Copyright Statement

The digital copy of this thesis is protected by the Copyright Act 1994 (New Zealand).

This thesis may be consulted by you, provided you comply with the provisions of the Act and the following conditions of use:

- Any use you make of these documents or images must be for research or private study purposes only, and you may not make them available to any other person.
- Authors control the copyright of their thesis. You will recognize the author's right to be identified as the author of this thesis, and due acknowledgement will be made to the author where appropriate.
- You will obtain the author's permission before publishing any material from their thesis.

General copyright and disclaimer

In addition to the above conditions, authors give their consent for the digital copy of their work to be used subject to the conditions specified on the Library Thesis Consent Form and Deposit Licence.
Early life high-fat diet and voluntary physical activity affect body composition, bone phenotype and gene expression in the male rat

Dharani Mahesh Sontam

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy in Biomedical Science

The University of Auckland, 2017.
Outputs arising from this thesis

Published papers


**Sontam DM**, Firth EC, Tsai P, Vickers MH, O’Sullivan JM. Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca$^{2+}$ signalling pathways in the male rat bone. Physiological Reports. 2015;3(10):e12568.


Conference poster presentations


Abstract

Background

Childhood and adolescence are characterised by rapid musculoskeletal development and offer a window of opportunity for maximising skeletal gains through physical activity. Physical activity has also long been considered a non-pharmacological strategy to combat diet induced obesity and its associated co-morbidities. The classically accepted opinion that obesity has a protective effect on bone due to the increased mechanical demand imposed by the excessive body weight on the skeleton has been challenged in recent times. Animal models used to investigate the effects of physical activity on bone development often involve the use of forced exercise models. Such studies have the potential for data confounding as they elicit a stress response in the animals.

A series of studies were conducted to understand how voluntary physical activity and high-fat diet affect the body composition, bone mass indices, bone and marrow transcriptome during early life using a male rat model. Firstly, two voluntary exercise modalities: bipedal stance (BPS) and wheel exercise (WEX), were evaluated for their ability to influence body composition and gene expression without eliciting a stress response during the prepubertal period. Next, gene expression changes in the diaphyseal bones of the exercised animals were determined. In the second study, we examined the effects of an early life high-fat diet on body composition, bone mass indices and marrow gene expression in a young adult male rat model. Next, the ability of voluntary physical activity to modulate the early life high-fat diet effects was evaluated. Finally, whether the bone marrow retains a memory of early life exercise following its cessation was investigated.

There was a modality-dependent response to prepubertal physical activity. The WEX group had significantly greater lean mass percentage and lower fat mass percentage compared to the control (CON) group. In the femoral diaphysis, the cortical cross-sectional thickness and closed porosity of the WEX group was significantly greater than the CON group. Such differences were not observed in the BPS group with respect to either body composition or cortical bone parameters. This modality-specific response was also seen in the cortical bone gene expression. We observed that genes with functions in integrin-linked kinase (ILK) and calcium (Ca\(^{2+}\)) signalling pathways, both prominent mechanotransduction pathways in bone, had a diametrically opposite response to wheel and bipedal stance exercises.
Early life high-fat diet positively affected body weight, total fat percentage and bone mass indices. In the bone marrow, it resulted in adipocyte hypertrophy and a pro-inflammatory and pro-adipogenic gene expression profile. Crucially, the bone marrow of the rats that exercised using the wheel while on a high-fat diet retained a memory of the early life exercise even when the exercise was stopped for 60 days. In the bone marrow, the adipocyte size of the rats that exercised did not exhibit hypertrophy. Several genes associated with mature adipocyte function were downregulated in the early-exercise group. Moreover, response of adiponectin gene expression in the marrow was different to that documented in white adipose tissues indicating that the the marrow adipose tissue may have a unique response to high-fat feeding.

These results show that short-term prepubertal voluntary physical activity affects body composition, bone mass indices and cortical bone gene expression in a modality dependent manner. Voluntary physical activity has the ability to modulate the body composition and gene expression changes induced by a high-fat diet. In the bone marrow, the transcriptome retains a memory of the early life physical activity 60 days after exercise has ceased. The potential mechanisms driving the gene expression response must be the focus of future studies.
Acknowledgements

The research presented in this thesis was carried out at the Liggins Institute, University of Auckland, New Zealand. I sincerely acknowledge the support and assistance of several people who made this thesis possible. I am grateful for the supervision I received from Dr. Justin O’Sullivan, Professor Elwyn Firth and Professor Mark Vickers. Their guidance, advice, support and feedback were indispensable in keeping me motivated and on track.

Thanks to Eric Thorstensen and Chris Keven for their assistance with mass spectrometry and biochemical analyses respectively. Thanks to Samuel Haysom for carrying out the adipocyte area assessment. I would also like to thank Jennifer Chin and Chris Hedges for their assistance with animal care and assessment. My sincere thanks to Amorita Petzer at the School of Biological Sciences Animal Care Facility for kindly providing me with rat bone samples for optimisation of my RNA extraction protocols. Thanks to HuiHui Phua for her tips and tricks regarding all things RNA. Thank you William Schierding for patiently answering my many questions regarding RNA-seq and Linux command line. I also would like to thank Jean Leonard for the research administrative support I received during my time at the Liggins Institute.

I would like to thank my fellow PhD students (past and present) at the Liggins Institute – particularly Emma McGoldrick, Veronica Boyle and Minglan Li for their friendship and support. To Martin, thank you for your unconditional support, understanding and patience. To my family, none of this would be possible without your love and encouragement.

This PhD was funded by GRAVIDA: National Centre for Growth and Development. I am also immensely grateful for the financial assistance from GRAVIDA that enabled me to present and discuss my research findings at two international conferences.
Co-authorship forms
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 3
Title: Different short-term mild exercise modalities lead to differential effects on body composition in healthy prepubertal male rats.
An original research article published in the journal "BioMed Research International".

| Nature of contribution by PhD candidate | Plasma and fecal analysis, data analysis, manuscript preparation. |
| Extent of contribution by PhD candidate (%) | 70 |

CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.H. Vickers</td>
<td>designed and supervised study, co-wrote manuscript</td>
</tr>
<tr>
<td>J.M. O'Sullivan</td>
<td>supervised study, co-wrote manuscript</td>
</tr>
<tr>
<td>M. Watson</td>
<td>ran pQCT, manuscript preparation (pQCT section)</td>
</tr>
<tr>
<td>E.C. Firth</td>
<td>designed and supervised study, co-wrote manuscript</td>
</tr>
</tbody>
</table>

Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>M.H. Vickers</td>
<td></td>
<td>10/1/17</td>
</tr>
<tr>
<td>J.M.O'Sullivan</td>
<td></td>
<td>05/1/17</td>
</tr>
<tr>
<td>M. Watson</td>
<td></td>
<td>13/01/17</td>
</tr>
<tr>
<td>E.C. Firth</td>
<td></td>
<td>16/1/17</td>
</tr>
</tbody>
</table>

Last updated: 19 October 2015
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 4
Title: Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca2+ signaling pathways in the male rat bone
An original research article published in the journal "Physiological reports".

<table>
<thead>
<tr>
<th>Nature of contribution by PhD candidate</th>
<th>RNA extraction, sequence analysis, statistical analyses, manuscript preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extent of contribution by PhD candidate (%)</td>
<td>90</td>
</tr>
</tbody>
</table>

CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elwyn C. Firth</td>
<td>supervised study, co-wrote manuscript</td>
</tr>
<tr>
<td>Peter Tsai</td>
<td>Guided data analysis</td>
</tr>
<tr>
<td>Mark H. Vickers</td>
<td>supervised study, co-wrote manuscript</td>
</tr>
<tr>
<td>Justin M. O'Sullivan</td>
<td>supervised study, co-wrote manuscript</td>
</tr>
</tbody>
</table>

Certification by Co-Authors

The undersigned hereby certify that:

✓ the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
✓ that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elwyn C. Firth</td>
<td></td>
<td>16/1/17</td>
</tr>
<tr>
<td>Peter Tsai</td>
<td></td>
<td>11/1/17</td>
</tr>
<tr>
<td>Mark H. Vickers</td>
<td></td>
<td>10/1/17</td>
</tr>
<tr>
<td>Justin M. O'Sullivan</td>
<td></td>
<td>16/1/17</td>
</tr>
</tbody>
</table>
Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

Please indicate the chapter/section/pages of this thesis that are extracted from a co-authored work and give the title and publication details or details of submission of the co-authored work.

Chapter 5
A memory of early life physical activity is retained in bone marrow of male rats fed a high-fat diet
An original research article published in ‘Frontiers in Physiology’

| Nature of contribution by PhD candidate | Data collection and analysis, interpretation of results, preparation of manuscript |
| Extent of contribution by PhD candidate (%) | 70 |

CO-AUTHORS

<table>
<thead>
<tr>
<th>Name</th>
<th>Nature of Contribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mark H. Vickers</td>
<td>10 Supervision, experiment model, manuscript</td>
</tr>
<tr>
<td>Elwyn C. Firth</td>
<td>10 Supervision, exp. design, exp. measures</td>
</tr>
<tr>
<td>Justin M. O'Sullivan</td>
<td>10 Supervision, Study design, Manuscript</td>
</tr>
</tbody>
</table>

Certification by Co-Authors

The undersigned hereby certify that:
- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

<table>
<thead>
<tr>
<th>Name</th>
<th>Signature</th>
<th>Date</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mark H. Vickers</td>
<td></td>
<td>24/8/17</td>
</tr>
<tr>
<td>Elwyn C. Firth</td>
<td></td>
<td>24/8/17</td>
</tr>
<tr>
<td>Justin M. O'Sullivan</td>
<td></td>
<td>23/8/17</td>
</tr>
</tbody>
</table>
Glossary

µCT - Micro-computed tomography
aBMD - Areal bone mineral density
ALP - Alkaline phosphatase
ALSPAC - Avon Longitudinal Study of Parents and Children
AN - Anorexia nervosa
ANOVA - Analysis of variance
AP-1 – Activator protein 1
ATP - Adenosine triphosphate
BAT - brown adipose tissue
BB/OK – BioBreeding/OttawaKarlsburg
BMC - Bone mineral content
BMD - bone mineral density
BMP - Bone morphogenetic protein
BPS - Bipedal stance
CATK - Cathepsin K
CCL5 - Chemokine ligand 5
CDO1 - Cysteine dioxygenase
CNS - Central nervous system
CON - Control
CRE - cAMP response element
CREB - cAMP response element binding protein
C-SED - Chow sedentary group
CSF-1 - Colony stimulating factor 1
CSS - Customary strain stimulus
CX43 - Connexin 43
DAG - Diacylglycerol
DEPC - Diethylpyrocarbonate
DETs - Differentially expressed transcripts
DLK1 - Delta-like non-canonical notch ligand 1
DSH - Disheveled
DXA - Dual energy x-ray absorptiometry
eNOS - Endothelial nitric oxide synthase
ERK - Extracellular signal regulated kinase
FAK – Focal adhesion kinase
FDR - False discovery rate
GEO - Gene expression omnibus
GMN - Global molecular network
GO - Gene ontology
GPCR – G protein coupled receptor
GSK-3β - Glycogen synthase kinase 3 beta
HF - High-fat
HF-EEX - High-fat early-exercise group
HF-LEX - high-fat late-exercise group
HF-SED - High-fat sedentary group
HPA - hypothalamic-pituitary-adrenal
HPLC - High performance liquid chromatography
IHH - Indian hedgehog
IKK - Inhibitor of nuclear factor kappa B
IL - Interleukin
IL1Ra - IL-1 receptor antagonist
ILK - Integrin-linked kinase
iNOS - Inducible nitric oxide synthase
IP3 - Inositol 1,4,5-trisphosphate
IPA - Ingenuity pathway analysis
IPKB - Ingenuity pathways knowledge base
JNK - c-Jun N-terminal kinase
Kcal - Kilocalories
LEF - Lymphoid enhancer factor
LRP5 - Low density lipoprotein 5
LTB - lymphotoxin beta
MAPK – Mitogen activated protein kinase
MAT - Marrow adipose tissue
MCP-1 - Monocyte chemoattractant protein 1
M-CSF - Macrophage colony stimulating factor
MSC - Mesenchymal stem cells
NGS - Next generation sequencing
nNOS - Neural nitric oxide synthase
NO - Nitric oxide
NOS - Nitric oxide synthase
Ocn - Osteocalcin
OPG - Osteoprotegerin
OSE - Osteoblast-specific cis-acting element
Osx - Osterix
PAI-1 - Plasminogen activator inhibitor-1
PEPCK-C - Phosphoenolpyruvate carboxykinase
PGE2 - Prostaglandin E2
PGI2 - Prostaglandin I2
PKA - Protein kinase A
PLC – Phospholipase C
pQCT - Peripheral quantitative computed tomography
PTH - Parathyroid hormone
PTHrP - Parathyroid hormone related peptide
RANK - Receptor activator of nuclear factor kappa B
RANKL - Receptor activator of nuclear factor Kappa B ligand
Ras – Rat sarcomeric monomeric protein
RCTs - Randomised controlled trials
RGD - Rat genome database
RIN - RNA integrity number
Rt - Retention time
RT - Room temperature
RUNX2 - Runt-related transcription factor 2
RXR - Retinoid-X receptor
SD - Sprague-Dawley
SNS - Sympathetic nervous system
TCF - T-cell factor
TGF-β - Transforming growth factor beta
TMD - Tissue mineral density
TNF-α - Tumour necrosis factor alpha
TRAF – Tumour necrosis factor receptor associated cytoplasmic factor
TRAP - Tartrate resistant acid phosphatase
VOI - Volume of interest
WAT - White adipose tissue
WEX - Wheel exercise
Contents

Outputs arising from this thesis ................................................................. ii

Abstract ...................................................................................................... iii

Acknowledgements .................................................................................... v

Co-authorship forms .................................................................................. vi

Glossary ....................................................................................................... vii

List of figures .............................................................................................. xvii

List of tables ............................................................................................... xviii

Chapter 1. Introduction ................................................................................. 1

1.1 Overview ............................................................................................... 1

1.2 Microstructure, growth and development of the long bone .................. 2

1.2.1 Osteoblasts ..................................................................................... 3

1.2.2 Osteoclasts ..................................................................................... 5

1.2.3 Osteocytes ....................................................................................... 7

1.2.4 Bone lining cells ............................................................................. 8

1.2.5 Structure of the long bone ............................................................... 8

1.2.6 Growth and development of the long bone ...................................... 9

1.2.7 Bone modelling .............................................................................. 9

1.2.8 Bone remodelling ......................................................................... 10

1.3 The mechanostat ................................................................................ 11

1.3.1 Cellular mechanotransduction ....................................................... 13

1.4 Physical Activity and the growing bone .............................................. 17

1.4.1 Physical activity during the prepubertal period – Evidence from studies in humans 17

1.4.2 Animal Studies ............................................................................. 18

1.5 Bone and fat ........................................................................................ 20

1.5.1 The hormonal link between bone and fat ....................................... 20

1.5.2 Inflammation ................................................................................ 22
1.5.3 Bone marrow adipose tissue ................................................................. 23
1.6 Effect of excess adiposity on bone development ........................................ 27
  1.6.1 Evidence from clinical studies ........................................................... 27
  1.6.2 Evidence from animal studies ........................................................... 28
1.7 Physical activity as an intervention strategy to mitigate adverse outcomes of a high-fat diet ................................................................. 29
1.8 Relevance of the laboratory rat as a model to study human skeletal biology. .... 31
Aims and Hypotheses ......................................................................................... 32
Chapter 2. Methods ......................................................................................... 33
  2.1 Overview of animal experimental design ................................................. 33
    2.1.1 Experimental design - Study 1 ......................................................... 33
    2.1.2 Experimental design – Study 2 ....................................................... 35
  2.2 Determination of body composition ......................................................... 36
  2.3 Euthanasia ............................................................................................... 36
  2.4 Tissue collection .................................................................................... 36
    2.4.1 Plasma ............................................................................................. 36
    2.4.2 Fat pads ........................................................................................... 36
    2.4.3 Bone and marrow .......................................................................... 37
    2.4.4 Faecal sample collection .................................................................. 37
  2.5 Biochemical analyses .............................................................................. 38
    2.5.1 Insulin and leptin ............................................................................. 38
    2.5.2 Fasting glucose ............................................................................... 38
    2.5.3 Corticosterone and testosterone ...................................................... 38
  2.6 Faecal corticosterone measurement using mass spectrometry ................. 38
  2.7 Micro-computed tomography (µCT) ......................................................... 39
  2.8 Peripheral quantitative computed tomography (pQCT) ............................ 39
  2.9 Determination of adipocyte area in the bone marrow ............................. 40
  2.10 RNA extraction .................................................................................... 41
    2.10.1 Tissue homogenisation and phase separation ............................... 41
2.10.2 RNA isolation .............................................................................................................41
2.10.3 DNase treatment........................................................................................................41
2.10.4 Washing and elution.................................................................................................42
2.11 RNA quality evaluation ............................................................................................42
2.12 RNA quantification ....................................................................................................42
2.12.1 Nanodrop ................................................................................................................42
2.12.2 Qubit® fluorometric quantitation ..........................................................................42
2.13 RNA-seq ....................................................................................................................43
2.14 Read quality control ..................................................................................................43
2.15 Read trimming ............................................................................................................44
2.16 Read alignment using TopHat ...................................................................................44
2.17 Differential gene expression analysis using Cuffdiff ..................................................45
2.18 Gene-annotation enrichment analysis ........................................................................45
2.19 Pathway analysis ........................................................................................................46
2.19.1 Canonical pathways ...............................................................................................47
2.19.2 Regulatory effects ..................................................................................................47
2.19.3 Downstream effects ...............................................................................................47
2.20 Statistical analyses ....................................................................................................48

Chapter 3. Different Short-Term Mild Exercise Modalities Lead to Differential Effects on
Body Composition and Gene Expression in Healthy Prepubertal Male Rats ......................49
3.1 Introduction ..................................................................................................................49
3.2 Materials and methods ...............................................................................................50
3.2.1 Study design ..............................................................................................................50
3.2.2 Whole body composition analyses ........................................................................51
3.2.3 Tissue collection ......................................................................................................51
3.2.4 Plasma and faecal analysis ......................................................................................52
3.2.5 Microcomputed tomography (µCT) .........................................................................52
3.2.6 Statistical analyses ..................................................................................................52
5.2.5 Adipocyte area in the bone marrow ........................................ 81
5.2.6 Peripheral quantitative computed tomography (pQCT) .............. 82
5.2.7 RNA extraction ........................................................................ 82
5.2.8 RNA-seq .................................................................................. 84
5.2.9 Sequence read processing, alignment and differential gene expression analysis .................................................. 84
5.2.10 Pathway analysis ...................................................................... 84
5.2.11 Statistical analyses ................................................................. 84
5.3 Results ......................................................................................... 85
5.3.1 Food intake ............................................................................... 85
5.3.2 Wheel activity ........................................................................... 87
5.3.3 Effect of HF diet on body composition ..................................... 88
5.3.4 Effect of physical activity in a HF diet environment ................ 89
5.3.5 Effect of physical activity cessation on body composition ....... 89
5.3.6 Bone marrow adipocyte area ................................................... 91
5.3.7 Imaging parameters ................................................................. 92
5.3.8 The bone marrow gene expression of HF-SED group resembled a pro-adipogenic and pro-inflammatory profile ............................................................... 94
5.3.9 Voluntary physical activity and lower caloric intake promoted an anti-inflammatory environment in the marrow ................................................................. 99
5.3.10 Bone marrow gene expression retained a memory of early-exercise .......... 102
5.4 Discussion .................................................................................. 105

Chapter 6. General discussion ................................................................. 111
6.1 Principal findings ......................................................................... 111
6.2 Discussion .................................................................................. 112
6.3 Strengths .................................................................................... 117
6.4 Limitations and future directions ................................................... 119
6.5 Concluding comments ............................................................... 121
Appendix I: Genes that were showed two-fold or greater differential expression in (A) HF-SED group compared to the C-SED group and (B) HF-LEX group compared to HF-SED group ........................................................................................................................................123

Appendix II ........................................................................................................................................................125

Appendix III ........................................................................................................................................................126

References ........................................................................................................................................................127
List of figures

Figure 1.1: A schematic diagram of osteoclastogenesis and bone resorption ........................6
Figure 1.2: The trajectory of bone gain and loss in humans ........................................17
Figure 1.3: Mesenchymal stem cell lineage allocation in the bone marrow ....................26
Figure 2.1: Femoral diaphyseal bone used for RNA extraction ....................................37
Figure 2.2: A representative image of summary result from FastQC analysis ..................44
Figure 2.3: Overview of a typical gene-enrichment software ......................................46
Figure 3.1: Body weights of prepubertal rats in control, BPS, and WEX exercise groups. ..53
Figure 3.2: Caloric intake of rats during the prepubertal exercise period .......................53
Figure 3.3: Comparison of wheel exercise (WEX) activity during the light and dark periods. ...........................................................................................................54
Figure 3.4: Comparison of body composition at D36 between the exercise groups ..........55
Figure 3.5: Epididymal (A) and retroperitoneal (B) fat weights of prepubertally exercised rats ..................................................................................................................55
Figure 3.6: Closed porosity (%) in bones from prepubertally exercised rats ...................58
Figure 4.1: Venn diagram showing the distribution of genes that responded to BPS and WEX exercises ...........................................................65
Figure 4.2: Molecular complexes involved in the integrin-linked kinase pathway responded to BPS and WEX treatments ............................................................73
Figure 4.3: Molecular complexes involved in the calcium signalling pathways responded to BPS and WEX treatments ............................................................75
Figure 5.1: Food consumption during the experimental period .......................................85
Figure 5.2: Body weights of the experimental groups .....................................................86
Figure 5.3: Wheel activity of HF-EEX and HF-LEX groups ........................................87
Figure 5.4: Total fat and lean mass percentage is affected by current but not previous physical activity .........................................................................................90
Figure 5.5: Mean marrow adipocyte area of the experimental groups at D120 .............91
Figure 5.6: A high-fat diet in early life upregulated genes that promote inflammation in the bone marrow ..............................................................98
Figure 5.7: Physical activity in high-fat fed rats downregulated genes involved in inflammation .................................................................100
Figure 5.8: Bone marrow gene expression retained a memory of physical activity following 60 days of physical activity cessation .................................................103
List of tables

Table 1.1: Differences between white adipose tissue and marrow adipose tissue..................25
Table 1.2: White and marrow adipose tissues differ in their response to pathological
conditions and nutritional insults ..................................................................................25
Table 2.1: Litter size and selection of animals for study 1 ....................................................33
Table 2.2: Composition of the diets used in the studies.......................................................34
Table 2.3: Diets and exercise regimens of the intervention and control groups in study 2 ...35
Table 2.4: Dilutions of standards for faecal corticosterone measurement using mass
spectrometry ..................................................................................................................39
Table 2.5: Phred quality scores and the probability of errors associated with them..........43
Table 3.1: Comparison of bone morphometric properties as determined by μCT ................57
Table 4.1: Alignment summary of RNA-seq reads to the rat transcriptome .........................64
Table 4.2: GO::TermFinder results for (A) upregulated genes and (B) downregulated genes
in response to WEX .......................................................................................................67
Table 4.3: GO::TermFinder results for (A) upregulated genes and (B) downregulated genes
in response to BPS .........................................................................................................69
Table 4.4: Canonical pathways enriched for differentially expressed transcripts in BPS and
WEX rats .......................................................................................................................72
Table 5.1: RNA quality and alignment summary of RNA-seq reads to the rat transcriptome
........................................................................................................................................83
Table 5.2 Body composition in experimental groups at D60 and D120.................................88
Table 5.3: Femoral cortical and trabecular bone properties in the experimental groups at the
end of the late exercise period .......................................................................................92
Table 5.4: Biological functions, physiological processes and diseases that were over
represented by genes that were differentially regulated due to the high-fat diet ..............94
Table 5.5: Genes with functions in ‘quantity of adipose tissue’ and ‘morphology of bone’
were significantly enriched among the differentially expressed genes in HF-SED group
compared to the C-SED group .......................................................................................95
Table 5.6: Biological functions, physiological processes and diseases that were over
represented by genes that were differentially expressed in HF-LEX group compared to HF-
SED group ....................................................................................................................99
Table 5.7: Genes that were differentially expressed greater than or equal to two fold in HF-EEX group compared to HF-SED group .................................................................102
Table 5.8: Biological functions, physiological processes and diseases that were over-represented by genes that were differentially expressed in the HF-EEX group compared to the HF-SED group ........................................................................................................102
Chapter 1. Introduction

1.1 Overview
Childhood and adolescence signify a period of rapid musculoskeletal development. Extrinsic factors such as nutrition and physical activity during this crucial period have the ability to affect bone mass acquisition when young and subsequent bone loss in later life. Mechanical loading is an important influencer of bone mass. It modifies bone structure and content by stimulating signalling pathways that regulate bone formation and resorption. The ability of bone to respond to exercise decreases with age (1). Cross-sectional (2–4) and intervention (5–8) studies in humans have shown that childhood offers a window of opportunity when the osteogenic effects of physical activity could be maximised. Skeletal gains acquired during the rapid growth period may mitigate bone loss associated with old age (9–14). Although several research groups have investigated the effects of early life exercise on bone development in animal models, many such studies involve the use of forced exercise models that elicit a physiological stress response which is known to affect visceral adiposity, insulin sensitivity and bone turnover (15–17). Even though there is a wealth of literature on bone’s histomorphometric and physiologic responses to early life exercise, the response of bone’s transcriptome to such an intervention has not been documented. Exploring the transcriptional landscape of bone in response to early life voluntary exercise has the potential to identify molecular candidates and signalling pathways that are crucial for such a response.

Historically considered as functionally exclusive tissues, bone and fat are intricately linked in a complex interaction that affects both energy and bone metabolism. In the bone marrow, adipocytes and osteoblasts share a common progenitor. The adipose tissue, traditionally regarded as a passive storehouse of excess energy, secretes pro-inflammatory cytokines, adipokines and other bioactive molecules that have an active role in bone metabolism. In turn, bone influences energy metabolism through osteocalcin, an osteoblast specific peptide which regulates insulin production and sensitivity. The alarming rise in childhood obesity rates and the increasing incidence of bone metabolic diseases such as osteoporosis in older adults make it imperative to understand the relationship between fat and bone metabolism and the associated molecular mechanisms.

While considered to be beneficial to bone health in post-menopausal women and ageing adults, the effects of obesity on bone growth and development during early years are unclear. While human studies have reported contradictory results owing to the complexity inherently associated with such studies (18,19), evidence from animal studies using rodent models is rapidly accumulating and points to an adverse bone response in the presence of excess adiposity (20–25). There have been several studies that examined the physical and physiological changes in bone due to increased adiposity.
However, there is a gap in the literature with regards to the molecular mechanisms that underlie the observed changes. In particular, there is a lack of studies that investigate the global gene expression changes in bone in response to obesity, which can shed light on the transcriptional changes and signalling pathways that are involved in bone-fat interaction.

Of critical consideration is the fact that physical activity has an opposing effect on many of the mechanisms through which high-fat diets affect bone. It is associated with a reduced pro-inflammatory profile (26) while having an enhancing effect on the production of anti-inflammatory cytokines (27). Mechanical stimulation has been shown to act as an osteogenic stimulus for bone marrow mesenchymal stem cells (MSCs) (28). As such, physical activity has the potential to act as a non-pharmacologic strategy to mitigate the adverse effects of diet induced obesity on bone metabolism.

The aims of this thesis are two-fold. Firstly, it seeks to investigate whether voluntary mild-moderate physical activity that begins and ends before puberty has the ability to influence body composition, bone phenotype and gene expression in a young male rat model. Secondly, it aims to examine the ability of voluntary physical activity to modulate the changes in body composition, bone parameters and bone marrow gene expression induced by an early life high-fat diet, also in a male rat model. The current chapter provides an overview of basic skeletal biology and physiology, evidence regarding early life physical activity on growing bone, evidence regarding the effects of a high-fat diet on growing bone, molecular mechanisms that underlie the observed changes, and where appropriate, highlights the gaps in the research area.

1.2 Microstructure, growth and development of the long bone

The skeleton serves several functions in the body. It provides protection to internal organs. It is a site of attachment for muscles and helps in locomotion. As a calcium and phosphate repository, it has a major role in mineral homeostasis and is an integral part of the endocrine system. As a site of blood cell production, it has a role in the circulatory and immune systems (29). The skeleton consists of two main parts: (1) the axial skeleton and (2) the appendicular skeleton. Depending on their shape and structure, bones are categorised into four types. The clavicles, humeri, radii, ulnae, metacarpals, femurs, tibiae, fibulae, metatarsals and phalanges are the long bones in the body. Short bones are the carpals, tarsals, patellae and sesamoid bones. The skull, mandible, scapulae, sternum and ribs are flat bones while the vertebrae, sacrum, coccyx and hyoid are irregular bones. Bone grows in size and changes shape through the process of modelling which is predominant during growth. Bone remodelling replaces old bone with new mechanically stronger bone. This helps repair microdamage and preserve bone strength. The three main types of bone cells: the osteoblasts, osteocytes and the osteoclasts work in conjunction to adapt bone to the body’s physical and physiological needs.
1.2.1 Osteoblasts

Osteoblasts are the cells responsible for bone formation or osteogenesis. Osteoblasts originate from multipotent mesenchymal precursors that are also capable of differentiating into myoblasts, adipocytes, chondrocytes and tendon cells (30). Runt-related transcription factor 2 [RUNX2 (also known as core-binding factor 1 (CBFA1), osteoblast-specific factor 2 (OSF2) and acute myeloid leukemia 3 (AML3)]) was the first identified osteoblast-specific transcription factor. It is a master regulator of osteoblast differentiation and maintenance of the mature osteoblast phenotype. It belongs to a family of transcription factors characterised by the presence of the DNA-binding runt domain. RUNX2 binds to osteoblast-specific cis-acting elements (OSEs) in the promoter regions of osteoblast precursors to initiate osteoblast differentiation. The importance of RUNX2 in skeletal development is demonstrated by the fact that null mutant mice for Runx2 die at birth due to a complete inability to form osteoblasts (31). RUNX2 is also important for the maintenance of the mature osteoblast phenotype. It is essential to allow replenishment of bone following bone loss. Osterix (OSX) is another transcription factor that is indispensable for osteoblast differentiation as Osx null mice have a completely cartilaginous skeleton and die at birth. The normal expression of Runx2 in the Osx−/− animals indicate that Osx acts downstream from Runx2 (32).

Discoveries that gain-of-function mutations in low density lipoprotein receptor-related protein 5 (Lrp5) were associated with a high bone mass phenotype while loss-of-function mutations led to osteoporosis-pseudoglioma syndrome led to the identification of the importance of the WNT signalling pathway in bone biology (33–35). Historically, WNT signalling can occur through three pathways: (1) the canonical WNT pathway, also known as WNT/β-catenin pathway (2) the noncanonical WNT-planar cell polarity (PCP) pathway and (3) WNT-calcium (WNT-Ca2+) pathway (36–38). WNT/β-catenin has emerged as a crucial pathway affecting several aspects of bone metabolism. The pathway is activated when a WNT ligand binds to a frizzled (FZD) receptor and LRP5/6 co-receptors. This leads to inhibition of glycogen synthase kinase-3β (GSK-3β) which usually phosphorylates β-catenin and targets it for degradation. Inhibition of GSK-3β leads to the stabilisation of β-catenin which then translocates into the nucleus. Interaction of β-catenin with T-cell factor/lymphoid enhancer factor (TCF/LEF) transcription factors leads to the activation of WNT responsive genes. WNT/β-catenin signalling is vital for the entire osteoblastic lineage. It represses the commitment of MSCs into adipogenic and chondrogenic lineages while promoting their entry into osteoblastic lineage (39–41). Canonical WNT signalling is also essential for osteoblast precursor proliferation and differentiation. WNT signalling between osteocytes (the terminally differentiated form of osteoblasts) and osteoblasts indirectly regulates osteoclast differentiation (42,43).

Mature osteoblasts are cuboidal mononuclear cells that contain bundles of actin, myosin and other cytoskeletal proteins involved in the maintenance of cell shape, attachment and mobility. They are
responsible for the synthesis and deposition of the organic component of the bone extracellular matrix. Osteoblasts synthesise and secrete precursors of type I collagen which constitutes 90-95% of the organic component or osteoid (44). Osteoid undergoes mineralisation through the deposition of hydroxyapatite crystals $[\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2]$ which form the inorganic component of the bone. While some osteoblasts get embedded in the mineralised matrix and undergo terminal differentiation into osteocytes, others undergo apoptosis or become bone-lining cells.

Osteoblasts also secrete non-collagenous structural proteins which can be broadly categorised into: (1) proteoglycans; (2) glycosylated proteins; (3) glycosylated proteins with cell attachment activities and (4) gamma-carboxyglutamic acid (Gla) containing proteins (45). Although the functions of many of these proteins have yet to be elucidated, they have multi-functional roles in matrix organisation, cell-matrix and mineral-matrix interactions and regulation of the mineralisation process (45). The synthesis of alkaline phosphatase (ALP) is a prominent feature of bone formation. It is produced by osteoblasts and is found either bound to the osteoblast cell surface or free within the bone matrix. It is essential for matrix mineralisation and also plays a role in the proliferation, migration and differentiation of osteoblasts (46,47). Serum alkaline phosphatase concentration is the most frequently used marker of bone metabolism. Osteocalcin, also known as bone gla protein (BGP), is one of the few osteoblast-specific proteins. It undergoes post-translational carboxylation on its glutamic acid residues in a vitamin K-dependent manner (48). In its γ-carboxylated form, osteocalcin regulates hydroxyapatite crystal size, binds to calcium and hydroxyapatite and is essential for bone mineralisation. Circulating osteocalcin is a classic marker for the degree of bone turnover. In recent years it has emerged as a key link between bone and whole body energy metabolism (49). Un- and undercarboxylated osteocalcin released into the blood affects insulin production from pancreatic β-cells, insulin sensitivity and production of adipokines by the adipose tissue (49). The endocrine role of osteocalcin in energy metabolism is discussed in detail later in this review.

