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

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Abstract

Divergent skeletal muscle phenotypes result from chronic resistance-type versus endurance-type contraction, reflecting the principle of training specificity. Our aim was to determine whether there is a common set of genetic factors that influence skeletal muscle adaptation to divergent contractile stimuli. 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 days of synergist ablation to induce functional overload of the plantaris muscle before comparison to non-overloaded controls of the same phenotype. RNA sequencing was performed to identify Gene Ontology biological processes with differential (LRT vs. HRT) gene set enrichment. We found that running distance, determined in advance of synergist ablation, increased in response to aerobic training in HRT but not LRT (65 ± 26 vs.

$-6 \pm 18\%$, mean $\pm$ SD, $P < 0.0001$). The hypertrophy response to functional overload was attenuated in LRT versus HRT (20.1 ± 5.6 vs. $41.6 \pm 16.1\%$, $P = 0.015$). 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. In conclusion, 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 might 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

1 | 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 the adaptive response to exercise has been reported in several studies (Roberts, Haun et al., 2018; Vellers et al., 2018), with a significant proportion of individual variation being 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 vs. anabolic) that are responsible for training-specific adaptation responses (Coffey & Hawley, 2017)? Answers to this question are important to our understanding of 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 et al., 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), which 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 (FO) 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 might be genetically predisposed for muscle growth compared with HRT, which might be 'programmed' for endurance adaptation through the selective breeding process. However, LRT have poor metabolism and angiogenesis in addition to dysregulated molecular signal transduction, which are processes that are important in adapting to mechanical overload (Lessard et al., 2013, 2018). Moreover, previous studies have shown that mitochondrial capacity and capillary density might 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.

2 | METHODS

2.1 | 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 (reference 1300000531). Experiments were carried out according to the guidelines laid down by the animal ethics/welfare committees and conform to the principles and regulations of this journal.

2.2 | Experimental animals

Rats were obtained from a bi-directional selective breeding programme that has been described in detail previously (Koch et al., 2013). Briefly, genetically heterogeneous rats from the upper

and lower 10th percentile for endurance adaptations to an 8-week treadmill training programme were selected as breeders for each subsequent generation. Endurance adaptation was defined as post-training exercise capacity minus pretraining 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 programme commenced at ~12 weeks of age and comprised 3 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 24 female rats [12 high responders to training (HRT) and 12 low responders to training (LRT)] from the 19th generation were used for this study owing to their larger responses to training 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-day experimental period at 14 months of age to maximize 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 a control group (n = 6/group). Rats were housed two per cage in temperature- and humidity-controlled facilities on a 14 h–10 h light–dark cycle with *ad libitum* access to standard chow (20% protein, 4.8% fat) and water.

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 that 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.

2.3 | 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). General anaesthesia was induced by inhalation of 2–4% isoflurane in an individual chamber, followed by inhalation via nose cone that was maintained throughout all surgical procedures. An incision was made to the lower hindlimb, 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 FO of plantaris. 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 of recovery by administration of Tramadol hydrochloride (Merck, Kenilworth, NJ, USA) in drinking water (25 mg/l). In the 14-day intervention period, animals were monitored daily for signs of pain or postoperative infection. None of the animals showed signs of undue discomfort or distress. Fourteen days after surgery, the rats were anaesthetized before being weighed, and intact plantaris muscles from the FO 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 killed 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).

2.4 | RNA extraction, library preparation and RNA sequencing

Total RNA from the plantaris muscle was isolated using the miRNeasy mini kit (Qiagen, Chadstone, VIC, Australia) according to the manufacturer's protocol. Briefly, ~50–80 mg of tissue was homogenized in QIAzol with 0.9–2.0 mm RNase-free steel beads in a Bullet Blender Gold at 4°C (Next Advance, Troy, NY, USA). Total RNA was purified further using RNeasy spin columns. The RNA yield was determined using a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Seventeen Mile Rocks, QLD, Australia), and RNA integrity was assessed using a 2100 Bioanalyzer (Agilent, Mulgrave, VIC, Australia). 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, according to 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 ~50 million paired-end 2 × 100 bp reads for each sample.

