive of changes in FXYD1
541 and its phosphorylation. These investigations will need to calibrate the potentially differing impacts of
542 training intensity, duration and training modalities. Studying both exercise intensity and duration as
543 differing regulators of NKA, would provide valuable understanding whether specific isoforms have a
544 particular threshold of physical activity for upregulation, whether one specific isoform is upregulated in
545 preference to, in concert with, in sequence with, or independent of other isoforms during training and
546 may reveal the mechanisms behind training induced NKA upregulation.

The limited available evidence with voluntary disuse in humans suggests that NKA content is surprisingly resilient to change with short-term inactivity. However, severe injury, which promotes long-term inactivity, such as observed with spinal injury, ACL injury and shoulder impingement clearly reduce skeletal muscle NKA content. These conclusions are all drawn however, from a limited number of studies, so further research is needed to better understand the NKA response to disuse. An important component of this should be a focus on the time course of responses in NKA isoforms with both training and inactivity, focusing on specific adaptations to disuse as well as their implications for muscle NKA activity and overall muscle function. Finally, molar quantification of each of the NKA α and β isoforms in human skeletal muscle is essential, particularly in the context of heterodimers, which determine NKA function. Understanding the relative distribution of these isoforms in muscle, in specific fiber-types, including through co-IP studies, could uncover their specific contributions to changes in muscle function and adaptability. Detailed understanding of the functional roles of the different NKA isoforms will enable the implications of their adaptability for understanding human musculoskeletal function, as well as exercise limitation through peripheral and respiratory muscles.

562 **LIST OF FIGURES.**

563 **Figure 1. Percentage changes in [^{3}H]ouabain binding site content (NKA content) in human**
564 **skeletal muscle with A) Injury, inactivity and chronic disease and B) exercise training**

565 Panel A, the data shows difference from pre and post inactivity and is presented as calculated percent
566 change ± 90% CI. Data is presented from four studies, of which two were models of injury, one of
567 paraplegia and one study comprized inactivity. Specifically, three references show percentage
568 compared to a control limb (67, 95, 96) while two others compared to control participants (13, 22).
569 Panel B, the data shows difference from pre and post training, calculated percent change ± 90% CI.

570

571 **Figure 2. Neither training intensity nor volume is specifically related to upregulation of**
572 **[^{3}H]ouabain binding site content in human skeletal muscle with training.**

573 Data is presented as percentage increases in [^{3}H]ouabain binding site content (NKA content) in
574 human skeletal muscle, plotted against A) training intensity, B) minutes trained per week and C) total
575 training minutes. Training intensity was expressed as percentage of maximum, using measures
576 utilizedin differing studies, which included maximum HR, maximum running speed and $VO_{2\,max}$. In
577 studies where training minutes or exercise intensity were gradually increased during the training
578 period, the average over the duration of the study was used and plotted.

579

580 **Figure 3. Inconsistent training adaptions of NKA isoforms measured in homogenates in human**
581 **skeletal muscle.**

582 Data for isoforms are compared to 'pre-training' and presented as calculated percent change ± 90%
583 CI for A) α_1, B) α_2 C) β_1

584 Isoforms not indicated were not measured, or reported in that study. Significance levels were $p<0.05$.

585

LIST OF TABLES.

Table 1. Table 1. General characteristics of different training types

ET, Endurance Training; HIT, High Intensity Training; AHIT, Aerobic High-Intensity Training, RT, Resistance Training; SET, Speed Endurance Training; ST, Sprint/Speed Training; RSE, Repeat Sprint Exercise.

Table 2. Adaptations in exercise performance and skeletal muscle [^{3}H]ouabain binding site content (NKA content) to intense exercise training in healthy young humans

NR not reported in that study. n.c= no significant difference pre-post training. ↑ = increase compared to pre-training. Significance levels were $p<0.05$. References. 1. Kjeldsen et al., 1990 (57) 2. McKenna et al., 1993 (82) 3. Green et al., 1993 (39) 4. Madsen et al., 1994 (73) 5. Green et al., 1999a (34) 6. Evertsen et al., 1997 (25) 7. Medbø et al., 2001 (83) 8. Harmer et al., 2006 (43) 9. Aughey et al., 2007 (6) 10. Green et al., 2008 (38) 11. Edge et al., 2013 (23) 12. Wyckelsma et al., 2017 (118).