One of the crucial functions of osteoblasts is to synthesise and secrete molecules that control osteoclast differentiation (50). They secrete the osteoclast differentiation factor, receptor activator of nuclear factor kappa B ligand (RANKL) [also known as TNF-related activation-induced cytokine (TRANCE)] which binds to receptor activator of nuclear factor kappa B (RANK) on the surface of haematopoietic precursor cells and induces osteoclastogenesis (51). Osteoblasts also produce osteoprotegerin (OPG), a soluble decoy ligand which binds to RANKL and prevents it from binding to RANK thereby inhibiting osteoclastogenesis. Expression of RANKL and OPG is a tightly controlled process through which bone resorption and thereby bone density is regulated. For this reason osteoblasts are the target for several hormones, cytokines, growth factors and local signalling molecules which regulate bone metabolic homeostasis. Under pro-resorptive conditions, for example, when triggered by parathyroid hormone (PTH), parathyroid hormone related peptides (PTHrP), 1, 25-dihydroxyvitamin D$_3$ and pro-resorptive
cytokines such as tumour necrosis factor (TNF-α), osteoblasts express RANKL which binds to its receptor RANK on osteoclast precursors and triggers osteoclast differentiation. Bone anabolic agents like oestrogens and bone morphogenetic proteins (BMPs) stimulate the production of OPG by osteoblasts. OPG binds to RANK and blocks RANKL-RANK interaction thereby by reducing the number of mature osteoclasts. This leads to an increase in bone mass.

1.2.2 Osteoclasts
Osteoclasts are bone-specific multinucleated macrophages of monocyte/macrophage haematopoietic lineage that reside on or near the bone surface (52). They are responsible for bone removal or resorption. Many adult skeletal diseases such as osteoporosis, periodontal disease and rheumatoid arthritis are the result of imbalanced bone resorption caused by excessive osteoclast activity. Two haematopoietic factors, RANKL and colony stimulating factor 1 (CSF-1, also known as macrophage colony stimulating factor, M-CSF) are both necessary and sufficient for osteoclastogenesis. Under normal physiological conditions, differentiation of osteoclasts is predominantly regulated by osteoblasts. In pathologic states that result in excess bone resorption such as sex steroid deficiency (e.g., menopause), rheumatoid arthritis and other acute and chronic inflammatory states, T and B lymphocytes also express RANKL and contribute to increased osteoclast numbers. Moreover, TNF-α has been shown to promote the proliferation of osteoclast precursors (53). Osteoclast precursors themselves secrete cytokines such as TNF-α, interleukin 1 (IL-1) and interleukin 6 (IL-6) whose production is increased in response to TNF-α. In this way, TNF-α has an auto-amplifying effect on osteoclastogenesis at sites of inflammation in and around bones.

The preliminary step in RANK/RANKL signalling is the binding of tumour necrosis factor receptor (TNFR)-associated cytoplasmic factors (TRAFs) to the cytoplasmic domain of RANK in osteoclast precursors. Subsequently, TRAFs act as adaptor proteins and facilitate the assembly of signalling proteins that direct osteoclast-specific gene expression that leads to their differentiation and activation. Five distinct signalling cascades have been identified that are induced in response to RANKL/RANK signalling: (1) inhibitor of nuclear factor kappa B (NF-κB) (IKK); (2) c-Jun N-terminal kinase (JNK); (3) p38; (4) extracellular signal regulated kinase (ERK) and (5) Src pathways (52). The RANK/RANKL pathway is subject to several levels of control to regulate bone resorption. Osteoclasts express interleukin 1 receptor (IL-1R), colony-stimulating factor-1 receptor (c-Fms), TNF-α, prostaglandin E2 (PGE2) and transforming growth factor beta (TGF-β) surface receptors that have been shown to stimulate bone resorption in vivo (52). IL-1R and tumour necrosis factor receptor 1 (TNFR1) signal through TRAF-6 and they act synergistically to promote RANK signalling (54). c-Fms and TGF-β upregulate the levels of RANK in osteoclast cell surface thereby increasing the potency of RANKL (55,56).
Figure 1.1: A schematic diagram of osteoclastogenesis and bone resorption

Osteoblasts and stromal cells produce RANKL and M-CSF and induce osteoclast differentiation. OPG is produced by osteoblasts and acts as a decoy receptor, inhibiting the commitment to osteoclast lineage. The mature osteoclast forms a tight junction with the bone surface. H\(^+\) ions are pumped into the sealed compartment along with lytic enzymes such as CATK. While the H\(^+\) ions dissolve the bone mineral, the enzymes degrade the organic matrix. RANKL: Receptor activator of NFκB ligand; M-CSF: Macrophage colony stimulating factor; OPG: osteoprotegerin; CATK: Cathepsin K

Activation of RANK on the surface of osteoclast precursors by RANKL triggers the activation of downstream signalling pathways that control various functions of osteoclasts. They induce the expression of genes that are characteristic of osteoclast lineage including tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor and β\(_3\)-integrin (52). Bone resorption is a multistep process that begins with the proliferation of osteoclast precursors, their commitment to the osteoclast lineage, and lastly, the degradation of bone. When a mature osteoclast is activated by resorptive signals, its cell body becomes polarised and it undergoes rearrangement of its actin cytoskeleton. A tight junction is formed between the bone surface and the basal membrane of the osteoclast creating a sealed compartment. Hydrogen (H\(^+\)) ions produced by the osteoclasts are pumped into this external vacuole by the H\(^+\) transporting vacuolar proton pump member I (ATP6i) complex. In addition, lytic enzymes such as TRAP, CATK and collagenases are secreted into the sealed vacuole. H\(^+\) ions acidify the compartment and dissolve the mineral while the enzymes breakdown the organic matrix. The breakdown products - collagen, calcium and phosphate are processed by the osteoclasts before being released into circulation. In addition to their central role in bone resorption, osteoclasts have been identified to play a role in the differentiation of osteoblasts, movement of haematopoietic...
stem cells from the bone marrow into the blood stream, immune responses and inflammatory processes affecting bone (53).

1.2.3 Osteocytes

Osteocytes are terminally differentiated osteoblasts that get embedded in the mineralised bone matrix. They are the most abundant cells in mature bone and reside in spaces called lacunae that are distributed throughout the matrix. Each osteocyte is connected to its neighbouring cells through dendritic processes that reside in narrow channels filled with extracellular fluid called canaliculi. The lacunocanalicular system facilitates the diffusion of nutrients, gases and waste products between the osteocytes and the blood vessels (57). The extended osteocytic network is made up of cells connected through multiple cell processes and gap junctions, their abundance and wide distribution in the bone matrix allows the osteocytes to sense changes in the local environment induced by mechanical signals (58). As such, osteocytes function as the mechanosensors of bone and have an active role in resorbing, forming and maintaining the bony matrix in response to changes in the mechanical environment. Mechanical signals produced by exercise or external loading are transmitted to the osteocytic cytoskeleton either directly through the solid matrix or through the load-induced fluid pressure and shear forces in the lacunocanalicular system which is ideal for signal transfer through mechanical, electrical and chemical mechanisms. Integrins, G-proteins, cytoskeleton, ion channels and cilia of the osteocytes all play a role in sensing and transduction of the mechanical signals into biochemical signals. The exact identity of the downstream signal transduction pathways leading to release of second messengers, transcription factors, and finally gene expression are yet to be elucidated and is an area of active research.

Osteocytes also play an important role in the adaptation of bone to mechanical disuse. When bone experiences lower than normal mechanical strain, for example during immobilisation, bed rest and microgravity, osteocytes undergo apoptosis triggering osteoclastogenesis and eventual bone resorption (59). The mechanism through which disuse brings about the death of osteocytes is not well understood. Knothe Tate et al. (1998) found that diffusion alone was not enough to transport large molecules such as proteins to osteocytes (60). Convective transport in the form of load-induced fluid flow was necessary for large molecule transportation. Thus, disuse may result in nutrient deficiency and inadequate waste removal from the osteocytic environment.

Osteocytes regulate bone formation through the production of sclerostin (SOST), a product of the Sost gene. SOST is a cysteine knot-secreted glycoprotein that has recently been identified as a potent negative regulator of bone formation. Mutations in Sost gene or its regulatory elements lead to sclerosing bone diseases such as sclerosteosis and Van Buchem disease characterised by a very high bone mass phenotype (61). SOST is structurally related to the DAN/Cerberus family of BMP
antagonists. In keeping with this structural similarity, SOST was shown to bind to BMPs and inhibit the proliferation and differentiation of osteoblasts. More recently, it has been shown to bind to LRP5/6 with high affinity and inhibits canonical WNT signalling (62,63). Robling et al. (2008) found that mechanical loading dramatically reduced Sost transcripts and consequently enhanced WNT/β-catenin signalling and bone formation (64). In contrast, bone disuse, induced through hindlimb unloading resulted in a significant upregulation of Sost gene expression. Sost-deficient mice subjected to hindlimb unloading resisted bone loss suggesting that sclerostin may have a role in osteocyte apoptosis and bone resorption (65).

1.2.4 Bone lining cells
Bone-lining cells are quiescent osteoblasts that form the endosteum on trabecular and endosteal surfaces and underlie the periosteum. They are found close to osteoblasts and are connected to them through adherens junctions – protein complexes that link neighbouring cells’ cytoskeleton. They are thought to be involved in ion transport of bone extracellular fluid. They can redifferentiate into osteoblasts when stimulated by PTH or mechanical forces (66).

1.2.5 Structure of the long bone
The long bone consists of a central hollow shaft called the diaphysis which is contiguous with a flared cone-shaped metaphysis. The growth plates sit above the metaphyses and are topped by rounded epiphyses. The skeleton is composed of two types of bone tissue. The cortical bone is compact, dense and forms the outer layer of the bone. It is covered on the outside by the periosteum, a fibrous connective tissue containing blood vessels, nerve fibres, osteoblasts and osteoclasts. On the inside, it is lined by the membranous endosteum. The diaphysis is predominantly made up of cortical bone surrounding a central medullary canal. The trabecular bone is the primary component of the metaphysis and epiphysis surrounded by a thin layer of cortical bone. The bony and latticed trabeculae are covered in endosteal tissue as they are adjacent to the marrow cavities. The differences in the morphology of the various anatomic regions of the bone signify their adaptation to the different types of mechanical forces acting on them. Capozza et al. (2010) found that while the distal ends of tibiae in humans which contain trabecular bone were more adapted to compression forces, the diaphyseal region was more resistant to bending and torsion (67).

The osteon is the primary microscopic unit of both cortical and trabecular bone. In the cortical bone, the osteons are organised into Haversian systems that are cylindrical in shape and arranged in concentric lamellae of a mineralised matrix of collagen fibres that surround a central haversian canal. Adult cortical bone also consists of circumferential lamellae composed of multiple layers of lamellae all or part of periosteal and endosteal surfaces. Interstitial lamellae are incomplete fragments of old lamellae that are found between osteons. The alternating arrangement of the collagen fibrils confers
significant strength to the lamellar bone. The Haversian systems are interconnected to each other and to the marrow cavity through Volkmann canals. While Haversian canals run almost parallel to the bone axis, Volkmann’s canals run perpendicular to the bone axis and connect the inner endosteal surface to the outer periosteal surface. This three dimensional arrangement of canals facilitates exchange of nutrients, nerve signals and metabolites between bone and its surrounding environment. Dispersed throughout the osteons are lacunae which house osteocytes. The trabecular bone osteons are called packets which are semilunar in shape and composed of concentric lamellae. The trabeculae usually do not contain blood vessels and the osteocytes in the trabecular bone rely on canalicular diffusion for nutrient exchange.

1.2.6 Growth and development of the long bone
Long bones develop through the process of endochondral ossification. It begins with the differentiation of mesenchymal cells into chondrocytes and the subsequent formation of a cartilaginous template of the bone. This template is surrounded by a fibrous connective tissue called the perichondrium. The perichondrium, upon vascularisation, becomes the periosteum containing osteoprogenitor cells. Chondrocytes in the primary ossification centre, located in the centre of the bone shaft or diaphysis, undergo rapid proliferation. The chondrocytes then mature and undergo hypertrophy. Mature chondrocytes undergo hypertrophy and secrete a specialised matrix composed of type X collagen and later, ALP, an essential enzyme for mineralisation (68). The chondrocytes then undergo apoptosis triggering the invasion of blood vessels carrying haematopoietic cells and osteoprogenitors. The endochondral ossification begins in the centre of the bone and spreads outward in both directions until all the cartilage is replaced by bone. The physes located at either end of the long bones are responsible for postnatal increase in bone length. Chondrocytes in the growth plate proliferate and mature which is followed by blood vessel invasion and ossification. This process occurs throughout childhood with a prominent spike during adolescence after which cartilage growth slows and ultimately ceases. The physis then undergoes complete ossification and only a thin physeal line remains in its place.

1.2.7 Bone modelling
Bone modelling is the primary process through which bone changes its size and shape and adapts to the physiological and mechanical cues acting on it. During modelling, osteoblasts and osteoclasts work independently and new bone is added on some surfaces while it is removed from others (69). Thus, as modelling can either refer to bone formation or resorption, Harold Frost coined the terms formation modelling and resorption modelling to distinguish between the two processes. ‘Formation modelling’ occurs when new bone is added along some surfaces and ‘resorption modelling’ refers to the removal of bone from others. The osteoblasts in the periosteum add new circumferential lamellae to the cortical bone. The osteoclasts remove bone from the endocortical, intra cortical and trabecular surfaces.
excavating the marrow cavity and establishing the cortical and trabecular architecture. The cone-like ends of the long bones are a result of resorption on the periosteal surface during growth. Thus modelling is involved in the establishment of normal bone architecture that is predetermined according to a genetic blue print (70). It is most prominent during growth when bone increases and alters its shape and size rapidly to withstand the forces of rapidly growing muscle mass. These processes result in expansion of the long bone diameter while the overall cortical thickness stays relatively consistent. As the cortex is now positioned away from the neutral axes, the resistance of bone to bending forces increases. Greater cortical bone size in boys is attributed to the enhancing effect of androgens on periosteal bone apposition. In girls, oestrogens limit periosteal bone formation while they do not influence bone size in boys (71–73). This results in increased bone strength in boys. Studies by Bass et al. (1999) (74) found that the femoral cortical wall thickness in girls increases due to endosteal apposition. This process is not seen in boys (75).

1.2.8 Bone remodelling

Bone remodelling is a tightly coupled process where osteoblasts and osteoclasts work in conjunction to renew old bone to maintain bone strength, repair microdamaged bone and maintain mineral homeostasis. Remodelling occurs throughout an organism’s lifespan (76) and is responsible for the replacement of immature woven bone with mature, biomechanically and metabolically competent lamellar bone, conversion of primary spongiosa woven bone into secondary spongiosa lamellar bone and repair of damaged bone. Due to its greater surface to volume ratio, trabecular bone is remodelled more actively than cortical bone. Bone resorption and formation are tightly controlled during bone remodelling. The osteoblasts and the osteoclasts, along with other assistant cells and regulating factors form temporary anatomical structures called basic multicellular units (BMUs). Remodelling can be targeted when it is activated by fatigue microdamage or non-targeted to maintain calcium homeostasis (77). Remodelling occurs in five sequential phases: (1) activation, (2) resorption, (3) reversal, (4) formation and (5) quiescence.

During the activation phase, osteoclast precursors are recruited to the site of remodelling where they fuse to form multinucleated preosteoclasts (76). The mechanosensing osteocytes detect damaged or unfit matrix and produce an activation signal to initiate the bone remodelling process. In response to the activation signal, capillaries and bone lining cells invade the remodelling site and break down the osteoid layer to expose the mineralised surface - the attachment sites for osteoclasts. Detached bone lining cells are thought to migrate into the adjacent marrow where they communicate with osteoclast precursors through direct cell-cell interactions. This leads to osteoclastogenesis. Alternatively, osteocyte apoptosis has also been known to induce osteoclast differentiation. Mature osteoclasts then migrate to the resorption site where they attach to the mineralised bone surface. In the resorption phase,
the attached osteoclasts form tight sealing zones around bone-resorbing compartments. These resorbing compartments are termed as Howship’s lacunae in trabecular bone while they are referred to as cutting cones in cortical bone. Reversal phase is the interval between resorption by osteoclasts and initiation of bone formation. During this phase, the osteoclasts undergo apoptosis. Mononuclear cells closely related to osteoclasts are thought to complete the final stages of resorption and clear the remnants of demineralised matrix. Preosteoblasts are then recruited to the resorption site to initiate bone formation (formation phase). Mature osteoblasts lay down type I collagen fibres which are later cross-linked. The gap regions between the fibrils are filled with mineral crystals. The osteocytes regulate the influx of mineral ions that form the hydroxyapatite crystals. The mineralisation begins at the junction of unmineralised osteoid and mineralised bone and proceeds towards the upper lamellar layer. Towards the end of the formation phase, the majority of the osteoblasts migrate to a layer of un-mineralised matrix on the bone surface where they either become bone lining cells or undergo apoptosis. Some osteoblasts get embedded in the lacunae of the bone matrix and differentiate into osteocytes. When bone formation is complete, the bone surface enters the quiescent or resting phase where the bone surface is covered by bone lining cells and an endosteal layer.

1.3 The mechanostat

The skeleton regulates its mass and architecture to meet the structural and metabolic demands of the body. The structural strength of the skeleton is a product of its mechanical properties. Bone must adapt its morphology according to the mechanical demand imposed on it. The importance of mechanical loading in regulating bone mass and strength is demonstrated by the fact that disuse such as immobilisation and microgravity leads to atrophy of bone while increased loading in the form of sport and exercise causes bone mass gain (38). In 1892, Julius Wolff postulated “Wolff’s law” which states that “every change in form and function of the bone is accompanied by changes in its internal architecture and external conformation in accordance to mathematical laws” (78). The mechanostat hypothesis, put forward by Harold Frost, is one of the most recognised theories that offer an explanation of how load-bearing bones adapt to changes in their mechanical environment. According to the mechanostat hypothesis, the post-natal skeleton is governed by a homeostatic mechanism - termed the mechanostat, which senses changes in the mechanical usage and alters the mass and strength of the bone accordingly (79). The baseline condition and baseline activities for the skeletal architecture and related biological mechanisms are established at birth (79). When mechanical forces exceed the set threshold, bone is added. Below a certain threshold of mechanical use, it is resorbed. Thus bone has an inherent ‘mechanostat’ that regulates bone functional adaptation.

Although the individual components that make up the bone mechanostat are yet to be identified, it is thought to comprise of a stimulus, a sensory mechanism capable of perceiving the stimulus, and an
effector component that is able to restore homeostasis (58). Strain magnitude was first proposed as the mechanostat’s stimulus. Strain is defined as the relative change in the length of the bone that occurs when it is subjected to loading. Bone tightly regulates the magnitude of strain experienced by it. This is inferred from the fact that the peak strains experienced by bone are kept within a close range across several different species (58). Since the proposal of the mechanostat theory, a number of other strain-related features have been identified. These include strain rate, frequency of loading cycles, rest between cycles, bouts of loading and distribution of strains within the bone all of which play a role in the functional adaptation of the bone. All these features together make up the ‘customary strain stimulus’ (CSS) (80). CSS is sex and site specific and it is genetically, biochemically and pharmacologically modified. As the central cellular participants of bone formation and resorption, osteoblasts and the osteoclasts are the natural candidates involved in the effector mechanisms. In response to CSS, osteoblasts and osteoclasts can either carry out bone modelling or remodelling. Bone modelling, as mentioned previously, is responsible for changing the size and shape of the bone. Resorption modelling is a much more localised process that involves the coupled action of osteoblasts and osteoclasts. Under normal physiological conditions, the amount of bone formed is generally equivalent to the amount resorbed. However, as the complete cycle of bone resorption followed by formation takes several weeks to months, there exists a transient period where the greater porosity of bone due to resorption results in a temporary decrease in bone strength.

While osteocytes, osteoblasts and bone lining cells have all been implicated in fulfilling the sensory role of the mechanostat, osteocytes have recently emerged as the primary mechanosensory cells in bone (81). When bone is deformed by loading, the resulting pressure gradient between areas of compression and areas of tension induces fluid flow from areas of high pressure to areas of low pressure. This fluid flow causes the perturbation of osteocytes and their dendritic processes (82). When the strain stimulus exceeds the CSS leading to the perturbation of osteocytes, factors such as nitric oxide (NO), prostaglandins E2 (PGE2) and I2 (PGI2), adenosine triphosphate (ATP) and intracellular calcium ([Ca^{2+}]_i) are released which have been shown to elicit an anabolic response in bone through formation modelling (58). Studies show that such formation modelling occurs on existing trabeculae and on the periosteal surface of the long bones (58). When the strain stimulus acting on the bone is lower than the CSS, for example, due to immobilisation, bed rest or space flight, the osteocytes undergo apoptosis which triggers bone loss through resorption modelling. Aguirre et al. (2006) showed that in mice, osteocytic apoptosis occurred within three days of tail suspension followed by osteoclastogenesis and bone resorption. Bone was lost from trabecular and endocortical surfaces of long bones (59).
1.3.1 Cellular mechanotransduction

Mechanosensitivity is not a property that is exclusive to bone cells. Almost all cell types in the body including myocytes, endothelial cells, platelets, chondrocytes and fibroblasts are able to sense changes in their biomechanical environment and respond accordingly (83). Although our understanding of the regulatory events that coordinate the mechanical responses of the inner ear (84), cardiovascular (85) and renal tissues (86) has advanced considerably, the mechanisms and signalling pathways that drive bone cells’ response to mechanical signals are still poorly understood. Cellular mechanotransduction is the process through which the biomechanical stimuli acting on a cell are converted to biochemical signals which eventually culminate in a cellular response (87). Mechanotransduction incorporates four distinct stages: (1) mechanocoupling during which the external mechanical signal is transduced into a local signal that is recognised by a sensor cell, (2) biochemical coupling during which the local mechanical signal is transformed into a biochemical signal. This step involves various cellular machinery and signalling pathways which ultimately lead to gene expression changes, (3) transmission of signal from sensor cell to the effector cell, and (4) effector cell response, during which the cells actually involved in bone formation and resorption (osteoblasts and osteoclasts respectively) initiate an appropriate response (88). The exact mechanism and players involved in the events leading to inducing intracellular signal transduction have not yet been elucidated in bone (89).

Mechanotransduction involves a series of discrete molecular events regulated by common, yet not clearly defined signalling networks (90,91). When bone tissue is subjected to mechanical loading, the tissue undergoes deformation generating local strains. The osteocytes embedded and dispersed throughout the bone tissue also undergo deformation due to the applied load. In addition, the pressure gradient created as a result of the bending forces induces extracellular fluid flow across the osteocytes (92,93). The resulting fluid shear stresses and electric potentials in osteocytes trigger the downstream steps in mechanotransduction. Mechanosensors, eg., the surface proteoglycan layer on the bone cells detect mechanical forces generated by the stretch and shear forces. The mechanical signal is then transmitted to the cell membrane or the cortical cytoskeleton (94).

Lipid rafts, focal adhesion complexes and adherens junctions act as mechanotransduction sites on the cell membrane (95). Integrins, G-proteins, the cytoskeleton and ion channels are involved in biochemical coupling (58,96). Integrins are transmembrane protein receptors and are activated by extracellular matrix elements such as osteopontin, collagen and fibronectin. They are heterodimeric and made up of α and β subunits. In human osteoblasts, fluid shear stress has been shown to induce α5β3 clustering and its increased association with Src homolog 2 domain containing protein (Shc), which is involved in the activation of rat sarcomeric monomeric protein (Ras), a small GTP-ase which is an upstream effector of ERK/JNK MAPK pathway (described below) (97). Ion channels, in particular, calcium channels are involved in load induced signal transduction. McDonald et al. (1996) found that
in a human osteoblast cell line, \([\text{Ca}^{2+}]_i\) rapidly increased upon flow stimulation (98). This increase was due to the influx of extracellular calcium caused by G-protein activation or through L-type voltage gated calcium channels (98,99). G proteins co-localise with integrin β1 and the cytoskeleton and modulate calcium channels (100). They may have multiple roles as they are also required for integrin mediated signalling which is independent of calcium and cytoskeleton mediated signalling (100).

The cytoskeleton has the ability to act as an independent mechanosensor (101). Pommerenke et al. (2002) showed that cyclic stress leads to an increase in cytoskeleton-bound phosphorylated proteins and translocation of focal adhesion kinase (FAK) to the cytoskeleton (102,103). A number of signalling molecules localise near the adhesion complexes (103). The microfilament component of the cytoskeleton is integral for signal transduction as cyclic forces enhance the anchorage of tyrosine-phosphorylated proteins and increased activation of FAK and mitogen activated protein kinase (MAPK). Nucleocytoplasmic DNA binding proteins can move between the adhesion complexes and the nucleus regulating mechanosensitive genes.

1.3.1.1 Signal transmission pathways in bone

Bone cells respond to mechanical stimuli through a number of methods including proliferation, matrix secretion, change in function and cytokine secretion (104). Multiple signalling pathways must be involved in order for mechanical forces to elicit such a diverse range of responses in bone cells. Mechanical forces have been shown to activate a number of signalling cascades. MAPK cascades, \(\text{Ca}^{2+}\) signalling, WNT/β-catenin pathway, integrin signalling, NO and prostaglandin pathways are some of the well characterised signalling pathways associated with mechanotransduction in bone and hence are described in detail below.

1.3.1.1.1 Mitogen activated protein kinase (MAPK) pathways

Mitogen activated protein kinases are a superfamily of serine/threonine kinases that are involved in a variety of physiological processes like growth, differentiation, stress transmission and apoptosis. There are three major groups of MAPK signal cascades: extracellular signal regulated kinase (ERK), c-Jun NH2-terminal kinase (JNK) and p38 cascade. ERK and JNK pathways are triggered by Ras activation and target transcription factors which ultimately result in expression modulation of members of Jun and Fos family of transcription factors. The Fos and Jun family of transcription factors heterodimerise to form activator protein (AP-1) which is vital for expression of genes involved in the early stages of osteoblast differentiation (105).

Several studies, both in vitro and in vivo have established the role of the MAPK cascade in bone biology and stress induced response in bone cells (106). In cultured osteoblast-like cells (ROS 17/2.8), mechanical strain and fluid movement increased the activation of ERK1/2 (107). In young Wistar rats,
altered mastication loads increased the expression and activation of the ERK/JNK pathways (108). In addition, increased loads on the rats’ temporomandibular joints induced the expression of Runx2 through the ERK/JNK axes.

1.3.1.1.2 Ca\(^{2+}\) Signalling

Ca\(^{2+}\) is a common second messenger in eukaryotic cells and regulates several basic functions including muscle contraction, neurotransmitter release, cell proliferation, mitosis, differentiation and cell motility (109–111). One of the earliest responses to both static and dynamic patterns of shear stress or strain in osteoblasts is the rapid influx of extracellular Ca\(^{2+}\) and mobilisation of [(Ca\(^{2+}\))]\(_i\) (112,113). Osteoblasts express several types of ion channels on their plasma membranes. Cell culture and in vivo studies indicate that the L-type voltage-sensitive Ca\(^{2+}\) channels are of the most functional significance in bone mechanobiology (114,115). In addition, G-protein coupled receptors (GPCRs) also contribute to [(Ca\(^{2+}\))]\(_i\) spikes (116). The resulting Ca\(^{2+}\) influx leads to the activation of phospholipase C (PLC). PLC, through hydrolysis, generates inositol 1,4,5-trisphosphate (IP\(_3\)) and diacylglycerol (DAG). These molecules act as second messengers and induce the release of [(Ca\(^{2+}\))]\(_i\) stores (116). Certain GPCRs also activate protein kinase A (PKA) which activates cAMP response element (CRE)-binding protein (CREB) through phosphorylation. CREB then binds to cyclooxygenase 2 (Cox-2) or FBJ murine osteosarcoma viral oncogene homolog B (FosB/ΔFosB) genes, which are well known to be mechanoresponsive in osteoblasts (117,118). In addition to the IP3 cascade, spikes in [(Ca\(^{2+}\))]\(_i\) trigger several mechanically regulated cascades including adenosine triphosphate (ATP) (119), NO (120), MAPK (121,122) and c-Fos (123). Thus Ca\(^{2+}\) occupies a central role in bone mechanotransduction.

1.3.1.1.3 WNT/β-catenin signalling

WNTs are a family of secreted glycoproteins that regulate several basic biological processes such as embryonic development, tissue morphogenesis, homeostasis, cell proliferation and apoptosis (124). WNT signalling can take place either through the canonical WNT/β-catenin pathway or through an alternative pathway that does not involve β-catenin. As mentioned in section 1.2.1 (page three), the canonical pathway has been increasingly shown to be crucial in skeletal physiology and thus has been the best studied and characterised. Please refer to section 1.2.1 for a description of the canonical WNT/β-catenin pathway.

In osteoblast cell lines and primary cultures, fluid shear stress promoted the phosphorylation of GSK-3β and induced β-catenin signalling (125). In vivo studies in tibiae of LRP5G171V-transgenic mice showed that loading increased the expression of Wnt10b, secreted Frizzled related protein 1(Sfrp1), cyclin D1 (Ccmd1), Fzd2, WNT1 Inducible Signalling Pathway Protein 2 (Wisp2) and connexin 43 (Cx43) supporting the role of canonical WNT pathway in mechanotransduction in osteoblasts (126). WNT/β-catenin pathway also indirectly affects osteoclast function. β-catenin, along with TCF1 and
TCF4 promotes the expression and secretion of OPG from osteoblastic/stromal cells. As mentioned previously, OPG binds to RANK and prevents RANKL mediated differentiation of osteoclasts (42).

1.3.1.4 Nitric oxide and prostaglandin signalling
Nitric oxide (NO) is a free radical that mediates several physiological processes including vascular relaxation, platelet aggregation, neurotransmission and immune regulation (127). It is produced by nitric oxide synthase (NOS) which exists in three distinct isoforms: the constitutively active neural (nNOS) and endothelial forms (eNOS) and an inducible isoform (iNOS). All three isoforms are expressed in bone cells. Aguirre et al. (2001) found that mice lacking eNOS exhibited reduced bone mass and suppressed osteogenic function thereby establishing a role for NO in normal bone metabolism (128). Osteoblasts show increased eNOS expression under physiological levels of mechanical stress (129). NO has a role in osteoclast function as well. While high NO concentrations promote osteoclast apoptosis, lower NO concentrations promote osteoclast formation and function (130).

Prostaglandin E2 is an anabolic agent that stimulates bone formation under various conditions including mechanical stimulation. Osteoblasts and osteocytes release PGE2 through CX43 hemichannels. Fluid shear stress has been shown to promote the translocation of CX43 to the cell membrane and the rapid opening of the channels which results in the release of intracellular PGE2. The downstream signalling cascade for PGE2 involves a cyclic adenosine monophosphate (cAMP)-dependent insulin like growth factor 1 (IGF-1), induction of Osx, and Tgf-1β (131,132).

1.3.1.5 Integrin signalling
Integrins have roles in mechanotransduction in a number of mechanosensitive cell types including fibroblasts, myoblasts, endothelial cells, bone cells and cartilage cells. They act as receptors for extracellular proteins that include collagen, laminin and fibronectin. The cytoplasmic tail of the β subunit interacts with intracellular signalling molecules and the actin cytoskeleton. A focal adhesion complex is formed in response to the extracellular signal. Focal adhesion kinase is recruited to the complex. Activation and autophosphorylation of FAK enables it to interact with Src and Fyn kinases. This triggers a downstream cascade of phosphorylation events which culminates in the activation ERK and JNK MAPKs. The complex also has been implicated in the activation of PLC-IP3 pathways and hence rapid release of [(Ca²⁺)ᵢ].
1.4 Physical Activity and the growing bone

At any given age, the difference between bone gained and lost is the key determinant of bone mass and risk of fracture. Childhood is characterised by rapid acquisition of bone mass in keeping with the rapid skeletal growth (Figure 1.2). Bone density continues to increase after the cessation of growth until maximum bone mass, termed peak bone mass (PBM), is achieved. Peak bone mass varies between sexes and also between skeletal sites in the body (134,135). The exact age at which PBM is achieved is unclear but is thought to occur around the end of the second decade of life (133). The years following the attainment of PBM are characterised by bone loss, which is further exacerbated by the onset of menopause in women. As bone mass and risk of fracture at any age is determined by the difference between bone gained and bone lost, maximising bone mass gain during the childhood years is a potential strategy to reduce fracture risk in children and potentially later in life (136,137). Indeed, several sources of evidence indicate that early childhood and adolescence are critical for bone health and therefore can be exploited to maximise bone mass acquisition (133). Although 60-80% of variation in bone mass is due to genetic factors, modifiable exogenous factors such as nutrition and physical activity can still be used as intervention strategies to maximise bone mass within the genetically predefined variance (136–138).

1.4.1 Physical activity during the prepubertal period – Evidence from studies in humans

Physical activity as an intervention to improve skeletal health in children and adolescents has been explored by several research groups [reviewed in (139,140)]. Indeed, a meta-analysis of 10 randomised controlled trials (RCTs) that evaluated the effects of exercise on bone strength concluded that regular...
weight-bearing exercise can bring about significant improvements in bone strength at the loaded sites (140). The prepubertal period in particular has been identified as a window of opportunity where skeletal gains through exercise can be maximised. Several lines of evidence add weight to this idea. In female squash players, larger side-to-side differences in bone mineral content (BMC) and bone mineral density (BMD) were observed in players who started training before or at menarche than those who began their training one year or more after menarche (141). In female racket-sport (tennis and squash) players, the greatest difference in BMC between the playing and non-playing arms was observed in players that started training prior to or at menarche (2). Bass et al. (1998) observed that areal BMD (\(\rho\)BMD) in prepubertal female gymnasts was 8-20% higher compared to controls (12).

