

Running title: Low responders to endurance training and functional overload

**Low responders to endurance training exhibit impaired hypertrophy and divergent biological process responses in rat skeletal muscle**

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## New Findings

What is the central question of this study?

The extent to which genetics determines adaptation to endurance versus resistance exercise is unclear. Previously, a divergent selective breeding rat model showed genetic factors play a major role in the response to aerobic training. Here, we asked: do genetic factors that underpin poor adaptation to endurance training affect adaptation to functional overload?

What is the main finding and its importance?

Our data show that heritable factors in low responders to endurance training generated differential gene expression that was associated with impaired skeletal muscle hypertrophy. A maladaptive genotype to endurance exercise appears to dysregulate biological processes responsible for mediating exercise adaptation, irrespective of the mode of contraction stimulus.

## Abstract

Divergent skeletal muscle phenotypes result from chronic resistance-type versus endurance-type contraction, reflecting the principle of training specificity. **Aim:** To determine whether there is a common set of genetic factors that influence skeletal muscle adaptation to divergent contractile stimuli. **Methods:** Female rats were obtained from a genetically heterogeneous rat population and were selectively bred from high responders to endurance training (HRT) or low responders to endurance training (LRT; n=6/group; generation 19). Both groups underwent 14-d synergist ablation to induce functional overload of the plantaris muscle prior to comparison to non-overload controls of the same phenotype. RNA sequencing was performed to identify Gene Ontology Biological Processes with differential (LRT vs HRT) gene set enrichment. **Results:** Running distance, determined in advance of synergist ablation, increased in response to aerobic training in HRT but not LRT ( $65 \pm 26\%$  versus  $-6 \pm 18\%$ , mean  $\pm$  SD,  $P < 0.0001$ ). The hypertrophy response to functional overload was attenuated in LRT versus HRT ( $20.1 \pm 5.6\%$  versus  $41.6 \pm 16.1\%$ ,  $P = 0.015$ ). Between-group differences were observed in the magnitude of response of 96 upregulated and 101 downregulated pathways. A further 27 pathways showed contrasting upregulation or downregulation in LRT versus HRT in response to functional overload. **Conclusions:** Low responders to aerobic endurance training were also low responders for compensatory hypertrophy, and attenuated hypertrophy was associated with differential gene set regulation. Our findings suggest that genetic factors that underpin aerobic

training maladaptation may also dysregulate the transcriptional regulation of biological processes that contribute to adaptation to mechanical overload.

**Keywords:** heritable factors, molecular networks, skeletal muscle plasticity, specificity of adaptation

## INTRODUCTION

Skeletal muscle is a highly malleable tissue with the capacity to modify its phenotype in response to contractile stimuli. The specificity of adaptation in skeletal muscle is evident in response to exercise training, whereby contractile overload with resistance-type compared with endurance-type exercise generates divergent morphological phenotypes and performance capacity (Coffey & Hawley, 2007; Hickson, 1980; Wilkinson et al., 2008).

Variation in the magnitude of adaptation response to exercise has been reported in several studies (Roberts, Haun, et al., 2018; Vellers, Kleeberger, & Lightfoot, 2018), with a significant proportion of individual variation attributable to genetic factors (Phillips et al., 2013; Timmons, 2011; Vellers et al., 2018). However, whether genetic factors underpinning variation in adaptation are specific to the mode of contraction is unclear. For example, do genetic factors play a role in activating distinct mechanisms (e.g., metabolic versus anabolic) that are responsible for training-specific adaptation responses (Coffey & Hawley, 2017)? Answers to this question are important to understand the interaction between the specificity of training adaptation and individual responses—such as the extent to which individuals are genetically predisposed to adapt to a given exercise mode.

The present study used a genetically heterogeneous rat population selectively bred for its distinct adaptive or maladaptive responses to aerobic endurance exercise (Koch, Pollott, & Britton, 2013). A previous study used this selective breeding model to demonstrate that high responders to endurance training (HRT), improved running capacity, and exhibited enhanced muscle- and whole-body metabolism, in contrast to the selectively bred low responders to endurance training (LRT), that *failed* to improve running capacity and exhibited metabolic dysfunction (Lessard et al., 2013). Here, we examined changes in skeletal muscle mass and the enrichment of gene sets in response to functional overload using this unique HRT and LRT model.

Given the lack of understanding about the genetic contribution to training specificity and potential for molecular interference (Coffey & Hawley, 2017) and interindividual responses (Vellers et al., 2018), LRT may be genetically predisposed for muscle growth compared with HRT, which may be 'programmed' for endurance adaptation through the selective breeding process. However, LRT have poor metabolism and angiogenesis as well as dysregulated molecular signal transduction, processes

which are important in adapting to mechanical overload (Lessard et al., 2018; Lessard et al., 2013). Moreover, previous studies have shown mitochondrial capacity and capillary density may determine, in part, the hypertrophy response (Roberts, Romero, et al., 2018; Snijders et al., 2017). Accordingly, we reasoned that the same heritable factors that induce low response to aerobic training would also interfere with mechanisms that facilitate hypertrophy. Thus, we hypothesized that LRT would exhibit attenuated hypertrophy compared with HRT, and that divergent responses would also be evident in distinct gene set enrichment maps of biological processes.

## METHODS

### Ethical approval

All experimental procedures were approved by the University Committee of Use and Care of Animals at the University of Michigan and Queensland University of Technology Animal Ethics Committee (Ref: 1300000531). Experiments were carried out according to the guidelines laid down by the animal ethics/welfare committee's and conform to the principles and regulations of this journal.

### Experimental animals

Rats were obtained from a bi-directional selective breeding program that has been described in detail previously (Koch et al., 2013). Briefly, genetically heterogeneous rats from the upper and lower 10<sup>th</sup> percentile for endurance adaptations to an eight-week treadmill training program were selected as breeders for each subsequent generation. Endurance adaptation was defined as post-training exercise capacity minus pre-training exercise capacity. Total running distance, work performed, and time-to-fatigue variables during treadmill tests were recorded and calculated as previously described (Koch et al., 2013). The training program commenced at ~12 wk of age and comprised three days of treadmill running each week (total 24 training sessions). The training protocol provided a total of 618 min (>10 h) of running time, a total distance of ~9.9 km, and a cumulative vertical gain of ~2.5 km. A total of twenty-four female rats (12 high responders to training [HRT] and 12 low responders to training [LRT]) from the 19th generation were used for this study due to larger training responses than males and to extend on our previous work using the LRT/HRT model (Koch et al., 2013; Lessard et al., 2013). The HRT and LRT groups were classified *a priori* on endurance training not hypertrophy responses. Rats began the 14 d experimental period at 14 months of age to maximise the latent period after endurance training before the contrasting stimulus of compensatory hypertrophy. Rats were randomly assigned to either a surgical ablation intervention group or control group (n=6/group). Rats were housed two per cage in temperature- and humidity-controlled facilities on a 14:10 h light-dark cycle with ad libitum access to standard chow (20% protein, 4.8% fat) and water.