Table 3. NKA isoform abundance in human homogenates and exercise performance changes following intense exercise training in healthy young humans

n.s = no significant difference pre-post training. ↑ = increase compared to pre-training. Significance levels were $p<0.05$. References.1. Dela et al., 2004 (20) (CON group) 2. Nielsen et al., 2004 (92) 3. Aughey et al., 2007 (6) 4. Mohr et al., 2007 (86) 5. Green et al., 2008 (38) 6. Iaia et al., 2008 (51) 7. Bangsbo et al., 2009 (8) 8. Thomassen et al., 2010 (106) 9. Gunnarsson et al., 2012 (41) 10. Gunnarsson & Bangsbo, 2012 (40) 11. Thomassen et al., 2016 (107) 12. Benziane et al., 2011 (9) 13. Vorup et al., 2016 (117).

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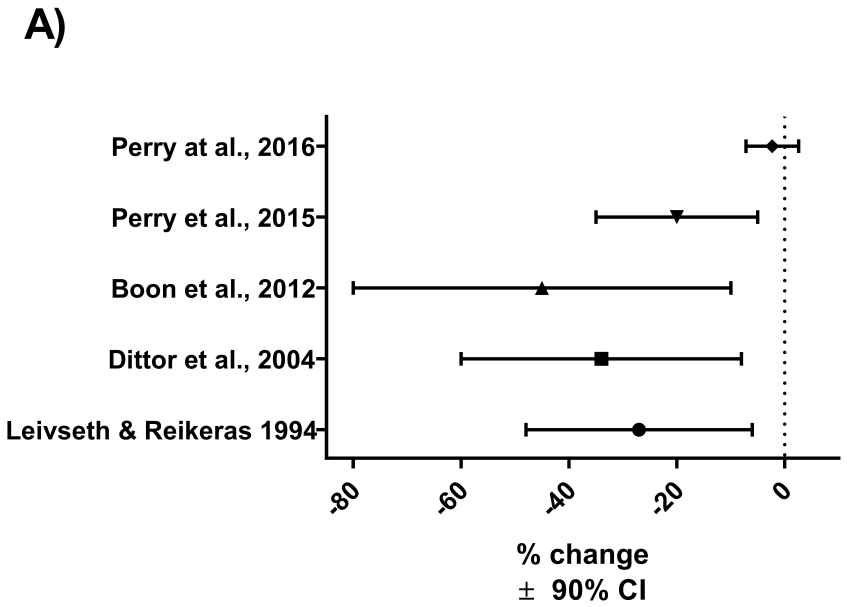
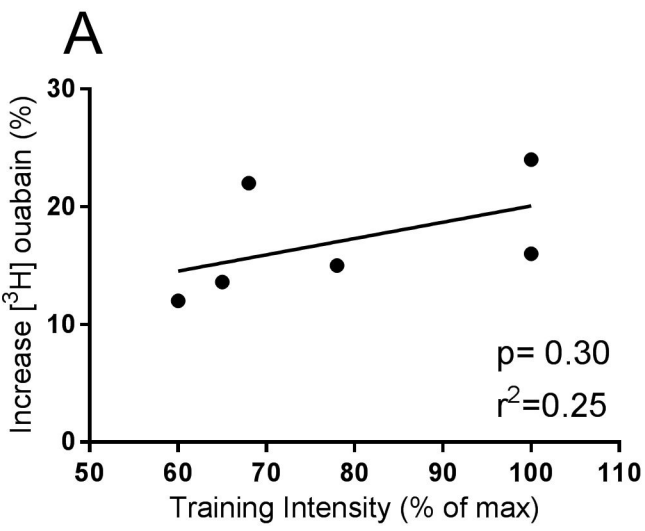
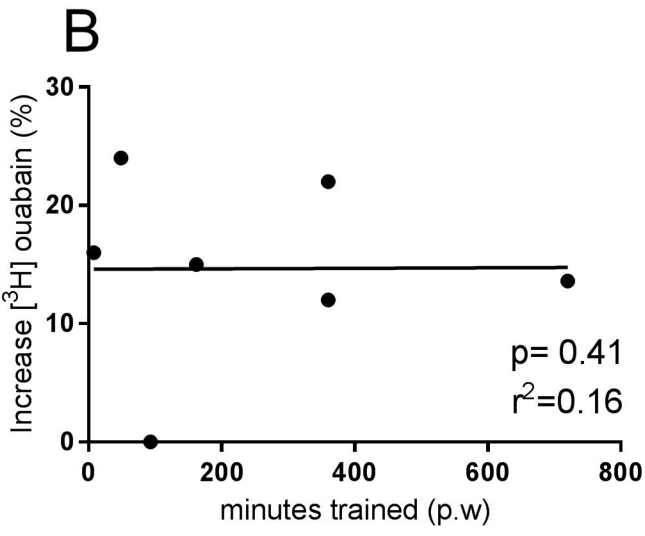
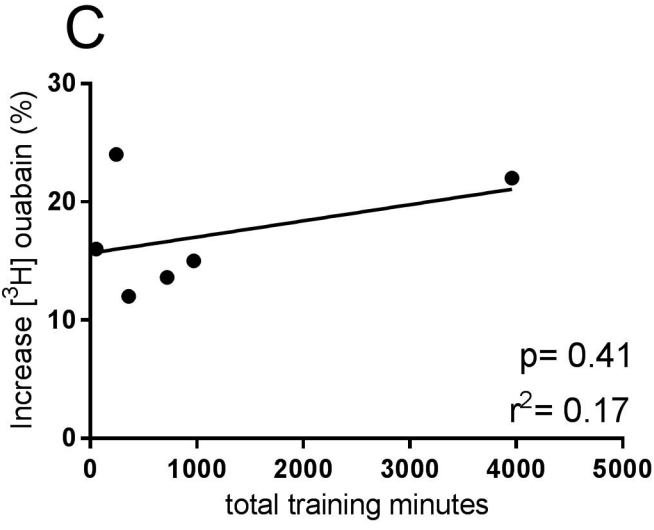
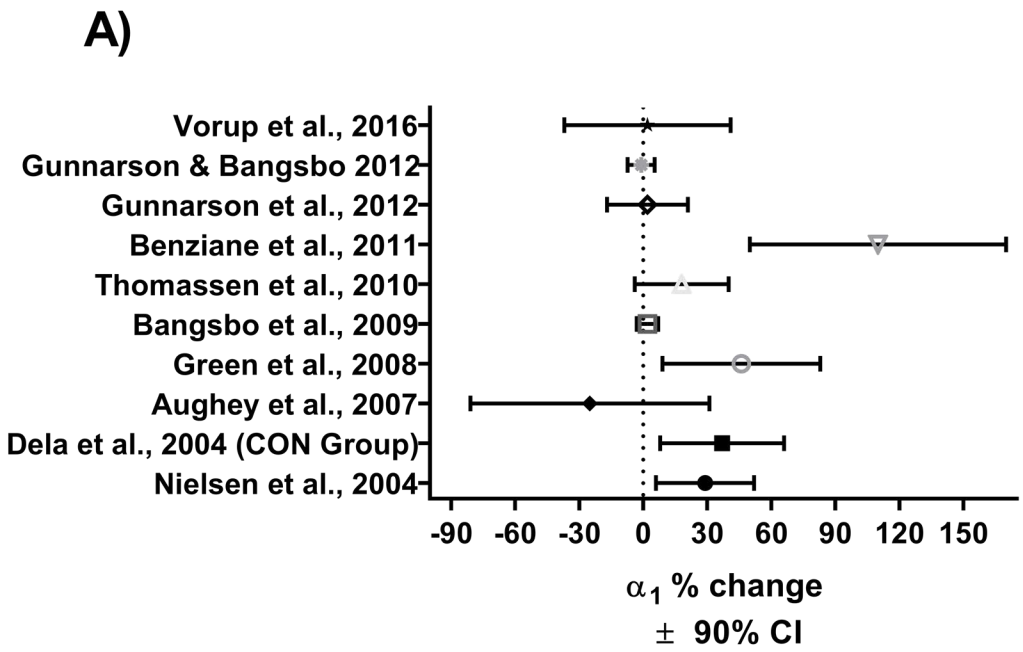
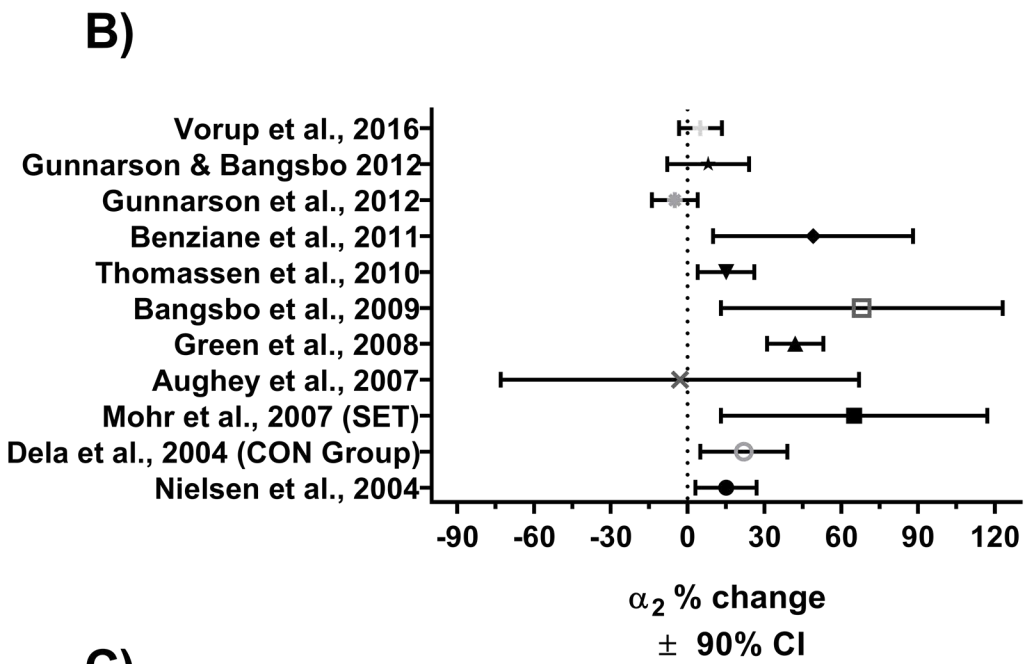