Evidence from intervention studies also suggests that the prepubertal period is ideal for maximising the effect of exercise on the skeleton. Bradney et al. (1998) conducted the first intervention study to determine if exercise could improve the bone mass, size, areal and volumetric BMD in prepubertal boys (6). After eight months of moderate weight-bearing exercise, the increases in \(\rho\)BMD of lumbar spine, legs and total body in exercised individuals was twice as much as in control subjects. The femoral midshaft cortical thickness, volumetric BMD (\(\rho\)BMD) and section modulus increased in exercised subjects while no such increases were observed in control subjects. In pre-menarcheal girls, 10 months of high-impact strength-building exercise increased BMC and BMD at multiple skeletal sites, improved grip strength and lean mass (142). In a systematic review of intervention studies in children and adolescents, the authors observed that bone was highly responsive to weight-bearing high-impact exercise during the very early stages of puberty (139). Results from a recent meta-analysis investigating bone response to weight-bearing activities in female children and adolescents showed that exercise has a site-specific response on bone in young girls (143).

Although there is evidence from both cross-sectional and intervention studies in humans that the period before puberty has the potential to maximise the effect of exercise on the skeleton, human studies allow for limited conclusions on the exact relationship between exercise and bone response. For instance, several parameters such as the exact type of exercise, its duration, intensity, number of loading cycles and frequency of loading cycles still remain undefined. Also, it is important to understand the long-term effects of early life physical activity, as evidence indicates that bone mass attained through physical activity during growth is retained even after exercise cessation (12,13,144). Animal models play a crucial role in understanding the role of each of these factors in eliciting an osteogenic response and have the potential to shed light on the biological mechanisms that drive this response.

1.4.2 Animal Studies

In laboratory animals, exercise of low-moderate intensity causes significant structural adaptations in trabecular and cortical bone (145). Controlled loading studies using extrinsic loading models (where
forces are generated by an external actuator) have helped investigators isolate the effects of frequency, loading cycles and load magnitude on skeletal response. These studies have demonstrated that static or isometric loading are inefficient stimuli for mechanotransduction. Also, activation of new bone formation requires that a threshold strain rate be exceeded meaning that activities that create high strain rates are more osteogenic, for example, jumping or plyometrics (145). Evidence from animals studies shows that growing bone is much more responsive to exercise than adult bone [reviewed in Forwood and Burr 1993 (146)]. For instance, Rubin et al. (1992) found that in an externally loaded turkey ulna model that after eight weeks of loading, the bone cross-sectional area of one year old animals was increased by 30% compared to the contralateral limb while that of the three year old animals remained unchanged (147).

Results from studies that investigated whether the skeletal gains accrued through exercise persist after a long period of detraining have been equivocal (14,148). Young Wistar rats trained for 10 weeks followed by a 10 week period of detraining showed increased bone length (femur, tibia, humerus and coxa) and BMC (femur, tibia and humerus) along with a significant increase in total and cortical areas (148). The bone mass acquired through exercise persisted 10 weeks after training cessation. Similarly, Warden et al. (2007) found that in five-week-old Sprague Dawley rats exercised using a forearm compression axial loading model, bone quantity was increased along with significant structural adaptation (14). While the bone quantity reverted to pre-exercise state, structural adaptations persisted after 92 weeks of no training. On the other hand, Jarvinen et al. (2003) found that loss of exercise-induced bone changes were not age-dependent in rats that underwent 14 weeks of training followed by 14 weeks of normal cage activity (149). Similarly, in another study, exercise induced benefits in the femoral neck of rats were eventually lost after 42 weeks of deconditioning (150).

It is well documented that mechanical loading affects signalling pathways in bone (91,151). Although there have been several studies to date that aimed to understand bone’s structural and mechanical adaptations to mechanical stimuli, only a few studies so far have attempted to understand the gene expression changes in bone in response to mechanical stimuli (152–156). Gene expression studies using high-throughput technologies such as microarrays and RNA-seq have the potential to identify previously unknown signalling pathways and molecular players that drive the anabolic response of bone. Roosa et al. (2010, 2012) exploited the power of microarray technology and predictive bioinformatics algorithms to understand the expression patterns of bone subjected to controlled loading and the regulatory mechanisms driving the response (152,153). Six distinct time-dependent gene expression patterns were observed that could be categorised into (1) genes that were up-regulated early (2) genes that were up-regulated during matrix formation and (3) genes that were down-regulated during matrix formation. Wasserman et al. (2013) identified osteocyte specific mechanoresponsive genes in the caudal vertebrae of mice subjected to controlled compression loading, also using
microarray technology (156). High-throughput sequencing technology such as RNA-seq has become the method of choice to analyse transcriptomic profile and unlike qPCR and microarrays, RNA-seq is unbiased and does not rely on a pre-determined set of genes. In one of very few studies that employed RNA-seq to analyse gene expression changes in bone, Kelly et al. (2016) identified gene expression differences between cancellous and cortical bone of young female mice that underwent a single bout of axial tibial compression (154).

1.5 Bone and fat

Adipose tissue regulates energy homeostasis in the body by acting as a storage site for excess energy and releasing the energy stores when needed. It also produces hormones that regulate energy metabolism. Formerly considered mutually exclusive processes, bone and energy metabolism have emerged as being closely interlinked. Recent findings establish bone as an endocrine organ (157) which influences glucose homeostasis through its effect on insulin secretion and sensitivity (158). In turn, pancreatic insulin, and adipokines affect bone metabolism.

1.5.1 The hormonal link between bone and fat

1.5.1.1 Osteocalcin

Osteocalcin is the most abundant non-collagenous protein secreted by mature osteoblasts. It is a 49 residue protein that undergoes carboxylation of its three glutamic acid residues before secretion by osteoblasts. In the resorption lacunae formed by osteoclasts during resorption, it is decarboxylated, upon which it enters the circulation. Lee et al. (2007) provided the first in vivo evidence that uncarboxylated osteocalcin has the ability to regulate energy metabolism by influencing insulin and adiponectin production from β-cells and adipocytes respectively (49). In a mutant mouse model that lacked osteocalcin (Ocn−/−), the authors noted reduced islet size and number, β-cell mass, pancreas insulin content and insulin immunoreactivity. In addition, the Ocn−/− mice were abnormally fat with increased fat mass, adipocyte number and serum triglyceride concentrations. Absence of osteocalcin affected adiponectin expression and also serum adiponectin concentrations.

In order to identify if the status of carboxylation affects the hormonal function of osteocalcin, Lee et al. (2007) performed two additional experiments (49). In the first experiment, osteoblasts were treated with warfarin which inhibits γ-carboxylation. Warfarin treated osteoblasts were more potent in inducing adiponectin expression compared to vehicle-treated osteoblasts. In the second experiment, carboxylated and uncarboxylated bacterial origin osteocalcin were used in cell-based assays to test their ability to affect adiponectin production. Only the uncarboxylated form was effective in inducing adiponectin expression in adipocytes and insulin expression in β-cells. These experiments conclusively proved that it is in fact the uncarboxylated osteocalcin that acts as a hormone.
Adipokines: Leptin and adiponectin

The adipose tissue, hitherto considered an inert fat repository, secretes over 50 hormones and signalling molecules collectively termed adipokines (159). Among these, leptin, a pleiotropic peptide hormone forms a complex link between adipose tissue and bone. Leptin is a product of the *Ob* (Obese) gene and is primarily secreted by the adipocytes (160). Circulating leptin helps to inform the central nervous system (CNS) of the status of energy stores in the body (161). It acts through the hypothalamus and inhibits feeding when the body has excess energy stores. A fall in leptin levels, conversely, stimulates feeding. Recent studies have shown that leptin receptors are not just limited to the hypothalamus but are found in several organs in the body.

A seminal study by Ducy et al (2000) found that adipose tissue regulates bone metabolism indirectly through leptin’s actions on the CNS. Hypogonadism and hypocortisolism is a common feature in mice that lack leptin (*Ob/Ob* mutants) and its receptors (*db/db* mutants). Even though gonadal failure is associated with bone loss, the authors found that the mutant mice had increased bone mass. The authors subsequently found that intracerebroventricular infusion of leptin caused bone loss in leptin-deficient mice.

Leptin receptors have since been found in several skeletal cell types including osteoblasts, bone marrow stromal cells and haematopoietic precursors (162–164). Data from Thomas et al (1999) and Cornish et al. (2002) showed that leptin had a positive effect on osteoblast proliferation and differentiation (162,165). Taken together, these findings indicate that leptin has different modes of action through which it affects bone metabolism. The negative effects of leptin acting through its central nervous system pathway could be counter-balanced by its peripheral positive effects (166).

Adiponectin is another peptide hormone secreted exclusively by the adipocyte and is present abundantly in the plasma. Messenger RNA (mRNA) and protein levels of adiponectin decrease in obesity and type 2 diabetes (167,168). Studies in humans and primates using hyperinsulinemic and euglycemic clamps showed that plasma adiponectin concentrations correlated well with insulin sensitivity (169,170). In addition, in peroxisome proliferator-activated receptor gamma (PPARγ)+/− mice treated with (PPARγ)+/−/retinoid-X receptor (RXR) inhibitors, treatment with a combination of adiponectin and leptin completely reversed the insulin resistance phenotype thereby establishing adiponectin as an insulin sensitising hormone (171).

The observation that bone-derived osteocalcin signals adipocytes and promotes the synthesis and secretion of adiponectin (49) prompted Kajimura et al. (2013) to investigate the effects of adiponectin on bone (172). The authors found that adiponectin affects bone mass accrual through two modes of antagonising mechanisms. On one hand, it acts directly on osteoblasts and prevents their proliferation while promoting their apoptosis. However, it also has a second more powerful mode of action through
which it reduces the activity of the sympathetic nervous system (SNS) and thereby promotes bone mass accrual.

1.5.1.3 Insulin

The discovery that osteocalcin and thereby osteoblasts regulate glucose metabolism and the finding that osteoblasts express insulin receptors (IRs) prompted Ferron et al. (2010) to investigate whether insulin affects osteocalcin synthesis and activity in a feed forward loop (173). The authors found that insulin signalling in osteoblasts promoted the secretion of RANKL and thereby increased bone resorption. It not only promoted osteoclastogenesis but also osteoclast function by increasing the expression of T-cell immune regulator 1 (Tcirg1), a gene implicated in bone resorption (174). The acidification of the ECM environment by osteoclasts promotes the decarboxylation of osteocalcin which in turn favours glucose metabolism. Thus insulin regulates osteoblast mediated glucose homeostasis in a bone resorption-dependent manner.

In an independent series of experiments, Fulzele et al. (2010) demonstrated the importance of insulin signalling in osteoblasts to skeletal and whole body metabolism (175). In vitro experiments by the research group showed that insulin receptor signalling in osteoblasts controls their development and osteocalcin production. It did so by inhibiting twist family BHLH transcription factor 2 (TWIST2), a known suppressor of osteoblast differentiation. Ob-ΔIR mice that lacked insulin receptors specifically in osteoblasts suffered metabolic dysregulation characterised by insulin resistance, increased fat accumulation and glucose intolerance. Infusion of Ob-ΔIR mice with undercarboxylated osteocalcin improved insulin sensitivity in these animals.

1.5.2 Inflammation

Although the adipocyte is the predominant cell type in the adipose tissue, the tissue is also host to pre-adipocytes, endothelial cells, fibroblasts, leukocytes and bone-marrow derived macrophages (176). In addition to being involved in the hormonal regulation of glucose homeostasis, the adipose tissue is the site of production of several pro- and anti-inflammatory cytokines which have important roles in metabolic diseases associated with obesity. One of the characteristic features of obesity is chronic low-grade systemic inflammation due to cytokine production and activation of inflammatory signalling pathways which are responsible for the pathogenesis of metabolic and chronic inflammatory disorders (177,178). The adipose tissue is the predominant site of residence for obesity triggered inflammation (179).

In lean individuals, adipose tissue preferentially produces anti-inflammatory molecules which include adiponectin, TGF-β, IL-10, IL-4, IL-13, IL-1 receptor antagonist (IL1Ra) and apelin (180). In obesity, adipocytes produce low levels of TNF-α stimulating the pre-adipocytes to secrete monocyte
chemoattractant protein-1 (MCP-1). This triggers macrophage infiltration of adipose tissue resulting in the production of pro-inflammatory cytokines such as TNF-α, IL-6, leptin, visfatin, resistin, angiotensin-II and plasminogen activator inhibitor-1 (PAI-1) (180–184). Pro-inflammatory molecules directly affect cellular metabolism which results in reduced insulin sensitivity, increased lipolysis and hyper-triglyceridemia (185–187).

Several pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-15 and IL-17 have a well-established role as mediators of osteoclast activity and thereby bone resorption (188–193). For instance, TNF-α plays a crucial role in bone and joint related inflammatory diseases such as rheumatoid arthritis, bone loss of periodontitis and other forms of chronic inflammatory osteolysis (194–198). It also mediates bone loss due to oestrogen deficiency (199,200). TNF-α exerts its influence on bone resorption by stimulating RANKL production in osteoblasts and consequently promoting osteoclast differentiation (201,202). It also activates osteoclasts in a direct RANKL-independent manner (203). Interleukin 6 has a similar osteoclastogenic effect on bone. Ishimi et al. (1990) showed that in cultured osteoblasts, addition of bone resorption agents induced the expression of IL-6 (191). It induced bone resorption in a dose dependent manner in cultured mouse calvaria. Interleukin 6 also mediates bone loss associated with oestrogen loss (204,205).

1.5.3 Bone marrow adipose tissue

The bone marrow stroma plays host to numerous cell types, each with a unique function. However, the function of the most abundant cell type in the human bone marrow stroma, the adipocyte (206), still remains to be elucidated. Marrow adipose tissue (MAT) is distinct from white adipose tissue (WAT). Firstly, it is considered that MAT arises from a unique progenitor compared to WAT (207,208). WAT is characterised by the presence of a small number of mitochondria. On the other hand, brown adipose tissue (BAT) has numerous mitochondria which facilitate its core role in adaptive thermogenesis (209). Marrow adipose tissue has a moderate number of mitochondria and is considered to be made up of a mixture of WAT and BAT. Studies in humans and animal models found that the marrow adipocytes are morphologically distinct from white adipose tissue with a unique lipid profile (210–212) (Table 1.1).

Marrow adipose tissue also differs in its response to pathological conditions associated with metabolic dysfunction and nutritional deprivation (Table 1.2). A striking example of this differential behaviour of MAT can be seen in patients with Anorexia nervosa (AN), a psychiatric condition which leads to severe loss of body weight and depletion of body fat, but increased levels of marrow fat (213). Similar observations were made in animal studies where nutritional deprivation led to decreases in WAT but induced adipocyte formation in the bone marrow (212,214,215).

For a long time, MAT was considered to be a passive tissue occupying space vacated by the bone. However, like extramedullary adipose tissue, marrow adipocytes secrete several endocrine, paracrine
and growth factors that influence bone remodelling, inflammation, haematopoiesis and skeletal muscle adaptation (216,217). Cawthorn et al. (2014) found that adiponectin secretion from MAT is greater than WAT (217). In mice, inhibition of MAT formation resulted in decreased circulating concentrations of adiponectin under calorie-deficient conditions. However, the production of adiponectin from WAT was not altered. In addition, human bone marrow adipocytes secrete adipokines and cytokines such as IL-1β, TNF-α and IL-6 that influence bone cell differentiation.
Table 1.1: Differences between white adipose tissue and marrow adipose tissue

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Subcutaneous WAT</th>
<th>Visceral WAT</th>
<th>MAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte cell size</td>
<td>+</td>
<td>2++</td>
<td>+</td>
</tr>
<tr>
<td>Polyunsaturated fatty acid content (%)</td>
<td>9-21</td>
<td>20</td>
<td>9-20</td>
</tr>
<tr>
<td>Monounsaturated fatty acid content (%)</td>
<td>46-63</td>
<td>45</td>
<td>48-58</td>
</tr>
<tr>
<td>Saturated fatty acid content (%)</td>
<td>23-36</td>
<td>35</td>
<td>29-33</td>
</tr>
<tr>
<td>Precursor cell type</td>
<td>Lin−CD29+CD34+Scal+CD24+</td>
<td>Lin−CD29+CD34+Scal+CD24+</td>
<td>Unknown, not CD24+</td>
</tr>
</tbody>
</table>

WAT: White adipose tissue; MAT: Marrow adipose tissue. Adapted from Fazeli et al. (2013).

Table 1.2: White and marrow adipose tissues differ in their response to pathological conditions and nutritional insults

<table>
<thead>
<tr>
<th>Condition</th>
<th>WAT</th>
<th>MAT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Caloric restriction/anemia</td>
<td>Decrease</td>
<td>Increase</td>
</tr>
<tr>
<td>Type I diabetes mellitus</td>
<td>Decrease</td>
<td>Increase</td>
</tr>
<tr>
<td>Obesity</td>
<td>Increase</td>
<td>Variable</td>
</tr>
<tr>
<td>Estrogen deficiency</td>
<td>Variable</td>
<td>Increase</td>
</tr>
<tr>
<td>Type 2 diabetes mellitus</td>
<td>Variable</td>
<td>Variable</td>
</tr>
<tr>
<td>Growth hormone deficiency</td>
<td>Increase</td>
<td>Increase</td>
</tr>
</tbody>
</table>

WAT: White adipose tissue; MAT: Marrow adipose tissue. Adapted from Fazeli et al. (2013).

1.5.3.1 Relationship between marrow fat and bone

Several lines of evidence indicate that MAT influences the skeleton. For instance, age-related loss of bone is accompanied by increased marrow adiposity (218). In childhood, very little fat exists in the marrow cavity. It gradually fills the marrow space as individuals age and by the third decade of life, it is the most abundant tissue in the marrow cavity. Treatment with 1,25 dihydroxy vitamin D₃ improved osteogenesis and reduced the expression of adipogenic genes in an accelerated ageing mouse model (219).

Patients with osteoporosis exhibit increased MAT volume and decreased trabecular bone volume compared to their age-matched controls (220). Rodriguez et al. (2000) analysed the ability of MSCs derived from osteoporotic donors to generate and maintain type I collagen under osteogenic conditions (221). They found that MSCs from osteoporotic donors synthesised 50% less type I collagen than
normal cells. Also, MSCs from osteoporotic women showed reduced expression of TGF-β and had greater adipogenic capacity.

Figure 1.3: Mesenchymal stem cell lineage allocation in the bone marrow

Activation of transcription factors such as RUNX2, BMP2, TGF-β and OSX will direct the mesenchymal stem cell in the bone marrow towards osteoblast lineage. Alternatively, activation of CCAAT α,β andδ Ppara, β and γ leads to adipocytic differentiation. DLX5: Distal-less homeobox 5; MSX1: MSH homeobox 1; RUNX2: Runt-related transcription factor 2; CEBP: CCAAT enhancer binding proteins; PPARγ: Peroxisome proliferator activator receptor gamma

Bone loss is a prominent feature in patients with Anorexia nervosa. Bredella et al. (2009) found that the lumbar and femoral fat content was greater in patients with Anorexia nervosa than the control group and that the marrow fat was inversely correlated with BMD (213). Moreover, recovery from the disease led to lower levels of MAT compared to subjects with AN (222). Beresford et al. (1992) first made the observation that stromal cells derived from adult rat marrow were capable of differentiating into both adipocytes and osteoblasts (223). Modification of culture condition through delayed addition of a glucocorticoid or continuous presence of vitamin D₃ altered the differentiation propensity of the stem cells such that they preferred one lineage over the other. Since this seminal discovery, several groups have added evidence that supports the paradigm that there exists an inverse relationship between adipocytes and osteoblasts of the marrow (224).

Peroxisome proliferator-activated receptor gamma is a central regulator of adipocyte differentiation (225) and MSCs require its activation to enter into adipocyte lineage (226,227). Although the endogenous ligand for PPARγ has yet to be identified, exogenous ligands include long chain fatty acids, oxidised derivatives of fatty acids and thiazolidinedione family of insulin sensitisers (228,229). In vitro studies using stromal cell lines have shown that activation of PPARγ promotes adipocyte differentiation and at the same it negatively regulates osteoblast differentiation through inhibition of Runx2 (230). Conversely, Runx2 expression blocks PPARγ activity (231). Lecka-Czernik et al. (2002)
found that, in fact, the pro-adipogenic and anti-osteoblastogenic properties of PPARγ depends on the type of ligand activating it (232). A similar complex response of PPARγ has been reported in in vivo studies (233,234).

1.6 Effect of excess adiposity on bone development

Childhood obesity induced by excess energy intake has reached epidemic proportions worldwide (235). Although obesity increases the risk of metabolic conditions such as type 2 diabetes, hypertension and cardiovascular disease (236), it was regarded to have a protective effect on bone due to the increased mechanical demand imposed by the excessive body weight on the skeleton (237). This widely accepted opinion has been challenged in recent times with several cross-sectional studies in humans and intervention studies in animals suggesting an unfavourable relationship between obesity and bone health (238–242). As such, understanding the effects of excess adiposity on skeletal integrity and unraveling the complex mechanisms driving the relationship has become an area of intense investigation. It is vital to understand the effects of excessive adiposity on the growing skeleton since achieving optimal bone mass and strength during growth has biologically relevant effects on skeletal competence in later life (243).

1.6.1 Evidence from clinical studies

In a cross-sectional study that aimed to determine if distal forearm fractures, the most common type of fracture in children, were associated with a lower BMD at the site, the authors found that in girls aged 8-10 years, fracture risk was positively associated with body mass index, fat mass and lean tissue mass (240). Despite the extra weight-bearing, the BMC was not greater in girls with fractures. Four years later, the same study group was assessed to determine the predictors for subsequent fracture risk. The authors found that total body weight was one of the predictors of fracture risk (239). Following this, research by the same group (238,241,244,245) and several other groups [reviewed in (18)] concluded that obesity is associated with increased fracture risk and/or compromised bone strength parameters. Although in some studies excessive body weight in children was linked to greater BMD (246–248), the increase however was not sufficient to overcome the significantly greater forces that are generated during a fall (248).

In contrast, Leonard et al. (2004) found that in male and female subjects aged 4-20 years, obesity was positively associated with several vertebral and whole body bone parameters even after adjusting for sexual maturation and gender differences (248). Ducher et al. (2009) measured the effect of fat and muscle mass on tibial and distal radius bone characteristics in prepubertal children. While a greater fat to muscle ratio benefited tibial bone strength, it resulted in reduced bone strength in the distal radius (249). In the Avon Longitudinal Study of Parents and Children (ALSPAC) project from Bristol in the UK, children at 9.9 years exhibited a positive correlation between total body fat and total-body-less-
head bone mass. However, in girls, progression through puberty saw this relationship deteriorate and subsequently reverse (250). Additional studies support the notion that the prepubertal period may respond positively to increased fat mass while the relationship between bone and fat mass turns negative after the onset of puberty (244,245,251).

Conflicting reports regarding the relationship between obesity and bone in children and adolescents can be attributed to several factors: (1) Bone mineral density and bone mass are the most common parameters that are used to determine skeletal strength in a clinical setting. Dual energy x-ray absorptiometry (DXA) is the method of choice for body and bone measurements in human studies. However, the accuracy of DXA measurements is affected by fat tissue thickness (252,253). (2) Also, the two-dimensional information provided by DXA does not provide an accurate picture of the three-dimensional nature of bone architecture. (3) Skeletal growth and maturity is site and age specific and is another factor that contributes to the complex interaction. (4) Controversy still remains as to the most suitable method to correct for body size in children to reflect true physiological changes in bone growth.

1.6.2 Evidence from animal studies

Human studies are limited by the fact that detailed bone histomorphometry and molecular analyses that are necessary to accurately evaluate the skeletal response to increasing adiposity are not feasible due to their invasive nature. In addition, longitudinal studies, which are vital to understand the changing relationship between fat and bone through childhood to adult stage, are lacking in clinical studies. As such, animal models, especially rodents, have been widely employed to determine the consequences of excess adiposity on the skeleton and establish the mechanisms driving the response.

Animal models of high-fat (HF) diet induced obesity have helped shed light on biological processes associated with skeletal response to excess fat accumulation. Unlike in human studies, evidence from animal studies heavily favours the hypothesis that obesity may be detrimental to bone health during growth. Several studies reported a negative relationship between HF diet induced obesity and bone quality (20–25). Excess adiposity has been shown to have a negative effect in the bone of both male and female mice. However, skeletal response to HF diet is also gender specific with regards to the magnitude of response (254).

One of the mechanisms through which a HF diet affects bone phenotype is by favouring a pro-resorptive bone micro-environment with increased osteoclastogenesis. In a study by Shu et al. (2015), five-week-old male C57BL/6J mice fed a fat rich diet (60% Kcal from fat) developed obesity and metabolic dysfunction that is associated with the condition (255). Compromised trabecular phenotype in the HF diet-fed animals was accompanied by an increase in RANKL/OPG ratio, elevated IL-1 and TNF-α levels, increased PPARγ expression and greater adiposity which are known stimulators of
osteoclastogenesis. Intriguingly, bone formation rates were also higher in the HF fed animals with the bone marrow MSCs showing increased adipogenic and osteogenic potential leading the authors to hypothesize that the obesogenic HF diet caused bone loss through its augmenting effect on osteoclastogenesis which overrode the compensatory bone formation response of the osteoblasts. Similar resorption-favouring effects of increased adiposity have also been reported by other groups (22,254). In all these studies, even though there was increased osteoblastogenesis and/or osteoblast activity, the ultimate loss of bone suggests that the rate of bone resorption overrides the effect of bone formation. Alternatively, increased osteoblastogenesis and function may be a compensatory response to accelerated remodelling.

Although we are beginning to tease out the physical effects of a HF diet on bone and the associated physiology, the global transcriptomic landscape of the bone in the presence of a HF diet is an area of sparse research. Recent gene expression studies in HF diet-fed animal models have uncovered several potential candidate genes and pathways that may be involved in the molecular mechanisms driving the bone’s response to altered adiposity. Xiao et al. (2010) analysed the global gene expression profile using microarray technology and RNA extracted from the proximal femurs of mice fed a HF diet (21.2% kcal from fat) (256). Analysis of 12,960 genes revealed that several genes involved in extracellular matrix formation like collagens and matrix metalloproteinases were downregulated in the HF fed group correlating with the observed reduction in plasma carboxyterminal propeptide of type 1 procollagen (P1CP) concentrations, a marker for bone formation. Conversely, osteoclast specific genes like CATK, integrin β3 and calcitonin receptor were upregulated in the HF diet group. Several genes involved in lipid metabolism, adipogenesis (Pparγ, Ccaat/Cebpβ, Twist1, Scd2), fatty acid oxidation (Pparγ, ApoE, Acat1 and Lipe) and anti-oxidation (Sod2, Gpx3 and Txnrd1) were downregulated in the HF diet group. These molecular events could underlie the decrease in femoral BMD and mechanical strength phenotypic changes observed in the HF fed animals.

1.7 Physical activity as an intervention strategy to mitigate adverse outcomes of a high-fat diet

Physical activity is one of the key and most commonly advocated non-pharmacological interventional strategies to combat metabolic diseases associated with excess energy intake (257). Not only is physical activity effective in combating excess adiposity through increased energy expenditure, it also has an antagonistic effect on adverse physiological processes triggered by excess caloric intake. Of crucial consideration is the fact that obesity and mechanical loading share common mechanisms through which they affect bone metabolism.

While obesity is associated with a pro-inflammatory state, exercise has an anti-inflammatory effect. This can occur through three possible mechanisms: (1) reduction in the visceral fat mass; (2)
production and release of anti-inflammatory cytokines; (3) reduction in the expression of Toll-like receptors on the surface of monocytes and macrophages (258–260). Regular exercise has the ability to reduce abdominal and visceral fat mass. It also increases the circulating levels of adiponectin and reduces the concentrations of pro-inflammatory cytokines such as IL-6, TNF-α, retinol-binding protein 4 and leptin (261). Exercise induces the transient release of IL-6 from contracting skeletal muscle. IL-6 release triggers the release of anti-inflammatory cytokines such as IL-10 and IL-1RA (262). IL-1RA is secreted by monocytes and macrophages and it has an inhibitory effect on the actions of IL-1β, a pro-inflammatory cytokine. IL-10 promotes an anti-inflammatory state by inhibiting the effects of several pro-inflammatory cytokines (263). The concentrations of circulating monocytes increase due to inflammation. Exercise induces an increase in inflammatory monocyte numbers. However, this increase lasts for a short duration and falls to baseline numbers in recovery. Regular exercise has been shown to reduce the number of inflammatory monocytes in the resting state (264). The anti-inflammatory effects of exercise have important implications for bone metabolism. As mentioned previously, pro-inflammatory cytokines promote osteoclast differentiation and function, and promote bone resorption.

In the bone marrow, MSCs physically interact with the components of their micro-environment. Physical factors such as cell shape, external mechanical forces, extracellular matrix and geometric cues all affect the lineage commitment of the stem cells (265–268). Mechanical forces can facilitate MSCs to commit to an osteogenic lineage while discouraging adipogenic differentiation (269–272). Li et al. (2015) showed that mechanical strain induced the expression of Runx2 and Osx in MSCs cultured in general and adipogenic media (269). The mechanical stimulus also simultaneously reduced the expression levels of Pparγ2 and C/ebpα. Sen et al. (2008) found that in C3H10T1/2 MSCs cultured in adipogenic medium, application of mechanical strains inhibited Pparγ and adiponectin expression and subsequent addition of osteogenic factors such as BMP2 increased the expression of Runx2 (273). The authors found that mechanical strain inactivated GSK-3β thereby stabilising and activating the β-catenin pathway.

*In vivo* studies in mice support the *in vitro* findings. In a study by Luu et al. (2009), male C57BL/6J mice were subjected to low magnitude mechanical signals for six weeks which resulted in the increase of both overall marrow stem cell population and the number of MSCs (28). Crucially, the MSCs showed significant upregulation of Runx2 and downregulation of Pparγ. The phenotype of the treated mice was in agreement with the observed gene expression changes. While the trabecular bone volume fraction increased, visceral adipose tissue formation was suppressed. Thus, exercise has the ability to influence both fat and bone through its action on MSCs.
1.8 Relevance of the laboratory rat as a model to study human skeletal biology.

Mice, rats, rabbits, dogs, pigs, cows, sheep, chicken, horses and non-human primates are the commonly used animal models to study bone metabolism, diseases (e.g., osteoporosis) and as preclinical models to test the efficacy of treatments and interventions (274,275). The relevance (comparability to the human condition), convenience and appropriateness should be given careful consideration before adopting an animal model (276). For these reasons, the rat has been a popular model in bone research. Its small size, ease of handling, short life-span, well-characterised skeleton and well-defined genetic background make it an attractive animal model (274,277).

The rat skeleton shares several features that are common with the human skeleton. Increase in bone mass occurs through the same biological mechanisms in rats and in humans (278). While longitudinal growth adds length to cortical bone, modelling drifts thicken trabeculae (278). The rapid growth phase in rats lasts until the first three months of age and reaches a peak shortly after puberty (279). Growth hormones and sex steroids mediate the gender specific differences in bone elongation, cancellous bone modelling and remodelling, radial bone growth and timing of epiphyseal closure. Thus, the growing rat model has been proposed as a useful model to investigate the effects of several endogenous and environmental factors on bone growth and development (277).

There are also striking differences between a rat and a human skeleton that need careful consideration when adopting the rat model. The fact that rats lack a well-developed haversian remodelling system and undergo limited endocortical bone turnover is important when conducting studies in adult rats (277). While in humans, the physes fuse shortly after sexual maturation, in rodents, longitudinal bone growth continues after sexual maturation albeit at a slower rate. However, there is evidence for physeal closure in rats, the timing of which depends on the bone and the location of the growth plate (277,279).
Aims and Hypotheses

The aims of this thesis are threefold. Firstly, it seeks to determine the ability of voluntary mild-moderate physical activity to influence the body composition, bone phenotype and the diaphyseal bone gene expression when physical activity is undertaken before puberty. Secondly, it aims to examine the effects of an early life obesogenic diet on body composition, bone metabolism and gene expression in the young adult male rat. Lastly, it aims to investigate the ability of voluntary exercise to modulate the effects of HF diet. Based on current evidence in the literature, we hypothesize an obesogenic HF diet has an adverse effect on skeletal health, and voluntary physical activity has the ability to rescue the negative response in bone elicited by such a diet.

After outlining in Chapter 2, the methodologies employed in the studies, in Chapter 3, we evaluated the ability of two types of voluntary exercises: bipedal stance and wheel running to affect body composition and bone micro-architecture in prepubertal male rats. We hypothesized that the young animals can successfully carry out the two types of voluntary exercises and that the exercises will affect the body and bone composition without eliciting a stress response.

Based on the results in Chapter 3, we aimed to investigate the gene expression changes in the diaphyseal bone in response to exercise in Chapter 4. We hypothesized that significant changes in gene expression should precede the phenotypic changes we observed in Chapter 3.

In chapter 5, we examined the effects of an early life HF diet on body composition, bone mass indices and bone marrow gene expression in young male rats. Then, we evaluated the ability of voluntary wheel exercise to modulate HF diet induced changes in the animals. We hypothesized that the HF diet positively affects the body weight and total fat percentage while having a negative effect on the bone mass indices and that voluntary physical activity rescues the adverse effects of the diet.

In Chapter 6, we discuss our findings in the context of the currently available literature highlighting the original contributions and strengths of the work presented in this thesis. Finally, future directions that will further the knowledge gained from the studies presented in this thesis are suggested.
Chapter 2. Methods

2.1 Overview of animal experimental design

This thesis consists of two studies:

Study 1 (Chapters 3 and 4), used a prepubertal male rat model to investigate the effects of voluntary exercise on body composition, bone parameters and gene expression profile in the femoral diaphysis.