### Surgical procedure and muscle collection

Unilateral ablation of the gastrocnemius and soleus muscles was performed to induce compensatory hypertrophy of the plantaris muscle as previously described (Hamilton et al., 2014). The rats were initially anaesthetized with 2–4% isoflurane inhalation in an individual chamber followed by nose cone inhalation that was maintained throughout all surgical procedures. An incision was made to the lower hind limb, exposing the ankle extensor muscles. The soleus and gastrocnemius muscles were isolated and severed at the Achilles tendon, and the soleus and the distal two-thirds of the gastrocnemius were removed to induce plantaris functional overload. The incision was sutured closed before moving the animals to a temperature-controlled room to recover. All animals appeared to return to normal activity within ~1 h and analgesia was provided for the first 48 h recovery through administration of Tramadol in drinking water (25 mg/L). In the 14 d intervention period prior to collection, animals were monitored daily for signs of pain or postoperative infection. None of the animals showed signs of undue discomfort or distress. 14 d after surgery, the rats were weighed and intact plantaris muscles from the functional overload and control limbs were excised, weighed and snap frozen in liquid nitrogen for further analyses. On the same day, rats from the LRT and HRT control groups (no synergist ablation) were anaesthetized and the plantaris muscle was removed using the same procedures as above, for comparison of RNAseq analysis with the intervention groups. Rats were terminated after removal of plantaris muscles at the conclusion of the experimental period under general anaesthesia by permanent cessation of circulation (Annex IV in the European Directive 2010/63/EU).

### **RNA extraction, library preparation and RNA sequencing**

Total RNA from plantaris muscle was isolated using the miRNeasy mini kit (Qiagen) according to the manufacturer's protocol. Briefly, ~50–80 mg tissue was homogenised in QIAzol with 0.9–2.0 mm RNase-free steel beads in a Bullet Blender Gold at 4<sup>o</sup> C (Next Advance, New York, USA). Total RNA was then further purified using RNeasy spin columns. RNA yield was determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific), and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent). RNA integrity scores were >8.0 for each sample. RNA sequencing was performed at the Australian Translational Genomics Centre (Queensland University of Technology) according to standard protocols. Briefly, 1 µg of each RNA sample was used for library construction using the Illumina TruSeq Stranded Total RNA Library kit with Ribo-Zero Gold, as per the manufacturer's instructions. Adapter-ligated fragments were amplified by PCR for 11 cycles. The quality and size of the final library preparations were analysed on a TapeStation (Agilent). Indexed samples were pooled and then sequenced on a NextSeq 500 system (Illumina), generating approximately 50 million paired-end 2 × 100-bp reads for each sample.

## Statistical and bioinformatics analysis

Two-way analysis of variance with Sidak's multiple comparisons test was used to analyse endurance capacity, plantaris muscle mass and myosin heavy chain gene expression. Body mass and percent changes were analysed by unpaired t tests; percent change in plantaris muscle mass was calculated from the overloaded plantaris versus the contralateral control. Statistical analyses were performed in GraphPad Prism 7.03 (GraphPad software, CA USA). The level of statistical significance was set at  $P < 0.05$  and phenotype data presented as mean  $\pm$  standard deviation and 95% confidence intervals (CI).

Quality control of the raw FASTQ files was performed with FASTQC (Andrews, 2010). Low-quality reads and 3' adapters were trimmed with the BBDUK tool (Bushnell, 2014). Transcript quantification and quasi-mapping against the rat reference genome (Ensembl Rnor\_6.0 release 91; cDNA and ncRNA) was performed using SALMON 0.9.1 (Patro, Duggal, Love, Irizarry, & Kingsford, 2017). Transcript reads were then imported into R/BIOCONDUCTOR and summarised at the gene level using the TXIMPORT package (Soneson, Love, & Robinson, 2015). Genes with a median count of  $< 0.5$  per million across all 24 samples were excluded from analysis. Trimmed Mean of M-values normalisation was used to correct for composition bias and library size before voom transformation, differential expression analysis and descriptive statistics in LIMMA (Conesa & Nueda, 2017; Ritchie et al., 2015). The linear model also incorporated RNA integrity as a covariate.

The following pairwise comparisons were investigated: LRT functional overload vs. LRT control (FOinLRT), HRT functional overload vs. HRT control (FOinHRT) and the difference in hypertrophic effect with functional overload (FOinLRT vs FOinHRT; DELTA). A false discovery rate (FDR) was applied to correct for multiple comparisons, with statistical significance accepted at  $FDR < 0.001$ . Gene Set Enrichment Analysis (GSEA) was used to detect coordinated changes in gene expression of functionally related sets of genes. Gene sets are prespecified using GO annotations – a categorization of gene function, cellular location, and what biological processes (pathways, programs) it helps to carry out. Using the GSEA algorithm, all analysed genes were ranked (from the most upregulated to the most downregulated) in order of evidence using the signed moderated t-statistic from LIMMA. It was tested whether prespecified sets of genes mapping to Gene Ontology (GO) Biological Process annotations were enriched at the top (i.e., upregulated) or at the bottom (i.e., downregulated) of this ranked list using the CLUSTERPROFILER package (10,000 permutations; gene set size range 25-500) (Yu, Wang, Han, & He, 2012). Gene set enrichments were visualised as networks in CYTOSCAPE using the ENRICHMENTMAP package (Merico, Isserlin, Stueker, Emili, & Bader, 2010). Conservative threshold parameters were used; specifically,  $FDR < 0.05$ , nominal P-value  $< 0.001$  and a combined similarity cut-off  $> 0.375$ . Network clusters were further summarised and annotated using the AUTOANNOTATE package, with additional manual editing (Kucera, Isserlin, Arkhangorodsky, & Bader, 2016). Co-expression networks of genes within specific clusters were

visualised using the GENEMANIA package (Warde-Farley et al., 2010). Hub genes were defined as the top 5% of genes with the highest connectivity in each co-expression network.

## RESULTS

### **Endurance capacity**

There was a significant interaction for endurance training response between LRT and HRT ( $P < 0.0001$ ) with distance run after training increasing in HRT only (LRT  $-50 \pm 129$  CI  $-73-175$  versus HRT  $+375 \pm 101$  CI  $251-499$  m,  $P < 0.0001$ ; Figure 1A). The change in pre-to-post training running capacity expressed as percent difference was also different in HRT versus LRT for distance (+70%), work performed (+82%) and time-to-fatigue (+43%; all  $P < 0.001$ ).

