Transcriptional adaptations following exercise in thoroughbred horse skeletal muscle highlights molecular mechanisms that lead to muscle hypertrophy

Beatrice A. McGivney  
*University College Dublin, Ireland*

Suzanne S. Eivers  
*University College Dublin, Ireland*

David E. MacHugh  
*University College Dublin, Ireland*

James N. MacLeod  
*University of Kentucky, jnmacleod@uky.edu*

Grace M. O’Gorman  
*University College Dublin, Ireland*

Follow this and additional works at: [https://uknowledge.uky.edu/gluck_facpub](https://uknowledge.uky.edu/gluck_facpub)

Repository Citation  
McGivney, Beatrice A.; Eivers, Suzanne S.; MacHugh, David E.; MacLeod, James N.; O’Gorman, Grace M.; Park, Stephen D.E.; Katz, Lisa M.; and Hill, Emmeline W., "Transcriptional adaptations following exercise in thoroughbred horse skeletal muscle highlights molecular mechanisms that lead to muscle hypertrophy" (2009). *Veterinary Science Faculty Publications*. 13.  
[https://uknowledge.uky.edu/gluck_facpub/13](https://uknowledge.uky.edu/gluck_facpub/13)
Transcriptional adaptations following exercise in thoroughbred horse skeletal muscle highlights molecular mechanisms that lead to muscle hypertrophy

Digital Object Identifier (DOI)
http://dx.doi.org/10.1186/1471-2164-10-638

Notes/Citation Information
Published in *BMC Genomics*, v. 10, 638.

© 2009 McGivney et al; licensee BioMed Central Ltd.

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Authors
Beatrice A. McGivney, Suzanne S. Eivers, David E. MacHugh, James N. MacLeod, Grace M. O’Gorman, Stephen D.E. Park, Lisa M. Katz, and Emmeline W. Hill

This article is available at UKnowledge: https://uknowledge.uky.edu/gluck_facpub/13
Transcriptional adaptations following exercise in Thoroughbred horse skeletal muscle highlights molecular mechanisms that lead to muscle hypertrophy

Beatrice A McGivney1, Suzanne S Eivers1, David E MacHugh1,2, James N MacLeod3, Grace M O’Gorman1, Stephen DE Park1, Lisa M Katz4 and Emmeline W Hill*1

Address: 1Animal Genomics Laboratory, UCD School of Agriculture, Food Science and Veterinary Medicine, UCD College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland, 2UCD Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland, 3Gluck Equine Research Center, Department of Veterinary Science, University of Kentucky, Lexington, KY 40546-0099, USA and 4University Veterinary Hospital, UCD School of Agriculture, Food Science and Veterinary Medicine, UCD College of Life Sciences, University College Dublin, Belfield, Dublin 4, Ireland

Email: Beatrice A McGivney - Beatrice.McGivney@ucd.ie; Suzanne S Eivers - Suzanne.Eivers@ucd.ie; David E MacHugh - David.MacHugh@ucd.ie; James N MacLeod - jnmacleod@uky.edu; Grace M O’Gorman - Grace.OGorman@ucd.ie; Stephen DE Park - Stephen.Park@ucd.ie; Lisa M Katz - Lisa.Katz@ucd.ie; Emmeline W Hill* - Emmeline.Hill@ucd.ie

* Corresponding author

Abstract

Background: Selection for exercise-adapted phenotypes in the Thoroughbred racehorse has provided a valuable model system to understand molecular responses to exercise in skeletal muscle. Exercise stimulates immediate early molecular responses as well as delayed responses during recovery, resulting in a return to homeostasis and enabling long term adaptation. Global mRNA expression during the immediate-response period has not previously been reported in skeletal muscle following exercise in any species. Also, global gene expression changes in equine skeletal muscle following exercise have not been reported. Therefore, to identify novel genes and key regulatory pathways responsible for exercise adaptation we have used equine-specific cDNA microarrays to examine global mRNA expression in skeletal muscle from a cohort of Thoroughbred horses (n = 8) at three time points (before exercise, immediately post-exercise, and four hours post-exercise) following a single bout of treadmill exercise.

Results: Skeletal muscle biopsies were taken from the gluteus medius before (T0), immediately after (T1) and four hours after (T2) exercise. Statistically significant differences in mRNA abundance between time points (T0 vs T1 and T0 vs T2) were determined using the empirical Bayes moderated t-test in the Bioconductor package Linear Models for Microarray Data (LIMMA) and the expression of a select panel of genes was validated using real time quantitative reverse transcription PCR (qRT-PCR). While only two genes had increased expression at T1 (P < 0.05), by T2 932 genes had increased (P < 0.05) and 562 genes had decreased expression (P < 0.05). Functional analysis of genes differentially expressed during the recovery phase (T2) revealed an over-representation of genes localized to the actin cytoskeleton and with functions in the MAPK signalling, focal adhesion, insulin signalling, mTOR signalling, p53 signalling and Type II diabetes mellitus pathways. At T1, using a less stringent statistical approach, we observed an over-representation of genes involved in the stress...
response, metabolism and intracellular signaling. These findings suggest that protein synthesis, mechanosensation and muscle remodeling contribute to skeletal muscle adaptation towards improved integrity and hypertrophy.

Conclusions: This is the first study to characterize global mRNA expression profiles in equine skeletal muscle using an equine-specific microarray platform. Here we reveal novel genes and mechanisms that are temporally expressed following exercise providing new knowledge about the early and late molecular responses to exercise in the equine skeletal muscle transcriptome.

Background
The Thoroughbred racehorse is an elite athlete, that for four hundred years has been selected for physiological traits enabling exceptional speed and stamina. As a highly adapted athlete the Thoroughbred is a suitable model for understanding the physiology of exercise [1]. Thoroughbreds have a very high aerobic capacity or maximal oxygen uptake (VO$_{2}$max) [2] relative to their body mass. A bout of intense exercise requires both aerobic and anaerobic energy production and a Thoroughbred may increase its metabolic rate from basal levels by up to 60-fold under racing conditions [3]. A critical component for athletic performance is muscle and it is notable that the Thoroughbred has a high skeletal muscle mass comprising over 55% of total body mass [4].

The biological importance of skeletal muscle is reflected in its remarkable structural and functional plasticity that enables rapid alterations to phenotype following repeated bouts of exercise [5]. A single bout of acute exercise induces multiple stresses in skeletal muscle, including increased demand for ATP and mechanical stress [6,7]. The responses to these stressors can be divided into two broad categories: the return to homeostasis, and the adaptive response. The principle processes associated with homeostatic recovery are glucose sparing, elevated fat oxidation, glycogen resynthesis and free radical quenching, as well as the repairing of free radical-mediated damage and restoration of intracellular electrolyte concentrations and pH [8-12]. The adaptive response is the process whereby skeletal muscle responds to repeated exercise bouts (conditioning or training) in ways that cumulatively lead to an enhanced ability to maintain muscle homeostasis during exercise. This conditioning response involves both morphological changes, such as hypertrophy, and metabolic responses such as an increased capacity for oxidative substrate metabolism in mitochondria and a shift toward oxidizing proportionately more fats and less glucose during exercise [13,14].

Exercise studies using human subjects have demonstrated that changes in the expression of a wide range of mRNA transcripts play a major role in the adaptive response of muscle to exercise [15-18]. Furthermore, microarray studies have shown that a large number of genes are differentially expressed in skeletal muscle following exercise [19]. A single bout of exercise has been shown to increase mRNA expression particularly in genes involved in mitochondrial biogenesis and metabolism [20].

While protein changes and mRNA quantified in small panels of genes by Western blotting and real time qRT-PCR [21-24] have been investigated, global mRNA expression during the immediate-response period (< 8 minutes) has not, to our knowledge, previously been reported in skeletal muscle following exercise in any species. Also, global gene expression changes in equine skeletal muscle following exercise have not been reported. Therefore to identify novel genes and key regulatory pathways responsible for exercise adaptation we have used equine-specific cDNA microarrays to examine global mRNA expression in skeletal muscle from a cohort of Thoroughbred horses (n = 8) at two time points (immediately, and four hours post-exercise) following a standardised incremental-step exercise test on a high-speed equine treadmill.

Results and Discussion

Experiment overview
Eight four-year old unconditioned Thoroughbred horses (castrated males) were exercised to maximum heart-rate or fatigue in a standardized incremental-step exercise test [25-27] on a high-speed equine treadmill. Skeletal muscle biopsy samples were collected at three time points: at rest pre-exercise ($T_0$), immediately post-exercise ($T_1$) and four hours post-exercise ($T_2$). In a direct comparison microarray experiment, equine cDNA microarrays were hybridized with samples from $T_0$ Vs $T_1$ and from $T_0$ Vs $T_2$ for each animal.

Exercise parameters
Following warm-up, the exercise test comprised an average of six (range 5 - 7) incremental steps achieving a mean maximum velocity of 12.4 ± 0.2 m/s and a mean distance of 4,362.9 ± 102.7 m for an average duration of 8.77 ± 0.5 min. Mean maximal heart rate was 218 ± 9 beats per minute. Mean peak post-exercise ($T_1$) lactate concentrations were 13.3 ± 1.2 mmol/l and were significantly increased compared to pre-exercise values ($P < 0.0001$).
**Microarray annotation and gene ontology**

Of the 9,333 ESTs on the microarray, 8,519 aligned to a single location on the equine genome (EquCab 2.0). 372 aligned to more than one location and the remaining 442 failed to align to any location with high confidence. Fewer than 50% (4,631) of the ESTs matched an Ensembl gene, the majority (4,166) of which had human orthologs. The human orthologs were used to create input files for gene ontology functional analyses using the DAVID software package [28,29].

The functional representation of ESTs on the microarray relative to all genes in the Equus caballus Ensembl database that had human orthologs (66%) was assessed using 15 broad GO categories (developmental process, multicellular organismal process, biological regulation, metabolic process, cellular process, macromolecular complex, organelle part, organelle, cell part, cell, transporter activity, transcription regulator activity, molecular transducer activity, catalytic activity, binding). A similar distribution pattern among GO categories was observed for ESTs on the microarray when compared to all Ensembl genes (Additional file 1).