Study 2 (Chapter 5) used a young adult male rat model to understand the effects of early life high-fat diet and voluntary wheel exercise on the body composition, bone phenotype and gene expression in the bone marrow.

All animal work was approved by the University of Auckland Animal Ethics Committee (Approval numbers: AEC 001068 and AEC 001432 for study 1 and study 2 respectively).

2.1.1 Experimental design - Study 1

Time-mated female Sprague-Dawley (SD) rats were used to generate 59 offspring derived from a total of four litters. The day of birth was defined as day 0 (D0). As the study involved only male animals, female animals were culled on D1. Litter size was limited to eight pups per dam to standardise nutrition until weaning. Pups and mothers were weighed daily and food and water consumption was recorded daily until D20.

<table>
<thead>
<tr>
<th>Litter</th>
<th>Male</th>
<th>Female</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8</td>
<td>7</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>8</td>
<td>8</td>
<td>7</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>9</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 2.1: Litter size and selection of animals for study 1

Fifty nine pups were born to four mothers. Twenty four male rats were allocated to three exercise groups based on their body weight and total fat percentage.

2.1.1.1 Assignment to exercise groups

The exercise groups consisted of control (CON), bipedal stance (BPS), and wheel exercise (WEX) groups. On D20, pre-exercise body composition was determined using dual energy X-ray absorptiometry (DXA, Lunar Hologic, GE, Waltham, MA, USA). Rats were allocated to one of three experimental groups such that there were no statistically significant differences with regards to starting
body weight and total fat percentage and controlling for litter of origin. All animals were housed in a temperature (19.9–21.8°C) and light-dark cycle (12:12, lights on at 6 a.m.) controlled facility. The animals had *ad libitum* access to food and water (Diet 2018, Teklad Global 18% Protein Rodent Diet, Harlan Teklad, USA) (Table 2.2).

### Table 2.2: Composition of the diets used in the studies

<table>
<thead>
<tr>
<th>Source</th>
<th>Standard chow (Harlan Teklad Diet 2018)</th>
<th>High-fat diet Research Diets #D12451</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kilo calories</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein</td>
<td>24</td>
<td>20</td>
</tr>
<tr>
<td>Fat</td>
<td>18</td>
<td>45</td>
</tr>
<tr>
<td>Carbohydrates</td>
<td>58</td>
<td>35</td>
</tr>
<tr>
<td>Energy density</td>
<td>3.1 kcal/g</td>
<td>4.73 kcal/g</td>
</tr>
</tbody>
</table>

#### 2.1.1.1 Control (CON) group
The CON group was housed in pairs in standard 419 x 279 x 152 mm cages with their food and water at a height of ~90mm from the cage floor. The rats were allowed unrestricted movement within their cage.

#### 2.1.1.2 Bipedal stance (BPS) group
The BPS group was housed in pairs in a specially modified BPS cage (280,281). During the initial three-day exercise familiarisation period (D\textsubscript{21–23}), the feed height was gradually raised from a standard height of 90 mm to its full height of 220 mm which required the BPS animals to fully extend their tibiotarsal and femorotibial joints. There were two individual exercise periods. The first individual exercise period lasted for 44 hours on D\textsubscript{27–29} and the second exercise period lasted for 36 hours on D\textsubscript{33–35} to facilitate the collection of faecal pellets and exercise data for individual animals. During the individual exercise period, the two rats in each cage were separated by a clear plastic ‘buddy barrier’ to minimise stress due to cage-mate separation. Custom made rat position sensors were installed in each cage with the intention of measuring the number of times and the duration for which the rats rise on their hind legs to access their food source. The sensor mimicked a house hold burglar alarm which is tripped when an invisible infrared line between an LED emitter and a photodiode sensor is broken.
2.1.1.3 Wheel exercise (WEX) group

The WEX group was housed in pairs in cages equipped with an activity wheel (Model 80859, Lafayette Instrument, Lafayette, IN, USA) connected to a wheel control and counter (Model 86070A). The counter was used to monitor the exercise levels in one-hour intervals throughout the experimental period. Data were recorded using dedicated monitoring software (Model 86065). The wheel was locked and the animals were allowed to explore their new environment on the first day of the familiarisation period (D21). The wheel was unlocked on D22 and resistance of the wheel set to zero and remained so for the rest of the trial period. There were two individual exercise periods: 47 hours on D27–29 and 36 hours on D33–35 during which each rat was in its own wheel-instrumented cage to facilitate the collection of faecal pellets and exercise data for individual animals. Cages were made of clear plastic, and the cage-mates were placed adjacent to each other, so that the rats could see each other during separation to minimise the stress response.

2.1.2 Experimental design – Study 2

<table>
<thead>
<tr>
<th>Table 2.3: Diets and exercise regimens of the intervention and control groups in study 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experimental Groups</td>
</tr>
<tr>
<td>C-SED</td>
</tr>
<tr>
<td>Diet</td>
</tr>
<tr>
<td>Exercise (D21-D60)</td>
</tr>
<tr>
<td>Exercise (D60-D120)</td>
</tr>
</tbody>
</table>

C-SED: chow sedentary; HF-SED: high-fat sedentary; HF-EEX: high-fat early-exercise; HF-LEX: high-fat late-exercise.

The study consisted of four experimental groups: chow sedentary (C-SED); high-fat sedentary (HF-SED); high-fat early-exercise (HF-EEX); high-fat late-exercise (HF-LEX) (Table 2.3). Eighty male SD rats were purchased at 21 days of age and randomised to the four experimental groups carefully controlling for their body weights and litter of origin (n=20 per group). All animals were housed in pairs in the same room with a constant room temperature (RT) of 25°C and a 12 hour light:dark cycle. Animals either received standard chow with 18% of total energy from fat (Diet 2018, Teklad Global 18% Protein Rodent Diet, Harlan Teklad, USA) (Table 2.2) or a high-fat (HF) diet with 45% of total energy intake from fat (D12451, Research Diets, USA) (Table 2.2). Food, water and body weights were recorded at regular intervals.
During the first half of the study which spanned from D21-D60, animals in C-SED, HF-SED and HF-LEX groups were housed in standard cages and allowed unrestricted movement within their cages. The HF-EEX group had access to a running wheel set at zero resistance. The animals’ body composition was determined through DXA scanning on D54. Between D60-D63, 10 animals from each group were culled. From D60 to D120, C-SED, HF-SED and HF-EEX animals were housed in standard cages. HF-LEX animals had access to a running wheel (zero resistance). The animals’ body composition was determined through DXA scanning on D112 following which they were culled between D120-D122.

2.2 Determination of body composition
Dual energy x-ray absorptiometry (DXA) was used to determine whole body fat mass, lean mass, fat to lean mass ratio, bone mineral content (BMC), and areal bone mineral density (aBMD). Animals were anaesthetised with 4% isoflurane at induction and once anaesthetised, the animals were maintained under 2.5% isoflurane for the duration of the scanning.

2.3 Euthanasia
In study 1, all animals were culled on the same day by decapitation under isoflurane anaesthesia following an overnight fast. In study 2, animals were fasted overnight, anaesthetised using sodium petabarbitalone (60mg/kg, IP) and killed by decapitation.

2.4 Tissue collection

2.4.1 Plasma
Plasma was separated through centrifugation of the trunk blood which was collected into heparinised and stored at −20°C until analysis.

2.4.2 Fat pads
Epididymal and retroperitoneal fat pads were removed, weighed, snap-frozen in liquid nitrogen and stored at -80°C.
2.4.3 Bone and marrow

Two cuts were made in the femur, one at the junction of the distal and middle thirds and the other, at middle and proximal thirds. Mid-diaphysis was obtained by gently snapping the bone. Marrow was either flushed out with saline or removed by centrifugation.

A sterile saw was used to make a cut in the femur at the junction of distal and middle thirds, and of middle and proximal thirds. The bone was gently snapped to obtain the mid-diaphysis. In study 1, medullary fat was flushed out using saline and a syringe. In study 2, in order for a cleaner separation of the bone and marrow, a sterile 1mL pipette tip was cut into thirds. The narrow part of the tip was inserted into the wider part. This was inserted into a 1.5mL micro-centrifuge tube. The diaphyseal bone was placed in the tip and centrifuged (10,000 rpm, 30s, 4°C). The bone and marrow were placed in separate tubes, snap frozen in liquid nitrogen and stored at -80°C until further analysis.

2.4.4 Faecal sample collection

Faecal samples were collected from animals in study 1. Sample collection was performed according to a previously described protocol (281). Faecal samples were collected during the individual exercise periods between D_{27–29} and D_{33–35}. Sample collection was performed four hours after the start of the dark period on D_{28}, D_{29} and D_{34} as the time period marks the time of the faecal corticosterone nadir and is ideal for detecting between-group differences in glucocorticoids (282). The buddy barrier system in BPS group and individual wheel cages in WEX group made it possible to collect samples from each animal separately. As the CON group remained in pairs, faecal sample collection was done for pairs of animals. Samples were frozen at -20°C immediately after collection.
2.5 Biochemical analyses

2.5.1 Insulin and leptin
Fasting plasma samples were analysed for insulin and leptin using rat-specific commercial ELISAs (90060 and 90040, respectively, Crystal Chem, Downers Grove, IL, USA) according to the manufacturers’ instructions.

2.5.2 Fasting glucose
Fasting blood glucose measurements were made on tail blood samples using a glucose meter (Precision Xtra, Abbott, USA).

2.5.3 Corticosterone and testosterone
Plasma corticosterone and testosterone concentrations were measured using LC MS/MS as previously described (283). In a glass tube, 200µL of plasma was mixed with 100µL (20ng/mL in H2O) of corticosterone-d8 which was used as an internal standard. Steroids were then extracted with 1mL of ethyl acetate. The supernatant containing the steroids was transferred into a new tube and vacuum dried. The contents were resuspended in 100µL of mobile phase made up of 80% methanol and 20% water and transferred into high performance liquid chromatography (HPLC) injector vials. Thirty microlitre aliquots were injected into an HPLC mass spectrometer system with Waters Alliance 2690 Separations Module (Waters Corporation, Milford, MA, USA), followed by an Ion Max APCI source on a Finnigan TSQ Quantum Ultra AM triple quadrupole mass spectrometer controlled by Finnigan Xcalibur software (Thermo Electron Corporation, San Jose, CA, USA). The mobile phase flow rate was 600µL/min through Luna 3u C18 (2) 100A 250x4.6mm column held at 40°C (Phenomenex, Auckland, New Zealand). Ionisation was in positive mode and Q2 had 1.2 mTorr of argon. The mass transition followed was 355.3-125.2 at 24V for corticosterone-d8.

2.6 Faecal corticosterone measurement using mass spectrometry
Steroids were extracted from faecal samples using a previously described method (284). Samples were vacuum dried (8 hours, RT) and ground into a fine powder using tissue grinding pestles. Two hundred milligrams of sample was mixed with 800mL of sterile water. Five millilitres of ethyl acetate (Merck Life Science, Auckland, New Zealand) was added and the vial containing the mixture was put on a rotator at 4°C for 40 minutes to ensure thorough mixing. The mixing allows the steroids to move from the faecal matrix into the solvent. The mixture was then centrifuged (1690g, 15 minutes, 4°C) The ethylacetate fraction was transferred to a fresh glass vial. One millilitre of 0.1M NaOH was added to the steroid extract and vortexed for 10s and centrifuged (1690g, 10 minutes, 4°C). The wash was repeated twice with water and the extract was transferred to a fresh tube and dried (40 minutes). Samples were reconstituted in 400µL of methanol mixture containing 65% methanol (CH3OH) and
35% H₂O by volume. The samples were cleaned using a PTFE phenomenex membrane filter (0.2μm pore size) and 1 cc syringe. Corticosterone-d8 was used as an internal standard. Serial dilutions of corticosterone and 11-deoxycorticosterone were used to obtain standard curves. One milligram per millilitre stock of the standard was used to generate the various concentrations required for the standard curve (Table 2.4).

<table>
<thead>
<tr>
<th>Table 2.4: Dilutions of standards for faecal corticosterone measurement using mass spectrometry</th>
</tr>
</thead>
<tbody>
<tr>
<td>Standard</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>A</td>
</tr>
<tr>
<td>B</td>
</tr>
<tr>
<td>C</td>
</tr>
<tr>
<td>D</td>
</tr>
<tr>
<td>E</td>
</tr>
<tr>
<td>F</td>
</tr>
<tr>
<td>G</td>
</tr>
<tr>
<td>H</td>
</tr>
</tbody>
</table>

2.7 Micro-computed tomography (µCT)

In study 1, the right femur of each animal was cleaned of all soft tissue and placed in 70% ethanol at 4°C. Femur length was measured using a sliding callipers. Femurs were scanned using SkyScan 1172 µCT scanner (Bruker, Aartselaar, Belgium) as previously described (285). An X-ray voltage of 65 kV, was employed along with a 1mm aluminium filter, and an isotropic voxel size of 8µm. SkyScan NRecon software was used for standardised reconstruction and the datasets were analysed using SkyScan CT-analyser software (CTAn, SkyScan, Aartselaar, Belgium). Volumes of interest (VOI) were selected and analysed in two regions of the distal femur: for trabecular imaging, VOI was 2.32mm proximal to the distal femoral physis and extended 2mm in the proximal direction; for cortical bone imaging, VOI was 5.6mm proximal to the physis and extended 0.8mm proximally.

2.8 Peripheral quantitative computed tomography (pQCT)

The scans were done with a XCT Research SA+ pQCT machine (StratecMedizinTechnik, Pforzheim, Germany). Femur length was measured from the most distal aspect of the lateral femoral condyle to the proximal extent of the trochanter major using a sliding calliper. The bone was lodged in a plastic tube.
filled with saline, taking care that no air bubbles were present, and secured in the pQCT machine. After placing the reference line at the distal aspect of the condyles in the scout view, the machine was programmed to make one scan at 50% of the femoral length, slice thickness 0 and 70 microns voxel size. Suitable outcome measures to determine cortical bone density and bone architecture were chosen, as previously described (286).

2.9 Determination of adipocyte area in the bone marrow

2.9.1.1 Sectioning
Wax-embedded sections of the proximal tibial trabecular bone immediately subjacent to the subchondral bone were obtained from serial sections processed for study of joint cartilages published elsewhere (287). Briefly, wax blocks of the tibio-femoral joints were decalcified and then sectioned in the sagittal plane (Leica RM2245 microtome, Leica BioSystems, Germany) and knife angle of 6° (Leica 891 Low Profile Microtome blade, Leica BioSystems, Germany). Beginning at the medial aspect of the joint, four serial 8μm-thick sections were sampled every 200μm across the whole joint resulting in approximately 96 samples per joint. Sections were collected on Superfrost Ultra Plus (Thermo Scientific, Germany) microscope slides coated with poly-L-lysine (Sigma-Aldrich, USA) which were then heated at 60°C for approximately two hours, and stored (RT) prior to staining. A midline section from the medial condyle was selected based on the following morphological features: (1) no connection between anterior and posterior horns of the menisci; (2) proximal discontinuation of the anterior femoral articular cartilage occurring at approximately the same level as the posterior femoral articular cartilage; (3) no evidence of cruciate ligament attachment into either tibia or femur. From the reference section in the medial condyle, a section at 400μm distance laterally was chosen for analysis.

2.9.1.2 Metasystems VSlide scanning
Toluidine blue-stained sections were scanned using Metasystems VSlide scanner (MetaSystems, Version 2.1.124) running Metafer4 (version 3.9.2) coupled with MetaXpress (Molecular Devices, Version 5.3.0.4). Sections were pre-scanned at a low-power (2x) magnification and the pre-scanned images were used for threshold-based segmentation of the sections and artefacts. All sections were re-scanned at 20x magnification and viewed using Image Viewer (VSViewer, MetaSystems, Version 2.0).

2.9.1.3 Image analysis
The region of marrow snapshotted was located at the anterior margin of the tibia, at the inferior portion of, or distally adjacent, to the trabecular bone. Snapshots were then opened on ImageJ and the images calibrated to convert pixels into distance (in μm). Images were thresholded to black and white (thresholding range between 228 and 255) followed by conversion to binary images. The ‘erode’ option
followed by the ‘fill gaps’ option were used to identify adipocytes. Ten adipocytes at random were selected and the area of each of these cells was measured using the ‘measure’ function and data were analysed in excel.

2.10 RNA extraction

RNA was extracted from the femoral diaphyseal bone and bone marrow using a hybrid protocol first described by Ayturk et al. (2013) (288) that used Trizol® reagent (#15596-026, Life Technologies, Carlsbad, CA) to isolate RNA which was subsequently purified using spin columns (RNeasy® Mini Kit, #74104, Qiagen, Hilden, Germany). All hardware was baked (4 hours, 200°C) to minimise RNase contamination. RNaseZAP™ (#R2020-250ML, Sigma-Aldrich), a cleaning agent for eliminating RNase contamination, was used to wipe down the surfaces prior to extraction. The extraction process consisted of the following steps: tissue homogenisation, phase separation, RNA binding, DNase treatment, washing and elution.

2.10.1 Tissue homogenisation and phase separation

Tissue was homogenised in liquid nitrogen using a ceramic mortar and pestle cooled using liquid nitrogen. The homogenate was immediately transferred into a 1.5mL micro-centrifuge tube containing 1mL trizol®. The micro-centrifuge tube was then dropped into a container with liquid nitrogen and placed on ice to facilitate further cell lysis. Samples were sonicated (10s, 5 cycles, 40% power) (Bandelin Sonopuls HD2070, Bandelin, Berling, Germany) twice, cooling on ice between sonications. The lysate was incubated (5 min, RT) to allow the complete dissociation of nucleoprotein complexes before chloroform (0.2mL) was added to the lysate. Samples were shaken vigorously by hand for 15s. Lysates were incubated (3 min, RT) and centrifuged (12,000g, 15 min, 4°C). The colourless upper aqueous phase (400µL) containing RNA was transferred into a fresh tube. An equal volume of 70% ethanol (made using DEPC treated H₂O) was added (35% final concentration) and samples were mixed by inversion.

2.10.2 RNA isolation

Seven hundred microlitres of the sample-ethanol mixture was added to an RNeasy® spin cartridge and centrifuged (12,000g, 30s, RT). The flow-through was discarded and the process was repeated until the entire sample was processed. Wash buffer RW I (350µL) was added to the spin cartridge containing the bound RNA and centrifuged (12,000g, 30s, RT), flow-through was discarded and the spin column was placed in a new collection tube.

2.10.3 DNase treatment

DNase was prepared for each sample by adding 10µL of DNase I to 70µL of RDD buffer (RNase-Free DNase Set, #79254, Qiagen, Hilden, Germany) in a separate tube and mixing gently. DNase mixture
(80µL) was added to the spin column and incubated (15 min, RT). Buffer RW I (350µL) was added to each spin column and centrifuged (12,000g, 30s, RT), the flow-through discarded, and the spin column was placed in a new collection tube.

### 2.10.4 Washing and elution

Buffer RW I (700µL) was added to the spin column, centrifuged (12,000g, 30s, RT) and the flow-through was discarded. Buffer RPE (500µL) was added to each spin column and washed as above. The spin column was then centrifuged (12,000g, 1 min, RT) to dry the membrane before the spin cartridge was placed in an RNase free recovery tube. Nuclease free water (15µL - 30µL warmed to 50°C) was added directly onto the membrane incubated (1 min, RT) and the RNA was eluted by centrifugation (12,000g, 2 min, RT). Eluted RNA was quantified, quality checked (Bioanalyser and Nanodrop™) and stored (-80°C) until required for further analysis.

### 2.11 RNA quality evaluation

The integrity and quantity of total RNA was determined using an Agilent bioanalyser (Model 2100, Agilent Technologies, Santa Clara, CA, USA) and an RNA 6000 Nano LabChip kit according to the Manufacturer’s instructions. The RNA integrity number (RIN) ranges from 1 to 10, with 1 being the most degraded and 10 being the least degraded RNA.

### 2.12 RNA quantification

#### 2.12.1 Nanodrop

The concentration of all RNA samples in study 1 was determined using 1.5µL of each sample and a micro-volume spectrophotometer (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE). The Nanodrop measures absorbance at 260, 280 and 230 nm (289). The ratio of absorbances at these wavelengths is used to determine the purity of both nucleic acids and proteins. A 260/280 ratio of ~2.0 is deemed as pure for RNA. Absorbance at 230 nm is considered a result of other contamination such as Trizol®, phenol or glycogen. A 260/230 ratio of 2.0-2.2 is considered acceptable (289).

#### 2.12.2 Qubit® fluorometric quantitation

The Qubit® was used to measure RNA concentrations for samples in study 2. The Qubit® measures nucleic acid and protein concentration with the help of fluorescent dyes that fluoresce only when bound to the target molecules. The RNA High Sensitivity Assay Kit (#Q32852, Life Technologies New Zealand Limited, Auckland, New Zealand) was used to accurately measure sample concentrations which were estimated to fall between 250 pg/µL and 100ng/µL. Where necessary, samples were diluted so that they were within the instrument detection range.
2.13 RNA-seq

For study 1, purified RNA was stored at -80°C until required for sequencing (50 bp, paired end; Illumina HiSeq; BGI China). The sequence data were deposited in NCBI’s Gene Expression Omnibus [GEO; (Edgar et al. 2002) (290)] and are accessible through GEO Series accession number GSE67787 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67787).

For study 2, purified total RNA from the bone marrow was aliquoted into a 96 well plate containing RNA stable® (Biomatrica, Inc. San Diego, CA, USA) in a laminar flow hood. The plate was vacuum dried (one hour, RT), sealed and placed in a zip seal pouch with desiccant according to the manufacturer’s instructions. The RNA stable® plate containing the RNA was sent to Novogene (Novogene Corporation, Beijing, China) for RNA-seq (150 bp, paired end; Illumina Hi-Seq 4000).

2.14 Read quality control

Table 2.5: Phred quality scores and the probability of errors associated with them

<table>
<thead>
<tr>
<th>Phred Score</th>
<th>Incorrect base call probability</th>
<th>Inferred base call Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1 in 10</td>
<td>90%</td>
</tr>
<tr>
<td>20</td>
<td>1 in 100</td>
<td>99%</td>
</tr>
<tr>
<td>30</td>
<td>1 in 1000</td>
<td>99.9%</td>
</tr>
<tr>
<td>40</td>
<td>1 in 10,000</td>
<td>99.99%</td>
</tr>
<tr>
<td>50</td>
<td>1 in 100,000</td>
<td>99,999%</td>
</tr>
<tr>
<td>60</td>
<td>1 in 1,000,000</td>
<td>99,999%</td>
</tr>
</tbody>
</table>

The quality of the sequenced reads was determined by the Phred quality score (291,292). The quality of value q assigned to a base call is defined as:

\[ Q_{\text{PHRED}} = -10 \times \log_{10}(p) \]

where \( p \) is the estimate of error probability. Thus, high quality scores represent low error probabilities and vice versa. For instance, a Phred score of 30 represents a probability of an incorrect base call 1 in 1000 times (Table 2.5). Thus the base call accuracy is 99.9% for a Phred score of 30.
Figure 2.2: A representative image of summary result from FastQC analysis

The x axis indicates the base position. The y axis indicates the phred scores. The central red line indicates the median Phred score value. The yellow boxes are the inter-quartile ranges (25-75%). The whiskers represent the 10% and 90% values. The blue line represents the mean quality.

FastQC [FastQC; (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)], a quality control tool for high throughput sequence data was used to assess the read quality (Figure 2.2).

2.15 Read trimming

Reads obtained from the RNA samples of animals in study 2 were of high quality (Phred≥28) and trimming was not required. PRINSEQ (http://prinseq.sourceforge.net/) was used to trim the first eight bases from sequences obtained for study 2 and all bases with Phred scores below 30 starting from the 3’ end of the reads. The following shell script was used:

```
prinseq-lite.pl -fastq filename_fwd.fq -fastq2 filename_rev.fq -trim_left 8 -trim_qual_right 30 -out_good output_filename
```

The resulting reads were filtered with reads that were < 50 bp in length discarded. The following script was used to filter the reads:

```
prinseq-lite.pl -fastq filename_fwd.fastq -fastq2 filename_rev.fastq -min_len 50 -out_good output_filename
```

2.16 Read alignment using TopHat

The Tuxedo suite of bioinformatics tools was used for read alignment and differential gene expression analysis. The Rattus norvegicus reference genome NCBI version Rnor_5.0 was downloaded from
Illumina igenomes webpage (http://support.illumina.com/sequencing/sequencing_software/igenome.html). Among the many read alignment programs available, Bowtie is the most popular and efficient alignment tool (293). However, as Bowtie does not allow alignment if there are large gaps between a read and the reference genome, TopHat was developed to overcome this issue (294). Reads are first mapped to the reference genome using Bowtie. Unmapped reads are broken into smaller segments that are mapped onto the genome. When a read’s segments map to the genome far apart, it indicates a splice junction and TopHat infers the location of the junction’s splice sites (294).

Reads were mapped to the genome using TopHat (version 2.0.12 for study 1 and version 2.1.0 for study 2) by providing the GTF file and parameters -r 200 -G (295). The -G option is provided to supply TopHat with a set of gene model annotations and/or known transcripts. TopHat will in this instance, align the reads to the supplied transcriptome first. Reads that do not fully map are then mapped to the genome. The following script was used for read alignment:

```
Tophat2 --o output_filename --r 200 --p 32 --G genes.gtf genome filename_fwd.fq filename_rev.fq
```

### 2.17 Differential gene expression analysis using Cuffdiff

Differential gene expression was determined (Cufflinks version 2.2.1) using Cuffdiff (296). Cuffdiff calculates expression in two or more samples and tests the statistical significance of observed expression changes. The program assumes that the number of reads generated by a transcript is proportional to its abundance but fluctuates due to technical and biological variability. The following script was used:

```
cuffdiff -o output_filename -p 32 -u -b genome.fa genes.gtf
<sample1_replicate1.bam[,....,sample1_replicateM.bam]>\n<sample2_replicate1.bam[,....,sample2_replicateM.bam]>...
[sampleN_replicate1.bam[,....,sampleN_replicateM.bam]]
```

### 2.18 Gene-annotation enrichment analysis

Gene-annotation enrichment analysis utilises information accumulated in public databases such as Gene Ontology (297) to help investigators identify the most relevant biological processes for their study (298).
Enrichment analysis tools have three major components: backend annotation database; data mining; and result presentation. Diagram adapted from Huang et al. (2008) (299).

Enrichment analysis is based on the idea that if a biological process is altered in a study, the genes involved in the process should have a higher potential to be selected as a relevant group by the screening technologies. Differentially expressed transcripts were subjected to term enrichment analysis using GO::TermFinder (http://go.princeton.edu/cgi-bin/GOTermFinder) (300). The P-value cut-off was set at 0.05 and gene lists queried against the Rat Genome Database (RGD).

2.19 Pathway analysis

Ingenuity pathway analysis (IPA Winter Release 2014, Qiagen, Redwood City, www.qiagen.com/ingenuity) was used to identify the signalling and metabolic pathways, downstream biological processes and/or diseases affected by the differentially expressed genes and the key upstream regulators that may be driving the response. IPA is a web-based software package which takes data generated from omics experiments such as RNA-seq, microarrays, metabolics and proteomics and transforms it into a set of relevant networks. The software uses the ingenuity pathways knowledge base (IPKB), a manually curated database of findings from peer-reviewed scientific publications that are systematically encoded into an ontology (301, 302). It also incorporates data from third party databases such as IntAct, BIND, DIP, MINT, MIPS, BIOGRID and COGNIA (302). The knowledge base is used to create a larger global molecular network (GMN), which contains information about the interactions between thousands of genes and gene products (303).
Differentially expressed transcripts were loaded into IPA. The dataset was filtered using the following attributes: P-value = 0.05; the user dataset was used as the reference set; and *Rattus norvegicus* was specified as the species.

2.19.1 Canonical pathways
IPA identifies the canonical metabolic and signalling pathways that are enriched in a given set of genes (304). It does so using the right-tailed Fisher exact test to calculate the likelihood that the association between a set of focus genes in the experiment and a given process or pathway is due to random chance. The smaller the P-value, the less likely that the association is random and the more significant the association. The P-value for a given process/pathway is calculated by considering the number of focus genes that are part of the process and all the known number of genes that are known to be associated with that process in the reference set. The more focus genes involved, the more likely the association is not due to random chance and thus the more significant the P-value. The P-value thus identifies focus genes that are significantly over-representation in a given process.

2.19.2 Regulatory effects
Regulatory effects analysis provides an insight into the causes and effects of differentially expressed genes in a dataset (305,306). It provides a putative explanation for how the predicted activation/inhibition of upstream regulators might affect the observed downstream functional outcomes. The networks are organised in three tiers. The top tier is made up of the predicted activated/inhibited upstream regulators. The dataset genes that connect the upstream regulators with the bottom tier of downstream functions make up the middle tier. Each regulatory effects network is assigned a ‘consistency score’ by the IPA regulatory effects algorithm. The formula used to calculate the consistency score is:

\[
\text{Consistency score} = \frac{(P_cW_c + P_iW_i + P_nW_n)}{(S)^{W_s}}
\]

\(P_c\) = total number of consistent paths from regulator to function; \(W_c\) = weight that rewards for consistent paths and is set to 1; \(P_i\) = total number of inconsistent paths; \(W_i\) = weight that penalises inconsistent paths and is set to -15; \(P_n\) = total number of non-causal paths; \(W_n\) = weight for non-causal paths and is set to 0. \(S\) = size; \(W_s\) = penalty weight for the size of the network and is set at 0.5 (305).

Higher consistency scores are assigned to networks in which most of the paths from regulator to target to disease/function are in agreement with findings from the literature. The Fisher exact test is used to calculate the P-value of the overlap between the regulator and function/disease dataset molecules and this must be <0.05.

2.19.3 Downstream effects
Downstream effects analysis within IPA is used to identify the biological functions that are expected to be increased or decreased based on the observed gene expression changes in a given dataset (307). The
analysis is based on expected causal effects between genes and functions stored in the IPKB. Genes in the dataset that are known to affect a biological function are taken and their direction of change is compared to what is expected from the literature. If the observed direction of change is consistent with a particular activation state of a function, then a prediction is made about that activation state. For each biological function, an activation z-score is computed. It is used to infer the activation state of the implicated biological functions. IPA considers z-scores greater than 2 or smaller than -2 as significant.

2.20 Statistical analyses
All statistical analyses were carried out using SigmaPlot (V12.5, SysStat Software Inc., Ca, USA) and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). Unless otherwise stated, the alpha value was set at 0.05. In study 1, one-way analysis of variance (ANOVA) was employed to determine significant differences between the three exercise groups. If the ANOVA showed a significant difference (P-value<0.05), multiple comparison testing was performed using the Holm-Sidak procedure. Where the data failed the equal variance test, ANOVA on Ranks followed by Tukey test for multiple comparisons was used to test between-group differences. Differences in caloric intake between groups over the trial period in study 1 were tested by mixed ANOVA using IBM SPSS Statistics 21 (release 21.0.0) with an alpha level of 0.05. Body weight data were also analysed using repeated measures ANOVA.
Chapter 3. Different Short-Term Mild Exercise Modalities Lead to Differential Effects on Body Composition and Gene Expression in Healthy Prepubertal Male Rats

3.1 Introduction

Bone is sensitive to the mechanical stimuli imposed on it and responds to the loads applied largely by changing its mass and morphology (308,309). Physical activity thus has a vital role in regulating and improving bone strength. The strength of the response is age-dependent with the prepubertal period being the most effective stage for exercise to have maximal effect on bone mass (2,12,280,310,311). Evidence is accumulating that supports the hypothesis that changes in bone mass and architecture as a result of exercise at a young age are retained even after the cessation of exercise. For instance, in a school-based impact exercise intervention program that spanned seven months and involved children who were 10.2 ± 0.6 years old, the bone mineral content (BMC) in the proximal femur of the group which participated in the exercise program was 3.6% higher than in the control group. Of note, the BMC of the intervention group remained 1.4% higher than that of the control group eight years after the cessation of the exercise program (312). Likewise, bone health parameters in 75-year-old Swedish men were significantly positively correlated with the amount of competitive sport they participated in between the ages of 10 and 35 years (9). Similar observations have been made in studies of bone health in gymnasts (12), tennis players (10), and weight lifters (11,13). Thus exercise not only has immediate positive effects on improving bone health in the young (reduction in child fracture risk) (313) but also appears to have the potential to mitigate bone loss in later life.

Consistent with studies in humans, work in animal models has shown that the effects of physical activity on bone are retained after exercise cessation. Four-week-old male Wistar rats that underwent treadmill training for 10 weeks had greater BMC and longer bones than the control group. The bone mass accrued through training was retained after 10 weeks of exercise cessation (detraining) (148). Five-week-old Sprague-Dawley rats that were exercised three days per week for seven weeks had improved bone quantity and strength; these changes were still present when the rats were examined at two years of age (14). However, other studies have shown that exercise-induced changes in bone are lost with detraining (314,315). Four-week-old female Sprague-Dawley rats that exercised on a treadmill for eight and twelve weeks had greater femoral wet weight and bone volume than the control groups. However, eight weeks of exercise followed by four weeks of cessation resulted in a decrease of bone mass to levels seen in sedentary controls (314). In five-week-old male Sprague-Dawley rats, 14 weeks of treadmill running improved the size, mass, and strength of the femoral neck. These changes
were partially retained during a subsequent deconditioning period of 14 weeks. But the changes
eventually disappeared after 42 weeks of detraining (315). The apparent contradictions in the results of
these studies (14,148,314,315) may be due to one or more factors which include age and gender of the
animals, exercise modality and duration, skeletal site examined, and experimental design (14,314,315).