### **Body mass and skeletal muscle mass**

Body mass was approximately 20% greater in LRT than HRT, both prior to endurance training and prior to functional overload (FO LRT  $301 \pm 33$  CI  $266-337$  g versus HRT  $253 \pm 29$  CI  $223-284$  g;  $P < 0.05$ ). Body mass decreased after endurance training in both LRT ( $-42$  g,  $P < 0.001$ ) and HRT ( $-31$  g,  $P < 0.001$ ), but the relative loss was not different between groups.

Functional overload increased plantaris muscle mass in HRT and LRT (HRT  $0.38 \pm 0.12$  CI  $0.27-0.49$   $\text{mg} \cdot \text{g}^{-1}$  versus LRT  $0.21 \pm 0.05$ , CI  $0.11-0.33$   $\text{mg} \cdot \text{g}^{-1}$ ; both  $P < 0.001$ ); however, the overload-induced increase in plantaris muscle mass was attenuated in LRT versus HRT ( $+21.4 \pm 4.8\%$  versus  $41.6 \pm 16.1\%$ , respectively;  $P = 0.015$ , Figure 1B). There were no differences in plantaris muscle mass between the control limb of the functional overload groups and the matched HRT and LRT control groups used for RNAseq analysis.

### **Myosin heavy chain gene expression**

Whereas the primary focus of transcriptomic analysis was distinctly toward mapping gene networks rather than characterizing the expression of individual genes, myosin heavy chain (*Myh*) expression was examined in order to characterize this fundamental phenotypic trait. Of the *Myh* genes, *Myh1*, *Myh2*, *Myh4* and *Myh7* were most highly expressed (Figure 1C). There were main effects for group and functional overload for *Myh2* expression ( $P < 0.05$ ; Figure 1C); the higher *Myh2* expression in functional overload versus control was similar for LRT (9.7%) and HRT (10.3%; both  $P < 0.001$ ). Lower

*Myh4* expression in functional overload versus control was significant in HRT (−18.6%,  $P < 0.05$ ) but not LRT (−11%,  $P = 0.22$ ). *Myh3* and *Myh7* were modestly higher after functional overload in HRT (*Myh3* 2.1%,  $P < 0.0001$ ; *Myh7* 3.7%,  $P < 0.05$ ) but not LRT (*Myh3* 0.5%,  $P = 0.10$ ; *Myh7* 3.1%,  $P = 0.09$ ).

### Principal components analysis of gene expression data

RNA sequencing generated an average of 30.9 million (range = 27.0–34.4 million) reads that were mapped to 14,956 genes. Principal component analysis (PCA) illustrated variance in gene expression within and between sample groups (Figure 2A). PC1 showed a clear separation of functional overload and control groups, demonstrating that the synergist ablation intervention had the greatest effect on gene expression variability (43%). PC2 indicated that the LRT and HRT phenotypes accounted for 6% of variability in gene expression.

### Identification of differentially expressed genes

From a total of 14,956 detectable genes, 1,866 differentially expressed genes (DEGs) were identified (2-fold or greater) in LRT functional overload relative to LRT controls (Table S1). Most of these DEGs (92.4%) were upregulated in LRT in response to hypertrophy (Figure 2B, 2C). By comparison, 2,460 DEGs were identified in HRT of which 79.6% were upregulated (Figure 2B, 2D; Table S2). The expression of a subset of 1,612 genes (Figure 2B) was common to functional overload in both groups, representing 86.4% DEGs in LRT and 65.5% DEGs in HRT skeletal muscle (Table S3). Select anabolic and catabolic genes of interest associated with regulation of muscle mass were not differentially expressed for the LRT and HRT delta response from the respective control groups (Table S4).

### Gene set enrichment analysis

All genes with an Entrez GeneID (12,340 genes) were ranked according to their t-statistic, then subjected to GSEA against the GO Biological Processes annotations (Figure 3; Table S5). 224 biological processes were different in LRT versus HRT after functional overload. Specifically, differences were evident in the magnitude of response for 96 upregulated and 101 downregulated biological processes. The remaining 27 (of 224 total) enriched biological processes were different in the direction of change (i.e., positively versus negatively enriched processes) in LRT versus HRT groups (Figure 4). Selected co-expression networks and GSEA plots for *response to ER stress* (GO:0034976) and *Golgi vesicle transport* (GO:0048193) gene sets show the downregulation of the specific genes that were enriched (Figures 5 and 6). These processes are mapped (presented) because the greatest proportion of their constitutive gene sets showed contrasting negative versus positive enrichments in LRT compared to HRT. Note: this somewhat arbitrary selection was related to the absence of a standard bioinformatics procedure for reporting GSEA, and because endoplasmic



reticulum (ER) stress has been shown to regulate functional overload-induced hypertrophy (Hamilton et al., 2014).

#### **Data availability**

RNA-Sequencing data have been deposited to Gene Expression Omnibus (GEO) under accession number GSE156724.

#### **DISCUSSION**

The major findings of the present study were that low responders to aerobic endurance training (LRT) were also low responders to functional overload and that the attenuated hypertrophy response was associated with distinct enrichments for gene sets in a broad range of biological processes. In response to functional overload, high responders to aerobic endurance training (HRT) and LRT showed different magnitudes of response for approximately 200 upregulated or downregulated processes. For 27 enriched biological processes, HRT and LRT responded in opposing directions of upregulation versus downregulation in response to functional overload. Contrasting regulation by LRT versus HRT corresponding with an attenuated hypertrophy response may provide important insight on key biological processes that contribute to blunted skeletal muscle adaptation to mechanical overload.

Only one other study has used the LRT/HRT rat model (generation 18) to examine adaptations to a skeletal muscle overload stimulus (climbing activity) (Ahtiainen et al., 2018). Ahtiainen and colleagues (2018) showed no notable hypertrophy and little change in physiological adaptation in both LRT and HRT in response to 6 wk training. The compensatory hypertrophy response of HRT in the present study was similar to previous work employing uni- or bi-lateral synergist ablation for 14 d duration (~40–45%) (Bodine et al., 2001; Pehme, Alev, Kaasik, Julkunen, & Seene, 2004; Sakuma, Watanabe, Sano, Uramoto, & Totsuka, 2000; Tamaki et al., 2009). Differences in hypertrophy responses between the present study and the work by Ahtiainen et al. (2018) reflects differences in the method of muscle overload. Accordingly, we show for the first time that LRT for endurance also have impaired skeletal muscle hypertrophy in response to functional overload. The “normal” hypertrophy response in HRT indicates that the adaptive potential to unfamiliar stimuli was maintained in the skeletal muscle, despite the underlying phenotype-genotype interaction generated through selective breeding for high adaptation response to endurance training. In contrast, hypertrophy was clearly attenuated in LRT versus HRT after 14 d overload with no discernible difference in ambulatory activity, and the modest response in LRT was similar to that observed with short-term synergist ablation (Terena, Fernandes, Bussadori, Deana, & Mesquita-Ferrari, 2017). We did not include a separate generic, control group given the comparable hypertrophy of HRT with previous studies (Bodine et al., 2001; Pehme et al., 2004; Sakuma et al., 2000; Tamaki et al., 2009),

and the relative LRT compared with HRT hypertrophy differences were obvious, providing a clear contrast in response upon which to examine biological processes affecting the divergent adaptation phenotypes.