2.5 | Statistical and bioinformatics analysis

Two-way ANOVA with Sidak's multiple comparisons test was used to analyse endurance capacity, plantaris muscle mass and myosin heavy chain gene expression. Body mass and percentage changes were analysed by Student's unpaired t tests; the percentage change in plantaris muscle mass was calculated from the overloaded plantaris versus the contralateral control. Statistical analyses were performed in GraphPad Prism v.7.03 (GraphPad Software, San Diego, CA, USA). The level of statistical significance was set at $P < 0.05$, and phenotype data are presented as the mean ± SD 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; complementary DNA and non-coding RNA) was performed using SALMON v.0.9.1 (Patro et al., 2017). Transcript reads were then imported into R/BIOCONDUCTOR and summarized at the gene level using the TXIMPORT package (Soneson et al., 2015). Genes with a median count of <0.5 per million across all 24 samples were excluded from analysis. Trimmed Mean of M-values normalization was used to correct for composition bias and library size before voom transformation, differential expression analysis and descriptive statistics in LIMMA (Conesa et al., 2006; Ritchie et al., 2015). The linear model also incorporated RNA integrity as a covariate.

The following pairwise comparisons were investigated: LRT FO versus LRT control (FOinLRT), HRT FO versus HRT control (FOinHRT) and the difference in hypertrophic effect with FO (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 Gene Ontology (GO) annotations; a categorization of gene function, cellular location and what biological processes (pathways, programmes) 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 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 et al., 2012). Gene set enrichments were visualized as networks in CYTOSCAPE using the ENRICHMENTMAP package (Merico et al., 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 summarized further and annotated using the AUTOANNOTATE package, with additional

manual editing (Kucera et al., 2016). Co-expression networks of genes within specific clusters were visualized 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.

3 | RESULTS

3.1 | Endurance capacity

There was a significant interaction for endurance training response between LRT and HRT ($P < 0.0001$), with the distance run after training increasing in HRT only (LRT, −50 ± 129 m, 95% CI −73, 175 m vs. HRT, +375 ± 101 m, 95% CI 251, 499 m, $P < 0.0001$; Figure 1a). The change in pre- to post-training running capacity expressed as a percentage difference was also different in HRT versus LRT for distance (+70%), work performed (+82%) and time to fatigue (+43%; all $P < 0.001$).

3.2 | Body mass and skeletal muscle mass

Body mass was ~20% greater in LRT than HRT, both before endurance training and before FO (FO LRT, 301 ± 33 g, 95% CI 266, 337 g vs. HRT, 253 ± 29 g, 95% 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 ± 0.12 mg/g, 95% CI 0.27, 0.49 mg/g vs. LRT, 0.21 ± 0.05 mg/g, 95% CI 0.11, 0.33 mg/g; both $P < 0.001$); however, the overload-induced increase in plantaris muscle mass was attenuated in LRT versus HRT (+21.4 ± 4.8 vs. 41.6 ± 16.1%, respectively, $P = 0.015$; Figure 1b). There were no differences in plantaris muscle mass between the control limb of the FO groups and the matched HRT and LRT control groups used for RNAseq analysis.

3.3 | Myosin heavy chain gene expression

Although 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 FO for *Myh2* expression ($P < 0.05$; Figure 1c); the higher *Myh2* expression in FO versus control was similar for LRT (9.7%) and HRT (10.3%; both $P < 0.001$). Lower *Myh4* expression in FO 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 FO 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%, $P = 0.09$).
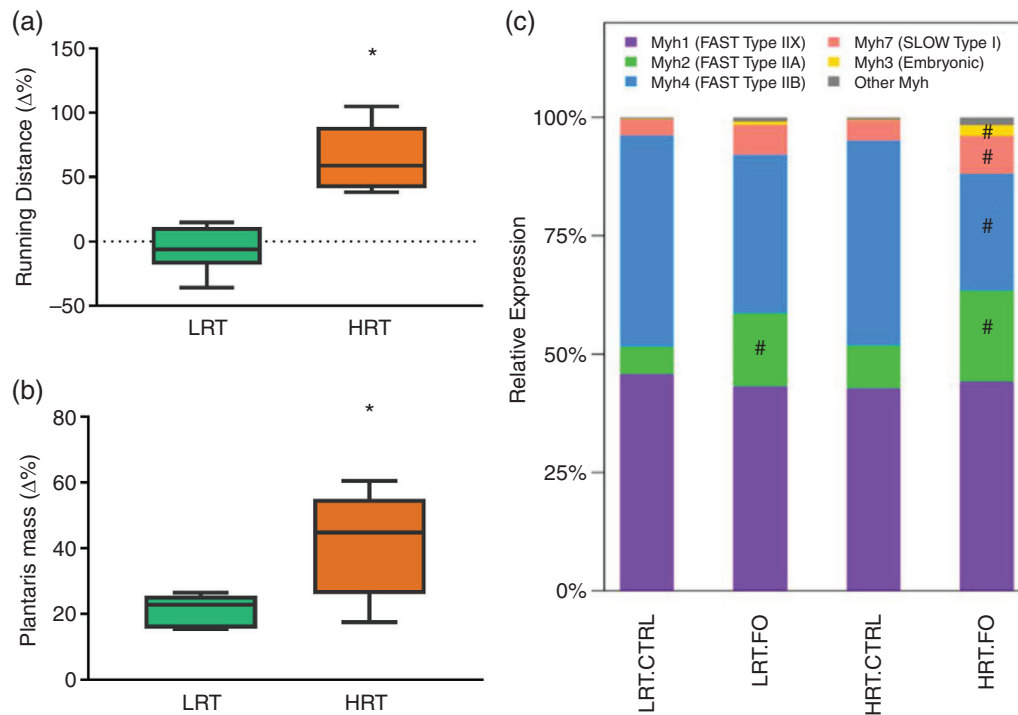
FIGURE 1 Changes in running distance after an 8-week treadmill training programme (a), plantaris muscle mass (overload vs. contralateral control; b) and myosin heavy chain (Myh) transcript expression (c) in functional overload (FO; 14 days) and control (CTRL) groups in selectively bred low and high responders to endurance training (LRT vs. HRT; $n = 6$/group). *Significantly different from LRT, $P < 0.05$; #significantly different from respective HRT/LRT control group, $P < 0.05$