There is a gap in our knowledge as to the ability of mild exercise to influence bone development when
the exercise begins and ends before puberty is reached. For example, although the studies mentioned
above (14,148,314,315) involved animals that may have been prepubertal when the studies
commenced, the duration of the exercise protocols meant that the animals were well past puberty by the
end of the experimental period. Therefore, to explore the effect of prepubertal exercise on bone
development, we conducted a controlled study where 21-day-old male Sprague-Dawley rats were
allowed to exercise voluntarily (i.e., wheel running or rising to a bipedal stance) for a period of 15 days
spanning the period between weaning and puberty.

3.2 Materials and methods

3.2.1 Study design.

Time-mated female Sprague-Dawley rats were used to generate 24 offspring derived from a total of
four litters. Litter size was limited to eight pups per dam to standardise nutrition until weaning. On D20,
pre exercise body composition was quantified, while under light isoflurane anaesthesia, using dual
energy X-ray absorptiometry with dedicated small animal software (DXA, Lunar Hologic, GE,
Waltham, MA, USA). Rats were allocated to one of three treatment groups ensuring no statistically
significant differences with respect to starting body weight and total fat percentage and controlling for
litter of origin. The exercise groups consisted of control (CON), bipedal stance (BPS), and wheel
exercise (WEX) groups. All animals were housed in a temperature (19.9°–21.8°C) and light-dark cycle
(12:12, lights on at 6 a.m.) controlled facility with ad libitum access to food and water (Diet
2018,Teklad Global 18% Protein Rodent Diet, Harlan Teklad, USA). Rats were housed in pairs except
for two intervals (on D27–29 and D33–35) during which the BPS and WEX rats underwent individual
exercise monitoring as detailed below. The experimental period lasted from D21 to D35 and the animals
were culled on D36.

Water and food intake and body weight were monitored regularly throughout the study in order to
examine possible effects of exercise on caloric intake. The amount of food consumed was determined
as the difference between the residual and initial feed weights. Caloric intake, defined as the number of
kilocalories (Kcal) consumed per gram of body weight, was calculated for each pair of rats using feed
consumption information and the caloric density of the diet (3.1 kcal/g).
The CON group was housed in pairs in standard cages. The BPS group was housed in pairs in a specially modified BPS cage (280, 281). There was an initial three-day exercise familiarisation period (D21–23) during which the feed height was gradually raised from a standard height of 90mm to its full height of 220mm. Raising the feed required the rats to fully extend the tibiotarsal and femorotibial joints. During the individual exercise period which lasted for 44 hours on D27–29 and 36 hours on D33–35, the two rats in each cage were separated by a clear plastic “buddy barrier” in order to minimise stress due to cage-mate separation.

WEX rats were pair-housed in cages containing an activity wheel (Model 80859, Lafayette Instrument, Lafayette, IN, USA) connected to a wheel control and counter (Model 86070A) which was used to monitor the exercise levels in one-hour intervals throughout the experimental period. Data were recorded using dedicated monitoring software (Model 86065). During the first day of the familiarisation period (D21), the wheel was locked and the animals were allowed to explore their new environment. The wheel was unlocked on D22 and resistance of the wheel set to zero which remained so for the rest of the trial period. During the individual exercise periods (47 hours on D27–29 and 36 hours on D33–35), each rat was in its own wheel-instrumented cage. Cages were made of clear plastic, and the cage-mates were placed immediately adjacent to each other, so that the rats could see each other during separation to minimise the stress response due to cage-mate separation. All animal work was approved by the University of Auckland Animal Ethics Committee.

3.2.2 Whole body composition analyses.

DXA was performed on all animals on D20 and D35 to determine whole body composition (lean and fat mass), fat to lean mass ratio, bone mineral content (BMC), and areal bone mineral density (aBMD). D20 DXA measurements were used to allocate the rats to exercise groups.

3.2.3 Tissue collection.

All animals were culled on D36 by decapitation under isoflurane anaesthesia following an overnight fast. Fasting blood glucose measurements were made on tail blood samples using a glucose meter (Precision Xtra, Abbott, USA). Trunk blood was collected into heparinized tubes stored on ice, centrifuged and the plasma was separated and stored at -20°C until analysis. Epididymal and retroperitoneal fat pads were removed, weighed, snap-frozen in liquid nitrogen and stored at -80°C. The left femur was dissected and sectioned into proximal, mid, and distal sections, snap-frozen in liquid nitrogen, and stored at -80°C. The right femur was stored in 70% ethanol and was used for µCT analysis.
3.2.4 Plasma and faecal analysis.

Fasting plasma samples were analysed for insulin and leptin using rat-specific commercial ELISAs (90060 and 90040, respectively, Crystal Chem, Downers Grove, IL, USA) according to the manufacturers’ instructions. Plasma corticosterone and testosterone concentrations were measured using LC MS/MS as previously described (283). In addition to plasma corticosterone, which exhibits a diurnal variation, faecal samples were also collected and corticosterone measured via mass spectrometry to reflect a more stable time course as per our previous publications (281).

3.2.5 Microcomputed tomography (µCT).

The right femur of each animal was cleaned to remove all soft tissue and placed in 70% ethanol at 4°C. Femur length was measured using sliding callipers. Femurs were scanned using SkyScan 1172 µCT scanner (Bruker, Aartselaar, Belgium) as previously described (316), with X-ray voltage 65kV, 1mm aluminium filter, and isotropic voxel size of 8µm. After standardised reconstruction using SkyScan NRecon software, the datasets were analysed using SkyScan CT-analyser software (CTAn, SkyScan, Aartselaar, Belgium). Volumes of interest (VOI) were selected and analysed in two regions of the distal femur: for trabecular imaging, the volume of interest (VOI) was 2.32mm proximal to the distal femoral physis and extended 2mm in the proximal direction; the cortical bone-imaging site was 5.6mm proximal to the physis and extended 0.8mm proximally.

3.2.6 Statistical analyses.

All statistical analyses were carried out using SigmaPlot (V12.5, SysStat Software Inc., Ca, USA) and GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) was employed to determine significant differences between the three treatment groups. The alpha value was set at 0.05. If the ANOVA showed a significant difference (P value < 0.05), multiple comparison testing was performed using the Holm-Sidak procedure. Differences in caloric intake between groups over the trial period were tested by mixed ANOVA using IBM SPSS Statistics 21 (release 21.0.0) with an alpha level of 0.05. Body weight data were also analysed using repeated measures ANOVA.
3.3 Results

3.3.1 Weight gain and food intake

Figure 3.1: Body weights of prepubertal rats in control, BPS, and WEX exercise groups.
Body weights of prepubertal rats in control, BPS, and WEX exercise groups. CON: control; BPS: bipedal stance; WEX: wheel exercise. Data are presented as mean ± SEM, n=8 per group.

Figure 3.2: Caloric intake of rats during the prepubertal exercise period.
Caloric intake was obtained by dividing the number of Kcal consumed by the body weight of the animals. All data are on a per cage basis and presented as mean ± SEM. CON: control; BPS: bipedal stance; WEX: wheel exercise.
The group mean weights of the animals on D21 were not significantly different (CON: 53.44 ± 1.18 g; BPS: 50.68 ± 1.49; WEX: 52.49 ± 3.88). The trajectory of weight gain over the course of the experimental period was similar between the groups (Figure 3.1) and the final body weights of the groups were CON: 150.20 ± 5.35 g; BPS: 142.65 ± 5.92; WEX: 141.61 ± 6.29. The total weight gain, defined as the difference between the final and initial body weights, was highest in CON (96.77 ± 4.52 g) followed by BPS (91.98 ± 4.71) and then WEX (89.12 ± 5.6). Neither final body weight nor the total weight gain was significantly different between the groups. There were no significant differences in caloric intake between the groups across the treatment period. Although the BPS group appeared to have a high caloric intake between D21 and D23 (Figure 3.2), this difference did not reach statistical significance.

3.3.2 Exercise.

![Figure 3.3: Comparison of wheel exercise (WEX) activity during the light and dark periods.](image)

For D21-24 and D30-32, data represent the mean exercise for pairs of rats (n= 4 pairs). For each of D28-29, D29-30, D32-33, and D33-34, data are means measurements for individual rats (n= 8). Data are presented as mean ± SEM.

The BPS and WEX animals exercised without any observable adverse health effects. The propensity for each animal to exercise varied among the animals. The total wheel exercise distance on the first day the wheel was unlocked was 1801 ± 493 m day⁻¹ cage⁻¹ (i.e., sum of distance for two rats, which were housed in pairs at this stage). WEX activity depended on the time of day and was considerably lower during the light than the dark period throughout the 15-day trial (Figure 3.3). For instance, on D22-23, the mean distance ran by the four pairs of WEX animals was 1453 ± 474 m during the dark period but only 348 ± 104 m between 6 a.m. and 6 p.m. in the succeeding light period on D23. The exercise records of the two periods in which the wheel exercise of each individual rat was quantified showed that the amount of exercise varied greatly between individual rats. For instance, the distances run
ranged from 881 m day$^{-1}$ to 6373 m day$^{-1}$ during the first individual exercise period and from 916 m day$^{-1}$ to 7376 m day$^{-1}$ during the second individual exercise period.

3.3.3 Body composition.

![Image of bar charts comparing body composition between exercise groups]

**Figure 3.4:** Comparison of body composition at D36 between the exercise groups.

(A) The lean mass percentage of WEX was significantly higher than CON. (B) The fat mass to lean mass ratio was significantly lower in WEX compared to CON. CON: control; BPS: bipedal stance; WEX: wheel exercise. Data are presented as mean ± SEM; n=8 per group. *P< 0.05 for CON versus WEX.

![Image of bar charts for epididymal and retroperitoneal fat weights]

**Figure 3.5:** Epididymal (A) and retroperitoneal (B) fat weights of prepubertally exercised rats. WEX rats had a significantly lower percentage of epididymal fat (A) and lower percentage of retroperitoneal fat (B). CON: control; BPS: bipedal stance; WEX: wheel exercise. Data are mean ± SEM; n=8 per group. *P< 0.05.

At the end of the exercise period (D35), the WEX group had small but significantly increased lean mass percentage than the CON and BPS groups (CON: 83.41 ± 0.4%; BPS: 85.16 ± 0.55; WEX: 86.56 ±
There were no significant differences in percentage total fat mass as quantified by DXA (15.51 ± 0.33%, 14.46 ± 0.72 and 13.33 ± 0.91 for CON, BPS, and WEX respectively). The fat/lean mass ratio was lower in WEX than CON and BPS groups (CON: 0.19 ± 0.01; BPS: 0.16 ± 0.01; WEX: 0.15 ± 0.01, Figure 3.4B). Absolute weights of epididymal fat pads were significantly decreased in the WEX group compared to CON and BPS groups (CON (mg): 660 ± 35; BPS: 677 ± 57; WEX: 450 ± 23, P<0.05 for CON and BPS versus WEX). When adjusted for body weight, the relative epididymal fat pad weights remained significantly decreased in the WEX group compared to CON and BPS groups (CON: 0.47 ± 0.02; BPS: 0.48 ± 0.03; WEX: 0.34 ± 0.01, Figure 3.5A). Similarly, absolute weights of retroperitoneal fat pads were significantly decreased in the WEX group compared to CON and BPS groups (CON (mg): 331 ± 18; BPS: 310 ± 32; WEX: 171 ± 27, P< 0.05 for CON and BPS versus WEX). When adjusted for body weight, the relative retroperitoneal fat pad weights remained significantly decreased in the WEX group compared to both CON and BPS groups (CON (%BW): 0.24 ± 0.03; BPS: 0.22 ± 0.02; WEX: 0.13 ± 0.021, Figure 3.5B).

3.3.4 Plasma and faecal Analysis.

Plasma concentrations of leptin (CON: 0.90 ± 0.14 ng/mL; BPS: 0.88 ± 0.06; WEX: 0.89 ± 0.11), insulin (CON: 0.17 ng/mL ± 0.02; BPS: 0.19 ± 0.03; WEX: 0.19 ± 0.03), and corticosterone (CON: 270.4 ± 66.1 ng/mL; BPS: 390.2 ± 53.4; WEX: 326.7 ± 88) were not significantly different between groups. Faecal corticosterone was not detectable in any of the samples analysed. Blood glucose concentrations (mmol/L) were also similar between the groups (CON: 3.85 ± 0.17; BPS: 4.25 ± 0.17; WEX: 4.14 ± 0.21). Testosterone concentrations were low in all the animals confirming that these animals were prepubertal (CON: 71.9± 33.0 pg/mL; BPS: 65.0 ± 21.6; WEX: 67.3 ± 20.4).
3.3.5 Bone parameters.

Table 3.1: Comparison of bone morphometric properties as determined by μCT

<table>
<thead>
<tr>
<th>Trabecular bone</th>
<th>CON</th>
<th>BPS</th>
<th>WEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bone volume (mm³)</td>
<td>0.71 ± 0.06</td>
<td>0.66 ± 0.08</td>
<td>0.70 ± 0.06</td>
</tr>
<tr>
<td>Percent bone volume (%)</td>
<td>4.41 ± 0.29</td>
<td>4.30 ± 0.47</td>
<td>4.35 ± 0.38</td>
</tr>
<tr>
<td>Bone mineral density (g/cm³)</td>
<td>0.089 ± 0.006</td>
<td>0.093 ± 0.009</td>
<td>0.095 ± 0.006</td>
</tr>
<tr>
<td>Trabecular thickness (mm)</td>
<td>0.04 ± 0.00</td>
<td>0.04 ± 0.00</td>
<td>0.04 ± 0.00</td>
</tr>
<tr>
<td>Trabecular separation (mm)</td>
<td>0.50 ± 0.04</td>
<td>0.49 ± 0.04</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>Trabecular number (mm⁻¹)</td>
<td>1.15 ± 0.07</td>
<td>1.08 ± 0.12</td>
<td>1.08 ± 0.09</td>
</tr>
<tr>
<td>Trabecular pattern factor (mm⁻³)</td>
<td>28.28 ± 0.79</td>
<td>28.02 ± 1.01</td>
<td>27.04 ± 0.9</td>
</tr>
<tr>
<td>Structure model index (no units)</td>
<td>2.07 ± 0.03</td>
<td>2.1 ± 0.04</td>
<td>2.07 ± 0.04</td>
</tr>
<tr>
<td>Connectivity density (1/mm³)</td>
<td>82.07 ± 20.02</td>
<td>74.30 ± 26.27</td>
<td>73.85 ± 26.11</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Cortical bone</th>
<th>CON</th>
<th>BPS</th>
<th>WEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Periosteal volume (mm³)</td>
<td>7.49 ± 0.15</td>
<td>7.27 ± 0.16</td>
<td>7.47 ± 0.24</td>
</tr>
<tr>
<td>Endosteal volume (mm³)</td>
<td>5.51 ± 0.14</td>
<td>5.26 ± 0.14</td>
<td>5.40 ± 0.20</td>
</tr>
<tr>
<td>Bone volume (mm³)</td>
<td>1.98 ± 0.03</td>
<td>2.01 ± 0.04</td>
<td>2.07 ± 0.05</td>
</tr>
<tr>
<td>Percent bone volume (%)</td>
<td>26.48 ± 0.54</td>
<td>27.67 ± 0.50</td>
<td>27.81 ± 0.46</td>
</tr>
<tr>
<td>Tissue mineral density (g/cm³)</td>
<td>1.084 ± 0.019</td>
<td>1.129 ± 0.018</td>
<td>1.126 ± 0.009</td>
</tr>
<tr>
<td>Mean polar moment of inertia (mm⁴)</td>
<td>6.48 ± 0.22</td>
<td>6.35 ± 0.29</td>
<td>6.73 ± 0.37</td>
</tr>
<tr>
<td>Cross-sectional thickness (mm)</td>
<td>0.098 ± 0.005</td>
<td>0.112 ± 0.004</td>
<td>0.113 ± 0.004*</td>
</tr>
<tr>
<td>Open porosity (%)</td>
<td>12.31 ± 1.19</td>
<td>9.68 ± 0.77</td>
<td>9.40 ± 0.66</td>
</tr>
<tr>
<td>Total porosity (%)</td>
<td>12.60 ± 1.15</td>
<td>10.07 ± 0.75</td>
<td>9.83 ± 0.66</td>
</tr>
</tbody>
</table>

Trabecular and cortical variables were measured at the distal physis of the right femur in the exercise groups. WEX rats had higher cortical cross-sectional thickness. There was a trend towards differences in open porosity and total porosity between CON and WEX but these did not reach statistical significance (P = 0.0610 and P = 0.0673, respectively). CON: control; BPS: bipedal stance; WEX: wheel exercise. Data are mean ± SEM; n=8 per group. *P< 0.05 for CON versus WEX.
Figure 3.6: Closed porosity (%) in bones from prepubertally exercised rats.
Percent closed porosity was significantly increased in WEX versus CON animals. CON: control; BPS: bipedal stance; WEX: wheel exercise. Data are mean ± SEM; n=8 per group. \*P < 0.05.

The trabecular and cortical parameters of the right femur are summarised in Table 3.1 and Figure 3.6. The three exercise groups had similar femur lengths (CON: 24.17 ± 0.2 mm; BPS: 23.80 ± 0.24 mm; WEX: 23.74 ± 0.35 mm). There were no significant between-group differences in the BMC (standardised to body weight) as measured by DXA. Cortical tissue mineral density (TMD) and trabecular bone mineral density (BMD) also showed no between-group differences. Cortical cross-sectional thickness was significantly greater in the WEX than the CON group. Numerically, cortical cross-sectional thickness was 15% higher in the WEX group, similar to the 12% higher cortical BMC in the WEX than the CON group when values were corrected to body weight. Open and closed porosity was defined using the SkyScan software as follows: closed pore; 3D connected assemblage of space (black) voxels that is fully surrounded on all sides in 3D by solid (white) voxels. Conversely an open pore was defined as any space located within a solid object or between solid objects which has any connection in 3D to the space outside the object(s). Percent of closed porosity was higher in the WEX than in the CON group (Figure 3.6). The differences between CON and WEX with respect to percent of open porosity and total porosity approached statistical significance (P = 0.062 and P = 0.067 for open and total porosity, respectively).
3.4 Discussion

The onset of puberty does not occur at a fixed age in rats. In male rats, the first spermatozoa appear in seminiferous tubules by 40-45 days (317) and the first significant increase in plasma testosterone has been reported to occur between 40 and 50 days of age (318). Evidence of balanopreputial separation, which occurs at the time of pubertal rise in androgen levels, is often used as an indicator of pubertal onset and this is known to occur at approximately 39 days of age in healthy male rats (319). This evidence, taken together with the low plasma testosterone concentrations observed in rats in the current study serves to validate the prepubertal status of the animals.

Despite their young age, the animals were able and willing to exercise throughout the trial period. Importantly, the low plasma corticosterone concentration observed, which was not different between the groups, indicated that the exercised animals did not experience additional stress due to the exercise regime. Moreover, the WEX rats rapidly adapted to the exercise environment and immediately showed a clear circadian rhythm in their activity, running considerably greater distances during the dark period, in keeping with their nocturnal nature (320). This exercise pattern persisted throughout the trial period, consistent with previous observations (321).

The amount of activity varied widely between the WEX rats as evidenced by the individual exercise records. For both periods in which exercise of each WEX individual was quantified, the individual which ran the greatest distance ran 7-8 times more than the individual that ran the least. Such differences in running activity are not uncommon even in rats of the same strain (322–324) and have been attributed to genetic, hormonal, physiological, and psychological factors (325).

Effects of voluntary exercise on body weight in rats are known to be highly variable (321,322) and have been attributed to gender, strain, age, or experimental conditions (321). Introduction of exercise (BPS or WEX) in the current study did not affect either the trajectory of weight gain during the trial period or the final body weight of the animals. Similar findings were reported elsewhere in studies in which young male rats (21 days-30 days old) voluntarily exercised on a wheel (321,326,327).

Although the absolute calorie consumption increased in all the groups as the experiment progressed, calorie consumption per gram of body weight remained similar over the course of the experiment. The fact that BPS animals’ caloric intake was similar to the other groups indicates that the animals had little difficulty reaching the elevated food source. A lack of significant changes in food intake across the different exercise modalities confirms that the effects observed on reduced fat pad weight in the WEX group is resultant from the exercise itself and not a reduction in caloric intake.

Although the mean body weights of the groups did not show significant differences, the body composition of the WEX group differed significantly from that of the CON and BPS rats. WEX rats
had a significantly higher percent lean mass and lower fat to lean mass ratio than the CON and BPS groups. The WEX group also had a significantly decreased absolute and relative epididymal and retroperitoneal fat mass compared to CON and BPS animals. Despite the changes in fat mass, plasma leptin concentrations, which have previously been shown to correlate positively to adipose tissue mass in both humans and rodents (328), were not significantly different between the exercise groups. This could relate to the young age of the animals and leptin-independent changes in body composition in rodents as reported by Cottrell et al. (2011) (329). In this context, although insulin and glucose play central roles in energy homeostasis, neither plasma insulin nor glucose was different across any of the groups studied and thus other markers of energy metabolism need to be examined in future independent studies.

In the present study, the femoral cortical cross-sectional thickness was significantly greater in the WEX than in the CON group. Cross-sectional thickness may increase due to an increase in periosteal volume alone, a decrease in endosteal/medullary volume alone, or a combination of both. There were no significant differences between WEX and CON groups with respect to these two variables when separately analysed, but when combined in the form of mean cortical thickness the small changes resulted in a significant difference in the latter morphological property of the WEX compared to the CON group. Despite the periosteal volume of the WEX group being almost identical to that of the CON group, when corrected to body weight the former was 6.7% greater than the latter; a similar but lesser difference was also present in endosteal volume. Although these changes were small, there appeared to be a biological effect on cortical bone mass and thickness, presumably associated with the different exercise regimen of the two groups. Because bending during locomotion is likely to be greater in the mid-diaphysis than at the site chosen for cortical bone scanning in this study, differences in cortical thickness and possibly other morphological features or properties may have been even more marked at the mid-diaphyseal site, and possibly in the BPS group also.

Cortical porosity is made up of resorption cavities, vascular, lacunar-canaliculi, and collagen-apatite porosities (330) and contributes to the mechanical properties of cortical bone (331). Small changes in porosity can result in considerable loss of bone strength (332). Percentage of closed porosity was significantly higher in the WEX than in the CON group. Related properties, percentage of open porosity, and total porosity tended to be lower in the WEX compared to the CON group, and these differences approached statistical significance.

We demonstrated differential effects in the two exercise groups as compared to the control group. We could not record all parameters of the two regimens, such as number of cycles, duration of exercise, rest insertion, and forces acting in the hindquarters of the animals. Thus standardisation of the two exercise regimens and comparison between them was not directly possible. Due to the short duration of
exercise (15 days), as dictated by the interval between weaning and puberty, this experiment was designed to determine if high frequency activity (running) in young rats in a free-running wheel or a low frequency activity (lifting body weight in bipedal standing) compared to control rats would be associated with detectable effects on phenotype.

3.5 Conclusions
Collectively our observations indicate that the cortical bone microenvironment was affected by the exercise stimulus and responded accordingly. Fifteen days of moderate voluntary exercise was sufficient to affect muscle mass, localised adiposity, and bone micro-architecture in prepubertal male rats. Importantly, these changes were independent from any adverse stress responses or changes in caloric intake of the animals. Response to exercise was modality dependent with wheel exercise having a significant effect on the musculoskeletal system and localised adipose reserves. On the other hand, bipedal stance, a form of resistance training, did not significantly affect any of the parameters examined. The knowledge that early life short-term voluntary exercise can affect three major components of body composition (muscle, bone, and fat) can be exploited to investigate its efficacy as an early life intervention to improve health outcomes later in life, particularly in those at risk for early obesity.
Chapter 4. Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca$^{2+}$ signalling pathways in the male rat bone

4.1 Introduction

Associational studies have established positive and long-lasting effects of early life exercise in people who engaged in competitive sport as youths or adolescents (2,13). Due to the intense level(s) of training required to achieve this competitive rank, such activity is unsuitable as a health recommendation for a majority of any community. However, despite the fact that mild or moderate early life exercise does not negatively affect bone (333) or joint development (334,335), the long-lasting effects of this form of exercise on the musculoskeletal and other body systems have been poorly described. Indeed, morphological studies into the effects of early exercise are further complicated by the fact that the effects on bone phenotype in young animals might be obscured by the normal musculoskeletal growth, and thus be difficult to detect.

Integrating physiological, morphological, and molecular responses of bone to spontaneous exercise is vital for exercise to be advocated as an effective strategy for maintaining/improving bone health parameters. Previously, we identified subtle morphological changes in the femoral diaphysis of prepubertal male rats that were allowed to undertake wheel running (WEX) or had to rise to an erect bipedal stance (BPS) when feeding, for a period of only 15 days (336). These exercise programs were chosen to minimise the confounding effects of stress, and the exercise period was limited to 15 days to ensure that the manipulation was specific to the prepubertal period.

We hypothesised that significant changes in gene expression of cells should precede significant measurable phenotypic changes in the tissues of which they are part. Here, we characterise and compare the global gene expression profile changes in the diaphysis of BPS or WEX rats to those of conventionally housed rats (Control; CON). We identify shared and exercise-specific changes to pathways involved in mechanotransduction, energy homeostasis and cell-cell junction signalling. We show diametrically opposite effects on transcripts for multiple genes within the integrin-linked kinase (ILK) and calcium (Ca$^{2+}$) signalling pathways such that they were upregulated in BPS and downregulated in WEX.
4.2 Materials and methods

4.2.1 Study design
Tissues that were analysed in this paper were collected simultaneously with those reported in Sontam et al. (2015) (336). All animal work was performed under approval R1068 (Animal Ethics Committee, University of Auckland). Twenty-four weanling (21 day old) male pups were obtained from four time-mated Sprague-Dawley rats and assigned to three exercise groups (n = 8): control (CON), bipedal stance (BPS), and wheel exercise (WEX) as previously described [Sontam et al. 2015 (336)]. Briefly, body composition of the animals was determined before the start of exercise, on day 20 (D20) using dual energy X-ray absorptiometry (DXA, Lunar Hologic, GE, Waltham, MA). The animals were assigned to one of the three exercise groups such that the body weight and total fat percentage were not significantly different between the groups at the outset of the experimental protocol. All animals had ad libitum access to food (Diet 2018, Teklad Global 18% Protein Rodent Diet, Harlan, Teklad) and water. All groups were pair-housed. CON group rats had unrestricted movement within their cage. BPS animals had to fully extend their tibiotarsal and femorotibial joints to reach their food which was 220 mm above the cage floor. WEX rats could voluntarily access a zero-resistance activity wheel. Food and water intake was measured daily from D20–27 and on alternative days from D27–35. The trial period lasted from D21 to D35. A further DXA scan was undertaken at the end of the exercise period (D35).

4.2.2 Tissue collection
On D36, animals were culled by decapitation under isoflurane anaesthesia following an overnight fast. Epididymal and retroperitoneal fat pads were collected and weighed. The left femur was immediately dissected from the surrounding soft tissue and was sectioned into proximal, middle, and distal thirds, which were snap-frozen in liquid nitrogen and stored at -80°C; the right femur was harvested, cleaned, and stored in 70% ethanol for micro-computed tomography (µCT) analysis.

4.2.3 RNA extraction
From the mid-femoral section, total RNA was extracted according to Ayturk et al. (2013) (288). Briefly, bone marrow was removed from the mid-diaphysis by centrifugation (10,000 rpm, 2 min, 4°C). The bone was then crushed into a fine powder and transferred to a 1.5 mL sterile microcentrifuge tube containing 1 mL of TRIzol® Reagent (#15596-026, Life Technologies, Carlsbad, CA), and homogenised by sonication (Bandelin Sonopuls HD2070, Bandelin, Berlin, Germany). Total RNA was extracted using TRIzol®-chloroform extraction and cleaned using the RNeasy Mini Kit (#74104, Qiagen, Hilden, Germany). Traces of genomic DNA were removed by on-column DNA digestion (RNase-free DNase Set; Qiagen). RNA quality/quantity was measured using spectrophotometry (Nanodrop ND-1000, Thermo Scientific, Wilmington, DE) and Bioanalyser (Model 200, Agilent)
Technologies, Santa Clara, CA). All sample RNA integrity numbers (RIN) numbers ranged from 6.1 to 8.7 (Table 4.1). Purified RNA was stored at -80°C until sequencing (50 bp, paired end; BGI China). The sequence data have been deposited in NCBI’s Gene Expression Omnibus [GEO; (Edgar et al. 2002) (290)] and are accessible through GEO Series accession number GSE67787 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE67787).

4.2.4 Read alignment and differential gene expression analysis
Four biological replicates from CON and five biological replicates each from BPS and WEX groups were included in the differential gene expression analysis. Sequenced read quality was assessed [FastQC; (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/)]. Phred quality scores were >28 and trimming was not necessary. Sequenced reads were aligned to the rat transcriptome (reference sequence NCBI version Rnor_5.0) using TopHat (version 2.0.12) by providing the GTF file and parameters -r 200 -G (295). The concordant pair alignment rate was 92.6 - 93.9% with 7.0 - 8.9% multiple alignments and 1.2 - 1.7% discordant alignment rates. Differential gene expression was determined (Cufflinks version 2.2.1) (296). Cuffdiff was run with the parameters -u (multiread correction algorithm) and -b (bias detection and correction algorithm). Differentially expressed transcripts were subjected to term enrichment analysis using GO::TermFinder (http://go.princeton.edu/cgi-bin/GOTermFinder) (300). The P-value cut-off was set at 0.05 and gene lists queried against the Rat Genome Database (RGD).

4.2.5 Pathway analysis
Differentially expressed transcripts were loaded into Ingenuity Pathway Analysis (IPA Winter Release 2014, IPA, QIAGEN, Redwood City, www.qiagen.com/ingenuity). The dataset was filtered using the following attributes: P-value = 0.05; the user dataset was used as the reference set; and Rattus norvegicus was specified as the species. IPA identified pathways with significant enrichments and ranked them according to P-values derived from a right tail Fisher exact test.

4.3 Results

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Replicate</th>
<th>RIN</th>
<th>Total read (pairs)</th>
<th>Aligned pairs</th>
<th>Concordant pair alignment rate (%)</th>
<th>Multiple alignments (%)</th>
<th>Discordant alignments (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>AA11</td>
<td>7.9</td>
<td>16180254</td>
<td>15354030</td>
<td>93.2</td>
<td>8.6</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>AA22</td>
<td>8.2</td>
<td>21510189</td>
<td>20408251</td>
<td>93.3</td>
<td>8.6</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>AA33</td>
<td>8.7</td>
<td>19985990</td>
<td>19004887</td>
<td>93.7</td>
<td>8.6</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>AA44</td>
<td>7.1</td>
<td>18219920</td>
<td>17249629</td>
<td>92.9</td>
<td>7.6</td>
<td>1.8</td>
</tr>
<tr>
<td>BPS</td>
<td>BB11</td>
<td>7.7</td>
<td>26109589</td>
<td>24724072</td>
<td>93.2</td>
<td>8.3</td>
<td>1.6</td>
</tr>
<tr>
<td></td>
<td>BB22</td>
<td>8.3</td>
<td>22931245</td>
<td>21753771</td>
<td>93.3</td>
<td>8.3</td>
<td>1.6</td>
</tr>
</tbody>
</table>
The phenotypic characteristics of animals within the BPS, CON, and WEX groups have been previously described by our group (336). Briefly, we showed that the diaphysis of femurs within the WEX group had a significantly higher cortical cross-sectional thickness and percent closed porosity than the CON group (336). We showed that the effects of exercise on these changes were independent of caloric intake, body weight, or measures related to stress. The initial changes that we saw led us to hypothesize that RNA expression patterns may be altered in response to the different exercise modalities. Therefore, we undertook a retrospective analysis of the RNA profiles in the femurs of these animals.

(A) 617 genes responded solely to BPS exercise while 133 genes were differentially expressed only in WEX. 191 genes were differentially expressed both in BPS and WEX compared to the CON group. (B) Among the 191 genes that were differentially expressed both in BPS and WEX, 51 were upregulated and 51 were downregulated in both exercise groups compared to the control. The behavior of the remaining 89 genes that were differentially expressed in just one of the exercise groups is shown in Figure 4.1.