**Immediate response to exercise**

**Differential expression of genes**

Immediately following exercise (T₁) two probes were significantly (P < 0.05) differentially regulated. Four hours (T₂) after exercise 1,485 probes were differentially expressed with fold changes ranging from +4.8-fold to -2.9-fold. At T₂, 923 probes were up-regulated and 562 probes were down-regulated. At the chosen significance threshold (α = 0.05) 74 of these probes are likely to be false positives. The probes with the greatest changes in expression (> +1.5-fold) immediately post-exercise are shown in Table 1. The probes with the greatest changes in expression (> +1.5-fold or -1.5-fold) four hours post-exercise are shown in Table 2 (up-regulated) and Table 3 (down-regulated). A full list of gene expression changes at T₁ and T₂ are available in additional files 2 and 3. The equine cDNA microarray expression data generated was deposited in the NCBI Gene Expression Omnibus (GEO) repository with experiment series accession [GEO:GSE16235].

Among the probes with the greatest expression changes (> +1.5-fold) at T₁ were seven probes representing four genes: three probes representing FOS (v-fos FBJ murine osteosarcoma viral oncogene homolog gene; mean +1.9-fold, unadjusted P = 0.004, 0.003, 0.039); two probes representing HSPA1A (heat shock 70 kDa protein 1A gene; mean +2.7-fold, unadjusted P = 1.50E-07, 2.42E-05); one probe located ~2kb upstream of PFKFB3 (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3 gene; +2.0-fold, unadjusted P = 4.71E-06) and one probe representing EGR1 (early growth response 1 gene; +1.6-fold, unadjusted P = 0.014).

The gene expression changes observed for the FOS and HSPA1A genes are consistent with previous mammalian studies that have shown increased expression of these genes in response to exercise [24,30]. HSPA1A, FOS and EGR1 are members of the immediate-early response (IER) gene family. These genes are early regulators of cell growth and differentiation signals, and are induced in response to a wide variety of stress stimuli [31]. The heat shock protein Hsp70, encoded by the HSPA1A gene, is known to protect skeletal muscle cells against the pathophysiological effects of oxidative stress. In transgenic mouse models this cytoprotection is brought about both through improvement in muscle function and decreased apoptosis [32-34]. It has been suggested that the cytoprotective effects of the Hsp70 protein are related to an ability to assist with the refolding of denatured or partially degraded proteins [35].

### Table 1: Genes ≥ +1.5-fold (up-regulated) differential expression immediately post-exercise compared to pre-exercise levels.

| Gene Symbol | Gene Name | GenBank ID | Fold change | P       | adj P | Annotation |
|-------------|-----------|------------|-------------|---------|-------|------------|
| HSPA1A      | Heat shock 70 kDa protein 1A | CX602571   | 3.11        | 1.50E-07| 0.001 | EquCab     |
| HSPA1A      | Heat shock 70 kDa protein 1A | CX600510   | 2.15        | 2.42E-05| 0.080 | EquCab     |
| FOS         | v-fos FBJ murine osteosarcoma viral oncogene homolog | CX597113   | 2.13        | 0.004   | 0.997 | chr24:20:679,377-20,681,089 |
| PFKFB3      | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase | CX594334   | 1.96        | 4.71E-06| 0.023 | chr29:27,672,694-27,678,314 |
| FOS         | v-fos FBJ murine osteosarcoma viral oncogene homolog | CX604427   | 1.87        | 0.003   | 0.997 | H. Sapien  |
| FOS         | v-fos FBJ murine osteosarcoma viral oncogene homolog | CX592361   | 1.59        | 0.039   | 0.997 | H. Sapien  |
| EGR1        | early growth response 1 | CX602573   | 1.55        | 0.014   | 0.997 | H. Sapien  |

The gene names provided are either HUGO approved or Equus caballus specific. Adj P is the P-value following adjustment for multiple testing. Predicted gene annotations were assigned to unannotated probes of interest based on the gene located closest to the probe and homology to mammalian genes, chromosomal locations are provided for these genes.
Table 2: Genes ≥ +1.5-fold (up-regulated) differential expression four hours post-exercise compared to pre-exercise levels.

| Gene Symbol | Gene Name                                      | Fold change | adj P  |
|-------------|------------------------------------------------|-------------|--------|
| HSPA1A      | heat shock 70 kDa protein 1A                  | 4.84        | 1.61E-05|
| HSP90AA1    | heat shock protein 90 kDa alpha (cytosolic), class A member 1 | 2.20    | 0.002   |
| USP36       | ubiquitin specific peptidase 36               | 2.17        | 0.001   |
| VWC2        | von Willebrand factor C and EGF domains       | 2.07        | 0.001   |
| CCDC6       | coiled-coil domain containing 6               | 1.91        | 0.003   |
| HSP90AA1    | heat shock protein 90 kDa alpha (cytosolic), class A member 1 | 1.88 | 0.003   |
| NFIC        | nuclear factor I/C (CCAT-binding transcription factor) | 1.85 | 0.003   |
| RCD1        | RCD domain containing 1                       | 1.80        | 0.001   |
| SEPT9       | septin 9                                      | 1.75        | 0.005   |
| TMEM145     | transmembrane protein 145                     | 1.71        | 0.002   |
| BAIAP2      | BAI1-associated protein 2                     | 1.70        | 0.001   |
| ATXN2L      | ataxin 2-like                                 | 1.70        | 0.003   |
| NUCB1       | nucleobindin 1                                | 1.67        | 0.004   |
| HSPA8       | heat shock 70 kDa protein 8                   | 1.67        | 0.005   |
| STRN4       | striatin, calmodulin binding protein 4        | 1.66        | 0.006   |
| PKM2        | pyruvate kinase, muscle                       | 1.66        | 0.001   |
| DLX5        | distal-less homeobox 5                        | 1.66        | 0.003   |
| CRTC2       | CREB regulated transcription coactivator 2    | 1.66        | 0.002   |
| C20orf112   | uncharacterised protein                        | 1.64        | 0.001   |
| LAMP2       | lysosomal-associated membrane protein 2       | 1.64        | 0.003   |
| E1F1        | eukaryotic translation initiation factor 1    | 1.64        | 0.018   |
| PKM2        | pyruvate kinase, muscle                       | 1.63        | 0.028   |
| PLOD1       | procollagen-lysine 1, 2-oxoglutarate 5-dioxygenase 1 | 1.63 | 0.003   |
| EML32       | echinoderm microtubule associated protein like 3 | 1.62 | 0.006   |
| C14orf93    | uncharacterised protein                       | 1.61        | 0.016   |
| PDCD6IP     | programmed cell death 6 interacting protein   | 1.61        | 0.001   |
| OGRF        | opioid growth factor receptor                 | 1.61        | 0.003   |
| ENP3        | epithelial membrane protein 3                 | 1.61        | 0.006   |
| DPRP1       | DRI-associated protein 1 (negative cofactor 2 alpha) | 1.61 | 0.001   |
| C14orf151   | uncharacterised protein                       | 1.59        | 0.004   |
| C20orf29    | uncharacterised protein                       | 1.59        | 0.001   |
| TUBB        | tubulin, beta                                 | 1.58        | 0.001   |
| RNF19B      | ring finger protein 19B                       | 1.58        | 0.017   |
| RCN3        | reticulocalbin 3, EF-hand calcium binding domain | 1.58 | 0.004   |
| PPI1        | proline/arginine-rich end leucine-rich repeat protein | 1.58 | 0.012   |
| PEX16       | peroxisomal biogenesis factor 16              | 1.58        | 0.003   |
| NRA41       | nuclear receptor subfamily 4, group A, member 1 | 1.58 | 0.006   |
| FTH1        | ferritin, heavy polypeptide 1                 | 1.58        | 0.004   |
| CRTC2       | CREB regulated transcription coactivator 2    | 1.58        | 0.004   |
| SUZ12       | suppressor of zeste 12 homolog (Drosophila)   | 1.57        | 0.001   |
| SF3B5       | splicing factor 3b, subunit 5, 10 kDa         | 1.57        | 0.003   |
| SLC16A3     | solute carrier family 16, member 3 (monocarboxylic acid transporter 4) | 1.57 | 0.017   |
| PKM2        | pyruvate kinase, muscle                       | 1.57        | 0.006   |
| DNAPC1      | Dnaj (Hsp40) homolog, subfamily C, member 1   | 1.57        | 0.002   |
| DLX5        | distal-less homeobox 5                        | 1.57        | 0.004   |
| DCHS1       | dachsous 1 (Drosophila)                       | 1.57        | 0.002   |
| SIPALI      | signal-induced proliferation-associated 1 like 1 | 1.56 | 0.014   |
| SNAI1       | snail homolog 1 (Drosophila)                  | 1.55        | 0.006   |
| PRKCSH      | protein kinase C substrate 80K-H              | 1.55        | 0.007   |
| LCN2        | lipocalin 2                                   | 1.55        | 0.002   |
| HOXAS5      | homeobox A5                                  | 1.55        | 0.004   |
| KDEL1       | KDEL (Lys-Asp-Glu-Leu) endoplasmic reticulum protein retention receptor 1 | 1.54 | 0.006   |
| IDH2        | isocitrate dehydrogenase 2 (NADP+), mitochondrial | 1.54 | 0.011   |
| ALP1        | ancient ubiquitously protein 1                | 1.54        | 0.048   |
| SLC16A13     | solute carrier family 16, member 13 (monocarboxylic acid transporter 13) | 1.53 | 0.008   |
| NIN1        | ninjurin 1                                   | 1.53        | 0.003   |
| HMOX2       | heme oxygenase (decycling) 2                  | 1.53        | 0.020   |
| HSP90AA1    | heat shock protein 90 kDa alpha (cytosolic), class A member 1 | 1.53 | 0.049   |
| CCDC12      | coiled-coil domain containing 12             | 1.53        | 0.011   |
ARHGEF19  Rho guanine nucleotide exchange factor (GEF) 19  1.52  0.002  
NAGPA  N-acetylglucosamine-1-phosphodiester alpha-N-acetylglucosaminidase  1.52  0.005  
PTTG1P  pituitary tumor-transforming 1 interacting protein  1.51  0.014  
NR1D1  nuclear receptor subfamily 1, group D, member 1  1.51  0.020  
CLSTN1  calcyntenin 1  1.51  0.002  
BAIAP2  BAI1-associated protein 2  1.51  0.005  
ATF4  activating transcription factor 4 (tax-responsive enhancer element B67)  1.51  0.005  
C1orf4  uncharacterised protein  1.50  0.005  
TSPAN4  tetraspanin 4  1.50  0.008  
GMPPA  GDP-mannose pyrophosphorylase A  1.50  0.016  
CHAD  chondroadherin  1.50  0.005  
BCKDK  branched chain ketoacid dehydrogenase kinase  1.50  0.014  
ACTN1  actinin, alpha 1  1.50  0.006  

Adj P is the P-value following adjustment for multiple testing.