3.4 | 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 components analysis illustrated variance in gene expression within and between sample groups (Figure 2a). Principal component 1 showed a clear separation of FO and control groups, demonstrating that the synergist ablation intervention had the greatest effect on gene expression variability (43%). Principal component 2 indicated that the LRT and HRT phenotypes accounted for 6% of variability in gene expression.

## 3.5 | Identification of differentially expressed genes

From a total of 14,956 detectable genes, 1866 differentially expressed genes (DEGs) were identified (twofold or greater) in LRT FO relative to LRT controls (Table S1). Most of these DEGs (92.4%) were upregulated in LRT in response to hypertrophy (Figure 2b,c). In comparison, 2460 DEGs were identified in HRT, of which 79.6% were upregulated (Figure 2b,d; Table S2). The expression of a subset of 1612 genes (Figure 2b) was common to FO in both groups, representing 86.4% of DEGs in LRT and 65.5% of DEGs in HRT skeletal muscle (Table S3). Select anabolic and catabolic genes of interest associated with the

regulation of muscle mass were not differentially expressed for the LRT and HRT delta response from the respective control groups (Table S4).

3.6 | 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; Table S6). Two hundred and twenty-four biological processes were different in LRT versus HRT after FO. 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 vs. 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 with HRT. 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 FO-induced hypertrophy (Hamilton et al., 2014).

FIGURE 2 RNA-Sequencing analysis from LRT and HRT rat skeletal muscle in response to functional overload. (a) Principal components analysis showing the two major components. (b–d) Venn diagram (b) and volcano plots (c,d) represent the number and magnitude of difference in expression in LRT and HRT overloaded muscle (14 days), respectively, relative to breeding line (LRT/HRT)-matched controls (fold change > 2; false discovery rate < 0.001). $n = 6$/group. Abbreviations: FO, functional overload of the plantaris muscle; HRT, high responders to endurance training; LRT, low responders to endurance training; PC, principal component
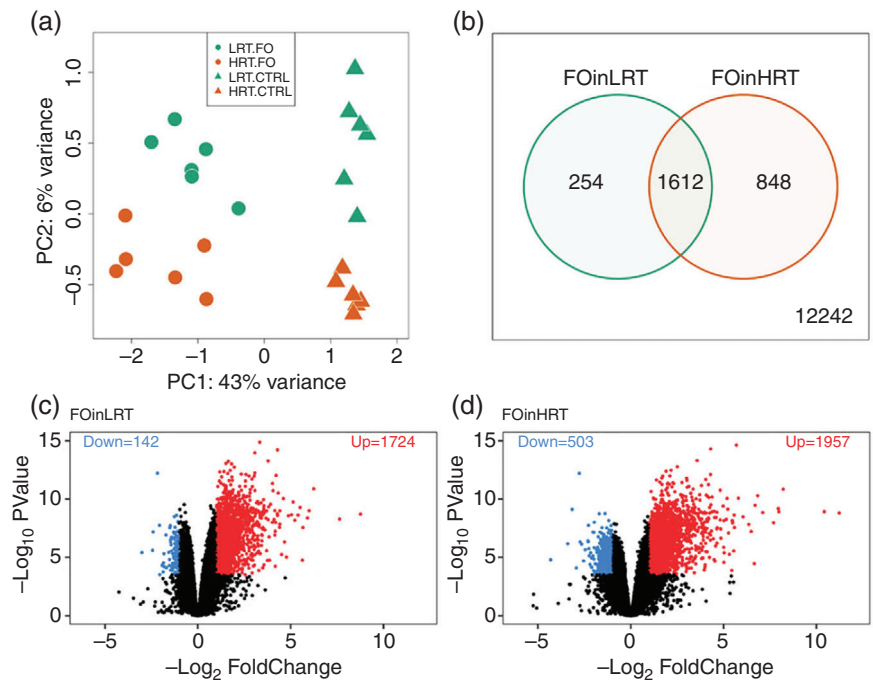
4 | DISCUSSION