Studies have demonstrated acute anabolic resistance in a variety of contexts and through a variety of mechanisms (Cuthbertson et al., 2005; Hodson, West, Philp, Burd, & Moore, 2019; Kumar et al., 2009). However, little is known about the potential genetic underpinning of the attenuated response to prolonged anabolic stimuli. Endoplasmic reticulum stress is associated with age-related impairments in skeletal muscle recovery following disuse (Baehr et al., 2016) and has been suggested as a molecular brake in response to functional overload (Hamilton et al., 2014). In the present study, two biological processes that were downregulated in LRT but upregulated in HRT were *Response to endoplasmic reticulum stress* and *Golgi vesicle transport/organisation and localisation*. The functional implications of downregulated endoplasmic reticulum and Golgi organisation pathways include disrupted synthesis, folding, and structural integrity of cellular proteins (Afroze & Kumar, 2019).

The mishandling of cell proteins induces cell stress and activation of the unfolded protein response (UPR) to maintain endoplasmic reticulum homeostasis by reducing accumulation of misfolded proteins (Deldicque, Hespel, & Francaux, 2012). In the present study, the endoplasmic reticulum UPR gene set was downregulated in LRT, whereas it was upregulated in HRT (Fig. 4). Wu and colleagues (2011) have shown that an appropriate UPR mediates skeletal muscle adaptation following exercise through activation of the transcriptional co-activator PGC-1 $\alpha$  (peroxisome proliferator-activator receptor gamma coactivator-1 alpha). Moreover, Marton and colleagues (2015) reported that factors associated with mitochondrial biogenesis, including PGC1- $\alpha$  protein content, were responsible for the magnitude of responses to endurance training in the HRT/LRT model used herein. In the present study, gene set enrichment responses in LRT to contractile overload were consistent with a maladaptive UPR and suppressed protein synthesis (Afroze & Kumar, 2019). Phillips and colleagues (2013) have suggested that inferior protein yield per unit RNA may contribute to low adaptation responses to resistance training in humans compared with the superior efficiency of high responders to upregulate global protein synthesis. Taken together, our biological process data show impaired gene pathways/programs for post translational protein assembly and transport in association with anabolic resistance in LRT rats. Consequently, we hypothesise that LRT have an inferior endoplasmic reticulum response-related molecular program that contributes to dysregulation of protein handling and suboptimal adaptation regardless of the mode of contractile activity.

Many nucleotide gene sets for which the magnitude, but not direction (up- versus downregulation), of expression was different between LRT and HRT rats compared to their respective control groups were in *Nucleotide metabolism* and *Mitosis* (Fig. 3). Specifically, when the magnitude of enrichment for nucleotide metabolism pathways was downregulated this was typically less

pronounced in LRT, while upregulation of mitosis-related pathways tended to be attenuated in LRT compared with HRT. Given that skeletal muscle is primarily comprised of post-mitotic muscle fibres, the extent to which variation in the magnitude of response in LRT versus HRT is meaningful to the ensuing hypertrophy response is unclear. Our analyses did not allow us to define mitotic versus post-mitotic contributions to the total transcript pool. Accordingly, it is possible that differences in mitotic pathway enrichment in HRT compared with LRT was related to greater numbers of muscle satellite cells (or other mitotic cell types). Terry and colleagues (2018) suggest that gene expression differences in skeletal muscle are not significantly contaminated by non-muscle mRNAs. Nonetheless, differentially regulated pathways in the present study included genes also commonly expressed in satellite cells, fibrogenic precursor cells, and neutrophils, and we cannot rule out the potential contributions of cell types other than post-mitotic muscle cells to expression profiles. Additional functions of specific cell cycle annotated genes or gene sets in terminally differentiated cells may also not be fully characterised. For example, protein kinase B/Akt1 and tumour suppressor p53 are amongst the most studied genes in biology (Dolgin, 2017) and contribute to regulation of the cell cycle (Giono & Manfredi, 2006; Liu et al., 2014). However, they are pleiotropic in a variety of cell types, including skeletal muscle where they promote hypertrophy and maintenance of the mitochondria, respectively (Beyfuss, Erlich, Triolo, & Hood, 2018; Bodine et al., 2001). Regardless, the transcription response of mitosis pathways in the present study may indicate poorer cell maintenance, repair and regulation in LRT compared with HRT in response to a hypertrophy stimulus.

Nucleotide synthesis is required to promote RNA production for synthesis of new proteins during adaptation. This energy-intensive process requires energy contributions from glycolytic and oxidative pathways (Lane & Fan, 2015; Vander Heiden, Cantley, & Thompson, 2009). In the present study, pathways related to global nucleotide metabolism were downregulated, an effect that was more prominent in HRT than LRT. Thus, metabolic dysfunction (Lessard et al., 2013) and/or attenuated mitochondrial biogenesis (Marton et al., 2015), both of which have been shown in the LRT model, may have contributed to the variation and extent of nucleotide pathway expression. How (or if) nucleotide metabolism downregulation contributed to hypertrophy is unclear. Wu and colleagues (2017) have shown increased flux through the pentose pathway leading to the accumulation of purines and pyrimidines at the metabolite level during hypertrophy. In turn, this may downregulate nucleotide synthesis pathways. Taken together, attenuated responses in LRT for nucleotide synthesis, DNA repair, and endoplasmic reticulum stress response may contribute to impaired transcription/translation programs and overall adaptability in LRT.

Lessard and colleagues (2013) employed microarray analysis of soleus muscle in LRT and HRT rats (generation 15), and identified clear contrasts in acute transcriptional responses to endurance exercise. Several hundred genes were up/down regulated, revealing that global gene expression, development, and cell cycle processes were altered in LRT but not HRT in response to

exercise. In the present study, and despite using conservative statistical approaches, RNA sequencing analysis identified over two thousand genes that were differentially expressed in LRT and HRT muscle. Given the complex gene interactions involved in exercise adaptation, and to avoid gene-specific bias, we focused on biological processes rather than individual gene expression, or the use of a transgenic model.