Table 3: Genes ≥-1.5-fold (down-regulated) differential expression four hours post-exercise compared to pre-exercise levels.

| Gene Symbol | Gene Name                                                                 | Fold change | adj P    |
|-------------|---------------------------------------------------------------------------|-------------|----------|
| ACTR10      | actin-related protein 10 homolog (S. cerevisiae)                          | -1.73       | 0.042    |
| ANXA7       | annexin A7                                                                | -1.73       | 0.039    |
| CBX3        | chromobox homolog 3 (HP1 gamma homolog, Drosophila)                      | -1.51       | 0.010    |
| C12orf57    | chromosome 12 open reading frame 57                                       | -1.52       | 0.008    |
| C17orf37    | chromosome 17 open reading frame 37                                       | -1.59       | 0.011    |
| COPB2       | coatomer protein complex, subunit beta 2 (beta prime)                     | -1.55       | 0.009    |
| C1orf2       | complement factor H                                                        | -1.63       | 0.035    |
| CWF19L2     | CWF19-like 2, cell cycle control (S. pombe)                               | -2.87       | 2.67E-03 |
| CWF19L2     | CWF19-like 2, cell cycle control (S. pombe)                               | -2.14       | 0.003    |
| C1orf37     | complement factor H                                                        | -1.52       | 0.012    |
| FBXW5       | F-box and WD repeat domain containing 5                                   | -1.57       | 0.007    |
| GALM        | galactose mutarotase (aldose 1-epimerase)                                 | -1.62       | 0.023    |
| GLB1        | galactosidase, beta 1                                                     | -1.54       | 0.006    |
| GNL3        | guanine nucleotide binding protein-like 3 (nucleolar)                     | -1.54       | 0.019    |
| HBS1L       | HBS1-like (S. cerevisiae)                                                  | -1.70       | 0.042    |
| HBS1L       | HBS1-like (S. cerevisiae)                                                  | -1.62       | 0.047    |
| KLHL2       | kelch-like 2, Mayven (Drosophila)                                         | -1.66       | 0.040    |
| LRRC8D      | leucine rich repeat containing 8 family, member D                         | -1.67       | 0.039    |
| ME1         | malic enzyme 1, NADP(+)-dependent, cytosolic                             | -1.59       | 0.003    |
| MUT         | methylmalonyl Coenzyme A mutase                                            | -1.59       | 0.014    |
| MIPEP       | mitochondrial intermediate peptidase                                       | -1.69       | 0.012    |
| MRPL39      | mitochondrial ribosomal protein L39                                        | -1.52       | 0.013    |
| NDUFA12     | NADH dehydrogenase (ubiquinone) I alpha subcomplex, 12                    | -1.50       | 0.020    |
| NDN         | neclin homolog (mouse)                                                    | -2.01       | 0.004    |
| NEDD1       | neural precursor cell expressed, developmentally down-regulated 1         | -1.52       | 0.022    |
| PCOLCE2     | procollagen C-endopeptidase enhancer 2                                    | -2.10       | 0.004    |
| PCOLCE2     | procollagen C-endopeptidase enhancer 2                                    | -1.62       | 0.010    |
| PCOLCE2     | procollagen C-endopeptidase enhancer 2                                    | -1.51       | 0.017    |
| QKI         | quaking homolog, KH domain RNA binding (mouse)                            | -1.54       | 0.020    |
| RN14        | reticulon 4                                                               | -1.59       | 0.039    |
| RPL22       | ribosomal protein L22                                                     | -1.61       | 0.048    |
| ROBO1       | roundabout, axon guidance receptor, homolog 1 (Drosophila)                | -1.83       | 0.007    |
| SIAH2       | seven in absentia homolog 2 (Drosophila)                                  | -1.55       | 0.023    |
| TXNDC17     | thioredoxin domain containing 17                                           | -2.15       | 0.005    |
| TRAM1       | translocation associated membrane protein 1                               | -1.87       | 0.008    |
| UTF5        | UDP-glucuronate decarboxylase 1                                           | -2.21       | 0.016    |
| C1orf8      | uncharacterised protein                                                    | -1.51       | 0.020    |
| VPS33A      | vacuolar protein sorting 33 homolog A (S. cerevisiae)                     | -1.70       | 0.048    |

Adj P is the P-value following adjustment for multiple testing.
Hsp70 can also interact with proteins involved in the regulation of cellular redox balance and Ca\(^{2+}\) homeostasis, and thus reduce oxidative stress and Ca\(^{2+}\) overload in response to physiological stress [36]. In addition Hsp70 protects against muscular degeneration and atrophy [37] through inhibition of caspase activation [38] and protein catabolism [37] and Hsp70 protein levels have been shown to correlate with muscular regeneration following injury [39]. Together these facts highlight the key role of Hsp70 in muscle protection following stress and as a modulator of muscular regeneration. The HSPA1A gene displayed a further increase in transcript expression at T\(_2\) (+4.8-fold, \(P < 0.001\)), whereas the expression of FOS, EGR1 and PFKFB3 had returned to resting levels. This suggests that while FOS, EGR1 and PFKFB3 responses may be immediate and transient, the HSPA1A response likely contributes to long term adaptation.

The probe upstream of the PFKFB3 gene shares strong homology to mammalian homologues of the gene thus it is likely that it represents expression of this gene product. The product of the PFKFB3 gene is involved in various aspects of energy sensing and metabolism, but has not previously been shown to be increased due to exercise. However, studies have shown increased expression of PFKFB3 in response to glucose deprivation [40] and hypoxia [41], both stimuli associated with exercise. The PFKFB3 protein is a powerful activator of glycolysis [42]. Surprisingly, in a panel of genes encoding glycolytic enzymes and other anaerobic metabolites, differential mRNA expression was not observed in this experimental cohort despite significant increases in plasma lactate concentrations [43]. Similar observations of a lack of transcriptional activation of glycolytic genes have been made in human exercise studies [44]. PFKFB3 is also involved in glucose-induced insulin secretion in pancreatic \(\beta\) cells [45] and a SNP in the 3’ untranslated region of the PFKFB3 gene is associated with obesity in humans [46]. The PFKFB3 gene promoter contains hypoxic response elements necessary for transactivation by hypoxia-inducible factor-1 alpha (HIF-1\(\alpha\)) in response to hypoxia [47]. This is relevant considering the observed increase in HIF-1\(\alpha\) protein in this cohort of horses immediately after exercise [43].

There was some overlap among probes differentially expressed at \(T_2\) and those tending towards differential expression at \(T_1\). Among the 434 probes tending towards differential expression (unadjusted \(P < 0.05\)) at \(T_2\) 154 were also among those at \(T_1\), which is more than twice as many expected by chance. Over 96% of the genes had both the same direction of regulation at both time-points and a greater magnitude of change at \(T_2\). Two genes had a greater magnitude of change at \(T_1\) and a different directionality was observed for four genes. The genes with the highest observed fold changes at both \(T_1\) and \(T_2\) included HSPA1A (heat shock 70 kDa protein 1A gene, \(T_1\): +2.6-fold (mean of two probes), unadjusted \(P = 1.22E-05\); \(T_2\): +4.8-fold, \(P = 1.61E-05\)); CRTC2 (CREB regulated transcription coactivator 2 gene, \(T_1\): +1.3-fold, adjusted \(P = 0.001\); \(T_2\): +1.7-fold, \(P = 0.003\)); and SLC16A3 (solute carrier family 16, member 13 gene, \(T_1\): +1.2-fold, adjusted \(P = 0.03\); \(T_2\): +1.6-fold, \(P = 0.012\)).

The CRTC2 protein is a potent activator of PGC-1\(\alpha\) (peroxisome proliferator-activated receptor gamma coactivator 1 alpha), the master regulator of mitochondrial biogenesis [48] and is also involved in the modulation of gluconeogenesis [49]. The SLC16A3 protein is found in greater abundance in fast twitch rather than slow twitch muscle [50] and plays a direct role in lactate efflux out of skeletal muscle. Thoroughbred horses have a strikingly high proportion of fast to slow twitch muscle fibres [51], which was also observed in this cohort of horses [43]. Increased mRNA levels of SLC16A3 were observed in "race fit" compared to moderately conditioned Standardbred horses [52]. SLC16A3 also plays a role in the transport of the performance enhancing drug gamma-hydroxybutyric acid (GHB) [53]. GHB is an endogenous metabolite but can also be administered orally as a performance-enhancing drug; therefore it is reasonable to hypothesize that endogenous GHB metabolism is associated with natural athletic ability. This hypothesis is supported by the observation that the alcohol dehydrogenase iron-containing protein 1 gene (ADHFE1), which is involved in GHB catabolism [54] is located in a genomic region that has been a target for positive selection during four hundred years of Thoroughbred evolution [55].

Overall, these data suggest that, in addition to a rapid and dramatic induction of a small number of stress response genes immediately after exercise, there are also more subtle early changes in gene expression that are difficult to detect but are functionally relevant. It is possible that many of the genes differentially expressed at \(T_2\) were also differentially expressed at \(T_1\), but show more gradual changes in gene expression and were not detectable at that time point.