The major findings of the present study were that LRT were also low responders to FO and that the attenuated hypertrophy response was associated with distinct enrichments for gene sets in a broad range of biological processes. In response to FO, HRT and LRT showed different magnitudes of response for ~200 upregulated or down-regulated processes. For 27 enriched biological processes, HRT and LRT responded in opposing directions of upregulation versus down-regulation in response to FO. Contrasting regulation by LRT versus HRT corresponding to an attenuated hypertrophy response might provide important insight into 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) and showed no notable hypertrophy and little change in physiological adaptation in both LRT and HRT in response to 6 weeks of training. The compensatory hypertrophy response of HRT in the present study was similar to previous work using uni- or bi-lateral synergist ablation for 14 days (~40–45%) (Bodine et al., 2001; Pehme et al., 2004; Sakuma et al., 2000; Tamaki et al., 2009). Differences in hypertrophy responses between the present study and the work by Ahtiainen et al. (2018) reflect 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 FO. 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 the high adaptation response to endurance training. In contrast, hypertrophy was clearly attenuated in LRT versus HRT after 14 days of FO

with no discernible difference in ambulatory activity, and the modest response in LRT was similar to that observed with short-term synergist ablation (Terena et al., 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 differences in hypertrophy of LRT compared with HRT 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 et al., 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 