Figure 4.1: Venn diagram showing the distribution of genes that responded to BPS and WEX exercises.
depended on the exercise imposed. While they were upregulated in the BPS group, the same set of genes was downregulated in the WEX group. The upregulated gene list was enriched for terms associated with skeletal system development. The downregulated gene list was enriched for immune system processes and the remaining 89 genes were involved in muscle contraction. CON, Control; BPS, Bipedal Stance; WEX, Wheel Exercise
Table 4.2: GO::TermFinder results for (A) upregulated genes and (B) downregulated genes in response to WEX

(A)

<table>
<thead>
<tr>
<th>ID</th>
<th>Term</th>
<th>P-value</th>
<th>Annotated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0050673</td>
<td>epithelial cell proliferation</td>
<td>4.51E-04</td>
<td><em>Fap, Col8a2, Nfib, Tbx18, WNT5a, Eya1, Bmp2, Twist1, Tnmd, Col8a1</em></td>
</tr>
<tr>
<td>GO:0001649</td>
<td>osteoblast differentiation</td>
<td>8.37E-04</td>
<td><em>Cat, Myoc, Fzd1, Ostn, Gnas, Bmp2, Twist1, Gdf10</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>outflow tract morphogenesis</td>
<td>2.80E-03</td>
<td><em>WNT5a, Eya1, Sema3c, Twist1, Fzd1</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>regulation of osteoblast differentiation</td>
<td>3.47E-03</td>
<td><em>Gnas, Bmp2, Twist1, Fzd1, Gdf10, Ostn</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>regulation of ossification</td>
<td>5.15E-03</td>
<td><em>WNT5a, Gnas, Bmp2, Twist1, Fzd1, Gdf10, Ostn</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>skeletal system development</td>
<td>7.05E-03</td>
<td><em>Myoc, Nfib, Ostn, Gnas, WNT5a, Eya1, Bmp2, Twist1, Lrrc17, Hapln1</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>ossification</td>
<td>8.18E-03</td>
<td><em>Cat, Myoc, Fzd1, Ostn, Gnas, WNT5a, Bmp2, Twist1, Gdf10</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>negative regulation of canonical WNT signalling pathway</td>
<td>2.18E-02</td>
<td><em>WNT5a, Bmp2, Fzd1, Dkk3, Tbx18</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>tube development</td>
<td>2.25E-02</td>
<td><em>Cat, Sema3c, Fzd1, Nfib, Ephb4, Tbx18, WNT5a, Eya1, Ephb3, Bmp2, Twist1</em></td>
</tr>
<tr>
<td>GO:0010811</td>
<td>positive regulation of cell-substrate adhesion</td>
<td>2.41E-02</td>
<td><em>Ndnf, Myoc, Edil3, Col8a1, Ccl21</em></td>
</tr>
<tr>
<td>ID</td>
<td>Term</td>
<td>P-value</td>
<td>Annotated genes</td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------------</td>
<td>---------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GO:0006936</td>
<td>muscle contraction</td>
<td>4.74E-13</td>
<td>Myl2, Actc1, Tmod4, Actn3, Myom2, Tn, Stac3, Cacna1s, Tnnt1, Mybpc1, Tnnc1, Pgam2, Actn2, Tnnt1, Myl3, Nef1, Hspb6, Myh7, Cav3, Scn4b, Mybpc2, Rcsd1, Myom1, Mylk2, Ryr1</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>muscle system process</td>
<td>1.33E-12</td>
<td>Myl2, Actc1, Tmod4, Actn3, Myom2, Tn, Stac3, Cacna1s, Hrc, Tnnt1, Mybpc1, Tnnc1, Pgam2, Actn2, Tnnt1, Myl3, Nef1, Hspb6, Myh7, Trim72, Cav3, Scn4b, Mybpc2, Rcsd1, Myom1, Mylk2, Ryr1</td>
</tr>
<tr>
<td>GO:0030218</td>
<td>erythrocyte differentiation</td>
<td>5.80E-09</td>
<td>Trim10, Gfi1b, Rhd, Bcl6, Dmnt, Ank1, Inpp5d, LOC287167, Tal1, Rhag, Alas2, Hmgb2, Isg15, Klj1, Ahsp</td>
</tr>
<tr>
<td>GO:0034101</td>
<td>erythrocyte homeostasis</td>
<td>2.31E-08</td>
<td>Trim10, Gfi1b, Rhd, Bcl6, Dmnt, Ank1, Inpp5d, LOC287167, Tal1, Rhag, Alas2, Hmgb2, Isg15, Klj1, Ahsp</td>
</tr>
<tr>
<td>GO:0061061</td>
<td>muscle structure development</td>
<td>5.07E-08</td>
<td>Jph2, Myl2, Actc1, Tmod4, Kel, Ankrd23, LOC100910104, Flnc, Tn, Pax5, Stac3, Cacna1s, Trdn, Srpk3, HistIh1b, Sntnl1, Tnni1, Tnnc1, Myl3, Ankrd2, Rbm38, Capn3, Myh7, Trim72, Cav3, Mynp, Mylk2, Ryr1, Ldb3</td>
</tr>
<tr>
<td>GO:0055002</td>
<td>striated muscle cell development</td>
<td>1.85E-07</td>
<td>Myl2, Actc1, Tmod4, Capn3, Kel, Cav3, Ankrd23, LOC100910104, Flnc, Tn, Mynp, Stac3, Cacna1s, Ryr1, Ldb3</td>
</tr>
<tr>
<td>GO:0051146</td>
<td>striated muscle cell differentiation</td>
<td>3.14E-07</td>
<td>Myl2, Actc1, Tmod4, Kel, Ankrd23, LOC100910104, Flnc, Tn, Stac3, Cacna1s, Trdn, Rbm38, Ankrd2, Capn3, Trim72, Cav3, Mynp, Ryr1, Ldb3</td>
</tr>
<tr>
<td>GO:0002262</td>
<td>myeloid cell homeostasis</td>
<td>3.35E-07</td>
<td>Trim10, Gfi1b, Rhd, Bcl6, Dmnt, Ank1, Inpp5d, LOC287167, Tal1, Rhag, Alas2, Hmgb2, Isg15, Klj1, Ahsp</td>
</tr>
<tr>
<td>GO:0030239</td>
<td>myofibril assembly</td>
<td>4.95E-07</td>
<td>Myl2, Actc1, Tmod4, Capn3, Cav3, Ankrd23, LOC100910104, Tn, Mynp, Ldb3</td>
</tr>
<tr>
<td>GO:0055001</td>
<td>muscle cell development</td>
<td>6.44E-07</td>
<td>Myl2, Actc1, Tmod4, Capn3, Kel, Cav3, Ankrd23, LOC100910104, Flnc, Tn, Mynp, Stac3, Cacna1s, Ryr1, Ldb3</td>
</tr>
</tbody>
</table>
### 4.3.1 Differential gene expression

#### Table 4.3: GO::TermFinder results for (A) upregulated genes and (B) downregulated genes in response to BPS

**(B)**

<table>
<thead>
<tr>
<th>ID</th>
<th>Term</th>
<th>P-value</th>
<th>Annotated Genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GO:0061061</td>
<td>Muscle structure development</td>
<td>2.05E-30</td>
<td>Ank2, Jph2, Myod1, Mamstr, Slc25a4, Flnc, Chrnb1, Cacna1s, Acadum, Neurl1, Myl3, Bin1, Sdhb, Klb41, Wnt5a, Sgcb, Ryr1, Lmod1, Nr4a1, Myh14, Meox2, Myh11, P2rx5, Lga5l1, Sfrk3, Trdn, Klb40, Jph1, Ppapdc3, Mylpf, Sgca, Xirp2, Rbp4, Lmod2, Myh7, Ankrd1, Bves, Mylk2, Popdc2, Emb3, Ky</td>
</tr>
<tr>
<td>GO:0003012</td>
<td>Muscle system process</td>
<td>2.53E-29</td>
<td>Ank2, Atplb1, Myl1, Myf2, Myod1, Myoc, Col14a1, Myl4, Map2k6, Actc1, Actn3, Tnn1, Slc25a4, Homer1, Chrnb1, Tn, Akap6, Acadum, Neurl1, Myl3, Myc, Hspa6, Tnn1, Cacna1s, Tnn2, Hspa6, Tnn1, Isp1, Scn4b, Act2, Ryr1, Tnn2, Chrnb, Tnn4, Lmod1, Lmod3, Myo2, Myh14, Myh11, Myh3, Lmod1, Stac3, Hr, Myh2, Actn2, Psg2, Pfy2, Mylpf, Tnn1, Sgca, Myh7, Cav3, Myog, Mb, Trm63, Mybpc2, Mylk2, Myo1, Casq2, Cmya5, Atpl2</td>
</tr>
<tr>
<td>GO:0006936</td>
<td>Muscle contraction</td>
<td>2.26E-24</td>
<td>Ank2, Atplb1, Myl1, Myf2, Myd1, Myl4, Map2k6, Actc1, Actn3, Homer1, Chrnb1, Tn, Akap6, Acadum, Neurl1, Myl3, Scn1b, Myh8, Myf5, Hspa6, Tnn1, Isp1, Scn4b, Act2, Ryr1, Tnn2, Chrnb, Tnn4, Lmod1, Lmod3, Myo2, Myh14, Myh11, Myh3, Stac3, Myh2, Actn2, Psg2, Pfy2, Mylpf, Tnn1, Sgca, Myh7, Mb, Trm63, Mybpc2, Mylk2, Myo1, Apol1a2</td>
</tr>
<tr>
<td>GO:0014706</td>
<td>Striated muscle tissue</td>
<td>3.89E-22</td>
<td>Jph2, Myf2, Myod1, Col14a1, Sema3c, Actc1, Sgsc, Scl25a4, Myf6, Smd1, LOC100910104, Homer1, Tn, Asb2, Akap6, Acadum, Neurl1, Tnn1, Vgl2, Tnn1, Myl3, Isga7, Sdha41, Wnt5a, Sgcb, Ryr1, Chrnb, Nr4a1, Myh14, Meox2, Myh11, S100b, Eyai, P2rx5, Nduf2, Stac3, Arnl, Sfrk3, Rxy, Khb40, Rhy4, Tbx1, Mylpf, Ankrd2, Xirp2, Cav3, Myh7, Eln, Ankr1, RGD1309821, Myog, Nbx, Vens, Mylk2, Popdc2</td>
</tr>
<tr>
<td>GO:0007517</td>
<td>Muscle organ development</td>
<td>5.09E-22</td>
<td>Jph2, Myf2, Myod1, Col14a1, Actc1, Isga7, Sgsc, Scl25a4, Myf6, Smd1, Homer1, Tn, Ass2, Akap6, Acadum, Neurl1, Tnn1, Vgl2, Tnn1, Myl3, Isga7, Trmn1, Sdha41, Wnt5a, Sgcb, Ryr1, Chrnb, Cmya5, Myf6, Ankrd2, Xirp2, Cav3, Myh7, Eln, Ankr1, RGD1309821, Myog, Mylk2, Ky</td>
</tr>
<tr>
<td>GO:0060537</td>
<td>Muscle tissue development</td>
<td>8.94E-22</td>
<td>Jph2, Myf2, Myod1, Col14a1, Sema3c, Actc1, Sgsc, Scl25a4, Myf6, Smd1, LOC100910104, Homer1, Tn, Asb2, Akap6, Acadum, Neurl1, Tnn1, Vgl2, Tnn1, Myl3, Isga7, Trmn1, Sdha41, Wnt5a, Sgcb, Ryr1, Chrnb, Cmya5, Myf6, Ankrd2, Xirp2, Cav3, Myh7, Eln, Ankr1, RGD1309821, Myog, Nbx, Vens, Mylk2, Popdc2</td>
</tr>
<tr>
<td>GO:005501</td>
<td>Muscle cell development</td>
<td>1.00E-18</td>
<td>Ank2, Myl2, Myod1, Col14a1, Actc1, Sgsc, Scl25a4, Myf6, Ankrd23, Flnc, LOC100910104, Homer1, Chrnb1, Tn, Akap6, Acadum, Klb41, Wnt5a, Sgcb, Ryr1, Lmod1, Klb41, Mb, Trm63, Mybpc2, Mylk2, Casq2, Nmyr2, Ldb3</td>
</tr>
<tr>
<td>GO:0042692</td>
<td>Muscle cell differentiation</td>
<td>2.25E-18</td>
<td>Ank2, Myl2, Myod1, Col14a1, Actc1, Mamstr, Sgsc, Csrp2, Scl25a4, Myf6, Ankrd23, Smd1, Flnc, LOC100910104, Homer1, Chrnb1, Tn, Ass2, Akap6, Acadum, Bin1, Trm63, Sdha41, Wnt5a, Sgcb, Ryr1, Rbm24, Tmn4, Lmod3, Lmod1, Myh11, Lgal1s, Stac3, Trdn, Khb40, Tbx1, Ppapdc3, Ankrd2, Casq3, Myo2, Ankrd1, RGD1309821, Myog, Cmya5, Isga8, Vens, Bves, Casq2, Popdc2, Ldb3</td>
</tr>
<tr>
<td>GO:0006091</td>
<td>Generation of precursor</td>
<td>3.04E-18</td>
<td>Sdhb, Grb10, Pgm1, LOC685596, Sucl2a, Pkm, Nduf2, Gpc1, Apl, Pdhb, Acadum, Cat, Prkag1, Pp1cb, Pflm, Chcl10, Fh, Sdhb, Nduf3, Coxib, Mxipl, Nduf5, Pkg2, Gys1, Sdhc, Nduf8, Idh2, Phka1, Etfb, Phkg1, Mdb1, Gylg1, RGD1230734, Nduf2, Phkb,</td>
</tr>
<tr>
<td>ID</td>
<td>Term</td>
<td>P-value</td>
<td>Annotated Genes</td>
</tr>
<tr>
<td>----------------</td>
<td>-----------------------------------------------</td>
<td>-----------</td>
<td>---------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>GO:0002376</td>
<td>Immune system process</td>
<td>5.27E-23</td>
<td>Hck, Oasl1b, Coro1a, Tbc1d10c, Siglec10, Il12rb1, Igam, Dhlx58, Olfm4, Prex1, Cd79b, Oasl, Hp, Blk, Unc13d, Csf3r, Syk, Bcl6, Rassf2, Irf7, Pik3cd, Spib, Jak3, Tf, Klh6, Gfi1, Spn, Inpp5d, Mcpt7, Myo1g, Siglec15, Mit1, Vpreb1, Cd19, Irf4</td>
</tr>
<tr>
<td>GO:0002682</td>
<td>Regulation of immune system process</td>
<td>2.51E-14</td>
<td>Pleg2, Sash3, Coro1a, Tgfr2, LOC100913063, Tbc1d10c, Isg15, Il12rb1, Sp1, C3, Dhx58, Olfm4, Cd74, Cd79b, Cxcl13, Irf7, Ptpn6, Ceacam1, Gsf, Bk, Jak3, Unc13d, Csf3r, Klh6, Syk, Gfi1, Spn, Bcl6, Rassf2, Irf7, Cfg2, Inpp5d, Sit1, Nckap1l, Mit1, Myo1g, Siglec15, Aim2, Cd19, Irf4</td>
</tr>
<tr>
<td>GO:0002520</td>
<td>Immune system development</td>
<td>6.56E-13</td>
<td>Pleg2, Sash3, Coro1a, Tgfr2, LOC100913063, Isg15, Siglec10, Sp1, Igam, C3, Dock2, Prex1, Cd74, Nbeal2, Cxcl13, Junb, Ptpn6, Ceacam1, Bk, Jak3, Spib, Tf, Csf3r, Syk, Spn, Bcl6, Rassf2, Irf7, Pik2b, Inpp5d, Ppt2f2, Nckap1l, Vpreb1, Siglec15, Irf4</td>
</tr>
<tr>
<td>GO:0048534</td>
<td>Hematopoietic or lymphoid organ development</td>
<td>8.85E-13</td>
<td>Pleg2, Sash3, Coro1a, Tgfr2, LOC100913063, Isg15, Siglec10, Sp1, Igam, Dock2, Prex1, Cd74, Nbeal2, Cxcl13, Junb, Ptpn6, Ceacam1, Bk, Jak3, Spib, Tf, Csf3r, Syk, Spn, Bcl6, Rassf2, Irf7, Pik2b, Inpp5d, Ppt2f2, Nckap1l, Vpreb1, Siglec15, Irf4</td>
</tr>
<tr>
<td>GO:0046649</td>
<td>Lymphocyte activation</td>
<td>1.21E-12</td>
<td>Pik3cd, Pleg2, Sash3, Blk, Jak3, Coro1a, Unc13d, Tfgr2, LOC100913063, Tbc1d10c, Syk, Hsh2d, Spn, Il12rb1, Sp1, Bcl6, Igam, Dock2, Pik2b, Inpp5d, Prex1, Cd74, Ppt2f2, Mit1, Nckap1l, Vpreb1, Ppt2n6, Irf4, Ceacam1</td>
</tr>
<tr>
<td>GO:0045321</td>
<td>Leukocyte activation</td>
<td>2.23E-12</td>
<td>Pik3cd, Pleg2, Sash3, Blk, Jak3, Coro1a, Unc13d, Tfgr2, LOC100913063, Tbc1d10c, Syk, Hsh2d, Spn, Il12rb1, Sp1, Bcl6, Igam, Dock2, Pik2b, Inpp5d, Prex1, Cd74, Ppt2f2, Mit1, Nckap1l, Vpreb1, Mit1, Ppt2n6, Irf4, Ceacam1</td>
</tr>
<tr>
<td>GO:0002252</td>
<td>Immune effector process</td>
<td>3.54E-12</td>
<td>Pleg2, Sash3, Oasl, Oasl1b, Jak3, Unc13d, Mx2, Isg15, Syk, Spn, Il12rb1, Bcl6, Dock2, C3, Pik2b, Dhlx58, Inpp5d, Cd74, Ppt2f2, Mxl1, Irf7, Mit1, Ppt2n6, Myo1g, Aim2, Irf4, Cfg2, Ceacam1</td>
</tr>
<tr>
<td>GO:0002521</td>
<td>Leukocyte differentiation</td>
<td>4.09E-12</td>
<td>Pleg2, Sash3, Blk, Spib, Jak3, Tf, Coro1a, Tgfr2, LOC100913063, Syk, Spn, Sp1, Rassf2, Bcl6, Igam, Dock2, Pik2b, Inpp5d, Prex1, Cd74, Junb, Ppt2f2, Nckap1l, Siglec15, Ppt2n6, Irf4</td>
</tr>
<tr>
<td>GO:0002684</td>
<td>Positive regulation of immune system process</td>
<td>5.20E-12</td>
<td>Pleg2, Sash3, Blk, Jak3, Coro1a, Unc13d, Tfgr2, LOC100913063, Klh6, Isg15, Syk, Gfi1, Spn, Il12rb1, Bcl6, C3, Pik2b, Dhlx58, Inpp5d, Cd74, Klh6, Cxcl13, Nckap1l, Irf7, Myo1g, Ppt2n6, Aim2, Irf4, Cd19, Ceacam1, Cfg2</td>
</tr>
</tbody>
</table>
There were 324 differentially expressed transcripts (DETs) in the mid-diaphysis of the WEX animals when compared to CON [false discovery rate (FDR) adjusted P-value < 0.05]. The gene transcripts that were upregulated in the WEX rats were enriched for Gene Ontology (GO) terms associated with skeletal system development and homeostasis (P-value ≤ 0.0218; Table 4.2A). These GO terms included genes that are involved in bone metabolism (e.g. *Bmp2*, *Cat*, *Dkk3*, *Ephp3*, *Epha4*, *Fap*, *Fzd1*, *Gdf10*, *Gnas*, *Lrc17*, *Myoc*, *Nfib*, *Ostn*, *Tbx18*, *Twist1*, and *WNT5a*). The gene transcripts that were downregulated in the mid-diaphysis of the WEX rats were enriched for GO terms associated with muscle and blood (P-value ≤ 6.44E-07; Table 4.2B).

There were 808 DETs in the mid-diaphysis of the BPS animals when compared to CON (FDR adjusted P-value < 0.05). The gene transcripts that were upregulated in the BPS group were associated with GO terms related to muscle cells and energy metabolism (P-value ≤ 5.10E-18; Table 4.3A). The gene transcripts that were downregulated in the BPS animals were enriched for GO terms associated with the immune system (P-value ≤ 5.20E-12; Table 4.3B).

There were 191 gene transcripts that were differentially regulated in both forms of exercise. Within this common set of gene transcripts, 51 were upregulated in both conditions and were enriched for GO terms associated with skeletal system development. Fifty-one transcripts were downregulated in both forms of exercise and were enriched for immune system processes. The remaining 89 gene transcripts were enriched for GO terms related to muscle contraction. The expression changes of these 89 gene transcripts were directionally dependent upon the exercise modality: specifically, these 89 transcripts were upregulated in the BPS and downregulated in the WEX group (Figure 4.1).

In order to determine if any of the common 191 differentially regulated transcripts were influenced by the magnitude of the exercise performed, the WEX animals were divided into two subgroups based on their individual total running distance within a monitored 36 hour period (D33). The mean (±SEM) distances run by the low (2130 ± 575.55 m) and high (3888.04 ± 825.9 m) running subgroups were significantly different (P-value: 0.04). Differential gene expression analysis between the high and low running subgroups identified six transcripts (*LOC100360843*, *Ankrd2*, *Myh7*, *Myl3*, *Moyz2*, and *Tnnt1*) that were significantly changed in the high, and two transcripts (*Fkbp5 and Ccl21*) that were significantly changed in the low running sub-groups.
### 4.3.2 Pathway analysis

**Table 4.4:** Canonical pathways enriched for differentially expressed transcripts in BPS and WEX rats

<table>
<thead>
<tr>
<th>No</th>
<th>BPS Canonical pathway</th>
<th>P-value</th>
<th>Ratio</th>
<th>BPS No</th>
<th>WEX Canonical pathway</th>
<th>P-value</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oxidative phosphorylation</td>
<td>1.66E-31</td>
<td>40/86 (0.465)</td>
<td>1</td>
<td>Epithelial adherens junction signalling</td>
<td>1.07E-04</td>
<td>10/136 (0.074)</td>
</tr>
<tr>
<td>2</td>
<td>Mitochondrial dysfunction</td>
<td>2.21E-25</td>
<td>44/144 (0.306)</td>
<td>2</td>
<td>Calcium signalling</td>
<td>1.24E-04</td>
<td>11/166 (0.066)</td>
</tr>
<tr>
<td>3</td>
<td>Calcium signalling</td>
<td>8.83E-10</td>
<td>28/166 (0.169)</td>
<td>3</td>
<td>ILK signalling</td>
<td>1.62E-04</td>
<td>11/171 (0.064)</td>
</tr>
<tr>
<td>4</td>
<td>Cellular effects of sildenafil</td>
<td>1.87E-18</td>
<td>22/123 (0.179)</td>
<td>4</td>
<td>Cellular effects of sildenafil</td>
<td>2.45E-04</td>
<td>9/123 (0.073)</td>
</tr>
<tr>
<td>5</td>
<td>ILK Signalling</td>
<td>3.27E-18</td>
<td>26/171 (0.152)</td>
<td>5</td>
<td>Remodelling of epithelial adherens junctions</td>
<td>5.67E-04</td>
<td>6/61 (0.098)</td>
</tr>
</tbody>
</table>

Enrichment was determined using IPA. The P-value was calculated using the Fisher’s exact test. The ratio gives an indication of the number of differentially expressed transcripts compared to the total number of genes in the dataset that correspond to a canonical pathway.

Ingenuity pathway analysis revealed that the WEX-affected transcripts were significantly enriched in pathways for epithelial adherens junction signalling, calcium signalling, integrin linked kinase (ILK) signalling, cellular effects of sildenafil, and remodelling of epithelial adherens junction signalling (Table 4.4). By contrast, BPS-affected transcripts were significantly enriched in calcium (Ca²⁺) signalling, ILK signalling, cellular effects of sildenafil, oxidative phosphorylation, and mitochondrial dysfunction pathways (Table 4.4).
Figure 4.2: Molecular complexes involved in the integrin-linked kinase pathway responded to BPS and WEX treatments.

(A) Schematic of the integrin-linked kinase signalling pathway. Complexes highlighted in blue responded to BPS exclusively. Complexes highlighted in yellow responded to WEX exclusively. Complexes highlighted in orange responded to both treatments. (B) Individual members of the complexes that responded to the different exercises are listed in the table. The direction of the arrow next to the genes indicates the upregulation (↑) or downregulation (↓) of the corresponding transcript.
Ca$^{2+}$, ILK and sildenafil pathways responded to both BPS and WEX. However, the genes that responded to BPS and WEX within these pathways exhibited exercise-dependent changes in transcript levels. For example, the transcript levels of nine genes in the ILK signalling pathway responded to both treatments (Figure 4.2). Of these, eight transcripts were upregulated in BPS and downregulated in WEX. Only one gene transcript (Actg2) was upregulated in both treatments (Figure 4.2).
Figure 4.3: Molecular complexes involved in the calcium signalling pathways responded to BPS and WEX treatments.

(A) Schematic of the calcium signalling pathway. Complexes highlighted in blue responded to BPS exclusively. Complexes highlighted in yellow responded to WEX exclusively. Complexes highlighted in orange responded to both treatments. (B) Individual members of the complexes that responded to the different exercise treatments are listed in the table. The direction of the arrow next to the genes indicates upregulation (↑) or downregulation (↓) of the corresponding transcript.
Transcripts associated with Ca\(^{2+}\) signalling pathways also exhibited exercise-dependent changes (Figure 4.3) for 10 genes. Again, these genes showed diametrically opposite responses to the exercise modalities being upregulated in BPS, and downregulated in WEX (Figure 4.3).

4.4 Discussion

We determined the \textit{in vivo} effects of two simple exercises on gene transcript levels within the femoral mid-diaphysis of prepubertal male rats on D\textsubscript{36} following 15 days of voluntary exercise. We used a 15 day regimen of voluntary mild exercise in prepubertal male rats because: (1) almost all literature concerning exercise in the young and its effect on adult musculoskeletal system phenotype deals with high exercise loads associated with competitive sport; (2) the regimen we implemented did not cause a stress response (336); (3) the 15 day period of exercise avoided the highly variable time of onset, effects, and endocrine status of animals becoming pubertal during the experiment; and (4) the use of male rats avoided the highly variable physical activity that is typical of female rats. The main limitation of our experiment was that it could not be established that such mild exercise would have phenotypic effects on bone and other tissues later in life. That gene transcript levels were so different between exercise groups was very notable.

Exercise induces a number of physiological responses within the body such as changes in blood flow and oxygenation (337), an endocrine response (338), and increased muscle and tendon forces (339) all of which, in concert, influence the overall response of bone to exercise (340). This is particularly relevant to our findings as the cortical bone of the mid-diaphysis is a complex heterogeneous tissue consisting of marrow, vasculature, epithelium, and nerves within an extracellular matrix that is mineralised to a variable degree. The changes we observed in transcript levels cannot be attributed to specific cell types within the bone. However, the data in the current study demonstrate some of the complex morphological, architectural, and gene expression changes that occur in bone in response to the mechanical loading associated with growth and physical activity.

Both BPS and WEX have previously been shown to elicit an osteogenic response in the rat (281,341). However, our results show significant modulation of transcript levels according to exercise modality. The two exercise modalities differed in their gene expression signatures with respect to: (1) the number of genes that responded; (2) the directionality of response (up- or downregulated); and (3) the magnitude of response. At the same time, there were changes
to common genes and signalling pathways. Crucially, the different exercise modalities had opposite effects on key components within the ILK and Ca\(^{2+}\) signalling pathways that are two prominent mechanotransduction pathways involved in bone’s adaptation to exercise (116,342). Therefore, while it remains possible that these signals are hormonal, the differences in transcript levels for mechano-responsive molecules (e.g. integrin β) indicate that the strain magnitude, strain frequency, and fluid shear forces may signal the exercise modality (343).

Both ILK and Ca\(^{2+}\) signalling pathways are known for their role in mechanotransduction (116,342). Integrins are heterodimeric transmembrane adhesion proteins that have the ability to relay signals between the extracellular matrix and the cytosol. Activation of the heterodimeric complex leads to its association with and reorganisation of the actin cytoskeleton. This reorganisation may influence osteoblastic gene expression through cytoskeletal-nucleoskeletal linkages (344). The actin cytoskeleton may also function as a scaffold for the translocation of signalling molecules from the membrane to the nucleus (344). Additionally, integrin activation leads to the modulation of activity of focal adhesion kinase (FAK) and ILK, which are involved in several signal transduction pathways in bone cells (342,345).

Ca\(^{2+}\) is a well-known second messenger in many cell types and plays a signalling role in bone metabolism. For example, in osteoclasts RANKL activation of RANK increases intracellular calcium ([Ca\(^{2+}\)]\(_i\)) which initiates downstream signalling processes that result in osteoclast differentiation (346). Increases in ([Ca\(^{2+}\)]\(_i\)) in response to fluid shear stress are vital for actin stress fibre formation and subsequent gene expression changes in osteoblasts (347). In osteocytes, increased ([Ca\(^{2+}\)]\(_i\)) is necessary for pulsating fluid flow-induced prostaglandin E2 (PGE2) release (348). Additionally, many of the transcripts that IPA identified to be part of sildenafil pathway play an integral role in calcium signalling. For example, the L-type and voltage-gated calcium channels, GNAS locus, and phospholipase C (PLC) are all necessary for calcium-mediated signalling in bone (349) and must, in the present context, be considered to be a part of the Ca\(^{2+}\) signalling pathways.

Genes traditionally associated with muscle development and function responded to one or both exercises. This is consistent with earlier studies that have shown downregulation of muscle genes within rat ulna in response to axial loading (153). Moreover, Paic et al. (2009) identified elevated expression of muscle-related transcripts in osteocytes compared to
osteoblasts (350). Therefore, it is possible that our observations reflect a change in the ratio of osteocytes relative to osteoblasts in response to BPS and WEX.

We hypothesized that mild to moderate exercise will elicit measurable changes in the gene expression profiles in the mid-diaphysis of prepubertal male rats whose bone micro-architecture and morphology showed minor changes in response to exercise. We identified and characterised several transcripts whose expression levels were affected by the exercise undertaken. The observation that the two exercise modalities elicited a diametrically opposite response for a subset of transcripts is particularly relevant given the perception that physical exercise promotes long and short-term bone health. Indeed, our results are consistent with earlier observations that the form and intensity of the exercise is critical for the bone-specific response that is elicited [e.g. Milgrom et al. (2000) (343)]. Direct comparisons regarding the activity type and activity cycles and therefore the strain magnitude and frequency required to elicit these changes are not currently possible, as there are no biomechanical models for these exercises in the rat. Moreover, determination of the actual exercise-dependent changes in the strain environment of bone cannot currently be empirically determined (340). We contend that future studies should characterise the exercise-dependent changes that occur at specific regions and to specific cells within bones, and at different stages of growth and development, in order that the full benefits of exercise can be harnessed to improve or maintain bone health.
Chapter 5. A memory of early life physical activity is retained in bone marrow of male rats fed a high-fat diet

5.1 Introduction

Childhood obesity induced by excess energy intake and reduced physical activity has reached epidemic levels worldwide (235). There is ample evidence that metabolic dysfunction induced by obesity in childhood persists into adulthood (351–354). Physical activity has long been considered as a non-pharmacological strategy to combat obesity and its associated co-morbidities (355,356).

Childhood and adolescence are periods of rapid bone growth and development. The attainment of optimal bone mass during early life is not only vital in reducing orthopaedic diseases such as fracture during a young age but also important for bone health in later life (313,357). The classically accepted opinion that obesity has a protective effect on bone due to the increased mechanical demand imposed by the excessive body weight on the skeleton has been challenged in recent times (241,244,358,359). Evidence from animal studies heavily favours the hypothesis that obesity may be detrimental to bone health during growth with several studies reporting an inverse relationship between excess adiposity and mechanical as well as micro-structural properties of bone (20–25). As such, it is vital to understand the effects of excessive adiposity on the growing skeleton since achieving optimal bone mass and strength during growth has biologically relevant effects on skeletal competence in both early and later life (243).

Mechanical loading in the form of physical activity and/or exercise has the ability to influence body composition and bone properties. Physical activity not only suppresses the accumulation of fat mass through increased energy expenditure, but also promotes an anti-inflammatory environment, thereby helping to reduce chronic low-grade inflammation associated with excess adiposity (27). In the bone marrow, the adipocytes and osteoblasts share a common mesenchymal progenitor whose lineage commitment can be influenced by environmental factors (223,272,360). Studies have reported opposing effects of high-fat (HF) diet and mechanical stimulation on lineage commitment of the bone marrow stem cells. For instance, an atherogenic HF diet reduced the ability of marrow stromal cells to differentiate into osteoblasts in a female mouse model (361). In contrast, application of low magnitude mechanical signals in mice resulted in a marrow environment that favoured osteogenesis.
(28). In addition, skeletal loading causes expression changes in genes involved in adipocyte and osteoblast differentiation and function (362,363). Previous gene expression studies in bone and/or marrow helped to understand the effects of HF diet or exercise separately (256,364,365). The question of how the bone marrow modulates its gene expression in response to mechanical loading in the presence of HF diet has not yet been addressed. Moreover, it is unclear if the beneficial effects of physical activity are retained or lost following cessation of the exercise program, with some studies reporting adverse outcomes in previously trained subjects compared to sedentary controls (149,366,367).

Here, we aimed to identify the gene expression changes associated with early life HF diet accompanied by voluntary physical activity. Using a young male Sprague-Dawley (SD) rat model, we measured the effects of HF diet (45% Kcal from fat) and voluntary wheel running, before and after puberty, on body composition, bone mass indices and gene expression in the marrow. We also determined if the diet and/or physical activity dependent changes in gene expression were retained after cessation of the wheel running and resumption of normal activity within the cage.

5.2 Methods

5.2.1 Study design

All animal work was approved by the University of Auckland Animal Ethics Committee (AEC001432).

Eighty male SD rats born from SD mothers were randomised into two dietary intervention groups controlling for their body weights and litter of origin, to either chow-fed [18% of total calories from fat, Diet 2018, Teklad Global 18% Protein Rodent Diet, Harlan Teklad, USA, and allowed only spontaneous movement within their standard cages (C-SED) (n=20)], or HF-fed groups (45% of total energy intake from fat, D12451, Research Diets, USA) (n=60). The rats were 21-23 (D21-23) days old at the start of the experiment. For ease of annotation, from here on, the age of the rats at weaning is denoted as D21. Rats were kept in pairs throughout the study in order to minimise stress induced by cage-mate separation (368). All animals had ad libitum access to food and water and were housed in a temperature controlled room (25°C) with a 12 hour light/dark cycle. Food and water intake was recorded at regular intervals.

The HF-fed group was further divided into three sub-groups. The high-fat sedentary (HF-SED, n=20) and high-fat late exercise (HF-LEX, n=20) groups which had only cage activity
up to \(D_{60}\). The high-fat early exercise group (HF-EEX, \(n=20\)) had access to a running wheel set at zero resistance from \(D_{22}\) to \(D_{60}\) (Model 80859, Lafayette Instrument, Lafayette, IN, USA). Wheel exercise data was recorded at 15 minute intervals using dedicated monitoring software (Model 86065). Following the early exercise period, five pairs of rats from each group were culled between \(D_{60}\) and \(D_{62}\). Between \(D_{67}\) and \(D_{120}\), the remainder of the HF-EEX group were allowed free movement within their cage. The HF-LEX group had access to a running wheel from \(D_{67}\) to \(D_{120}\). At the end of the late exercise period, animals were fasted overnight, anaesthetised using sodium petabarbitone (60mg/kg, IP) and culled by decapitation.