Overrepresentation of functional ontologies among differentially expressed genes

The relatively small number of probes (\(n = 434\), unadjusted \(P < 0.05\)) tending towards significant differential expression immediately after exercise suggested that deriving meaningful functional information may be problematic given an expected false discovery rate of approximately 400 probes in this experiment. Therefore the FatiScan gene enrichment test, which incorporates all transcriptional data rather than limiting to only significantly differentially expressed probes was used to analyse...
the transcriptional profile immediately after exercise [56]. Genes were ranked by differential expression and functional blocks that were significantly up-regulated and down-regulated immediately after exercise were identified (Table 4). Overrepresented GO functional groups associated with up-regulated genes included response to stress, RNA metabolism and developmental processes. The overrepresentation of genes involved in the stress response suggests that exercise-induced muscle repair may be a particular requirement for the maintenance of structural integrity in Thoroughbred skeletal muscle following disruption of muscle fibres. This may be understood in the light of very high aerobic and anaerobic capacities in Thoroughbreds, which enable high intensity exercise even in the unconditioned state. The principal GO functional groups associated with down-regulated genes were those involving the ribosome, oxidative phosphorylation and proton-transporting ATP synthase complex. The strong overrepresentation of down-regulated ribosomal genes suggests an inhibition of protein synthesis. Previous studies have reported a reduced rate of protein synthesis [57,58] and observed the disaggregation of polysomes to ribosomes immediately post exercise [59]. The down-regulation of genes associated with oxidative phosphorylation may represent a shift from aerobic towards anaerobic respiration.

The majority of exercise studies investigating the immediate response to exercise have focussed on post-transcriptional or post-translational modifications or have used real time qRT-PCR to investigate a limited number of genes [21-23,60]. Because of the lack of literature documenting the immediate transcriptional response to exercise in skeletal muscle it is not clear whether the responses detected in this study are specific to horses, or indeed Thoroughbred horses.

### Delayed response to exercise

#### Differentially expressed genes

A significantly larger number of genes were differentially expressed four hours following exercise. Sixteen genes had very significant \( P < 0.01 \) increases in expression with magnitudes > +1.8-fold and 104 had had significant \( P < 0.05 \) expression differences > +1.5-fold. Among the 16 most differentially expressed genes at T2 were HSPA1A (heat shock 70 kDa protein 1A gene, +4.8-fold, \( P < 0.001 \); TPM4 (tropomyosin 4 gene; +1.9-fold, \( P = 0.008 \)); HSP90AA1 (heat shock protein 90 kDa alpha (cytosolic), class A member 1 gene; +2.2-fold, \( P = 0.002 \)) and USP36 (ubiquitin specific peptidase 36 gene; +2.16-fold, \( P = 0.001 \)). Other notable genes present among those differentially upregulated (> +1.5-fold) were HSPA8 (heat shock 70 kDa protein 8 gene; +1.9-fold, \( P = 0.003 \)); CR7C2 (CREB-regulated transcription coactivator 2 gene; +1.7-fold, \( P = 0.002 \)) and LAMP2 (lysosome-associated membrane glycoprotein 2 precursor gene; +1.6-fold, \( P = 0.028 \)). Notably genes encoding three heat shock proteins (HSPA1A, HSP90AA1 and HSPA8) were among the most highly differentially regulated transcripts. The Hsp70 (HSPA1A) and Hsp90 (HSP90AA1) proteins have been

### Table 4: Significantly up-regulated and down-regulated GO categories immediately post-exercise compared to pre-exercise levels.

| GO ID          | GO Term                              | GO Term No | Genes | \( P \) |
|---------------|--------------------------------------|-------------|-------|--------|
| GO:0006950    | response to stress                   | 3           | 0.001 |       |
| GO:0007275    | multicellular organisom development  | 6           | 0.001 |       |
| GO:0048731    | system development                   | 5           | 0.002 |       |
| GO:0048856    | anatomical structure development     | 5           | 0.003 |       |
| GO:0009986    | cell surface                         | 12          | 0.005 |       |
| GO:0007242    | intracellular signaling cascade       | 195         | 0.009 |       |
| GO:0009897    | external side of plasma membrane     | 5           | 0.026 |       |
| GO:0005794    | Golgi apparatus                      | 76          | 0.034 |       |
| GO:0044459    | plasma membrane part                 | 53          | 0.039 |       |
| GO:0016070    | RNA metabolic process                | 4           | 0.040 |       |
| GO:003735     | structural constituent of ribosome   | 21          | 0.000 |       |
| GO:0005840    | ribosome                             | 26          | 0.000 |       |
| GO:0044445    | cytosolic part                       | 33          | 0.001 |       |
| GO:0043228    | non-membrane-bound organelle         | 187         | 0.001 |       |
| GO:0015934    | large ribosomal subunit              | 15          | 0.003 |       |
| GO:0043232    | intracellular non-membrane-bound organelle | 187 | 0.004 |       |
| GO:0005829    | cytosol                              | 130         | 0.007 |       |
| GO:0006119    | oxidative phosphorylation            | 28          | 0.039 |       |
| GO:0045259    | proton-transporting ATP synthase complex | 8     | 0.043 |       |

The FatiScan gene enrichment test was used to analyse the data.
shown to be associated with the transport of TOM (Translocases of the outer membrane) complex proteins to the mitochondrial surface [61,62] in response to contractile activity. These proteins in turn are responsible for the import of the hundreds of nuclear encoded proteins that function in the mitochondria [63,64]. It clearly follows that the reliance on nuclear encoded proteins for mitochondrial function is subject to the efficiency of protein translocation to the mitochondria While numerous studies have reported Hsp70 and Hsp90 induction in skeletal muscle in response to exercise [65,66], to our knowledge, no study has reported an induction of constitutively expressed HSPA8 protein. On the other hand, HSPA8 has been reported to be induced in rat cardiac muscle following hypoxic exposure [67] and may provide a protective effect following oxidative stress [68]. LAMP-2 is a lysosomal receptor involved in the elimination of misfolded proteins. It has been demonstrated that proteasomal inhibition results in an accumulation of Hsp70, LAMP-2 and ubiquitin aggregates [69]. Similarly, the TPM4 protein plays a major role in Ca^{2+}-regulated skeletal muscle contraction and is upregulated in muscle undergoing regeneration and focal repair [70]. Presumably the up-regulation of the heat shock genes, TPM4, LAMP2 and USP36 reflects activity in the reparation or degradation of damaged and misfolded proteins [69].

Fourteen probes representing 12 genes had very significant (P < 0.01) decreases in expression at T1, with magnitudes greater than -1.8-fold. One hundred and twenty-six genes had significant (P < 0.05) expression differences greater than -1.5-fold. The most differentially expressed genes were CWFI9L2 (CWF19-like protein gene); represented by two probes, mean -2.5-fold, P = 0.003; UXS1 (UDP-glucuronic acid decarboxylase 1 gene; -2.2-fold, P = 0.016); TXNL5 (thioredoxin domain-containing protein 17 gene; -2.2-fold, P = 0.005); PCOLCE2, (procollagen C-endopeptidase enhancer 2 precursor gene; represented by two probes, mean -1.9-fold, P = 0.004, P = 0.01); NDN (neclin gene; -2.0-fold, P = 0.004); TRAM1 (translocation-associated membrane protein 1 gene; -1.9-fold, P = 0.008); and ROBO1 (roundabout homolog 1 precursor gene; -1.8-fold, P = 0.007). Six probes also had decreased expression at T1 (unadjusted P < 0.05) representing GLB1 (T1; -1.2-fold; T2; -1.5-fold), SETD7 (T1; -1.1-fold; T2; -1.5-fold) and four unannotated probes.

**Overrepresentation of functional ontologies among differentially expressed genes**

At T2, there was an observed overrepresentation of genes that localised to the actin cytoskeleton, actin filament bundle and cortical actin cytoskeleton (Table 5). The overrepresentation of genes associated with the actin cytoskeleton may be indicative of responses to contraction and mechanical stimuli and may be associated with muscle remodelling via sarcomerogenesis. This is consistent with an observed overrepresentation of genes in the focal adhesion pathway. Actin remodelling has also been shown to be responsible for an increase in GLUT4 translocation in skeletal muscle [71]. An overrepresentation of actin-related gene ontologies following exhaustive exercise has not previously been reported. On the other hand, the observed overrepresentations of genes with intramolecular oxidoreductase activity, unfolded protein binding and heat shock protein binding molecular functions are consistent with human exercise studies that predict replenishment of intramuscular energy stores and a stress response during recovery from intense exercise because of ROS production, inflammation and intramuscular microtendons [8,19].

Because of the larger number of genes with assigned biological processes (497) the returned GO classes had more general higher level functions (e.g. protein folding, regulation of catalytic activity and regulation of the cell cycle) providing little insight into the underlying adaptive mechanisms. Therefore, we searched for overrepresented KEGG pathways among the significantly differentially regulated genes at T2. These included the well established exercise response pathways, insulin signalling [68], Type II diabetes mellitus [72], mTOR signalling [73] and MAPK signalling [74-76] as well as focal adhesion and p53 signaling pathway. A list of genes differentially expressed in these pathways is provided in additional file 4. The overrepresented KEGG pathways are associated with different but overlapping aspects of exercise stimuli and support the hypothesis that the genes governing these cellular pathways have been targets for selection for exercise adaptation in Thoroughbreds [55].