A)
Perry at al., 2016
Perry et al., 2015
Boon et al., 2012
Dittor et al., 2004
Leivseth & Reikeras 1994
-80
-60
-40
-20
0
% change
± 90% CI
B)
Wyckelsma et al., 2017
Edge et al., 2013
Green et al., 2008
Aughey et al., 2007
Harmer et al., 2006
Medbo et al., 2001
Green et al., 1999a
Evertsen et al., 1997
Madsen et al.,1994
Green et al., 1993
McKenna et al., 1993
-10
0
10
20
30
40
50
% change
± 90% CI


A
Increase [3H] ouabain (%)
30
20
10
0
50
60
70
80
90
100
110
Training Intensity (% of max)
p= 0.30
r2=0.25


B
Increase [3H] ouabain (%)
30
20
10
0
0
200
400
600
800
minutes trained (p.w)
p= 0.41
r2=0.16


C
Increase [3H] ouabain (%)
30
20
10
0
0
1000
2000
3000
4000
5000
total training minutes
p= 0.41
r2= 0.17


A)
Vorup et al., 2016
Gunnarson & Bangsbo 2012
Gunnarson et al., 2012
Benziane et al., 2011
Thomassen et al., 2010
Bangsbo et al., 2009
Green et al., 2008
Aughey et al., 2007
Dela et al., 2004 (CON Group)
Nielsen et al., 2004
-90 -60 -30 0 30 60 90 120 150
α1 % change
± 90% CI