5.2.2 Body composition
In the week preceding the culls, with animals under light isoflurane anaesthesia, body composition was determined using dual energy x-ray absorptiometry and dedicated small animal software (DXA, Lunar Hologic, GE, Waltham, MA, USA).

5.2.3 Bone and marrow collection
A sterile saw was used to partially saw the left femur at the junction of: (1) the distal and middle thirds; and (2) the middle and proximal thirds. The bone was then snapped to obtain the mid-diaphysis. Bone marrow was obtained from the diaphyseal bone by centrifugation (10,000 rpm, 4°C, 30s). The marrow was immediately snap frozen in liquid nitrogen and stored (-80°C) until analysis.

5.2.4 Biochemical analyses
Fasting blood glucose concentration was determined from a tail blood sample with a glucose meter (Precision Xtra, Abbott, USA). Plasma insulin and leptin concentrations were determined using rat-specific commercial ELISAs (90060 and 90040, respectively, Crystal Chem, Downers Grove, IL, USA) according to the manufacturer’s protocol. Plasma lipoprotein, cholesterol and triglyceride concentrations were determined using a Hitachi 902 autoanalyser (Roche Diagnostics, Basel, Switzerland). Plasma cytokine concentrations were measured using BD™ cytometric bead arrays (BD Biosciences, Haryana, India) according to the manufacturer’s protocol.

5.2.5 Adipocyte area in the bone marrow
Wax-embedded sections of the proximal tibial trabecular bone immediately subjacent to the subchondral bone were obtained from serial sections processed for study of joint cartilages published elsewhere (287). The imaged region of marrow was located at the anterior margin.
of the tibia, at the inferior portion of, or distally adjacent, to the trabecular bone. Images were opened using ImageJ software (version 1.51f) and calibrated to convert pixels into distance (µm). Images were thresholded to black and white (thresholding range between 228 and 255) and converted into binary images. The ‘erode’ and ‘fill gaps’ options were used to identify adipocytes. Ten adipocytes per rat (100 adipocytes per group) were selected at random and the area of each cell was measured using the ‘measure’ function.

5.2.6 Peripheral quantitative computed tomography (pQCT)
Femur length was measured from the most distal aspect of the lateral femoral condyle to the proximal extent of the trochanter major using a sliding calliper. The bone was lodged in a plastic tube filled with saline, taking care that no air bubbles were present, and secured in the pQCT machine (XCT Research SA+ pQCT machine, StratecMedizinTechnik, Pforzheim, Germany). After placing the reference line at the distal aspect of the condyles in the scout view, the machine was programmed to make one scan at 50% of the femoral length, voxel size 70 µm. Outcome measures were chosen to determine cortical bone density and bone architecture, as previously described (286).

5.2.7 RNA extraction
Total RNA was extracted from the bone marrow using a protocol modified from Ayturk et al. (2013) (288). Briefly, bone marrow was ground into a fine powder using a mortar and pestle cooled in liquid nitrogen. The frozen bone marrow powder was transferred to a sterile microcentrifuge tube containing 1mL TRIzol® (#15596-026, Life Technologies, Carlsbad, CA, USA) and mixed thoroughly by shaking. The sample was homogenised using sonication (Bandelin Sonopuls HD2070, Bandelin, Berlin Germany). RNA was extracted using TRIzol®-chloroform extraction and purified using spin cartridges (RNeasy Mini Kit, #74104, Qiagen, Hilden, Germany). On-column DNA digestion was performed to remove traces of genomic DNA (RNase-free DNase Set, #79254, Qiagen, Hilden, Germany). RNA quantity was measured using a Qubit™ 3.0 fluorometer with the RNA High Sensitivity Assay Kit (#Q32855, ThermoFisher Scientific, Waltham, MA, USA). RNA quality was assessed using a Agilent Bioanalyser (Model 2100; Agilent Technologies, Santa Clara, CA, USA). The RNA integrity numbers (RIN) for the samples ranged from 6.1 – 8.0 (Table 5.1).
### Table 5.1: RNA quality and alignment summary of RNA-seq reads to the rat transcriptome

<table>
<thead>
<tr>
<th>Group</th>
<th>Rat ID</th>
<th>RIN</th>
<th>Total read pairs</th>
<th>Aligned pairs</th>
<th>Concordant pair alignment rate (%)</th>
<th>Multiple alignments (%)</th>
<th>Discordant alignments (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C-SED</td>
<td>15</td>
<td>6.7</td>
<td>16400160</td>
<td>12810745</td>
<td>77.5</td>
<td>5.7</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>63</td>
<td>7.4</td>
<td>20460740</td>
<td>16141151</td>
<td>78.0</td>
<td>5.7</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>64</td>
<td>6.1</td>
<td>15181769</td>
<td>11974157</td>
<td>78.3</td>
<td>5.3</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>72</td>
<td>7.0</td>
<td>15831916</td>
<td>12487443</td>
<td>77.9</td>
<td>5.4</td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>79</td>
<td>7.5</td>
<td>14986215</td>
<td>11786369</td>
<td>78.2</td>
<td>7.2</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>6.5</td>
<td>14338747</td>
<td>10739900</td>
<td>73.8</td>
<td>5.8</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>7.6</td>
<td>12765046</td>
<td>9865364</td>
<td>75.8</td>
<td>5.9</td>
<td>1.9</td>
</tr>
<tr>
<td>HF-SED</td>
<td>29</td>
<td>7.3</td>
<td>18040968</td>
<td>14970936</td>
<td>82.6</td>
<td>6.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>7.5</td>
<td>18422550</td>
<td>15214910</td>
<td>81.9</td>
<td>5.4</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>7.3</td>
<td>18670642</td>
<td>15378292</td>
<td>82.0</td>
<td>5.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>46</td>
<td>7.2</td>
<td>17610594</td>
<td>14513939</td>
<td>82.0</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>61</td>
<td>7.5</td>
<td>15608673</td>
<td>13063035</td>
<td>83.4</td>
<td>6.2</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>62</td>
<td>6.7</td>
<td>15187945</td>
<td>12248170</td>
<td>80.3</td>
<td>4.9</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>37</td>
<td>7.4</td>
<td>13591531</td>
<td>11106307</td>
<td>81.3</td>
<td>5.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>38</td>
<td>7.2</td>
<td>18130925</td>
<td>14889895</td>
<td>81.6</td>
<td>5.0</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>53</td>
<td>7.8</td>
<td>14588952</td>
<td>11473040</td>
<td>78.0</td>
<td>6.2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>54</td>
<td>7.4</td>
<td>15036020</td>
<td>11677801</td>
<td>76.5</td>
<td>5.6</td>
<td>1.6</td>
</tr>
<tr>
<td>HF-EEX</td>
<td>9</td>
<td>7.5</td>
<td>14168861</td>
<td>12795752</td>
<td>80.0</td>
<td>5.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>7.4</td>
<td>15620760</td>
<td>12522086</td>
<td>79.7</td>
<td>5.9</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>49</td>
<td>7.1</td>
<td>17080289</td>
<td>13826557</td>
<td>80.6</td>
<td>6.5</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>7.5</td>
<td>21754907</td>
<td>17633701</td>
<td>80.7</td>
<td>6.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>33</td>
<td>6.6</td>
<td>14106232</td>
<td>11267763</td>
<td>79.5</td>
<td>4.7</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>7.1</td>
<td>20328275</td>
<td>16263921</td>
<td>79.6</td>
<td>6.2</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>7.1</td>
<td>16126943</td>
<td>12738016</td>
<td>78.5</td>
<td>5.6</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>7.5</td>
<td>17324954</td>
<td>13802004</td>
<td>79.3</td>
<td>5.9</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>65</td>
<td>7.3</td>
<td>14388493</td>
<td>11457989</td>
<td>79.1</td>
<td>5.5</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>66</td>
<td>7.7</td>
<td>14104123</td>
<td>11284043</td>
<td>79.6</td>
<td>5.8</td>
<td>0.5</td>
</tr>
<tr>
<td>HF-LEX</td>
<td>3</td>
<td>6.9</td>
<td>12458551</td>
<td>10042081</td>
<td>80.2</td>
<td>6.1</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>7.4</td>
<td>13404783</td>
<td>10785675</td>
<td>80.1</td>
<td>5.6</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>7</td>
<td>18249588</td>
<td>14977785</td>
<td>81.7</td>
<td>5.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>44</td>
<td>7.5</td>
<td>16701631</td>
<td>13770993</td>
<td>82.0</td>
<td>5.9</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>67</td>
<td>7.5</td>
<td>18406507</td>
<td>15196744</td>
<td>82.2</td>
<td>6.0</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>68</td>
<td>6.9</td>
<td>17946935</td>
<td>14872165</td>
<td>82.5</td>
<td>5.3</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>51</td>
<td>7.3</td>
<td>17056287</td>
<td>13952239</td>
<td>81.0</td>
<td>5.4</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>52</td>
<td>7.2</td>
<td>16857159</td>
<td>13849895</td>
<td>81.8</td>
<td>5.7</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>75</td>
<td>7.2</td>
<td>20113702</td>
<td>16658376</td>
<td>82.4</td>
<td>5.8</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>76</td>
<td>7.4</td>
<td>16514911</td>
<td>13588472</td>
<td>81.5</td>
<td>6.2</td>
<td>0.9</td>
</tr>
</tbody>
</table>

Groups: C-SED, Chow + sedentary; HF-SED, High-fat diet + sedentary; HF-EEX, High-fat diet + early-exercise; HF-LEX, High-fat diet + late-exercise.
5.2.8 RNA-seq
For shipping, purified total RNA samples were mixed with RNAsable® in a 96-well plate (#90220-001, Biomatrica, San Diego, CA, USA) and dried according to manufacturer’s instructions. RNA samples were sequenced using an Illumina Hi-seq 4000 (150 bp paired end; Novogene, Beijing, China).

5.2.9 Sequence read processing, alignment and differential gene expression analysis
Sequencing reads were trimmed (phred score<30 discarded) using PRINSEQ (http://prinseq.sourceforge.net/). Resulting reads that were <50 bp in length were discarded before downstream analyses.

Processed reads were aligned to the rat reference genome (NCBI version Rnor_5.0) using TopHat (version 2.1.0) (Table 5.1) (295). Gene model annotations were provided in a GTF file. Differential gene expression was determined using Cuffdiff (Cufflinks version 2.2.1) (296). Ten biological replicates from HF-SED, HF-EEX and HF-LEX conditions and seven biological replicates from the C-SED condition were sequenced and used for differential gene expression analysis.

5.2.10 Pathway analysis
Pathway analysis was completed using the Ingenuity pathway analysis (IPA, Ingenuity Systems Inc., Redwood City, CA, USA) software package. IPA was used to identify the biological functions, physiological processes and diseases associated with the differentially expressed genes. IPA downstream effects analysis was employed to identify biological functions that were predicted to be up- or downregulated based on the observed gene expression changes. The software assigns an ‘activation z-score’ which was used to infer the activation states of the identified biological functions. A z-score of greater than 2 or smaller than -2 is the recommended value to determine the significance of the predicted affected biological functions (307). Furthermore, regulatory effects analysis was carried out to identify potential upstream regulators that may explain the observed gene expression changes in the dataset. Data was uploaded into IPA with the ingenuity knowledge base as the reference set and a P-value < 0.05.

5.2.11 Statistical analyses
Statistical analyses were carried out using SigmaPlot 13.0 (SysStat Software Inc., CA, USA). One-way analysis of variance (ANOVA) was used to determine statistically significant differences between the groups. The Holm-Sidak method was used for multiple comparison
testing. Where the data failed the equal variance test, ANOVA on Ranks followed by Tukey test for multiple comparisons was used to determine the between-group differences.

5.3 Results

5.3.1 Food intake

Figure 5.1: Food consumption during the experimental period
Kcal consumed per gram of body weight obtained by dividing the number of calories consumed per pair of rats per day by the average body weight of the rats. C-SED = Control+sedentary; HF-SED = High-fat diet+sedentary; HF-EEX = High-fat+early-exercise; HF-LEX = High-fat+late-exercise.
Figure 5.2: Body weights of the experimental groups


All data are presented as means ± S.E.M with an n=10 per group unless otherwise stated. The average daily food intake of the animals in all groups increased with their growth (Figure 5.1). Hyperphagia due to prolonged ingestion of a high-fat diet as reported by other groups was not observed in the study (369).
5.3.2 Wheel activity

Previous studies by our group (336) and others (321) observed a distinct circadian pattern in the wheel activity of young rats. Preliminary analysis of exercise data showed that the HF-EEX group used the wheel preferentially during the dark period (18:00 hours to 6:00 hours) with minimal activity during the day. For this reason, only dark period wheel exercise data was analysed to estimate the amount of exercise carried out by each rat pair in the HF-EEX and HF-LEX groups.

At the beginning of the early exercise period, the mean dark period distance ran by the HF-EEX group was $1988.59 \pm 347.05$ m cage$^{-1}$ night$^{-1}$. This distance gradually increased as the animals became accustomed to the wheel (Figure 5.3). At the end of the early exercise period, the mean dark period distance run was $9971.97 \pm 1933.34$ m cage$^{-1}$ night$^{-1}$. There was a large variation with respect to the distance run per pair. For example the pair that recorded the greatest distance (cage-1) on D$_{22-23}$ ran 14 times further than the pair that recorded the least distance (cage-6) (3460.6m vs. 240.53m). The difference between the greatest and least running pairs fluctuated over the course of the early exercise period. Although cage-1 pair started off with the greatest distance, their daily dark distance showed little variation as the experiment progressed ($2493.33 \pm 134.23$m). The pair in cage-1 also consistently recorded
the lowest distances during the exercise period. Meanwhile, rats in cage-8 frequently ran the greatest distances on 18 out of the 37 nights on which distances were recorded.

At the beginning of the late exercise period (D_{67}), the animals ran an average daily distance of 1377 ± 254 m. The highest distance was recorded on D_{94-96}, when the animals ran a mean daily distance of 9907 ± 2550 m cage^{-1} night^{-1} (Figure 5.2). The mean daily distance gradually showed a decline after this point and at the end of the exercise period (D_{115-120}), the mean daily distance ran by the rat pairs was 3254 ± 1169m cage^{-1} night^{-1} (Figure 5.2). While the variation within cages across the days remained fairly small, the variation between cages was large, similar to that observed for the HF-EEX group. For instance, there was ~8 fold difference between the rat pair that ran the greatest distance compared to that which ran the least (9991.16 ± 786.44 m vs. 1254.33 ± 55.16 m).

5.3.3 Effect of HF diet on body composition

Table 5.2 Body composition in experimental groups at D_{60} and D_{120}

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age</th>
<th>C-SED</th>
<th>HF-SED</th>
<th>HF-EEX</th>
<th>HF-LEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>D_{60}</td>
<td>289.90±5.98 b,d</td>
<td>320.70±6.28 a,d</td>
<td>305.80±10.21 d</td>
<td>361±9.05 a,b,c</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>560.6±17.3 b,c</td>
<td>704±25.82 a,d</td>
<td>688.10±22.71 a,d</td>
<td>528.20±16.81 b,c</td>
</tr>
<tr>
<td>Total fat %</td>
<td>D_{60}</td>
<td>15.67±0.97 b,d</td>
<td>24.32±1.42 a,c</td>
<td>18.65±1.68 b,d</td>
<td>28.65±1.75 a,c</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>28.93±1.99 b,c</td>
<td>51.32±2.86 a,d</td>
<td>49.67±1.56 a,d</td>
<td>30.59±3.14 b,c</td>
</tr>
<tr>
<td>Fat:Lean</td>
<td>D_{60}</td>
<td>0.19±0.01 b</td>
<td>0.33±0.03 a</td>
<td>0.23±0.03 d</td>
<td>0.41±0.04 a,c</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>0.42±0.04 b,c,d</td>
<td>1.12±0.12 a,d</td>
<td>1±0.06 a,d</td>
<td>0.47±0.08 b,c</td>
</tr>
<tr>
<td>Lean mass %</td>
<td>D_{60}</td>
<td>84.37±0.98 b,d</td>
<td>75.73±1.41 a,c</td>
<td>81.45±1.70 b,d</td>
<td>71.43±1.76 a,c</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>70.97±1.97 b,c</td>
<td>48.73±2.87 a,d</td>
<td>50.36±1.56 a,d</td>
<td>69.41±3.14 b,c</td>
</tr>
<tr>
<td>BMC/BW</td>
<td>D_{60}</td>
<td>0.0186±0.00 b,c,d</td>
<td>0.0200±0.00 a</td>
<td>0.0197±0.00 a</td>
<td>0.0199±0.00 a</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>0.0239±0.00 c,d</td>
<td>0.0251±0.00 a</td>
<td>0.0265±0.00 a</td>
<td>0.0259±0.00 a</td>
</tr>
<tr>
<td>BMD (g/cm²)</td>
<td>D_{60}</td>
<td>0.11±0.00 b,c,d</td>
<td>0.12±0.00 a,d</td>
<td>0.12±0.00 a,d</td>
<td>0.13±0.00 a,b,c</td>
</tr>
<tr>
<td></td>
<td>D_{120}</td>
<td>0.17±0.00 b,c</td>
<td>0.18±0.00 a</td>
<td>0.19±0.00 a,d</td>
<td>0.17±0.00 c</td>
</tr>
</tbody>
</table>

Values are mean±S.E.M. P<0.05 compared to *C-SED; *HF-SED; *HF-EEX, *HF-LEX. Groups: C-SED, Chow + sedentary; HF-SED, High-fat diet + sedentary; HF-EEX, High-fat diet+ early exercise; HF-LEX, High-fat diet + late exercise. Statistical analyses were performed using one-way ANOVA and Holm-Sidak method for multiple comparison.
At D_{60}, the body weight, total fat% and fat:lean ratio of the HF-SED and HF-LEX groups, both sedentary at this point and fed the HF diet, were significantly greater while the lean mass% was significantly lower than that of the C-SED group (Table 5.2). As there were no statistically significant differences between any of the experimental groups with respect to the amount of food consumed between D_{22} and D_{60}, the greater body weight, total fat% and fat:lean ratio can be attributed to the high-caloric content of the HF diet. A similar effect of HF diet was seen at D_{120} as HF-diet fed sedentary groups (HF-SED and HF-EEX) had greater body weight, higher total fat%, and lower lean mass% than the C-SED group.

5.3.4 Effect of physical activity in a HF diet environment
The HF-EEX group voluntarily exercised using a running wheel from D_{22} to D_{60}. Even though the animals were on a HF diet, the voluntary wheel activity had a significant effect on the body weight and body composition of the HF-EEX group (Table 5.2). The body weight and total fat% of the HF-EEX group was significantly lower and the lean mass% significantly greater than HF-SED and HF-LEX groups. Such differences were not observed between HF-EEX and C-SED groups. The HF-LEX group were allowed access to running wheels from D_{67} to D_{120}. At D_{120}, the body weight, total fat% and fat:lean ratio of HF-LEX group were significantly lower than HF-SED and HF-EEX groups while there was no difference between HF-LEX and C-SED groups. The lean mass% of the HF-LEX group was significantly higher than HF-SED and HF-EEX groups. The food intake of HF-LEX group was significantly lower than HF-SED (P<0.01) and HF-EEX (P<0.01) groups during the late exercise period (Figure 5.1). Thus the observed differences between sedentary (HF-SED and HF-EEX) and wheel running (HF-LEX) HF-diet fed groups may be attributed to voluntary physical activity combined with a lower caloric intake during the exercise period.

5.3.5 Effect of physical activity cessation on body composition
The HF-EEX group had access to wheel running from D_{22} to D_{60} after which they were transferred to standard cages in which they remained until D_{120}. The observed effect of wheel activity on body weight and body composition in HF-EEX at D_{60} was not retained 60 days after the cessation of wheel exercise. At D_{120}, the body weight, total fat%, lean mass% and fat:lean ratio of HF-EEX group resembled that of HF-SED group and were significantly different than the C-SED group (Table 5.2 and Figure 5.4).
Figure 5.4: Total fat and lean mass percentage is affected by current but not previous physical activity

(A) Total fat percentage at D60 (B) Total fat percentage D120 (C) Lean mass percentage at D60 (D) Lean mass percentage at D120. * = P<0.05. All parameters were analysed using one-way ANOVA followed by Holm-Sidak method for multiple comparison. SED, sedentary; EEX, early-exercise; LEX, late-exercise
5.3.6 Bone marrow adipocyte area

The HF-SED group had the largest mean adipocyte area and C-SED group the smallest (C-SED: 782.86 ± 54.89 µm²; HF-SED: 965.76 ± 93.04 µm²; HF-EEX: 783.03 ± 60.07 µm²; HF-LEX: 906.74 ± 34.01 µm²) (Figure 5.5). The adipocyte area of HF-EEX group was almost identical to that of the C-SED group. The difference in adipocyte area between the HF-EEX and HF-SED groups approached statistical significance (P-value = 0.054).

Figure 5.5: Mean marrow adipocyte area of the experimental groups at D120.
A. Representative images of bone marrow adipocytes in the experimental groups. B. Graphical representation of mean adipocyte area. There were no statistically significant differences between any of the experimental groups with respect to their bone marrow adipocyte area. However, the mean marrow adipocyte area of the HF-SED group was larger than C-SED, HF-EEX and HF-LEX groups. The adipocyte area of HF-EEX group was almost identical to that of C-SED group.
5.3.7 Imaging parameters

Table 5.3: Femoral cortical and trabecular bone properties in the experimental groups at the end of the late exercise period

<table>
<thead>
<tr>
<th>Diaphysis</th>
<th>C-SED</th>
<th>HF-SED</th>
<th>HF-EEX</th>
<th>HF-LEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total area, mm²</td>
<td>15.28 ± 0.57</td>
<td>15.53 ± 0.53</td>
<td>16.58 ± 0.27</td>
<td>15.52 ± 0.47</td>
</tr>
<tr>
<td>Cortical BMC, mg</td>
<td>11.51 ± 0.35</td>
<td>12.93 ± 0.26</td>
<td>13.45 ± 0.29</td>
<td>12.46 ± 0.34</td>
</tr>
<tr>
<td>Cortical (\text{BMD}, \text{mg/cm}^3)</td>
<td>1371.42 ± 3.34</td>
<td>1397.67 ± 5.14</td>
<td>1391.53 ± 3.78</td>
<td>1392.17 ± 4.73</td>
</tr>
<tr>
<td>Cortical area, mm²</td>
<td>8.40 ± 0.25</td>
<td>9.26 ± 0.21</td>
<td>9.66 ± 0.20</td>
<td>8.95 ± 0.25</td>
</tr>
<tr>
<td>Cortical thickness, mm</td>
<td>0.73 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.81 ± 0.01</td>
<td>0.78 ± 0.01</td>
</tr>
<tr>
<td>Periosteal circumference, mm</td>
<td>13.84 ± 0.25</td>
<td>13.95 ± 0.23</td>
<td>14.43 ± 0.12</td>
<td>13.95 ± 0.21</td>
</tr>
<tr>
<td>Endosteal circumference, mm</td>
<td>9.28 ± 0.22</td>
<td>8.85 ± 0.24</td>
<td>9.32 ± 0.07</td>
<td>9.07 ± 0.19</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Metaphysis</th>
<th>C-SED</th>
<th>HF-SED</th>
<th>HF-EEX</th>
<th>HF-LEX</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total BMC, mg</td>
<td>18.33±2.02</td>
<td>18.4±1.81</td>
<td>19.34±1.92</td>
<td>19.34±1.92</td>
</tr>
<tr>
<td>Total (\text{BMD}, \text{mg/cm}^3)</td>
<td>591.52±63.71</td>
<td>547.48±52.59</td>
<td>530.50±51.5</td>
<td>530.50±51.50</td>
</tr>
<tr>
<td>Total area, mm²</td>
<td>31.10±3.47</td>
<td>33.61±3.28</td>
<td>36.48±3.58</td>
<td>33.66±3.27</td>
</tr>
<tr>
<td>Trabecular area, mm²</td>
<td>16.50±2.04</td>
<td>19.54±1.93</td>
<td>22.15±2.24</td>
<td>19.04±1.92</td>
</tr>
<tr>
<td>Trabecular BMC, mg</td>
<td>6.03±0.74</td>
<td>6.05±0.63</td>
<td>7.32±0.75</td>
<td>6.29±0.64</td>
</tr>
<tr>
<td>Trabecular (\text{BMD}, \text{mg/cm}^3)</td>
<td>366.21±38.96</td>
<td>308.97±30.26</td>
<td>330.06±31.74</td>
<td>331.69±33.46</td>
</tr>
</tbody>
</table>

Results are mean ± SEM. Groups: C-SED, Chow + sedentary; HF-SED, High-fat diet + sedentary; HF-EEX, High-fat diet + early exercise; HF-LEX, High-fat diet + late exercise. Statistical analyses were performed using one-way ANOVA and Holm-Sidak method for multiple comparison. Superscript letter in any column shows P<0.05 significance with respectively vs aC-SED; bHF-SED; cHF-EEX; dHF-LEX. BMC, bone mineral content; \(\text{BMD}, \text{volumetric bone mineral density.}\)

In the diaphysis, there were no differences in total area, periosteal circumference and endosteal circumference between any of the groups (Table 5.3). All the HF diet-fed groups (HF-SED, HF-EEX and HF-LEX) had greater cortical thickness and \(\text{\(\text{\text{BMD}}\)}\) than the C-SED group (Table 5.3). In HF-SED and HF-EEX groups, cortical BMC was higher than C-SED (Table 5.3). Only HF-EEX has greater cortical area than C-SED whereas this difference was not observed in HF-SED and HF-LEX compared to C-SED.

In the metaphysis, there was no statistically significant difference in total BMC between any of the groups (Table 5.3). Total \(\text{\(\text{\text{BMD}}\)}\) and trabecular \(\text{\(\text{\text{BMD}}\)}\) of all three HF-fed groups
(HF-SED, HF-EEX and HF-LEX) was significantly lower than that in the C-SED group (Table 5.3). The trabecular bone area within the HF-SED and HF-EEX groups was significantly larger than that in the C-SED group. This difference in trabecular area was not seen in comparisons of the HF-LEX and C-SED groups. In addition, the trabecular area of HF-EEX was also greater than that observed for the HF-LEX group. Total area and trabecular BMC was significantly higher in HF-EEX group compared to C-SED group, and trabecular BMC of HF-EEX group was also higher than that in the HF-SED group.
5.3.8 The bone marrow gene expression of HF-SED group resembled a pro-adipogenic and pro-inflammatory profile.

RNA-seq revealed that 538 genes were differentially expressed in the HF-SED group when compared to the C-SED group. While 328 genes were upregulated, 210 genes were downregulated in the HF-SED group. IPA functional analysis revealed that genes involved in inflammation, metabolism and connective tissue disorders were significantly enriched in the gene expression dataset for the HF-SED group (Table 5.4). IPA downstream effects analysis found that ‘quantity of adipose tissue’ function was positively activated based on the direction of changes of the genes in the dataset (z-score: +1.815) (Table 5.5). Genes belonging to the ontology category ‘morphology of bone’ were also identified as significantly over-represented in the set of differentially expressed genes (Table 5.5).

Table 5.4: Biological functions, physiological processes and diseases that were over-represented by genes that were differentially regulated due to the high-fat diet

<table>
<thead>
<tr>
<th>Physiological system development and function</th>
<th>P-value range</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hematological system development and function</td>
<td>5.12E-04 - 2.88E-19</td>
<td>164</td>
</tr>
<tr>
<td>Immune cell trafficking</td>
<td>5.12E-04 - 2.88E-19</td>
<td>102</td>
</tr>
<tr>
<td>Tissue morphology</td>
<td>4.63E-04 - 3.02E-19</td>
<td>136</td>
</tr>
<tr>
<td>Lymphoid tissue structure and function</td>
<td>4.55E-04 - 1.81E-14</td>
<td>104</td>
</tr>
<tr>
<td>Humoral immune response</td>
<td>3.88E-04 - 2.82E-14</td>
<td>62</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diseases and biological function</th>
<th>P-value range</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inflammatory response</td>
<td>5.13E-04 - 8.25E-14</td>
<td>161</td>
</tr>
<tr>
<td>Immunological disease</td>
<td>5.23E-04 - 3.10E-12</td>
<td>121</td>
</tr>
<tr>
<td>Metabolic disease</td>
<td>4.63E-04 - 4.62E-12</td>
<td>89</td>
</tr>
<tr>
<td>Connective tissue disorders</td>
<td>4.63E-04 - 9.29E-12</td>
<td>87</td>
</tr>
<tr>
<td>Inflammatory disease</td>
<td>5.13E-04 - 9.29E-12</td>
<td>108</td>
</tr>
</tbody>
</table>
Table 5.5: Genes with functions in ‘quantity of adipose tissue’ and ‘morphology of bone’ were significantly enriched among the differentially expressed genes in HF-SED group compared to the C-SED group

<table>
<thead>
<tr>
<th>Biological function</th>
<th>Gene ID</th>
<th>Experimental log ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quantity of adipose tissue</td>
<td>Acta1</td>
<td>4.25</td>
</tr>
<tr>
<td></td>
<td>Adipoq</td>
<td>1.435</td>
</tr>
<tr>
<td></td>
<td>Angptl4</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>Apoe</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>Arntl</td>
<td>0.557</td>
</tr>
<tr>
<td></td>
<td>Cebpa</td>
<td>0.371</td>
</tr>
<tr>
<td></td>
<td>Cidec</td>
<td>1.553</td>
</tr>
<tr>
<td></td>
<td>Cxcl14</td>
<td>0.286</td>
</tr>
<tr>
<td></td>
<td>Dlk1</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td>Hdc</td>
<td>0.293</td>
</tr>
<tr>
<td></td>
<td>Hrh3</td>
<td>0.419</td>
</tr>
<tr>
<td></td>
<td>Hspa5</td>
<td>-0.359</td>
</tr>
<tr>
<td></td>
<td>Lpl</td>
<td>0.452</td>
</tr>
<tr>
<td></td>
<td>Pck1</td>
<td>1.914</td>
</tr>
<tr>
<td></td>
<td>Plin1</td>
<td>1.506</td>
</tr>
<tr>
<td></td>
<td>Plin2</td>
<td>0.397</td>
</tr>
<tr>
<td></td>
<td>Plvap</td>
<td>0.391</td>
</tr>
<tr>
<td></td>
<td>Ucp3</td>
<td>0.692</td>
</tr>
<tr>
<td></td>
<td>Adipoq</td>
<td>1.435</td>
</tr>
<tr>
<td></td>
<td>Apoe</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>Arntl</td>
<td>0.557</td>
</tr>
<tr>
<td></td>
<td>Btd</td>
<td>0.627</td>
</tr>
<tr>
<td></td>
<td>Ccr1</td>
<td>0.559</td>
</tr>
<tr>
<td></td>
<td>Cdo1</td>
<td>0.876</td>
</tr>
<tr>
<td></td>
<td>Col1a1</td>
<td>-0.352</td>
</tr>
<tr>
<td></td>
<td>Col1a2</td>
<td>-0.354</td>
</tr>
<tr>
<td></td>
<td>Ddr1</td>
<td>0.688</td>
</tr>
<tr>
<td></td>
<td>Dlk1</td>
<td>0.719</td>
</tr>
<tr>
<td></td>
<td>Eln</td>
<td>0.441</td>
</tr>
<tr>
<td></td>
<td>Evc2</td>
<td>-1.548</td>
</tr>
<tr>
<td></td>
<td>Fos</td>
<td>-0.648</td>
</tr>
<tr>
<td></td>
<td>H19</td>
<td>3.053</td>
</tr>
<tr>
<td></td>
<td>Hdc</td>
<td>0.293</td>
</tr>
<tr>
<td></td>
<td>Hbsp</td>
<td>-0.413</td>
</tr>
<tr>
<td></td>
<td>Igh3</td>
<td>-0.546</td>
</tr>
<tr>
<td></td>
<td>Kif3a</td>
<td>-0.536</td>
</tr>
<tr>
<td></td>
<td>Kif2</td>
<td>0.603</td>
</tr>
<tr>
<td></td>
<td>Lfn</td>
<td>0.294</td>
</tr>
<tr>
<td></td>
<td>Lmo4</td>
<td>0.289</td>
</tr>
<tr>
<td></td>
<td>Mgp</td>
<td>0.48</td>
</tr>
<tr>
<td></td>
<td>Mmp13</td>
<td>-0.71</td>
</tr>
<tr>
<td></td>
<td>Ndrg2</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>P2ry6</td>
<td>0.761</td>
</tr>
<tr>
<td></td>
<td>Pax5</td>
<td>0.413</td>
</tr>
<tr>
<td></td>
<td>Plxnd1</td>
<td>0.274</td>
</tr>
<tr>
<td></td>
<td>Ptprc</td>
<td>0.309</td>
</tr>
<tr>
<td></td>
<td>S contributed text here</td>
<td>-0.456</td>
</tr>
<tr>
<td></td>
<td>Smad6</td>
<td>0.814</td>
</tr>
<tr>
<td></td>
<td>Sox4</td>
<td>0.39</td>
</tr>
<tr>
<td></td>
<td>Tfeb</td>
<td>0.442</td>
</tr>
<tr>
<td></td>
<td>Thbs1</td>
<td>-0.533</td>
</tr>
<tr>
<td></td>
<td>Tyrobp</td>
<td>0.288</td>
</tr>
</tbody>
</table>
The expression of genes coding for adiponectin (Adipoq), actin alpha 1 (Acta1), Cell death inducing DFFA like effector C (Cidec), phosphoenolpyruvate carboxykinase (Pck1) and peripilin 1 (Plin1) was upregulated more than two-fold in the HF-SED group compared to the C-SED group. CIDEC (a.k.a FSP27) and PLIN1 are proteins found on the surface of lipid droplets. Lipid droplets are the main long-term energy storage organelles in mammalian adipocytes (370). CIDEC is primarily found in white adipocytes and plays an important role in the regulation of lipid droplet size and triglyceride storage (370). PLIN1 is highly expressed in adipose tissue and is required for optimal storage and lipolysis. PCK1 is a key regulatory enzyme that controls glyceroneogenesis in adipose tissue, a process through which glycerol-3-phosphate, required for the esterification of fatty acids is produced.