The less well described focal adhesion and p53 signaling pathways are of particular note for their roles in muscle hypertrophy and metabolic improvements. Muscle stretch gives rise to the generation of focal adhesion complexes through the induction of actin polymerisation at focal adhesions and an increase in focal adhesion complex associated proteins has been found in hypertrophic muscle [77,78]. For instance, the mechanosensitive extra cellular matrix protein tenascin-C has been identified as a critical regulator of gene expression relating to repair and growth in muscle following damaging exercise [79]. Furthermore, focal adhesion kinase (FAK) has been shown to be an upstream regulator of the control of muscle mass via p70S6K [80] which may signal mTOR [81] independent of Akt. The central role of FAK in muscle growth and differentiation has been recently been demonstrated. Overexpression of FAK led to a shift towards slow twitch muscle generation and an up-regulation of genes involved in mitochondrial metabolism and contraction [82]. Therefore, an overrepresentation of focal adhesion molecules
Table 5: GO categories and KEGG pathways overrepresented among differentially expressed genes ($P \leq 0.05$) four hours post-exercise compared to pre-exercise levels.

| GO ID               | Molecular Function                      | GO Term                          | No. Genes | $P$       | Fold Enrichment |
|---------------------|----------------------------------------|----------------------------------|-----------|-----------|-----------------|
| GO:0005515          | protein binding                        |                                  | 352       | 0.004     | 1.09            |
| GO:0016853          | isomerase activity                     |                                  | 19        | 0.007     | 1.85            |
| GO:0005200          | structural constituent of cytoskeleton  |                                  | 7         | 0.026     | 2.72            |
| GO:0003700          | transcription factor activity           |                                  | 39        | 0.031     | 1.36            |
| GO:0016860          | intramolecular oxidoreductase activity  |                                  | 8         | 0.037     | 2.33            |
| GO:0031072          | heat shock protein binding              |                                  | 9         | 0.078     | 1.91            |
| GO:0051082          | unfolded protein binding                |                                  | 16        | 0.096     | 1.49            |
| **Cellular Compartment** |                                      |                                   |           |           |                 |
| GO:0044428          | nuclear part                           |                                  | 83        | 0.009     | 1.27            |
| GO:0044446          | intracellular organelle part           |                                  | 194       | 0.015     | 1.13            |
| GO:0044422          | organelle part                         |                                  | 195       | 0.016     | 1.13            |
| GO:0031974          | membrane-enclosed lumen                |                                  | 75        | 0.018     | 1.26            |
| GO:0043233          | organelle lumen                        |                                  | 75        | 0.018     | 1.26            |
| GO:0005667          | transcription factor complex            |                                  | 19        | 0.024     | 1.67            |
| GO:0015629          | actin cytoskeleton                     |                                  | 15        | 0.029     | 1.78            |
| GO:004451           | nucleoplasm part                       |                                  | 37        | 0.032     | 1.37            |
| GO:0005886          | plasma membrane                        |                                  | 79        | 0.042     | 1.20            |
| GO:0016020          | membrane                               |                                  | 222       | 0.049     | 1.09            |
| GO:0005654          | nucleoplasm                            |                                  | 40        | 0.061     | 1.29            |
| GO:0031981          | nuclear lumen                          |                                  | 51        | 0.064     | 1.24            |
| GO:0001725          | stress fiber                           |                                  | 4         | 0.066     | 3.79            |
| GO:0032432          | actin filament bundle                   |                                  | 4         | 0.066     | 3.79            |
| GO:0030864          | cortical actin cytoskeleton            |                                  | 4         | 0.066     | 3.79            |
| GO:0030863          | cortical cytoskeleton                  |                                  | 5         | 0.066     | 2.96            |
| GO:0030054          | cell junction                          |                                  | 15        | 0.074     | 1.58            |
| GO:004433           | cytoplasmic vesicle part               |                                  | 10        | 0.077     | 1.82            |
| GO:0030659          | cytoplasmic vesicle membrane           |                                  | 10        | 0.077     | 1.82            |
| GO:004448           | cell cortex part                       |                                  | 6         | 0.087     | 2.37            |
| GO:0031410          | cytoplasmic vesicle                    |                                  | 28        | 0.090     | 1.33            |
| GO:0012506          | vesicle membrane                       |                                  | 10        | 0.095     | 1.75            |
| GO:0016023          | cytoplasmic membrane-bound vesicle     |                                  | 24        | 0.098     | 1.35            |
| GO:0005770          | late endosome                          |                                  | 7         | 0.100     | 2.07            |
| **Biological Process**     |                                      |                                   |           |           |                 |
| GO:0051640          | organelle localization                 |                                  | 5         | 0.021     | 3.92            |
| GO:0006457          | protein folding                        |                                  | 29        | 0.029     | 1.45            |
| GO:0051650          | establishment of vesicle localization  |                                  | 4         | 0.032     | 4.70            |
| GO:0051648          | vesicle localization                   |                                  | 4         | 0.032     | 4.70            |
| GO:0006903          | vesicle targeting                      |                                  | 4         | 0.032     | 4.70            |
| GO:0050790          | regulation of catalytic activity       |                                  | 26        | 0.044     | 1.44            |
| GO:0051726          | regulation of cell cycle               |                                  | 34        | 0.045     | 1.36            |
| GO:0000074          | regulation of progression through cell cycle |                                | 34        | 0.045     | 1.36            |
| GO:0048523          | negative regulation of cellular process|                                 | 64        | 0.050     | 1.22            |
| GO:0016568          | chromatin modification                 |                                  | 19        | 0.058     | 1.51            |
| GO:0051656          | establishment of organelle localization|                                 | 4         | 0.067     | 3.76            |
| GO:0065009          | regulation of a molecular function      |                                  | 29        | 0.076     | 1.34            |
| GO:0048519          | negative regulation of biological process|                                 | 65        | 0.080     | 1.19            |
| GO:007049           | cell cycle                             |                                  | 49        | 0.080     | 1.23            |
| GO:0031324          | negative regulation of cellular metabolic process | | 25        | 0.082     | 1.37            |
| **KEGG ID**               |                                      |                                   |           |           |                 |
| hsa04010            | MAPK signaling pathway                 |                                  | 13        | 0.060     | 1.69            |
| hsa04510            | Focal adhesion                         |                                  | 17        | 0.099     | 1.45            |
| hsa04115            | p53 signaling pathway                  |                                  | 5         | 0.163     | 2.24            |
| hsa00590            | Arachidonic acid metabolism            |                                  | 3         | 0.182     | 3.70            |
| hsa04910            | Insulin signaling pathway              |                                  | 9         | 0.183     | 1.59            |
| hsa05211            | Renal cell carcinoma                   |                                  | 7         | 0.198     | 1.73            |
| hsa04670            | Leukocyte transendothelial migration   |                                  | 7         | 0.233     | 1.64            |
| hsa04150            | mTOR signaling pathway                 |                                  | 4         | 0.265     | 2.19            |
| hsa00720            | Reductive carboxylate cycle (CO2 fixation) |                              | 3         | 0.265     | 2.96            |
| hsa04930            | Type II diabetes mellitus              |                                  | 3         | 0.265     | 2.96            |

The FatiScan gene enrichment test was used to analyse the data.
indicates the importance of mechanical force altering skeletal muscle gene regulation towards muscle growth and remodelling [83]. Moreover, we have previously determined that focal adhesion molecules may represent targets for recent artificial selection in the Thoroughbred and therefore may be critical to the development of the muscle strength phenotype for which Thoroughbreds are renowned [55]. The p53 protein is best known for its role in apoptosis, however, recent studies have suggested that the p53 signaling pathway may play a role in regulation of aerobic metabolism with significant reductions in COX4 activity in KO mice [84,85]. Importantly, p53 may regulate the expression of PGC-1α [86].

Other KEGG pathways that were overrepresented were: arachidonic acid metabolism, involved in the modulation of function of voltage gated ion channels, primarily in neurons and muscle cells [87]; leukocyte transendothelial migration, associated with the inflammatory response and largely coordinated by chemokines [88,89]; reductive carboxylate cycle (CO₂ fixation), a metabolic pathway; and the renal cell carcinoma signalling pathway, which involves increased cell proliferation, energy demand and O₂ usage and is stimulated by hypoxia and HIF-1α [90,91].

Validation of a panel of genes by real time qRT-PCR

Nine genes that were found to be differentially expressed in the microarray experiment were selected for validation by real time qRT-PCR. These genes were chosen based on their involvement in muscle contraction or the response to hypoxia. Two probes (Genbank IDs: CX594334 and CX598227) that showed differential expression, but were not found within an annotated gene were also included for validation. CX594334 lies ~ 2 kb upstream of PFKFB3 and was upregulated immediately post-exercise. CX598227 lies ~ 1 kb downstream of calmodulin 1 (Calm1) and was upregulated four hours post exercise. The average gene expression of seven of the nine probes studied reached significance (P < 0.05) and six [(basic helix-loop-helix family, member e40 (BHLHE40), calmodulin 3(CALM3), HSPA1A, FOS, CX594334 and CALM1] were concordant with the microarray data (Table 6, Figure 1). A point of major concern in microarray studies is the presence of false positives within a gene list. Although the use of qRT-PCR is essential to validate the overall dataset it is not feasible to interpret the experimental findings by evaluating each gene individually. As genes function in co-operation within complex networks we report principally the expression patterns of functionally related groups of genes.

There were however, some interesting findings among the validated genes. For instance, the expression of FOS showed a high inter-sample variance in gene expression change estimates (+18.2-fold to +506.0-fold increase). The mean expression of the FOS gene mRNA transcript increased +198-fold. The biological significance of the high gene expression variance for this gene is not clear at present but warrants further investigation.

Transcripts for the HSPA1A gene and the probe CX594334 (which may represent the PFKFB3 gene) were significantly increased at T₁ (2.3-fold and 2.7-fold fold respectively). While both genes have quite different physiological roles [42,92] both contain hypoxic response elements (HRE) and have been shown to be transcriptionally activated by the HIF-1α protein under hypoxic conditions [93].

During the recovery period, four hours post exercise, HSPA1A mRNA levels remained elevated (+5.9-fold) while CX594334 transcript levels returned to baseline (Figure 1). The BHLHE40 gene, which increased in expression +2.3-fold is a transcription factor involved in the hypoxic response, contains a HRE and is inducible in hypoxic conditions through interaction with HIF-1α [94,95]. CALM3 and CX598227 which lies ~ 1 kb downstream of CALM1 showed directionally different changes in gene expression. CALM3 was downregulated -0.82-fold while CX598227 was upregulated +2.4-fold. This is of particular interest as little is known regarding the differential regulation of the individual genes within the Calmodulin gene family. Calmodulin is a calcium binding protein which acts as a calcium sensor [96] and plays an important role in mediating many cellular processes including muscle contraction [97,98].

Conclusion

The Thoroughbred horse provides a singular model system to understand exercise adaptations. For the first time following exhaustive exercise we have identified a large number of genes with functions in mechanosensation, muscle hypertrophy, repair and remodelling. The induction of the large numbers of genes with such functions may be explained by the extraordinary innate aerobic and anaerobic capacity of Thoroughbreds enabling high intensity exercise even in an unconditioned state leading to proportionally greater stresses on peripheral systems than in other species. Importantly it is unlikely this knowledge could be readily gained from human studies as the sustained "all out" effort required to elicit such molecular responses is difficult to attain from untrained/sedentary human subjects, but is naturally achieved by Thoroughbreds.