after disuse (Baehr et al., 2016) and has been suggested as a molecular brake in response to FO (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 organization 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 et al., 2012). In the present study, the endoplasmic reticulum UPR gene set was downregulated in LRT, whereas it was upregulated in HRT (Figure 4). Wu and colleagues (2011) have shown that an appropriate UPR mediates skeletal muscle adaptation after exercise through activation of the transcriptional co-activator
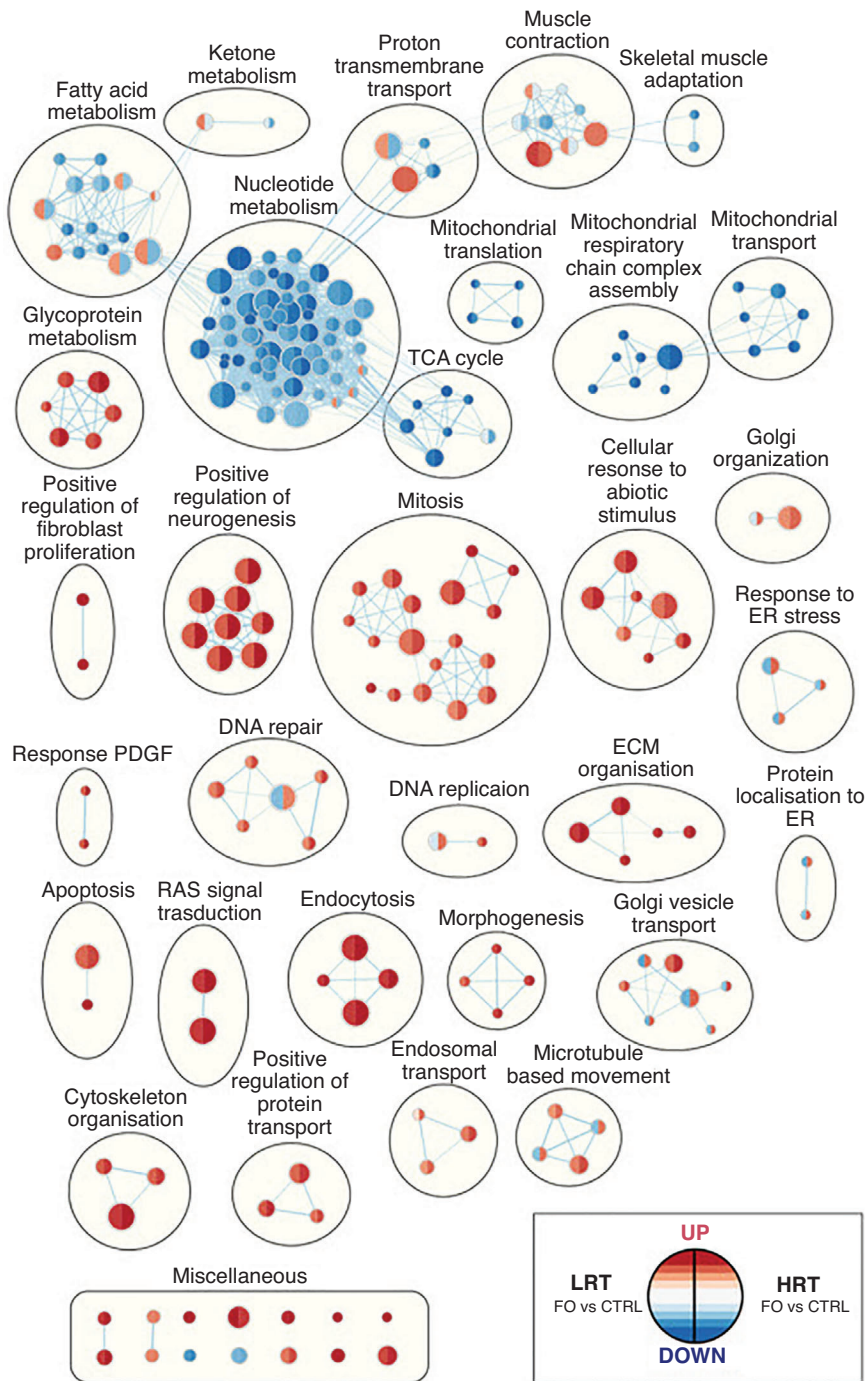
FIGURE 3 Enrichment map of Gene Ontology Biological Processes differentially expressed in LRT and HRT in response to FO. Enrichment results were mapped as a network of gene sets (nodes) related by mutual gene overlap (edges). The enrichment map reflects relative differences (FO vs. control) between LRT and HRT. Red identifies upregulated and blue downregulated gene sets after 14 days of functional overload. Differential expression (LRT vs. 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 LRT ($n = 6$/group) and the right side HRT ($n = 6$/group). Node size is proportional to the percentage of enriched genes per set, and colour intensity represents the magnitude of change in expression. Clusters of functionally related gene sets were circled and labelled manually to highlight prevalent biological functions among a set of related gene sets (FDR < 0.05; $P < 0.001$). Abbreviations: ECM, extra cellular matrix; ER, endoplasmic reticulum; FDR, false discovery rate; FO, functional overload of the plantaris muscle; HRT, high responders to endurance training; LRT, low responders to endurance training; PDGF, Platelet-derived growth factor; TCA, Tricarboxylic acid cycle