B)
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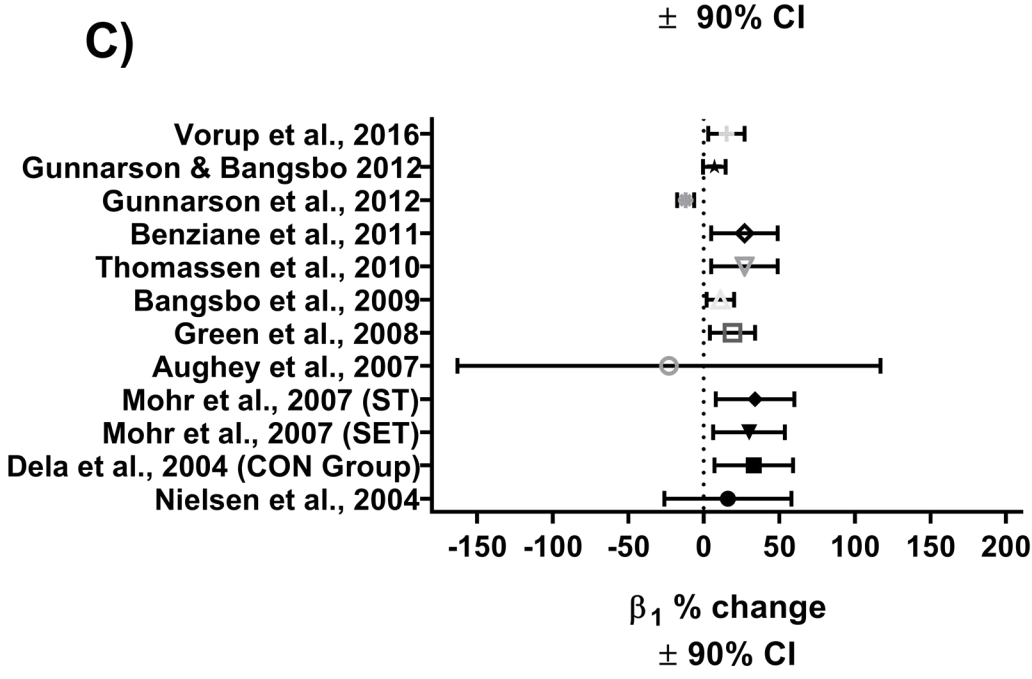
Gunnarson & Bangsbo 2012
Gunnarson et al., 2012
Benziane et al., 2011
Thomassen et al., 2010
Bangsbo et al., 2009
Green et al., 2008
Aughey et al., 2007
Mohr et al., 2007 (SET)
Dela et al., 2004 (CON Group)
Nielsen et al., 2004
-90 -60 -30 0 30 60 90 120
α2 % change
± 90% CI


C)
Vorup et al., 2016
Gunnarson & Bangsbo 2012
Gunnarson et al., 2012
Benziane et al., 2011
Thomassen et al., 2010
Bangsbo et al., 2009
Green et al., 2008
Aughey et al., 2007
Mohr et al., 2007 (ST)
Mohr et al., 2007 (SET)
Dela et al., 2004 (CON Group)
Nielsen et al., 2004
-150 -100 -50 0 50 100 150 200
β1 % change
± 90% CI

	ET	HIT				RT
		AHIT	SET	ST	RSE	
Nature	Continuous	Intermittent	Intermittent	Intermittent	Intermittent	Intermittent
Bout Number	1*	4-10	4-10	4-10	4-6	3-5 sets
Bout Duration	20-120 min	1-5 min	10-40s	2-10s	2-6s	5-12 reps
Bout Intensity	50-80%	80-100%	90-100%	≥100%	≥100%	60-80% 1RM
Work:Recovery ratio	1:0-1:1	1:0.5-1:2	1:5-1:6	1:10	1:4	1:2-1:5