Although RNA sequencing with enrichment mapping has many advantages, it was not conducive to time course analysis. Thus, a limitation of this study is that findings reflect a snapshot at 14 d of overload. Previous studies of functional overload show continued hypertrophy beyond 14 d, and compensatory hypertrophy of ~75–80% following 21–28 d overload has been reported (Hamilton et al., 2014; Sakuma et al., 2000). Indeed, the trajectory of muscle hypertrophy can be maintained, in a nearly linear fashion, through 30 d of overload (Plyley, Olmstead, & Noble, 1998). Thus, our gene set enrichment analysis data may reflect biological processes that perpetuate rather than initiate hypertrophy (Chailou, Lee, England, Esser, & McCarthy, 2013). Caution is also warranted when attempting to directly compare the supraphysiological animal model hypertrophy of the present study to human exercise training adaptations (Marsh, Thomas, Naylor, Scurrah, & Green, 2020; Thomas et al., 2020), which is influenced by many factors beyond mechanical loading. The present model is unique and provides important new information on how heritable factors and gene expression networks within biological processes influence muscle adaptation, but it does not take into account the complexity of the human response to training (Thyfault & Bergouignan, 2020). Future studies employing the LRT-HRT model would also benefit from additional molecular analyses such as translational efficiency and/or proteomics. Nonetheless, the research model and next generation sequencing analysis in the present study provide new insights into biological processes that underpin skeletal muscle plasticity, including protein folding and cell-development, structure, metabolism and stress responses.

In summary, selectively bred low responders to endurance training also exhibit an impaired hypertrophy response. Our findings indicate that the ability to adapt to diverse contractile stimuli may share a common set of heritable, genetic underpinnings, with profound effects on determining skeletal muscle adaptation. Gene set analysis revealed contrasting positive/negative enrichment as well as significant differences in the extent to which many biological processes are up- or downregulated. Whether divergence in the *magnitude* of biological process enrichment or *contrasting* positive/negative regulation of only a few processes has a greater influence on the adaptation response is intriguing and requires further research. Altogether, our novel approach using a unique rat model system, shows that low responders to endurance training exhibit compromised responses to muscle overload that are likely attributable to dysregulated activation of biological processes associated with a maladaptive genotype.

## **Additional information**

### **Competing interests**

The authors have no competing interests financial or otherwise to declare.

### **Author contributions**

DWDW, SJL, LGK, SLB, JMP, RS and VGC contributed to the conception and design of the study, DWDW, TWD, JMT, BPB, RS, NMB, MAB, KJA and VGC undertook acquisition, analysis or interpretation of the data, DWDW, TMD, KJA and VGC produced the initial draft of the manuscript, and all authors critically revised the manuscript and provided important intellectual content. All authors approved the final version.

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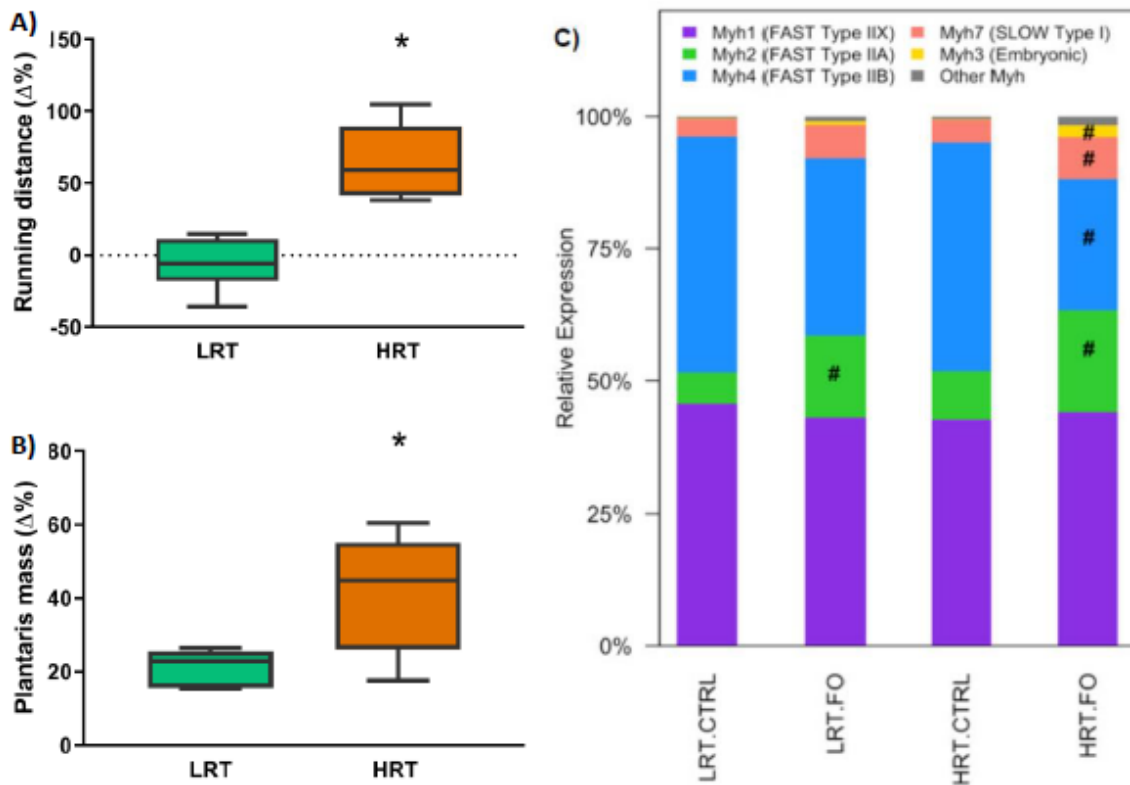
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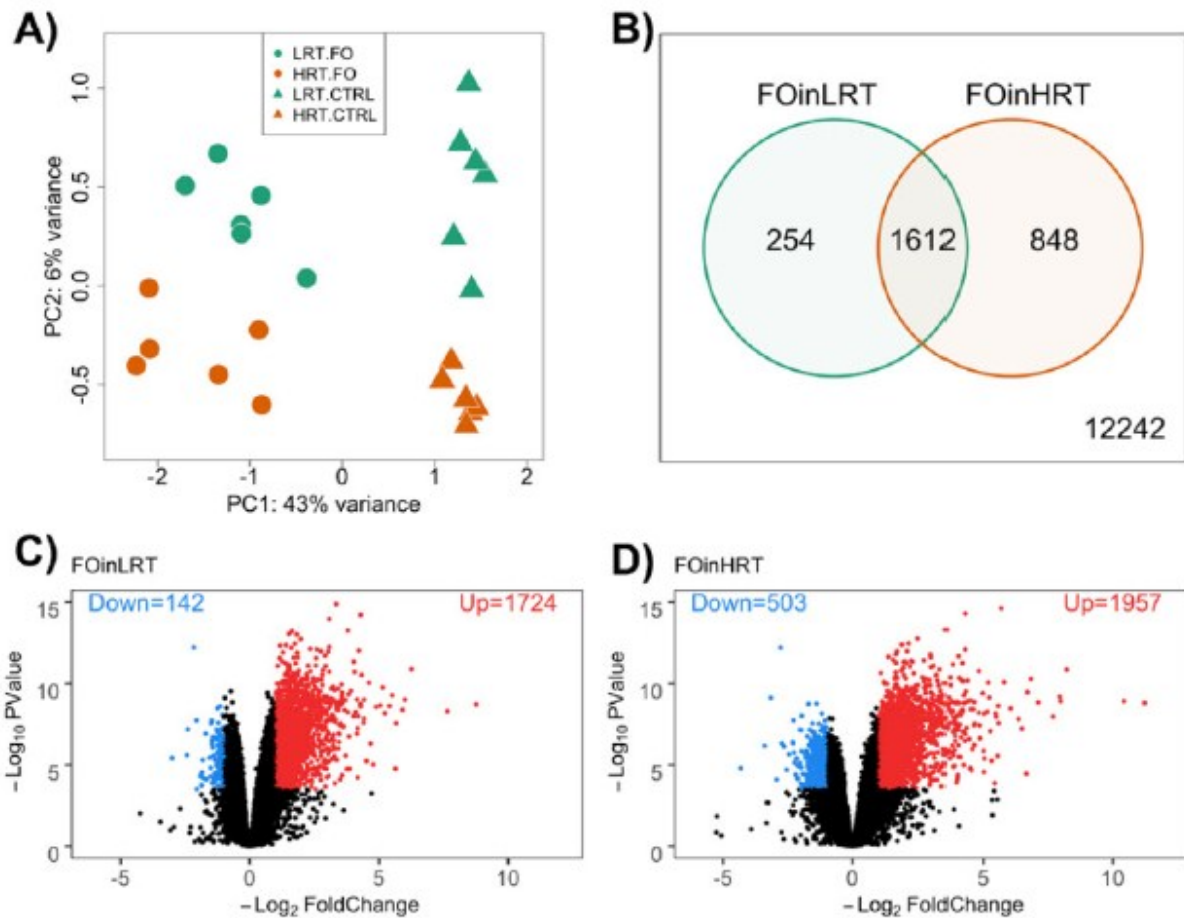