PGC-1α (peroxisome proliferator-activator receptor gamma coactivator-1 alpha). Moreover, Marton and colleagues (2015) reported that factors associated with mitochondrial biogenesis, including PGC1-α 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 might 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/programmes for post-translational protein assembly and transport in association with anabolic resistance in LRT rats. Consequently, we hypothesize that LRT have an inferior endoplasmic reticulum response-related molecular programme 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- vs. downregulation), of expression was different between LRT and HRT rats compared with their respective control groups were in *Nucleotide metabolism* and *Mitosis* (Figure 3). Specifically,
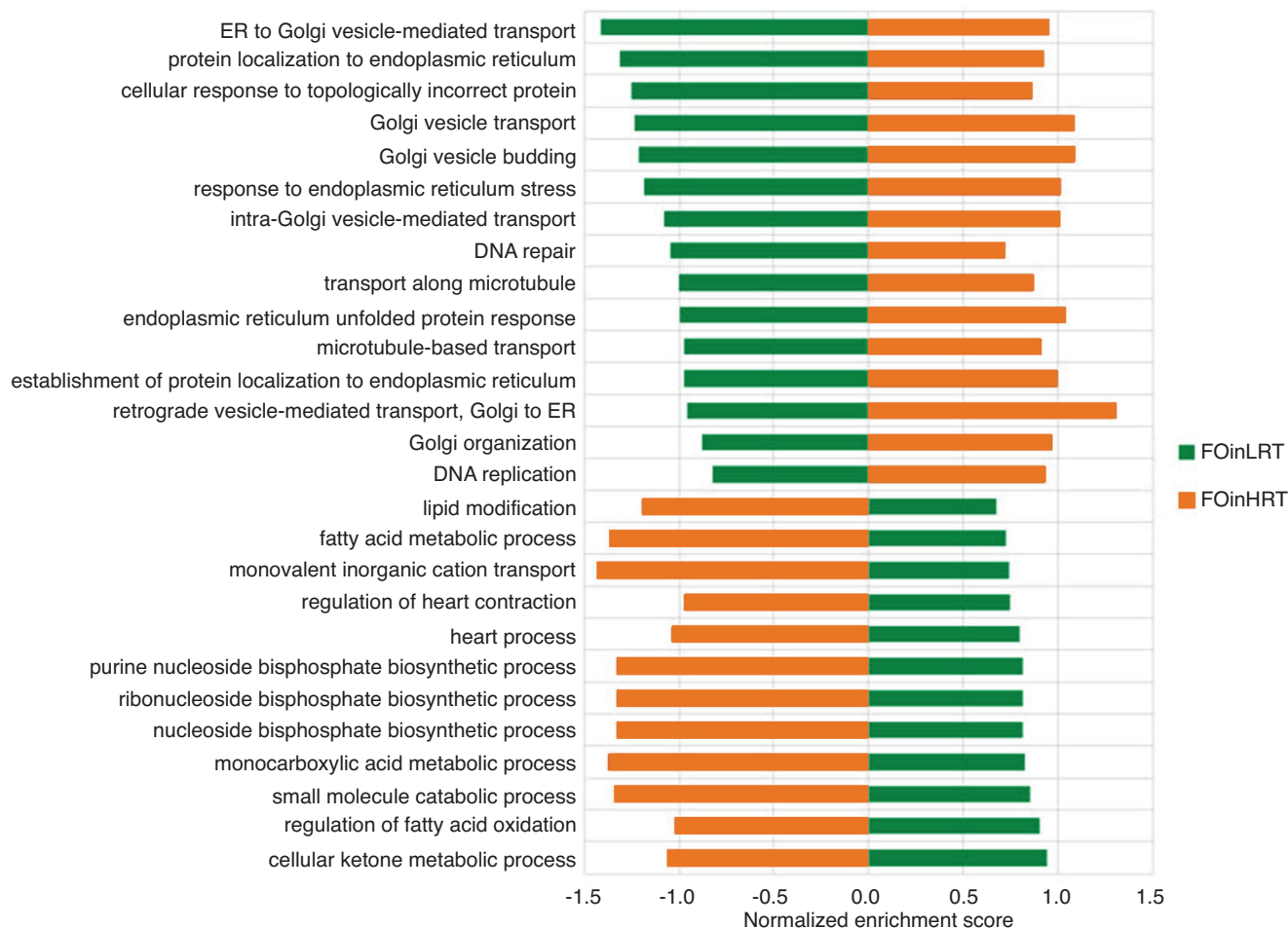
FIGURE 4 Gene set enrichment analysis. Bar plot depicting the normalized enrichment score of the 27 positively and negatively enriched gene sets after 14 days of functional overload in LRT and HRT relative to control animals. Analysis of enriched Gene Ontology biological pathway gene sets in LRT (green) 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. A false discovery rate < 0.05, P-value < 0.001 were used as the significance threshold. n = 6/group. Abbreviations: FO, functional overload of the plantaris muscle; HRT, high responders to endurance training; LRT, low responders to endurance training