The standard exercise test employed in equine exercise physiology studies requires both endurance and strength, a combination that is not easily reconstructed in other exercise models. The result is that immediately after exercise ribosomal genes are down-regulated indicating
decreased protein synthesis, a signature of endurance exercise. However, established responses associated with resistance exercise such as muscle repair and hypertrophy are observed four hours after exercise. Although the inhibition of protein synthesis and muscle hypertrophy are established responses to endurance and resistance exercise respectively here we detect both responses at a global transcriptional level from a single exercise bout combining both endurance and resistance stimuli.

This study has provided a snapshot of the transcriptional response to exercise in skeletal muscle from a highly adapted system. Genes that were differentially expressed immediately after exercise are likely to be directly involved in metabolism and the stress response. Four hours following exercise a more general transcriptional response associated with recovery and adaptation was observed, in particular highlighting the roles of genes in metabolism and muscle hypertrophy. Further studies are needed to clearly distinguish between the mechanisms associated with the recovery from exercise and return to homeostasis and those that are involved in the long term adaptive response to recurring bouts of exercise conditioning.

Methods
All animal procedures were approved by the University College Dublin, Animal Research Ethics Committee. In addition, a licence was granted from the Department of

Figure 1
Real time qRT-PCR results for genes used to validate microarray data. The standard $2^{-\Delta\Delta CT}$ method was used to determine mean fold changes in gene expression [116]. All Ct values were normalised using the NSUN6 gene. The Student’s t-test was used to identify significant differences in mRNA abundance between time-points. Each point on the graph represents the relative fold change in gene expression compared to pre-exercise levels. * signifies a P-value of <0.05 **, signifies a P-value of <0.01. *** signifies a P-value of <0.001.
Health and Children (Ireland) and owners’ consent was obtained for all horses.

Subjects
Eight four-year old unconditioned Thoroughbred horses (castrated males), raised at the same farm for the previous 12 months and destined for National Hunt racing with the same trainer comprised the study cohort. The horses had a mean height of 165.25 cm (± 1.44) and a mean pre-exercise weight of 565.75 kg (± 13.71). All horses participated in a standardized incremental-step exercise test [25-27] on a high-speed equine treadmill (Sato, Sato AB, Knivsta, Sweden). Before the exercise test, all horses were judged to be clinically healthy based on a veterinary examination that included a lameness assessment, resting upper airway endoscopy and basic bloodwork (complete blood count and serum biochemistry). Prior to entering the study, all of the horses had been raised together and had been kept in a grass field and fed 1.8 kg of 14% Race horses cubes (Gain horse feeds, Clonroche, Co. Wexford, Ireland) three times a day. During the study week the horses were housed in a stable and provided ab libitum access to water and fed grass hay and 2 kg of 10% Cool-n-Cooked Horse and Pony Mix (Connolly’s Red Mills, Baggelstown, Co. Carlow, Ireland) twice daily. Horses were fed approximately 3 hours and 55 minutes (235 ± 0.11 minutes) prior to the exercise test. All exercise tests were performed between 1000 - 1130 am.

Standardised exercise test
The treadmill was housed in an insulated room with temperature and humidity monitors. Prior to the exercise test, all horses were acclimatized to stand quietly and to comfortably transition gaits on the treadmill. The treadmill was set to a 6° incline for all of the exercise tests. The warm-up period consisted of 2 minutes at 2 m/s, followed by 2 minutes at 4 m/s and then 2 minutes at 6 m/s. This was then followed by an increase in treadmill velocity to 9 m/s for 60 seconds, and then a 1 m/s increase in treadmill velocity every 60 seconds until the animal was no longer able to maintain its position on the treadmill at that speed or until the heart rate reached a plateau (HRmax). Following completion of the test, the horses were quickly brought back to a walk, taken off the treadmill and washed down with cold water.

Instrumentation
Any instrumentation was performed 30 minutes to 1 hour prior to the exercise test. Heart rate (HR) was measured continuously before, during and after exercise by telemetry (Polar Equine S810i heart rate monitor system, Polar Electro Ltd, Warwick United Kingdom). Venous blood samples were collected immediately after exercise, 5 minutes after exercise and 4 hours post-exercise. Blood samples were collected and placed into fluoride oxalate tubes for the determination of plasma lactate concentrations. All tubes were stored on ice with the plasma separated within 1 hour of collection and analyzed within 30 minutes using the YSI 2300 STAT Plus™ lactate analyzer (YSI UK Ltd, Hampshire, UK).

Muscle biopsy sampling
Percutaneous needle muscle biopsies [99] were obtained from the dorsal compartment of the gluteus medius muscle according to Dingboom and colleagues [100] using a 6 mm diameter, modified Bergstrom biopsy needle (Jørgen KRUUSE, Veterinary Supplies). Biopsies were taken approximately 15 cm caudodorsal (one-third of the distance) to the tuber coxae on an imaginary line drawn from the tuber coxae to the head of the tail. The biopsies were obtained at a depth of 80 mm. Each biopsy site was shaved, scrubbed with an antiseptic and desensitized by a local anaesthetic. The biopsy samples were washed with sterile PBS (BD Biosciences, San Jose, CA) and preserved in RNAlater (Ambion, UK) for 24 hours at 4°C and then stored at -20°C.

| Gene symbol | GenBank ID | Microarray |
|-------------|------------|------------|
|             | FC T1      | P          | FC T1      | P          |
| HSPA1A      | CX602571   | 3.11       | 0.001      | 2.68       | 2.87E-04   |
| PFKFB3      | CX594334   | 1.84       | 0.023      | 2.30       | 0.010      |
| FOS         | CX604427   | 2.13       | 0.997      | 198.90     | 0.031      |
| ATF4        | CX603997   | 1.50       | 0.005      | -2.04      | 0.014      |
| BHLHE40     | CX599005   | 1.44       | 0.008      | 2.25       | 0.014      |
| CALM3       | CX600504   | -1.40      | 0.045      | -1.12      | 0.031      |
| CRTC2       | CX604423   | 1.58       | 0.004      | -1.12      | 0.483      |
| Calm1       | CX598227   | 1.48       | 0.004      | 2.36       | 0.010      |
| HSPA1A      | CX602571   | 4.84       | 1.6E-05    | 5.91       | 4.08E-04   |
| PKM2        | CX594899   | 1.63       | 0.028      | 1.05       | 0.648      |

FC T1 represents the fold change in gene expression immediately post-exercise. FC T2 represents the fold change in gene expression four hours exercise post-exercise.
Muscle biopsy samples were collected at three time points: at rest pre-exercise (T₀), immediately post-exercise (T₁) and four hours post-exercise (T₂). Pre-exercise biopsies were collected within 93 minutes (range 68 - 93 mins) before the commencement of exercise and between 155 and 170 minutes post feeding. The immediately post-exercise (T₁) biopsies were collected within seven minutes 30 seconds (range 5 mins 45 sec - 7 mins 30 sec) following cessation of exercise and four hour post-exercise (T₂) biopsies were collected within 262 minutes (range 242 - 262 mins) following cessation of exercise.

**RNA isolation and purification**

Approximately 100 mg of each muscle biopsy sample was removed from RNAlater and homogenized in 3 ml TRIzol using a Kinematica Polytron Homogeniser PT 1200 C Drive unit, 230 V (AGB, Dublin, Ireland) and the aqueous and organic phases were separated using 200 μl of chloroform. Total RNA was precipitated using isopropyl alcohol (0.6 times the volume of the aqueous phase). The remaining pellet was washed once in 75% ethanol, and redissolved in 35 μl of nuclease-free water (Promega UK Ltd, Southampton, UK). Each sample was purified using the RNeasy *Mini* kit (Qiagen Ltd, Crawley, UK) and DNase treated with RNase free DNase (Qiagen Ltd, Crawley, UK). To elute the total RNA, 35 μl of nuclease-free water were applied to the silica-gel membrane of the column to elute the total RNA, which was stored at -80°C. RNA was quantified using a NanoDrop® ND1000 spectrophotometer V 3.5.2 (NanoDrop Technologies, Wilmington, DE) and RNA integrity was subsequently assessed using the 18S/28S ratio and RNA integrity number (RIN) on an Agilent Bioanlyser with the RNA 6000 Nano LabChip kit (Agilent Technologies Ireland Ltd, Dublin, Ireland) according to the manufacturer’s instructions. The RNA isolated from these samples had an average RNA integrity number (RIN) of 8.43 ± 0.08 (range 8.0 - 9.0).

**Microarray description and annotation**

Microarray slides were printed with clones selected from a cDNA library generated using mRNA purified from the articular cartilage of a 15-month old male Thoroughbred horse [101]. Probe sets on the microarray slides were prepared and printed as previously described [102,103].

The cDNA sequences for all annotated genes on the *Equus caballus* Version 2.0 (EquCab2.0) genome sequence [http://www.broad.mit.edu/mammals/horse/](http://www.broad.mit.edu/mammals/horse/) were downloaded from Ensembl [http://www.ensembl.org](http://www.ensembl.org), release 50. The expressed sequence tag (EST) sequences of all the probes on the array were masked to remove repeats using Repeat-Masker [104] and blast searched against the cDNAs. BLAST hits were filtered to retain only hits with e values ≤ 10⁻¹⁰, ≥ 50 bp long, above 95% match-target identity, and where the best hit e value was ≥ 10⁻¹⁰ better than the next best. The EST sequences were cross-matched to horse Entrez gene IDs and to human Ensembl and Entrez gene IDs via accessions. EST matches to multiple horse or human genes were excluded. Because fewer than 50% of ESTs matched an Ensembl gene predicted gene annotations were assigned to unannotated probes of interest based on the gene located closest to the probe and homology to mammalian genes. Following a BLAST of RefSeq and protein databases search hits with an e value of < 1⁻¹⁰ and hits on non-mammalian species were eliminated. If the predicted gene annotations based on location and homology did not match, the probe was not assigned a predicted annotation.