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**Figure 1.** Changes in (A) running distance after an 8 wk treadmill training program, (B) plantaris muscle mass (overload versus contralateral control) and (C) myosin heavy chain (Myh) transcript expression in functional overload (FO; 14 d) and control (CTRL) groups in selectively bred low- and high responders to endurance training (LRT vs. HRT; n=6/group). \*significantly different from LRT P<0.05; # significantly different from respective HRT/LRT control group P<0.05.

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**Figure 2. RNA-seq analysis from LRT and HRT rat skeletal muscle in response to functional overload.** (A) Principal component analysis showing the two major components (PC). Venn diagram (B) and volcano plots (C and D) represent the number and magnitude of difference in expression in LRT and HRT overloaded muscle (14 d), respectively, relative to breeding line (LRT/HRT)-matched controls (fold change >2; FDR <0.001). n=6/group. LRT, low responders to endurance training. HRT, high responders to endurance training. FO = functional overload of the plantaris muscle.

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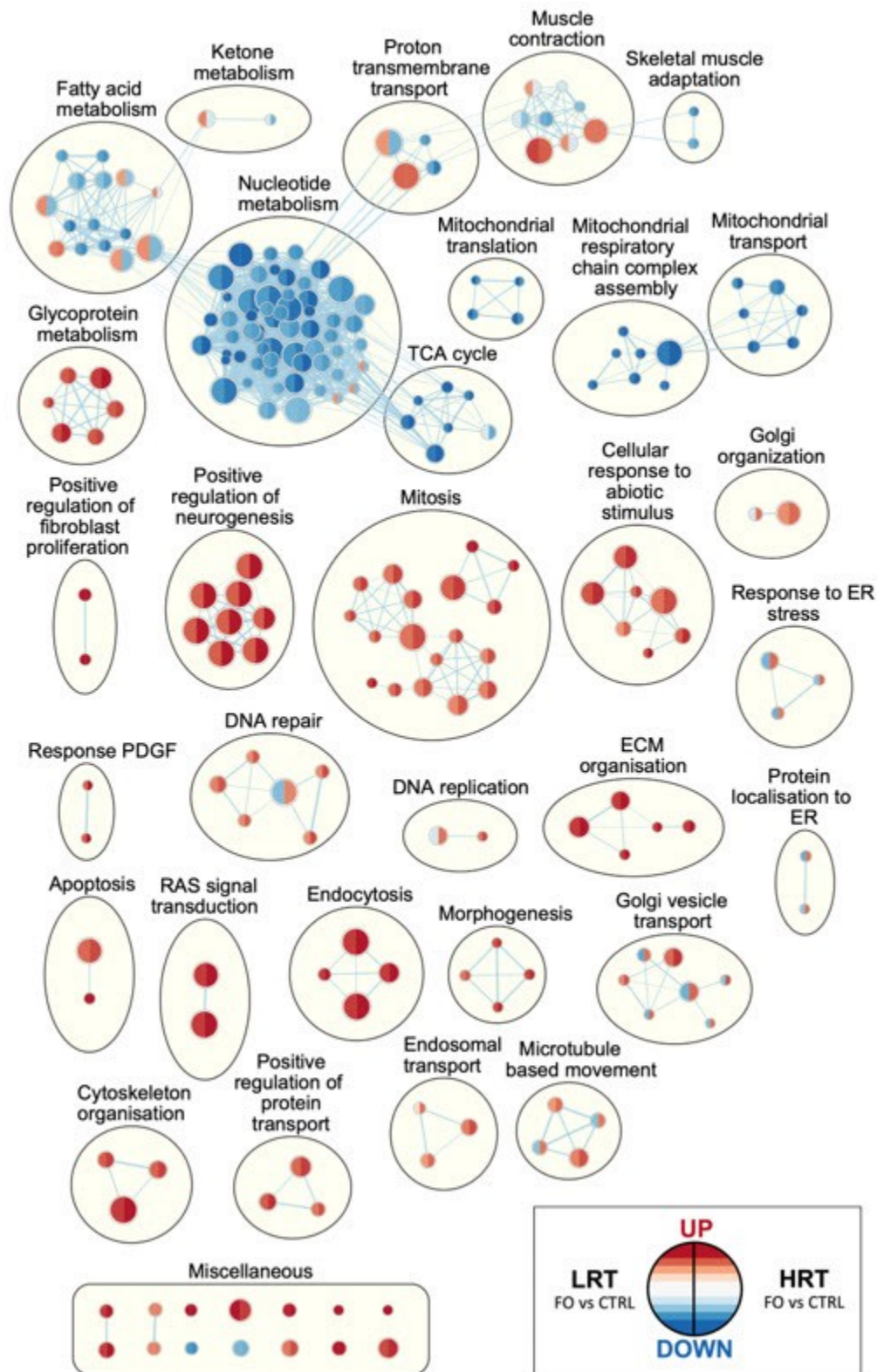
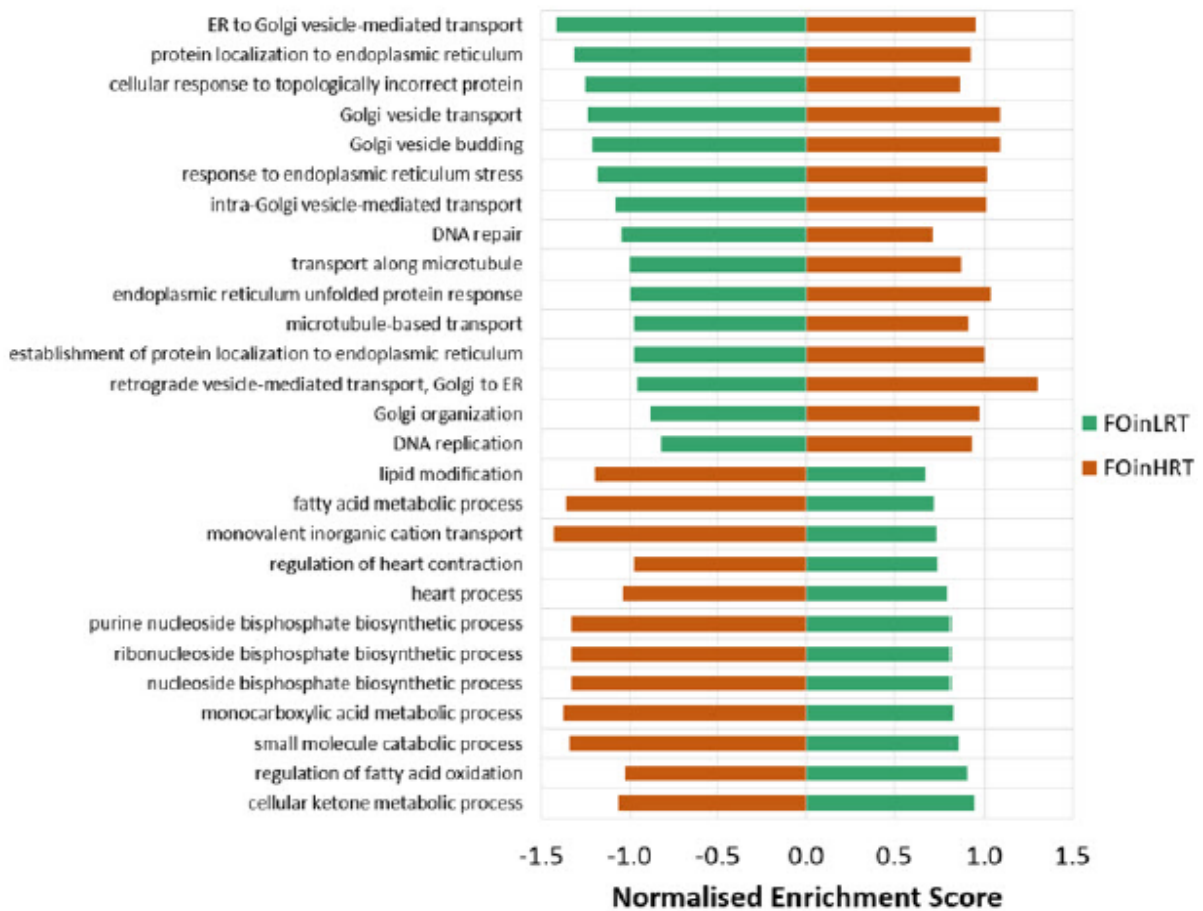