* may also include multiple bouts each of long duration

Ref.	Participant Characteristics			Training characteristics			Outcomes	
	N	Pre-train $VO_{2\ peak}$ ($ml.kg^{-1}.min^{-1}$)	Mean Age (yr)	Type/frequency of training	Session Intensity and duration	Duration (wk)	Performance measure (%Δ)	NKA content (%Δ)
1	15	NR	20	Military Moderate Physical Training Details NR	NR	10	↑ 7% distance during 12 min run test	n.c
2	6	51.1	18.8	SET x3 p/wk Wk1- 3 x30s bouts Wks 4-7 10x30s ST	30s maximal cycle sprints	7	↑ 11% work output	↑ 16%
3	9	47.5	19.7	ET	65% VO_2 max 2 hours	0.9	↑ 6.5% $VO2_{max}$	↑ 13.6%
4	39		30	Combined HIT+ ET x3 p/wk HIT x1-3 p/wk ET	93% of HR max Low-intensity run < 60% HR max	6	↑ 5%$VO_{2\ max}$	↑ 15%
5	16	45	21.4	x3 p/wk ET	ET group ~68% $VO_{2\ peak}$ ~ 2 hours	11	↑$VO_{2\ peak}$	↑22%
			19.9	RT	RT Group 3 sets 8-10 reps	12	n.c	↑16%
6	20	66.6	18	Skiing, Running	86% of training at 60-70%	~21	↑ Distance in 20 min	↑16% in both

				7 days p/wk MI group-	$VO_{2\ max}$		treadmill test	groups
				HI group	83% of training 80-90% $VO_{2\ max}$			
7	21	58	27	RT Group 1 x1 p/wk Group 2 x2 p/wk Group 3 x3 p/wk		3 month	↑ max strength all groups	n.s x1 p/wk ↑ x2 p/wk ↑x3 p/wk
8 (CON)	7	3.1 ($L.min^{-1}$)	24	SET x3 p/wk	x4-10, 30s maximal cycle sprints	7	↑ $VO_{2\ peak}$ ↑ Peak incremental power	↑8.2%
9	12	4.98 ($L.min^{-1}$)	31	HIT Wk 1- x3 p/wk Wk 2- x2 p/wk Wk 3 x2 p/wk	8x5min at 80% peak power output	3	↑ Peak power output 3%	n.c
10	12	44.8	19.2	ET	~60% VO_2 max 2hrs	3 d	NR	↑ 12%
11	12	49.5	21	HIT	6-10 x2 min intervals Cycle ergometer ~140-170% of LT_{Dmax} or 92-111% pre-training power at VO_{2peak}	5	↑ VO_2 peak ↑ power at VO_2 peak ↑ power at LT_{Dmax}	↑ 22-26%
12	8	24.7	65	HIT x3 p/wk	4x4 min cycle ~90-95% peak HR	12	↑ VO_2 peak ↑ Work (J) ↑ Peak HR	↑11%

Reference	Participant Characteristics			Training Characteristics			Outcomes	
	n	Pre-train VO_2 peak ($ml.kg^{-1}.min^{-1}$)	Age (yr)	Type /frequency	Intensity and duration	Duration (wk)	Performance measure (%Δ)	Isoform abundance
1.	7	NR	61	RT	Wk 1-2. 3x10 reps 50% 1RM Wk3-6. 8-12 reps 70-80% 1RM	6	↑ maximal leg press	↑ α_1 37% ↑ α_2 22% ↑ β_1 33%
2	6	50.2	25.3	wk 1-2, x3 p/wk wk 3-4, x4 p/wk wk 5-7, x5 p/wk	Intermittent knee extensor exercise- Single leg, 15 work intervals ~150% of thigh VO2 max.	7	↑16% power output ↑Time to fatigue 27%	↑α_1 29 ↑ α_2 15.1% n.s β_1
3	12	4.98 ($L.min^{-1}$)	31	Wk 1- x3 p/wk Wk 2- x2 p/wk Wk 3 x2 p/wk	HIT 8x5min at 80% peak power output	3	↑ Peak power output 3%	n.s- $\alpha_1, \alpha_2, \alpha_3$ n.s- $\beta_1, \beta_2, \beta_3$
4	13	Sprint train group (ST) 50.2	26.7 24.6	Wk 1-2, x3p/wk Wk 2-5, x4 p/wk Wk 6-8 x 5p/wk	ST 15 x 6s 95% max running speed	8	↑10%Yo-Yo IR2 ST & 30% SET ↑~18% time to	n.s - α_1 in either group ↑ α_2 speed