**Microarray hybridisation and experimental design**

Total RNA was amplified using a MessageAmpTM amplified RNA (aRNA) linear amplification kit (Ambion). 2 μg of aRNA was reverse transcribed and directly labelled with Fluor647 or Fluor555 using the SuperScript™ Plus Direct cDNA Labeling System (Bio-sciences, Dublin Ireland) according to the manufacturer’s instructions. Labelled samples were combined and co-hybridised on equine cDNA microarrays using SlideHyb Glass Array Hybridisation Buffer #3 (Applied Biosystems, Cambridgeshire, UK). Microarray hybridisations were performed on an automated HS400 hybridisation station (Tecan Group Ltd. Seestrasse 103 CH-8708 Männedorf, Switzerland) with the following protocol - wash: 75°C, runs 1, wash 10 s, soak 20 s; probe injection: 85°C; denaturation: 95°C, 2 min; hybridisation: 42°C, agitation frequency medium, 4 h; hybridisation: 35°C, agitation frequency medium, 4 h; hybridisation: 30°C, agitation frequency medium, 4 h; wash: 37°C, runs 2, wash 10 s, soak 20 s; wash: 25°C, runs 2, wash 15 s, soak 30 s; wash: 25°C, runs 2, wash 20 s, soak 40 s; slide drying: 25°C 2 min.

The experimental design was a direct comparison for each animal between pre- and both post-exercise time points. Each slide was hybridised with samples from T₀ Vs T₁ and from T₀ Vs T₂ for each animal. Technical replicates in the form of a dye swap were performed for each investigation.

**Microarray scanning and data acquisition**

Hybridised and dried slides were scanned using a GenePix 4000B scanner (Molecular Devices, Berkshire, UK) and image acquisition, first-pass data analysis and filtering were carried out using the GenePix 6.0 microarray image analysis package (Molecular Devices, Berkshire, UK). As a first step of feature extraction spots that were flagged as ‘poor’ by the GenePix software (due to signal foreground or background contamination, shape irregularity or poor spot quality) were assigned a weight of zero and were excluded from differential expression analyses. Images of the slides were visually examined and any obvious irregularities were also flagged, assigned a weight of zero and
excluded from differential expression analyses. All spots with a mean signal of less than background plus two standard deviations were flagged and were also excluded from differential expression analyses.

**Microarray data analyses**

All statistical analyses on the gene expression data were performed using the R language, version 2.5.1 [105] and the packages statmod and LIMMA from the Bioconductor project [106]. Robust multichip average (RMA) [107] and print tip lowess normalization [108,109] were performed on the data before differential expression analyses were performed using the lmFit function in LIMMA. Fluor647:Fluor555 log₂ ratios were calculated for each spot on the microarray and duplicate spots were averaged. The function duplicateCorrelation [110] was used to estimate the correlation between technical replicates (dye swaps) by fitting a mixed linear model by REML individually for each probe. The function also returned a consensus correlation, which is a robust average of the individual correlations. This was used to fit a linear model to the expression data for each probe taking into account the inter-technical replicate correlation between each microarray hybridisation.

Differentially expressed targets were determined using a Bayes moderated t-test [111]. Multiple testing was addressed by controlling the false discovery rate (FDR) by using the FDRmoderated t-test [111]. Multiple testing was addressed by controlling the false discovery rate (FDR) by using the FatiScan [56,114] gene enrichment test was used to analyse the transcriptional profile immediately after exercise. FatiScan is part of the Babelomics Suite Genes and tests for the asymmetrical distribution of biological labels in an ordered list of genes. Genes were ranked by differential expression and FatiScan was used to detect functional blocks (GO and KEGG pathways) that were significantly up-regulated and down-regulated immediately after exercise.

**Quantitative real time RT-PCR**

Selected cDNA samples from seven of the eight animals were quantified by real time qRT-PCR. One of the animals was omitted due to a shortage of RNA in the pre-exercise sample. 1 μg of total RNA from each sample was reverse transcribed into cDNA with oligo-dT primers using a SuperScript™ III first strand synthesis SuperMix kit according to the manufacturer’s instructions (Invitrogen Ltd, Paisley, UK). The converted cDNA was diluted to 2.5 ng/μl working stocks and stored at -20°C for subsequent analyses. Oligonucleotide primers for real time qRT-PCR were designed using Primer3 version 3.0 [http://www.primer3.sourceforge.net] and commercially synthesized (MWG Biotech, Germany), details of these primers are available in additional file 5. Each reaction was carried out in a total volume of 20 μl with 2 μl of cDNA (2.5 ng/μl), 10 μ SYBR® Green PCR Master Mix (Applied Biosystems, Cambridgeshire, UK) and 8 μl primer/H₂O. Optimal primer concentrations were determined by titrating 50, 300 and 900 nM final concentrations and dissociation curves were examined for the presence of a single product. qRT-PCR was performed using a 7500 Fast Real-Time PCR machine (Applied Biosystems, Cambridgeshire, UK).

A panel of four putative reference or ‘housekeeping’ genes were selected for a reference gene study. This panel comprised two frequently used reference genes (HPRT1, hypoxanthine phosphoribosyltransferase 1 gene; and GAPDH, glyceraldehyde-3-phosphate dehydrogenase gene) and two genes (NSUN6, NOL1/NOP2/Sun domain family, member 6 gene; and PIGO, phosphatidylinositol glycan anchor biosynthesis, class O gene) that were selected based on minimal variation across the time points observed in the microarray results. The panel of genes was evaluated using geNorm version 3.4 for Microsoft Excel [115]. Briefly, the gene expression stability measure ‘M’ for each control gene was calculated as the pairwise variation for that gene with all other tested reference genes across the exercise time-course (T₀, T₁, T₂). The candidate reference genes were ranked in order of decreas-
ing ‘M’ values or increasing mRNA expression stability [85]. Based on the geNorm analyses, the NSUN6 gene was the optimal reference gene and pre-exercise (T0) values were used to normalise the data. The 2^−ΔΔCT method (where CT is cycle threshold) was used to determine mean fold changes in gene expression [116]. The Student’s t-test was used to identify significant differences in mRNA abundance between time-points.

Authors’ contributions
EH, DMacH and LK conceived and designed the experiments. BMcG, EH, LK and SE participated in the exercise experiment and collection of samples. SE performed the RNA extractions. JMacL provided the microarrays. BMcG and GOG performed the microarray and qRT-PCR experiments. SP was responsible for the annotation of the microarray. BMcG and SP analysed the data. DMacH assisted with manuscript preparation. BMcG and EH wrote the paper. All authors read and approved the final manuscript.

Additional material

Additional file 1
The relative distributions of gene ontology (GO) categories (Level 1) on the equine cDNA microarray. A list of all available human orthologues to equine genes was compared to a list of human orthologues of probes on the microarray using the Database for Annotation, Visualization and Integrated Discovery (DAVID) [28,29] for functional clustering and overrepresentation analyses. The gene ontologies represented on the graph are: 1) gene expression, 2) metabolic process, 3) cellular process, 4) membrane-enclosed lumen, 5) macromolecular complex, 6) organelle part, 7) organelle, 8) cell part, 9) catalytic activity, 10) binding. Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-638-S1.PPT]

Additional file 2
Gene expression a T0. Excel file containing GenBank IDs, log fold changes, unadjusted and adjusted p-values and annotation where available for all probes on the array. Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-638-S2.XLS]

Additional file 3
Gene expression a T0. Excel file containing GenBank IDs, log fold changes, unadjusted and adjusted p-values and annotation where available for all probes on the array. Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-638-S3.XLS]

Additional file 4
Genes differentially expressed in KEGG pathways over-represented at T2
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-638-S4.XLS]

Additional file 5
Equine oligonucleotide primers used for real time qRT-PCR.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2164-10-638-S5.XLS]

Acknowledgements
The authors thank Mr P.J. Rothwell and Mr G. Burke for access to horses. We thank the University Veterinary Hospital yard staff for assistance with exercise experiments. We thank Dr J.A. Browne for helpful advice and assistance with the laboratory work. This work was financed by a Science Foundation Ireland President of Ireland Young Researcher Award (04-Y11-B539) to EH.

References
1. Poole DC: Current concepts of oxygen transport during exercise. Equine and Comparative Exercise Physiology 2004, 1(1):5-22.
2. Young LE, Marlin DJ, Deaton C, Brown-Feltner H, Roberts CA, Wood JL: Heart size estimated by echocardiography correlates with maximal oxygen uptake. Equine Vet J Suppl 2002:467-471.
3. Guthrie AJ, Lund RJ: Thermoregulation. Base mechanisms and hyperthermia. Vet Clin North Am Equine Pract 1998, 14(1):45-59.
4. Gunn HM: Muscle, bone and fat proportions and muscle distribution of Thoroughbreds and other horses. Equine exercise physiology 2 Davis, ICEEP, CA 1987:253-264.
5. Booth FW, Tseng BS, Fluck M, Carson JA: Molecular and cellular adaptation of muscle in response to physical training. Acta Physiol Scand 1998, 162(3):343-350.
6. Martineau LC, Gardiner PF: Insight into skeletal muscle mechanotransduction: MAPK activation is quantitatively related to tension. J Appl Physiol 2001, 91(2):693-702.
7. Sahlin K, Tonkonogi M, Soderlund K: Energy supply and muscle fatigue in humans. Acta Physiol Scand 1998, 162(3):261-266.
8. Pileggaard H, Ordway GA, Salton B, Neuffer PD: Transcriptional regulation of gene expression in human skeletal muscle during recovery from exercise. Am J Physiol Endocrinol Metab 2000, 279(4):E806-814.
9. Jeukendrup AE: Modulation of carbohydrate and fat utilization by diet, exercise and environment. Biochem Soc Trans 2003, 31(Pt 6):1270-1273.
10. Ji LL: Exercise-induced modulation of antioxidant defense. Ann N Y Acad Sci 2002, 959:82-92.
11. Green HJ: Membrane excitability, weakness, and fatigue. Can J Appl Physiol 2004, 29(3):291-307.
12. Richter EA, Derave W, Wojtaszewski JF: Glucose, exercise and insulin: emerging concepts. J Physiol 2001, 535(Pt 2):313-322.
13. Adhihetty PJ, Irrecher I, Joseph AM, Ljubicic V, Hood DA: Plasticity of skeletal muscle mitochondria in response to contractile activity. Exp Physiol 2003, 88(1):99-107.
14. Carter SL, Rennie C, Tarnopolsky MA: Substrate utilization during endurance exercise in men and women after endurance training. Am J Physiol Endocrinol Metab 2001, 280(6):E898-907.
15. Fluck M, Hoppeler H: Molecular basis of skeletal muscle plasticity--from gene to form and function. Rev Physiol Biochim Pharma col 2003, 146:159-216.
16. Booth FW, Baldwin KM: Muscle plasticity: energy demanding and supply processes. In Handbook of physiology Volume Chap 24. Issue Sect 12 Edited by: Rowell LB, Shepherd JT. Oxford University Press, New York; 1996:1075-1123.
24. Puntschart A, Wey E, Jostarndt K, Vogt M, Wittwer M, Widmer HR, Rose RJ, Gibala MJ, McGee SL, Garnham AP, Howlett KF, Snow RJ, Hargreaves Hoppeler H, Klossner S, Fluck M, Mahoney DJ, Parise G, Melov S, Safdar A, Tarnopolsky MA: Analysis of global mRNA expression in human skeletal muscle during recovery from endurance exercise. FASEB J 2005, 19(11):1498-1500.