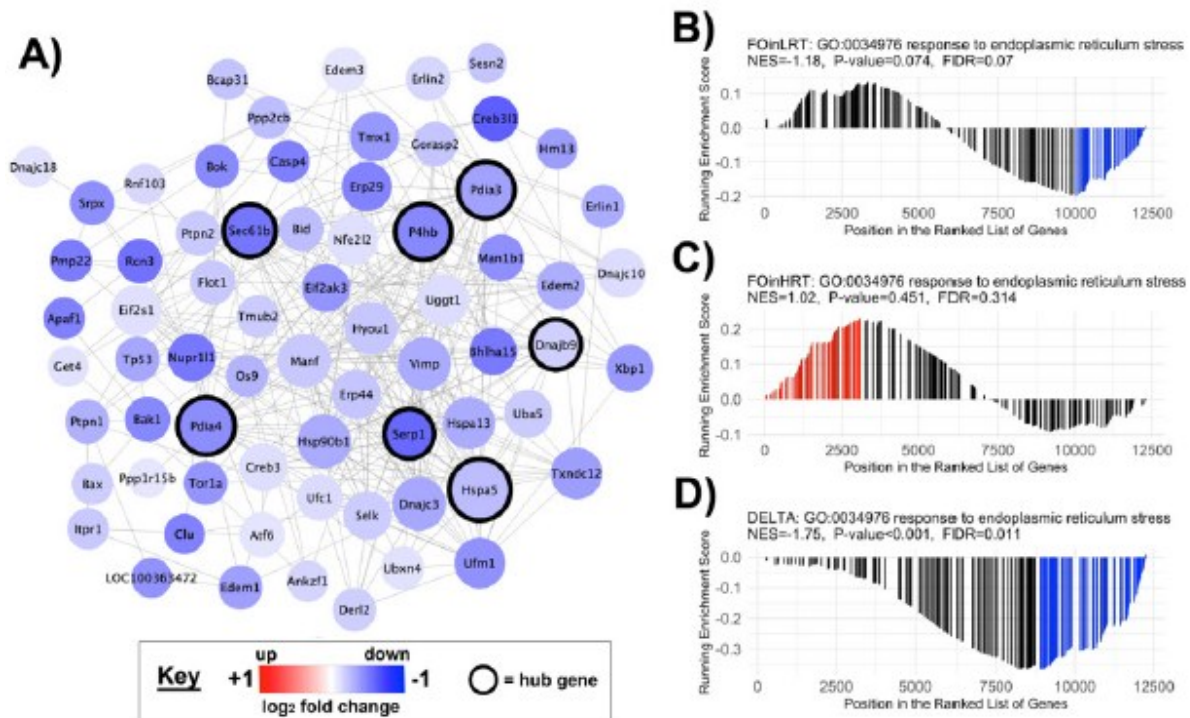
Figure 3. Enrichment map of Gene Ontology Biological Processes differentially expressed in LRT and HRT in response to functional overload (FO). Enrichment results were mapped as a

network of gene sets (nodes) related by mutual gene overlap (edges). The enrichment map reflects relative differences (FO versus control) between LRT and HRT. Red identifies up-regulated and blue down-regulated gene sets following 14 d of functional overload. Differential expression (LRT versus HRT) was analysed after accounting for the effect of hypertrophy in each group relative to their own genotypic control. The left side of each node indicates low responders to endurance training (LRT, n=6/group) and the right side indicates high responders to endurance training (HRT, n=6/group). Node size is proportional to the percent of enriched genes per set, and colour intensity represents magnitude of change in expression. Clusters of functionally related gene sets were circled and manually labelled to highlight prevalent biological functions among a set of related gene sets (FDR <0.05; P <0.001). LRT, low responders to endurance training. HRT, high responders to endurance training. FO, functional overload of the plantaris muscle.