when the magnitude of enrichment for nucleotide metabolism pathways was downregulated this was typically less pronounced in LRT, whereas upregulation of mitosis-related pathways tended to be attenuated in LRT compared with HRT. Given that skeletal muscle is primarily composed 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 were 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 might also not be fully characterized. 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 et al., 2018; Bodine et al., 2001). Regardless, the transcription response of mitosis pathways in the present study might 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 et al., 2009). In the present study, pathways related to global nucleotide metabolism were
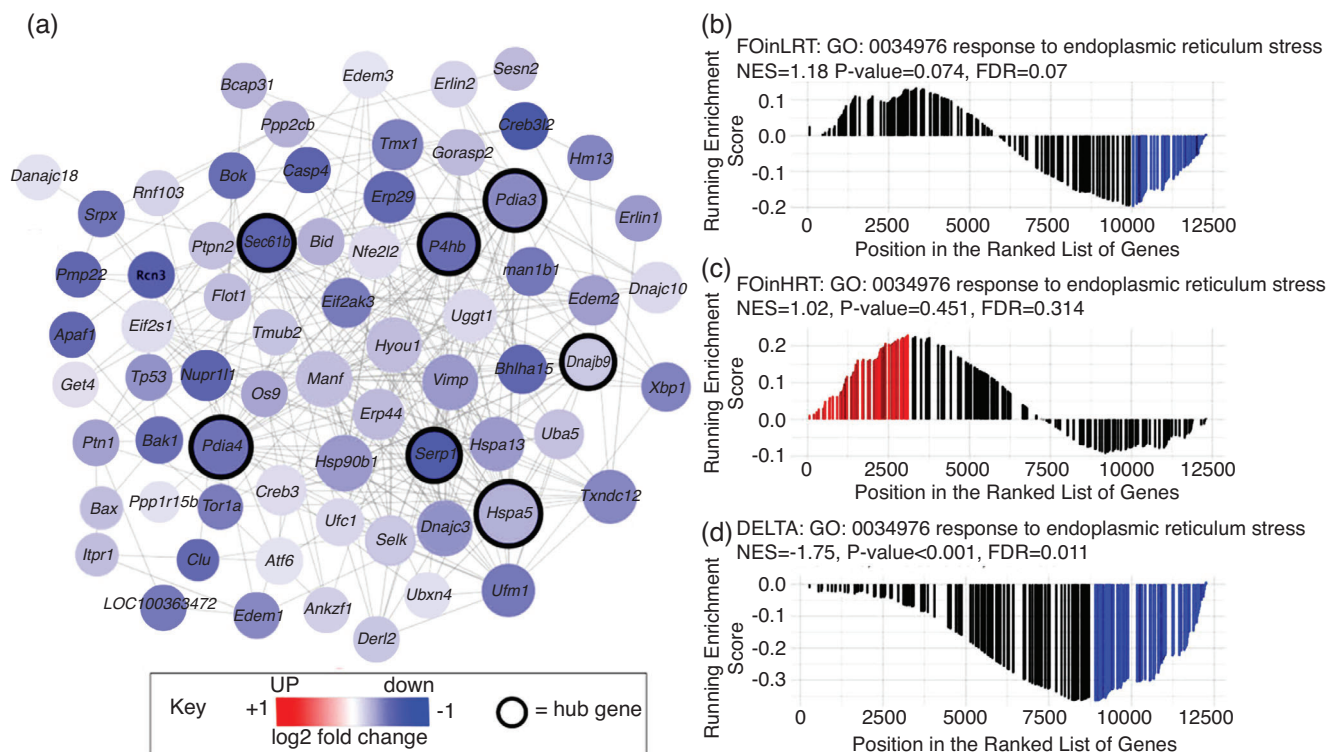
(a)

(b) FOinLRT: GO: 0034976 response to endoplasmic reticulum stress
NES=1.18 P-value=0.074, FDR=0.07

(c) FOinHRT: GO: 0034976 response to endoplasmic reticulum stress
NES=1.02, P-value=0.451, FDR=0.314

(d) DELTA: GO: 0034976 response to endoplasmic reticulum stress
NES=-1.75, P-value<0.001, FDR=0.011

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. (b–d) Gene set enrichment analysis rank plots shown for comparisons of FOinLRT (b), FOinHRT (c) and DELTA HRT-LRT (d). 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 NES, P-value and FDR are also shown. n = 6/group. Abbreviations: FDR, false discovery rate; FO, functional overload of the plantaris muscle; HRT, high responders to endurance training; LRT, low responders to endurance training; NES, normalized enrichment score

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, might have contributed to the variation and extent of nucleotide pathway expression. How (or whether) downregulation of nucleotide metabolism 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 might downregulate nucleotide synthesis pathways. Taken together, attenuated responses in LRT for nucleotide synthesis, DNA repair and the endoplasmic reticulum stress response might contribute to impaired transcription/translation programmes and overall adaptability in LRT.