		speed endurance training (SET) group 49.0		Final week- 6 times p/wk.	SET 8x30s 130% VO_2 max		exhaustion (SET) ↓~5.8%- 50m sprint (ST) ↓ 30m time (both)	endurance training only (68±26%) ↑ β_1 both ~38% ST ~35% SET
5	12	44.8	19.2	ET	~60% VO_2 max 2hrs	3 d	NM	↑ α_1 46% ↑ α_2 42% ↑ β_1 19%
6	15	55.8	33.4	SET 3-4 sessions per week CON 3-5 days per week	ST. 8-12 x30s runs at 90-95% max running speed. CON- normal training (9-12km, 45-60 min/day	4	↑ Yo-Yo IR2 19%- ST	↑ α_1 ~29% (ST) n.s α_2 n.s β_1
7	17	63.0	34.8	SET a) 2-3 p/wk b) 1 p/wk c)1-2 p/wk	SET sessions a) 30s bouts at ~95% of max running speed. b) 4x4 min at >85% max HR c) <75% max HR or 75-85% max HR	6-9	n.c $VO_{2\ max}$ ↓ 3km run performance ↑ mean speed during 3km run	n.s α_1 ↑ α_2 68% (SET) n.s β_1
8	18	55.0	23.4	5 sessions of aerobic high-intensity (AHI) & 5 sessions SET	AHI 8x2 min-4 vs.4 small sided soccer drills. 1 min rec SET 10-12 x 25-30s	2	↑ performance in 4th, 6th and 10th sprint in repeat sprint test ↓Total sprint time	n.s α_1 ↑ α_2 14.5 n.s β_1 ↑27.3% FXYD1^{ser68}

9	18	60.6	23.9	SET x1 per week + regular soccer commitments	6-9 intervals at 90-95% maximal intensity		↓ O_2 consumption at 10 $km.h^{-1}$ ↑ Yo-Yo IR2 11%	n.s α_1 n.s α_2 ↓ β_1 13%
10	18	52.2	33.8	HIT	3-4 x 5 minute running. Each 5 min consisting of 1 min intervals at <30%, <60% and 90-100% of running speed	7	↑10-20-30 performance by 6% ↑ $VO_{2\ max}$ 4%	n.s α_1 n.s α_2 n.s β_1
11	8	59	33	Cycle (outdoor) 2-3 x p/wk SET 1-2 sessions per week HIT Reduction in ~70% training volume from regular training	SET 10-12 x ~30-s maximal uphill ~6% gradient. Interspersed 4.5 min low-intensity exercise HIT 4-5 x ~4 min at 90-95% maximal HR 0% gradient. Interspersed with two days of recovery	7	n.c VO_2 ↑Time to exhaustion ↑mean power 4% ↑peak power 3%	↑FXYD1 30% n.s α_1 (~11%) n.s α_2 (~8%) n.s β_1 (~3%)

12	8	~44.3	23	END & HIT	END ~75% VO_2 peak Days 1,5,6 & 10 60min Day 3- 60 minutes Day 8- 90 minutes HIT 6x5 min ~90-100% VO_2 peak Days 2, 4, 7, 9	10 d	9% increase VO_2 peak	↑ α_1 113% ↑ α_2 49% n.s α_3 ↑ β_1 27% n.s FXYD1 n.s Ser^{68}, Ser^{63} or Thr^{69}
13	8	60.1	39	Combined RT and SET	x2 Strength p.wk 1x10 wk 1 2x8 wk 2 3x6 wk 3 4x4 wk 4-8 x2 SET p.wk 30s at 90-95% maximal speed x4 efforts wk 1 x6 efforts wk 2 x8 efforts wk 3-4 x10 efforts wk 5-8 58% ↓ training volume	8	↑ Yo-Yo IR2 (18.5%) ↓ 400m time (4.8%) ↑ Maximal Aerobic Speed (0.6 km hr^{-1}) ↑ 4RM (Squat, deadlift and Leg Press)	n.s α_1 n.s α_2 ↑ β_1 (15%)