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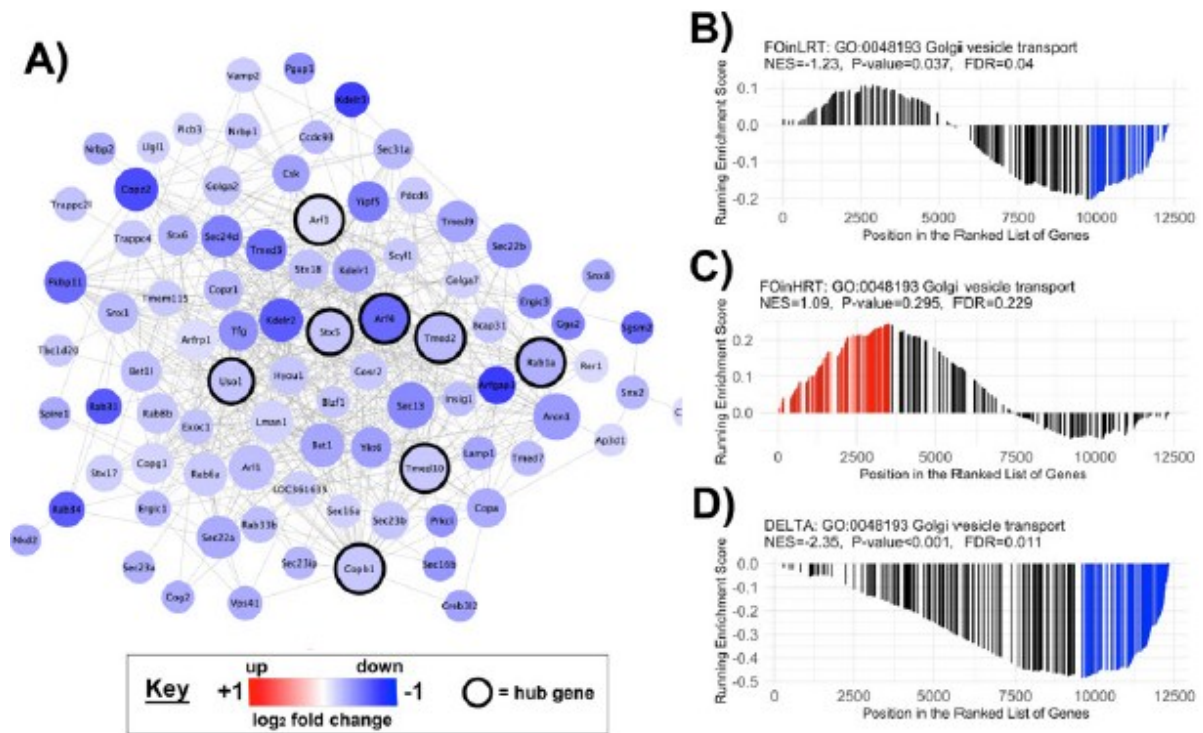


**Figure 4. Gene set enrichment analysis.** Bar plot depicting the normalized enrichment scores (NES) of the 27 positively and negatively enriched gene sets after 14 d functional overload in LRT and HRT relative to control animals. Analysis of enriched Gene Ontology Biological pathway gene sets in LRT (green) and HRT (orange) rats are shown. Names of significantly enriched gene sets are shown on the y-axis. Positively and negatively enriched gene sets are shown in bars to the right and left of the zero line, respectively. An FDR <0.05, P-value <0.001 were used as the significance threshold. n=6/group. LRT, low responders to endurance training. HRT, high responders to endurance training. FO, functional overload of the plantaris muscle.



**Figure 5. Gene Set Enrichment Analysis of the Response to Endoplasmic Reticulum Stress gene set.**

**A)** Co-expression network of genes involved in the *response to endoplasmic reticulum stress* (GO:0034976) gene set. Nodes correspond to individual genes enriched in the delta comparison between LRT and HRT. All genes were downregulated in LRT compared with HRT (FDR<0.05, p<0.001). Edge lines between two genes represent a co-expression relationship. Colour intensity (blue) represents the magnitude of downregulation, and black borders show 'hub' genes in the highest 5% of connectivity within the gene set. GSEA rank plots shown for **B)** FOinLRT, **C)** FOinHRT and **D)** DELTA HRT-LRT comparisons. On each plot the vertical lines (barcode) indicate the position of each gene in the GO:0034976 gene set within the ranked gene list. The height of each gene is proportional to the running enrichment score. Core genes that drive the enrichment score are shown in red (positive enrichment) or blue (negative enrichment). Corresponding normalised enrichment scores (NES), p-value and FDR are also shown. n=6/group. LRT, low responders to endurance training. HRT, high responders to endurance training. FO, functional overload of the plantaris muscle.



**Figure 6. Gene Set Enrichment Analysis of the Golgi vesicle transport gene set.** A) Co-expression network of genes involved in the *Golgi vesicle transport* (GO: 0048193) gene set. Nodes correspond to individual genes enriched in the delta comparison between LRT and HRT with all genes downregulated in LRT compared with HRT (FDR<0.05, p<0.001). Edge lines between two genes represents a co-expression relationship. Colour intensity (blue) represents the magnitude of downregulation, and black borders show 'hub' genes in the highest 5% of connectivity within the gene set. GSEA rank plots shown for B) FOinLRT, C) FOinHRT and D) DELTA HRT-LRT comparisons. On each plot the vertical lines (barcode) indicate the position of each gene in the GO:0048193 gene set within the ranked gene list. The height of each gene is proportional to the running enrichment score. Core genes that drive the enrichment score are shown in red (positive enrichment) or blue (negative enrichment). Corresponding normalised enrichment scores (NES), p-value and FDR are also shown. n=6/group. LRT, low responders to endurance training. HRT, high responders to endurance training. FO, functional overload of the plantaris muscle.