25. Hoppeler H, Klossner S, Fluck M: Gene expression in working skeletal muscle. Adv Exp Med Biol 2007, 618:245-254.

26. Gething MJ, Sambrook J: Protein folding in the cell. J Biol Chem 2004, 279(51):53562-53570.

27. Tsuchiya, Ryu Y, Dentin R, Chen C, Coletti D, S Ravnskjaer K, Schnel Milne, Meyers DJ, Cole P, Yates J, et al.: A fasting inducible switch modulates gluconeogenesis via activator/coactivator exchange. Nature 2008, 456(7219):269-273.

28. Liu Y, 411(1):137-144.

29. Snow DH, Guy PS: Muscle fibre type composition of a number of limb muscles in different types of horse. Res Vet Sci 1980, 28(3):137-144.

30. Koho NM, Hyyppa S, Poso AR: Monoaminergic transporters (MCT) as lactate carriers in equine muscle and red blood cells. Equine Vet J Suppl 2006, 38:248-358.

31. Wang Q, Morris ME: The role of monoaminergic transporters in the transport of dopamine, norepinephrine and serotonin in the brain. Drug Metab Dispos 2007, 35(8):1393-1399.

32. Lyon RC, Johnston SM, Panopoulos A, Alzeer S, McGarvie G, Ellis EM: Enzymes involved in the metabolism of gamma-hydroxybutyrate in SH-SYSY cells: identification of an iron-dependent alcohol dehydrogenase ADHFe1. Chem Biol Interact 2009, 178(1):283-287.

33. Gu J, Orr N, Park SD, Kazt LM, Sulimova G, MacHugh DE, Hill EW: A genome scan for positive selection in thoroughbred horses. PLoS ONE 2009, 4(6):e6576.

34. Al-Shahrour F, Ariza L, Dopazo H, Huerta-Cepas J, Minguez P, Montaner D, Dopazo J: From genes to functional classes in the study of biological systems. BMC Bioinformatics 2007, 8:114.
57. Dohm GL, Kasperek GJ, Tapscott EB, Beecher GR: Effect of exercise on synthesis and degradation of muscle protein. Biochem J 1980, 188:155-162.

58. Gausch TA, Anthony JC, Kimball SR, Paul GL, Layman DK, Jefferson LS: Availability of eIF4E regulates skeletal muscle protein synthesis during recovery from exercise. Am J Physiol 1998, 274(2 Pt 1):C406-414.

59. Williamson DA, Kubicz, N, Kimball SR, Jefferson LS: Exercise-induced alterations in extracellular signal-regulated kinase 1/2 and mammalian target of rapamycin (mTOR) signalling to regulatory mechanisms of mRNA translation in mouse muscle. J Physiol 2006, 573(Pt 2):497-510.

60. Hunt CR, Parsian AJ, Goswami PC, Kozak CA: Characterization and expression of the mouse Hsc70 gene. Biochim Biophys Acta 1999, 1444(3):315-325.

61. Humphries AD, Streitmann IC, Stojanovski D, Johnston AJ, Yano M, Hoogenraad NJ, Ryan MT: Dissection of the mitochondrial import and assembly pathway for human Tom40. J Biol Chem 2005, 280(12):11533-11543.

62. Takahashi M, Chesley A, Freyssenet D, Hood DA: Contractile activity-induced adaptations in the mitochondrial import system. Am J Physiol 1998, 274(5 Pt 1):CL380-1387.

63. Bottinelli R, Fluck M, Herrmann JM: pleiotropism: Multi- pathway for sorting mitochondrial precursor proteins. EMBO Rep 2008, 9(1):42-49.

64. Neupert W, Herrmann JM: Translocation of proteins into mitochondria. Annu Rev Biochem 2007, 76:723-749.

65. Vissing K, Overgaard M, Schiering P, Raadst T: Heat shock protein translocation and expression response is attenuated in response to repeated eccentric exercise. Acta Physiol (Oxf) 2009, 196(3):283-293.

66. Fluck M, Mund SI, Schiering P, Raadst T: Maximal eccentric exercise induces a rapid accumulation of small heat shock proteins on myofibrils and a delayed HSP70 response in humans. Am J Physiol Regul Integr Comp Physiol 2007, 293(2):R844-853.

67. Ikeda T, Abe K, Ota A, Inoue T: Heat shock protein 70 and heat shock cognate protein 70 messenger ribonucleic acid induction in the brains, hearts, and livers of neonatal rats after hypoxic stress. Am J Obstet Gynecol 1998, 179(2 Pt 1):457-461.

68. Srivastava KS, Edelstein K, Lille S, Khardori R, Lai CC: Constitutive Hsp70 attenuates hydrogen peroxide-induced membrane lipid peroxidation. Biochim Biophys Acta Res Commun 1999, 265(2):279-284.

69. Hyten T, Hyytinen JM, Kopitz J, Rilla K, Kuisisto E, Mannermaa E, Viiri J, Holmer MJ, Clinnen I, Meri S, et al.: Crosstalk between Hsp70 molecular chaperone, lysosomes and proteasomes in chondria. Physiol Regul Integr Comp Physiol 2007, 293(2):R844-853.

70. Vlassovich N, Schevovz G, Nair-Shaliker V, Ilkovski B, Arscott ST, Joys JE, Kee AJ, North KN, Gunning PW, Schiering P, Raadst T: Tropomyosin 4 defines novel filament in skeletal muscle associated with muscle remodelling/regeneration in normal and diseased muscle. Cell Motil Cytoskeleton 2008, 65(1):73-85.

71. Klip A: The many ways to regulate glucose transporter 4. Appl Physiol Nutr Metab 2009, 34(3):481-487.

72. Boden G: Pathogenesis of type 2 diabetes. Insulin resistance. Endocrinol Metab Clin North Am 2001, 30(4):801-815.

73. Reynolds TH, Bodine SC, Lawrence JC Jr: Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. J Biol Chem 2002, 277(20):17657-17662.

74. Wretman C, Lionikas A, Widegren U, Lannergren J, Westerblad H, Henriksson J: Effects of concentric and eccentric contractions on phosphorylation of MAPK(erk1/2) and MAPK(p38) in isolated rat skeletal muscle. J Physiol 2001, 535(Pt 1):155-164.

75. Johnson GL, Laparat R: Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. Science 2002, 298(5600):1911-1912.

76. Osorio-Fuentealba C, Valdes JA, Riquelme D, Hidalgo J, Hidalgo C, Carrasco MA: Hypoxia stimulates via separate pathways ERK phosphorylation and NF-kappaB activation in skeletal muscle cells in primary culture. J Appl Physiol 2009, 106(4):1301-1310.
99. Lindholm A, Piehl K: Fibre composition, enzyme activity and concentrations of metabolites and electrolytes in muscles of standardbred horses. Acta Vet Scand 1974, 15(3):287-309.

100. Dingboom EG, Dijkstra G, Enzerink E, van Oudheusden HC, Weijts WA: Postnatal muscle fibre composition of the gluteus medius muscle of Dutch Warmblood foals; maturation and the influence of exercise. Equine Vet J Suppl 1995:95-100.

101. MacLeod JN: Equine articular cartilage microarray abstract. Plant & Animal Genome Conference XIll: 2005; San Diego, CA, USA 2005.

102. Coleman SJ, Gong G, Gaile DP, Chowdhary BP, Bailey E, Liu L, MacLeod JN: Evaluation of Compass as a comparative mapping tool for ESTs using horse radiation hybrid maps. Anim Genet 2007, 38(3):294-302.

103. Band MR, Olmstead C, Everts RE, Liu ZL, Lewin HA: A 3800 gene microarray for cattle functional genomics: comparison of gene expression in spleen, placenta, and brain. Anim Biotechnol 2002, 13(1):163-172.

104. Smitt A, Hubley R, Green P: RepeatMasker Open-3.0. 1996.

105. Team RDC: R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2005.

106. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Ellis B, Gautier L, Ge Y, Gentry J, et al.: Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004, 5(10):R80.

107. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP: Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003, 4(2):249-264.

108. Bolstad BM, Irizarry RA, Astrand M, Speed TP: A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. Bioinformatics 2003, 19(2):185-193.

109. Cleveland WS: Robust locally weighted regression and smoothing scatterplots. Journal of the American Statistical Association 1979, 74(368):829-836.

110. Smyth GK, Michaud J, Scott HS: Use of within-array replicate spots for assessing differential expression in microarray experiments. Bioinformatics 2005, 21(9):2067-2075.

111. Smyth GK: Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004, 3:Article3.

112. Benjamini Y, Hochberg Y: Controlling the false discovery rate - a practical and powerful approach to multiple testing. J R Stat Soc Ser B-Methodol 1995, 57(1):289-300.

113. Hosack DA, Dennis G, Sherman BT Jr, Lane HC, Lempicki RA: Identifying biological themes within lists of genes with EASE. Genome Biol 2003, 4(10):R70.

114. Al-Shahrour F, Mingué P, Tarraga J, Montaner D, Alloza E, Uaquerizas JM, Conde L, Blaschke C, Vera J, Dopazo J: BABELOMICS: a systems biology perspective in the functional annotation of genome-scale experiments. Nucleic Acids Res 2006:W472-476.

115. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman F: Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 2002, 3(7):RESEARCH0034.

116. Livak KJ, Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) Method. Methods 2001, 25(4):402-408.