Lessard and colleagues (2013) used 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-/downregulated, 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 >2000 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 the present study is that the findings reflect a snapshot at 14 days of overload. Previous studies of FO show continued hypertrophy beyond 14 days, and compensatory hypertrophy of ~75–80% after 21–28 days of 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 days of overload (Plyley et al., 1998). Thus, our gene set enrichment analysis data might reflect biological processes that perpetuate rather than initiate hypertrophy (Chaillou et al., 2013). Caution is also warranted when attempting to compare the supraphysiological animal model hypertrophy of the present study directly with human exercise training adaptations (Marsh et al., 2020; Thomas et al., 2020), which
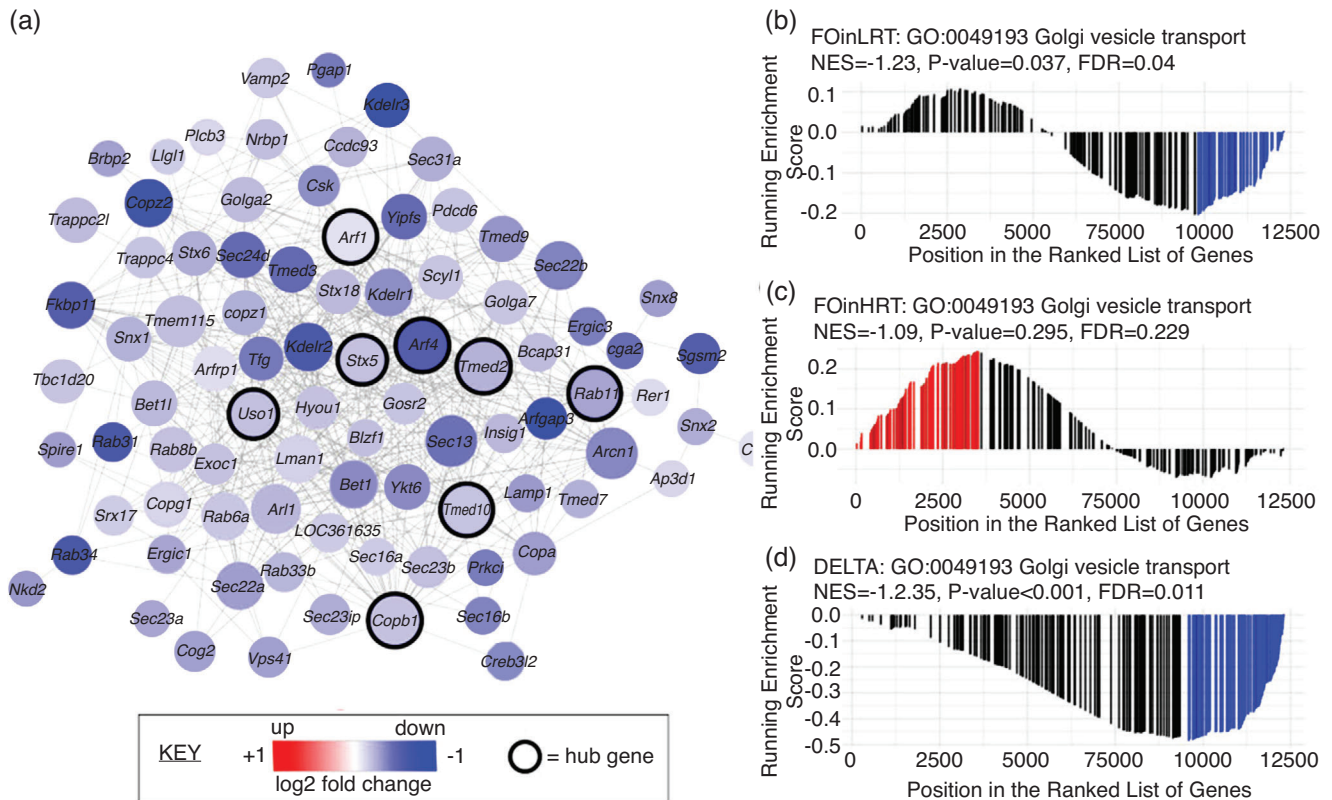
FIGURE 6 Gene set enrichment analysis of the Golgi vesicle transport gene set. (a) Co-expression network of genes involved in the *Golgi vesicle transport* (GO:0048193) gene set. Nodes correspond to individual genes enriched in the delta comparison between LRT and HRT, with all genes downregulated in LRT compared with HRT (FDR < 0.05, P < 0.001). Edge lines between two genes 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. (b–d) Gene set enrichment analysis rank plots shown for comparisons of FOinLRT (b), FOinHRT (c) and DELTA HRT-LRT (d). 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 NES, *P*-value and FDR are also shown. *n* = 6/group. Abbreviations: FDR, false discovery rate; FO, functional overload of the plantaris muscle; HRT, high responders to endurance training; LRT, low responders to endurance training; NES, normalized enrichment score

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 using 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 might share a common set of heritable genetic underpinnings, with profound effects on determining skeletal muscle adaptation. Gene set analysis revealed contrasting positive/negative enrichment and significant differences in the extent to which many biological processes are up- or down-

regulated. 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 to be attributable to dysregulated activation of biological processes associated with a maladaptive genotype.

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