Adiponectin is one of the key adipokines secreted by adipose tissue. It has been shown to play a regulatory role in insulin sensitivity and energy homeostasis (371). Both in humans and rodents, Adipoq mRNA and circulating protein levels have been shown to be inversely associated with high-fat diet induced obesity (167,371). In addition, adiponectin has also been shown to influence bone metabolism (172,372,373). Adiponectin receptors, AdipoR1 and R2 are expressed both in bone forming osteoblasts and bone resorbing osteoclasts (372,373). Kajimura et al. (2013) reported that adiponectin has two opposing mechanisms through which it influences bone metabolism (172). While its peripheral action inhibits osteoblast proliferation and promotes osteoblast apoptosis, its second mode of action through the sympathetic nervous system increases bone mass. However, in contrast to previous reports which reported an inverse relationship between adiposity and Adipoq mRNA levels, we observed greater Adipoq gene expression in the HF-SED group compared to the C-SED group (Table 5.5). Another candidate with dual roles in lipid and bone metabolism whose gene expression in the marrow was modulated by HF diet was apolipoprotein E (APOE) (Table 5.5). APOE is a glycoprotein with a central role in lipid metabolism. It mediates the binding of lipoproteins in the plasma to cell-surface receptors and thereby facilitates the distribution of lipids among different organs in the body. It has recently been identified as a novel player in bone metabolism (374). Bachner et al. (1999) observed the upregulation of ApoE in response to Bmp-2 in a murine mesenchymal progenitor cell line (375). Niemeier et al. (2012) found that mice lacking ApoE exhibited a high bone mass phenotype due to an increased rate of bone formation (374).

Cysteine dioxygenase (CDO1), an enzyme essential for taurine biosynthesis, was also found to be upregulated in response to HF diet. CDO1, in addition to its enzymatic role, interacts
with PPARγ and promotes adipogenesis in MSCs of the bone marrow (376). Zhao et al. (2016) found that in murine bone marrow stem cells, over-expression of Cdo1 suppressed osteogenesis by inhibiting the expression of Runt-related transcription factor 2 (Runx2) and distal-less homeobox 5 (Dlx5), transcriptional factors essential for osteoblast differentiation (376). Delta-like non-canonical notch ligand 1 (Dlk1) was another gene with roles in both adipocyte and osteoblast formation that was upregulated in the HF-SED group compared to the C-SED group. DLK1 is also known as pre-adipocyte factor 1 (Pref1) as it was originally identified as an adipogenesis inhibitor in NIH 3T3-L1 preadipocyte cell line (377). Abdallah et al. (2001) identified DLK1 as a novel player in the regulation of bone mass (378). In mice over-expressing Dlk1, both trabecular and cortical bone volume fraction was lower compared to wild-type control mice. Not only did DLK1 inhibit osteoblastogenesis, it also promoted osteoclastogenesis and bone resorption indirectly through osteoblast-dependent increase of pro-inflammatory cytokine production (378).
Figure 5.6: A high-fat diet in early life upregulated genes that promote inflammation in the bone marrow

The regulatory network pictured consists of three tiers. The top tier consists of predicted upstream regulators that might explain the gene expression changes observed in the experiment. NLRP3 and IRF1 are the upstream regulators whose predicted activated state may explain the expression changes in LIG4, LTB, PARP1, IL1B, CCL5 and CTSS (middle tier). The observed upregulation of LIG4, LTB, IL1B, CCL5 and CTSS and the downregulation of PARP1 leads to a predicted increase in T cell homeostasis, activation of T lymphocytes and cell movement of monocytes (bottom tier).

Among the most significant regulatory networks identified by IPA, four were associated with inflammatory pathways (Table 5.4). ‘Activation of T-lymphocytes’, ‘T cell homeostasis’ and ‘cell movement of monocytes’ were all predicted to be upregulated in the bone marrow of HF-SED rats (Figure 5.6). The expression of DNA ligase 4 (Lig4), lymphotoxin beta (Ltb), interleukin 1β (IL-1β), C-C motif chemokine ligand 5 (Ccl5) and cathepsin S (Ctss) was upregulated while poly (ADP-ribose) polymerase 1 (Parp1) expression was downregulated in the HF-SED group compared to the C-SED group. LTB belongs to TNF superfamily of pro-inflammatory cytokines and is primarily expressed on T-cells (379). IL-1β, a major pro-inflammatory cytokine produced by macrophages and associated with obesity and reduced insulin sensitivity was upregulated in the HF-SED group. We observed almost a two fold increase in Ccl5 transcripts in HF-SED group. CCL5 binds to its G-protein coupled receptors CCR1, CCR3 and CCR5 and promotes recruitment of mononuclear cells to sites of inflammation (380). It has been shown to recruit...
macrophages and promote their survival in human adipose tissue (380). CTSS belongs to a family of cysteine proteases and has been reported as being elevated in response to obesity (381). Parp1 is a transcriptional cofactor. Parp1-KO mice were found to be susceptible to diet induced obesity, fat tissue accumulation, hyperleptinemia and insulin resistance compared to WT controls (382). In addition to their roles in inflammation, LTB, IL-1β and CCL5 have been shown to participate in bone metabolism. Osteoclasts show increased activity in the presence of LTB (188,383). IL-1β is a pro-resorptive cytokine that promotes osteoclast resorption both in vitro and in vivo (384). In addition to its role in adipose tissue inflammation, CCL5 is a direct target of non-canonical WNT signalling in osteoblasts (385). CCL5 has been shown to promote chemotaxis and survival of osteoblasts in vitro and Ccl5 KO mice were found to be osteopenic with increased osteoclastogenesis (385,386).

5.3.9 Voluntary physical activity and lower caloric intake promoted an anti-inflammatory environment in the marrow

Table 5.6: Biological functions, physiological processes and diseases that were over-represented by genes that were differentially expressed in high-fat late-exercise (HF-LEX) group compared to high-fat sedentary (HF-SED) group

<table>
<thead>
<tr>
<th>Physiological system development and function</th>
<th>P-value range</th>
<th>N°. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immune Cell Trafficking</td>
<td>6.36E-03 - 6.37E-12</td>
<td>42</td>
</tr>
<tr>
<td>Hematological System Development and Function</td>
<td>6.36E-03 - 3.50E-11</td>
<td>55</td>
</tr>
<tr>
<td>Organismal Functions</td>
<td>2.72E-03 - 2.65E-07</td>
<td>9</td>
</tr>
<tr>
<td>Cardiovascular System Development and Function</td>
<td>6.07E-03 - 7.00E-07</td>
<td>37</td>
</tr>
<tr>
<td>Connective Tissue Development and Function</td>
<td>5.74E-03 - 7.25E-06</td>
<td>30</td>
</tr>
</tbody>
</table>

**Diseases and biological function**

<table>
<thead>
<tr>
<th>Diseases and biological function</th>
<th>P-value range</th>
<th>N°. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunological Disease</td>
<td>4.70E-03 - 4.58E-13</td>
<td>50</td>
</tr>
<tr>
<td>Inflammatory Response</td>
<td>6.36E-03 - 1.07E-09</td>
<td>65</td>
</tr>
<tr>
<td>Connective Tissue Disorders</td>
<td>5.94E-03 - 1.75E-09</td>
<td>34</td>
</tr>
<tr>
<td>Inflammatory Disease</td>
<td>4.70E-03 - 1.75E-09</td>
<td>42</td>
</tr>
<tr>
<td>Skeletal and Muscular Disorders</td>
<td>6.47E-03 - 1.75E-09</td>
<td>48</td>
</tr>
</tbody>
</table>

In HF-LEX group, voluntary physical activity between D₆₇ and D₁₂₀ combined with lower food intake induced changes in the D₁₂₀ bone marrow gene expression. Hundred and seventy nine genes were differentially expressed in the D₁₂₀ bone marrow samples from HF-LEX animals when compared to HF-SED, with 76 genes upregulated due to the exercise, and 102 genes downregulated. Among the five most significantly affected biological functions and diseases identified by IPA, three were related to inflammation and related ontologies (immunological disease, inflammatory response and inflammatory disease) (Table 5.6). The most significantly affected regulatory networks also belonged to the ‘inflammation’ category. Vimentin (Vim), glial fibrillary acidic protein (Gfap), TNF receptor superfamily member 1B (Tnfrsf1b), versican (Vcan), carcinoembryonic antigen
related cell adhesion molecule 1 (*Ceacam1*), low density lipoprotein receptor (*Ldlr*), Interferon-γ receptor 2 (*Ifngr2*), C-C motif chemokine receptor 1 (*Ccr1*), galectin 1 (*lgals1*), colony stimulating factor 1 receptor (*Csf1r*), and CD 14 molecule (*Cd14*) were downregulated in response to late exercise and lower caloric intake while the expression of cytochrome c (*Cycs*) and solute carrier family 6 member 4 (*Slc6a4*) was upregulated (Figure 5.7).

The majority of genes in the regulatory network were downregulated in the HF-LEX group compared to HF-SED group (Figure 5.7). Thus, exercise reduced the expression of genes that were involved in inflammation within HF-LEX animals while HF-SED animals exhibited upregulation of pro-inflammatory genes.

**Figure 5.7: Physical activity in high-fat diet fed rats downregulated genes involved in inflammation**

The regulatory network that is illustrated consists of three tiers. The top tier consists of predicted upstream regulators that might explain the gene expression changes observed in the experiment. IL2 and IL10 are the upstream regulators whose predicted activated state may explain the altered regulation of the middle tier genes. The observed gene expression changes lead to a predicted decrease in ‘generation of cells’, ‘migration of phagocytes’, ‘activation’ and ‘differentiation of leukocytes’ (bottom tier).

In the HF-LEX group, wheel running combined with lower caloric intake between D_{67} and D_{120} resulted in the downregulation of genes involved in inflammation. In chronic low grade inflammation triggered by obesity, macrophages that infiltrate the adipose tissue are derived from the hematopoietic cells in the marrow and are dependent on macrophage colony stimulating factor (M-CSF) for their development and survival (183). CSF1R, the receptor through which M-CSF induces the differentiation of macrophages (387), was downregulated in the HF-LEX group. MIP-1α is a CC chemokine that has been implicated in adipose
inflammation through induction of monocyte/macrophage infiltration (388,389). We observed the downregulation of CCR1, a receptor that mediates the action of macrophage inflammatory protein (MIP-1α). CSF1R also mediates the effects of M-CSF in osteoclasts, the bone resorbing cells from the monocyte/macrophage lineage. M-CSF and RANKL have been shown to be essential and sufficient to induce osteoclastogenesis (50).

In addition to CSF1R, we also observed the downregulation of other genes that have various roles in osteoclast formation and function. RANKL-mediated induction of osteoclastogenesis requires a co-stimulatory immunoreceptor tyrosin-based activation motif (ITAM) pathway which is activated in response to ligation of osteoclast associated receptors such as OSCAR. We observed a downregulation in the expression of Oscar in HF-LEX group. Recent studies show that the complement system may enhance the inflammatory response of osteoblasts by promoting osteoclastogenesis (390,391). Ignatius et al. (2011) showed that in a pro-inflammatory environment, the complement C3 and C5 bind to their receptors C3AR and C5AR respectively on osteoblasts and promote the expression of RANKL and induce osteoclastogenesis (391). In our current study, the expression of C5aR1 was downregulated in HF-LEX group.

In addition to an anti-osteoclastogenic gene expression profile, we observed an upregulation of genes that confer an osteoprotective effect. Neuropilin 1 (NRP1), which, in association with semaphorin 3A (SEMA3A), inhibits RANKL mediated activation of osteoclasts, was upregulated in the HF-LEX group (392). Simultaneously, SEMA3A and NRP1 stimulate osteoblast differentiation through the canonical WNT/β-catenin signalling pathway (392). Wnt5b was observed to be downregulated in the HF-EEX group. While the canonical WNT/β-catenin pathway is essential for osteoblast formation, WNT5B has been shown to cause increased adipogenesis presumably by activating the non-canonical WNT pathway (393).
5.3.10 Bone marrow gene expression retained a memory of early-exercise

Table 5.7: Genes that were differentially expressed greater than or equal to two fold in high-fat early-exercise (HF-EEX) group compared to high-fat sedentary (HF-SED) group

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Experimental log ratio</th>
<th>FDR adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RT1-Ba</td>
<td>4.91205</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC257642</td>
<td>2.76297</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102553290</td>
<td>2.63363</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102553223</td>
<td>2.0183</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
<tr>
<td>Lyc2</td>
<td>1.52106</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Acta1</td>
<td>1.48393</td>
<td>0.0034366</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.44246</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC102555237</td>
<td>1.4395</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC310926</td>
<td>1.32289</td>
<td>0.0034366</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.21214</td>
<td>0.0416151</td>
</tr>
</tbody>
</table>

Table 5.8: Biological functions, physiological processes and diseases that were over-represented by genes that were differentially expressed in the high-fat early-exercise (HF-EEX) group compared to the high-fat sedentary (HF-SED) group

<table>
<thead>
<tr>
<th>Physiological system development and function</th>
<th>P-value range</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Connective tissue development and function</td>
<td>6.16E-03 - 1.64E-08</td>
<td>23</td>
</tr>
<tr>
<td>Tissue morphology</td>
<td>6.32E-03 - 1.64E-08</td>
<td>33</td>
</tr>
<tr>
<td>Hematological system development and function</td>
<td>6.40E-03 - 3.27E-07</td>
<td>29</td>
</tr>
<tr>
<td>Immune cell trafficking</td>
<td>6.26E-03 - 3.27E-07</td>
<td>24</td>
</tr>
<tr>
<td>Organismal development</td>
<td>6.12E-03 - 3.46E-07</td>
<td>32</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Diseases and biological function</th>
<th>P-value range</th>
<th>No. of genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolic disease</td>
<td>4.41E-03 - 2.23E-11</td>
<td>29</td>
</tr>
<tr>
<td>Endocrine system disorders</td>
<td>4.41E-03 - 1.65E-07</td>
<td>29</td>
</tr>
<tr>
<td>Immunological disease</td>
<td>6.25E-03 - 1.13E-06</td>
<td>30</td>
</tr>
<tr>
<td>Inflammatory response</td>
<td>6.26E-03 - 1.25E-06</td>
<td>32</td>
</tr>
<tr>
<td>Gastrointestinal disease</td>
<td>6.12E-03 - 1.66E-06</td>
<td>27</td>
</tr>
</tbody>
</table>
Figure 5.8: Bone marrow gene expression retained a memory of physical activity following 60 days of physical activity cessation

Aquaporin 7 (Aqp7), Glycerol-3-Phosphate Dehydrogenase 1 (Gpd1), perilipin 1 (Plin1), adiponectin (Adipoq), fatty acid binding protein 4 (Fabp4) and phosphoenolpyruvate carboxykinase (Pck1) are all genes expressed by mature adipocytes. They were downregulated in HF-EEX group compared to HF-SED group following 60 days of wheel exercise cessation.

Hundred and twenty eight genes were differentially regulated (48 up- and 80 downregulated) in the HF-EEX group compared to the HF-SED group. 14 genes showed two-fold or greater upregulation while 16 genes showed two-fold or greater downregulation. Genes with roles in adipogenesis and adipocyte function were downregulated and formed the only regulatory network that was significantly differently expressed between HF-EEX and HF-SED conditions (Figure 5.8). This regulatory network consisted of Plin1, Adipoq, Pck1, aquaporin 7 (Aqp7), glycerol-3-phosphate dehydrogenase 1 (Gpd1) and fatty acid binding protein 4 (Fabp4) which were all downregulated in HF-EEX group compared to HF-SED group at D120.

AQP7 is an aquaglyceroporin that facilitates the the transport of water and glycerol across the cell membrane (394). It is known to be involved in multiple aspects of adipocyte formation and function. In the 3T3-L1 pre-adipocyte cell line, expression of Aqp7 is negligible and markedly increases upon stimulation of adipocyte differentiation by PPARγ.
AQP7 also has a role in the biosynthesis of triglycerides and maintenance of fasting glucose levels. It is one of the main channels through which glycerol, a product of lipolysis, exits adipocytes. Deficiency of AQP7 leads to accumulation of glycerol which subsequently triggers triglyceride synthesis. As such, fasting increases the levels of AQP7 while refeeding suppresses its expression.

Fatty acid binding proteins are a family of lipid chaperone proteins that have been recently shown to co-ordinate lipid responses in cells and also participate in inflammatory processes. Fabp4 (a.k.a. aP2) is mainly expressed in adipocytes and macrophages. Like Aqp7, it is highly regulated during adipocyte differentiation and is subjected to transcriptional control by fatty acids, PPARγ and insulin. FABP4-deficient obese mice showed reduced hyperinsulinemia and insulin resistance while the insulin sensitivity effect was not observed in lean mice. In macrophages, FABP4 modulates inflammatory responses including production of inflammatory cytokines such as TNF-α, IL-1β, IL6 and MCP1. GPD1 is an enzyme that catalyzes the reaction needed for the production of glycerol-3-phosphate, a molecule crucial for triglyceride production. Swierczynski et al (2003) found that GPD1 activity was positively associated with obesity in humans. In obese human subjects, the levels of GPD1 were two fold higher compared to lean controls.

In addition to the above genes associated with adipocyte formation and function, we also observed the differential expression of genes with roles both in adipocyte and bone metabolism. Crucially, we observed the downregulation of Adipoq (log ratio: -1.58), Cdo1 (log ratio: -0.87) and Dlk1 (log ratio: -0.47) in the bone marrow of HF-EEX group compared to HF-SED group. The same genes were shown to be upregulated in HF-SED group. Thus, our observations indicate that the early-exercise in the HF-EEX group influenced the gene expression of HF-EEX group such that at D120, their gene expression profile was significantly different from that of the HF-SED group.


5.4 Discussion

In the current study, we show that an early life HF diet has the ability to affect body composition, bone micro-architecture and bone marrow gene expression in young male rats. The HF diet resulted in greater body weight and total fat percentage accompanied by upregulation of adipocyte and inflammatory genes in the bone marrow. The HF diet also resulted in a pro-resorptive environment in the bone marrow. Additionally, we observed that early life voluntary physical activity between D23 and D60 was sufficient to bring about significant changes in body composition of the HF-EEX group such that the total fat percentage, lean mass percentage and fat:lean ratio resembled that of the C-SED group at D60. Following 60 days of wheel exercise cessation, the changes in body composition induced by early-exercise were not retained in the HF-EEX group. However, the bone micro-architecture and gene expression profile in the HF-EEX group were distinctly different from that of the HF-SED group at D120. Thus, the bone and the marrow retained a memory of the early life voluntary exercise undertaken by the HF-EEX group.

A high-fat diet resulted in greater body weight, total fat percentage and lower lean mass percentage in HF-SED compared to the C-SED group. This is consistent with previous reports that used rodent models of HF-diet induced obesity (23,405,406). Adipocytes respond to excess energy intake by increasing both their cell number (hyperplasia) and cell size (hypertrophy). In the current study, HF-SED group had the largest mean marrow adipocyte area that was positively associated with their whole body composition. In the presence of a HF diet, genes involved in mature adipocyte function and lipid metabolism (Adipoq, Cidec, Pck1 and Plin1) were upregulated more than two-fold in the bone marrow of HF-SED group. Mature white adipocytes are characterised by the presence of a unilocular lipid droplet (407). Both PLIN1 and CIDEC are lipid droplet associated proteins that are predominantly expressed in adipose tissue (370). They control many aspects of lipid metabolism including regulation of lipid droplet size, triglyceride storage and lipolysis (370,407). Upregulation of Cidec and Plin1 gene expression in the bone marrow is consistent with the greater total fat percentage observed in the HF-SED group compared to the C-SED group. Upregulation of Cidec gene expression in response to a high-fat diet has been reported previously by other studies. Reynolds et al. (2015) found that feeding wild-type C57B6 mice a HF diet (60% of total energy from fat) led to a significant increase in Cidec mRNA levels in the epididymal adipose tissue (405).

In the current study, we observed an upregulation of Adipoq gene expression in response to HF diet. This is in contrast to previous reports that observed a reduction in Adipoq gene
expression in mice fed a HF diet (371,408). Bullen et al. (2006) found that in perigonadal white adipose tissue, the expression of Adipoq mRNA levels decreased in response to HF feeding in C57BL/6J mice (371). The bone marrow adipose tissue is distinctly different from white adipose tissue with respect to its origin, morphology and cellular composition (210–212). It also differs in its response to pathologies related to metabolic dysfunction (409). In Anorexia nervosa, a psychiatric condition that results in drastic weight loss and depletion of body fat, the bone marrow adipose tissue responds with increased levels of marrow fat. Cawthorn et al (2014) found that in mice subjected to caloric restriction, the mRNA and protein levels of Adipoq were significantly greater in bone marrow adipose tissue compared to that in white adipose tissue depots (217). Our observation indicates that the bone marrow adipose tissue may respond differently to HF diet in terms of adiponectin expression.

High-fat diet also resulted in a pro-inflammatory gene expression profile in the bone marrow. It is well established that obesity induced by excessive calorie consumption is associated with chronic low grade inflammation characterised by an increase in the production of pro-inflammatory cytokines (410). In the current study, the gene expression changes in the HF-SED group show that the bone marrow also developed a pro-inflammatory environment in response to a HF diet, similar to other adipose tissues depots in the body. We found that the expression of Il-1β, Ltb and Ccl5, inflammatory cytokines that also have roles in bone metabolism was upregulated in the HF-SED group compared to the C-SED group. IL-1β is known to stimulate bone resorption and inhibit bone formation as shown by both in vitro and in vivo studies (411,412). LTB is another pro-inflammatory cytokine with pro-resorptive properties that was upregulated in response to the HF diet. While IL-1β is more potent than LTB in stimulating bone resorption, LTB acts synergistically with TNF-α and IL-1β and may become a potent stimulator of bone resorption (413). Shu et al. (2014) found that in MSCs isolated from mice fed HF diet for six weeks, IL-1β mRNA levels were higher than the control group (255). This increase in IL-1β, along with the increase in the expression of other pro-resorptive cytokines such as TNF-α and RANKL was positively associated with increased osteoclast precursor formation and bone resorption activity.

In the current study, indices of bone mass including cortical BMC, cortical 𝑠BMD, trabecular area and trabecular 𝑠BMD were significantly higher in the HF-SED group than in the C-SED group. While our observations are in contrast to some previous reports that found an adverse effect of HF diet on bone traits (20,255,414), they are consistent with
other reports that showed a positive association between HF diet and indices of bone mass (415,416). Ma et al. (2010) found that in seven-week-old male C57BL/6J mice fed HF diet (60% Kcal from fat), the heavier body mass induced by the HF diet was positively associated with diaphyseal and metaphyseal bone measures in the femur (415). Lecka-Czernik et al. (2015) found that in 12-week-old C57BL/6 male mice fed HF diet for 11 weeks, the bone mass was higher than in the control group (416). However, the rate of bone formation was lower in the HF diet-fed group. The authors proposed a two-phase effect of high-fat diet which includes an early beneficial effect of diet induced bone mass acquisition, mediated by the increased mechanical loading and/or bone anabolic adipokine production. This initial phase is followed by a second phase where bone formation is decreased as a result of metabolic impairment. It is possible that a similar mechanism might be at play in the HF-SED group in the current study. The greater Adipoq transcript levels in HF-SED group point to the possibility of adiponectin mediated augmentation of bone mass indices. This, combined with osteoblast stimulating effects of CCL5 may explain the observed improvements in the femoral bone traits in HF-SED group.

An unanticipated confounder in the current study was the reduced caloric intake in the HF-LEX group between D67 and D120 compared to HF-SED and HF-EEX groups. The reduced caloric intake combined with wheel running could explain why the HF-LEX group was similar to C-SED group in terms of the body weight, total fat and lean mass percentages. However, the HF-LEX group had greater whole body BMC, cortical and trabecular \(\beta\)BMD, cortical thickness and total metaphyseal \(\beta\)BMD than the C-SED group. Critically, there were no statistically significant differences between HF-EEX and HF-SED groups with respect to any of the bone parameters measured. This is in contrast to some studies that reported a protective effect of exercise on bone microstructural properties in rats fed a HF diet (414). Cao et al. (2015) showed that when six-week-old SD rats fed HF diet (45% Kcal from fat) were subjected to forced treadmill training, the bone eroding effects of HF diet were partially countered by the exercise (414).

Even though we did not observe any phenotypic differences between the HF-LEX and HF-SED groups in their bone micro-architecture, the marrow gene expression profiles of the two groups were distinctly different. While HF diet triggered a pro-inflammatory gene response, voluntary wheel running combined with lower caloric intake between D67 and D120 resulted in downregulation of genes involved in inflammation. Some of these genes, such as Csf1r, Ccr1 and C5aR1 encode receptors that play a crucial role in promoting osteoclastogenesis. Conversely there was upregulation of Nrpl, which is osteoproteective
through its enhancing effect on osteoblast differentiation. As such, voluntary wheel running and lower caloric intake led to an anti-osteoclastogenic and pro-osteoblastogenic gene expression in the bone marrow of the HF-LEX group.

HF-SED and HF-EEX groups received identical treatments throughout the study except that HF-EEX group was allowed access to a running wheel between D23 and D60. This early-exercise by HF-EEX group affected the total fat and lean mass percentages such that they were more similar to the C-SED group than to the HF-SED group at D60 even though the animals had been fed a HF diet. In the 60 days after exercise cessation, the differences in the body composition diminished, with the HF-EEX group not being significantly different from the HF-SED group with respect to their total fat and lean mass percentages. Our observation is agreement with the principle of training reversibility according to which the physiological adaptations induced by physical training are partially or completely lost when the training is stopped or markedly reduced (417).

Yasari et al. (2007) reported an increase in the mesenteric and intra-abdominal fat mass in female SD rats trained for eight weeks on a treadmill and then untrained for four weeks with/without a HF diet (366). Sertie et al. (2012), found that six-week-old male Wistar rats that performed treadmill running for eight weeks followed by four weeks of detraining had greater peri-epididymal adipocyte cross-sectional area than rats that remained sedentary throughout the experimental period (418). In the peri-epididymal adipose tissue of the trained group, the expression of Dlk1, a suppressor of adipogenesis, was greater than in the sedentary controls. The expression levels of Dlk1 were lower in the detrained group indicating a withdrawal of the adipogenesis suppressing factor. In contrast, the gene expression of adiponectin, a marker of mature adipocytes, was greater in the detrained group compared to both the sedentary and trained groups.

However, in the current study, the marrow adipocytes in the HF-EEX group did not display the hypertrophy as was observed in the periepidermidial adipocytes of the detrained group in the study by Sertie and colleagues (367). Moreover, the adipocyte cross-sectional area of the HF-EEX group at D120 was identical to that of the C-SED group, but the gene expression of both Dlk1 and adiponectin in HF-EEX was lower than the HF-SED group. Dlk1, in addition to its adipogenesis-inhibiting properties, also has an important role in skeletal development and function (419). Abdallah et al. (2011) found that transgenic mice over-expressing Dlk1 (Col1-Dlk1) had lower body weight and bone mineral density (378), and lower trabecular and cortical bone volume fractions in the tibiae of five-month-old transgenic mice. We observed that the total body BMD and femoral trabecular BMC of HF-EEX group were
significantly greater than that of the HF-SED group at D_{120}. The downregulation of Dlk1 thus may have contributed, among other factors, to the observed differences in the bone properties of the HF-EEX group. It is well known that the bone marrow adipose tissue responds differently from other adipose depots in the body to conditions of severe nutrition deprivation. In patients with Anorexia nervosa, while fat mass in the rest of the body decreases, the marrow adipose tissue mass accumulates. Our observations indicate that the marrow fat may also have a distinct response to conditions of nutrient excess and exercise detraining.

Pre-pubertal exercise was associated with significant differential transcript levels for 128 genes when compared to HF-fed sedentary animals. The difference is notable, as these two groups of animals received identical diets and exercise regimes between D_{67} and D_{120}, and gene expression changes in responses begin hours not days or weeks after novel physical activity begins. This is consistent with a “programmed” and long-lived memory of the early-life voluntary exercise. “Exercise memory” has been shown to occur in muscle where the muscle tissue retains memory of previous strength training even after a long period of detraining (420). Bruusgaard et al. (2010) observed that in muscle cells, new myonuclei were added in response to overload exercise in mice (420). These new myonuclei were retained even after a long period of denervation. Marini et al. (2008) found that enhanced angiogenesis in the myocardium was partially preserved in rats that were trained on a treadmill for 10 weeks followed by four weeks of detraining (421). Future experiments that aim to investigate the cellular changes in the bone marrow may shed light on the observed gene expression changes in the HF-EEX animals. Additionally, changes in the cellular composition also could contribute to the observed changes (422).

A third possible mechanism that needs to be taken into consideration is the epigenetic changes that alters programmed gene expression in response to physical activity (423). Epigenetic alterations can change gene expression through changes in chromatin proteins that alter the accessibility of DNA sequence for transcription. The epigenetic changes induced are preserved during cell division and therefore allow for a cellular memory (424). It is well established that physical exercise has the ability to influence several body systems through epigenetic alterations to DNA and/or the histone proteins (425). Our observation that several genes that are predominantly expressed in mature adipocytes (Aqp7, Gpd1, Plin1, Adipoq, Fabp4 and Pck1) were downregulated in the HF-EEX group compared to the HF-SED group suggests that mechanical stimulation in the form of voluntary wheel running may have caused epigenetic alterations in the stem cells of the bone marrow such that their ability to differentiate into adipocytes was affected by the altered mechanical environment.
Mesenchymal stem cells in the bone marrow undergo changes in their DNA methylation when stimulated by mechanical signals (424). Kim et al. (2015) showed that the Adipoq promoter underwent hypermethylation in response to obesity which resulted in lower Adipoq expression in obese mice (426). However, to date, the ability of mechanical stimulation to influence the epigenetic profile of Adipoq and the subsequent gene expression changes has not been elucidated. Our observations indicate that Adipoq gene expression may be epigenetically modulated by mechanical stimulation. As such, investigating the epigenetic profile of MSCs in the bone marrow milieu subjected to mechanical stimulation will inform us of the potential epigenetic factors driving the response to early-exercise and subsequent exercise cessation we observed in the HF-EEX group.

The bone marrow stroma is host to numerous cell types with functions in haematopoiesis, immune system regulation bone and energy homeostasis. Although it is preferable to use homogenous cell population to investigate gene expression changes, whole bone marrow with a mixed cell population was used in the current study so as not to affect the mechanical environment of the cells. The possible mechanisms driving the memory of early exercise need to be investigated. Furthermore, it needs to be seen how the white adipose tissue in the body responds to the diet and exercise intervention used in the current study.
Chapter 6. **General discussion**

### 6.1 Principal findings

The results presented in Chapter 3 support the hypothesis that low-impact voluntary physical activity during the prepubertal period has the ability to alter body composition and femoral micro-architecture in the male rat (336). Many currently utilised protocols have the potential for data confounding due to the impact of stress, thus making it difficult to delineate the effects of exercise per se (e.g., involuntary protocols including forced swimming and use of shock plates on treadmills). In our studies, we employed two forms of voluntary exercise that have been shown not to induce a physiological stress response in rats (281,341): wheel exercise (WEX) and rising to an erect bipedal stance (BPS). Neither of the exercise modalities we used raised plasma corticosterone concentrations in the rats, indicating that the exercise modalities chosen were successful in not triggering a stress response.

We observed a distinct modality-dependent response to exercise, both with respect to body composition and bone micro-architecture in the rats. The WEX resulted in greater lean mass percentage compared to the CON group and lower epididymal and retroperitoneal fat pad weights compared to both the CON and the BPS groups. The WEX group had a significantly greater cortical cross-sectional thickness and closed porosity compared to the CON group. The BPS group, on the other hand, did not differ significantly with respect to body composition or cortical bone micro-architecture compared to the CON group.

Next, we set out to determine whether the subtle phenotypic changes that were observed in the diaphyseal bone of the WEX group were accompanied by gene expression changes (Chapter 4). As hypothesized, the gene expression profiles of the diaphyseal bone in the BPS and the WEX groups were distinct from the CON group. An unexpected yet crucial outcome of the experiments outlined in Chapter 4 was the observation that a subset of transcripts showed diametrically opposite response to BPS and WEX. Out of 191 transcripts that were differentially regulated by both BPS and WEX, 89 transcripts were upregulated in BPS and downregulated in WEX. This modality-distinct effect was also manifest in key components of ILK and Ca\(^{2+}\) signalling pathways; both prominent mechanotransduction pathways in bone (116,342).

The results in Chapter 5 support the hypothesis that there is a memory of early life physical activity in the bone marrow of HF diet-fed rats that undertook voluntary wheel running.
Since wheel running elicited a change in body composition and bone phenotype in our previous experiments involving young male rats (Chapter 3), this experimental approach was employed to investigate the effects of physical activity in a HF diet environment. Our results showed that early life HF diet positively affected the body weight and total fat percentage of the HF-SED group. In the bone marrow of the HF-SED group, adipocyte hypertrophy was accompanied by a pro-adipogenic gene expression profile. Indices of bone mass including cortical BMC and vBMD, and trabecular area and vBMD were significantly greater in the HF-SED group compared to the C-SED group, indicating that despite the pro-resorptive gene expression, the HF diet had a positive effect on bone mass indices. It needs to be investigated whether this initial bone-augmenting effect might later be dominated by bone resorption. While previous studies reported a decrease in the levels of Adipoq mRNA in WAT in response to a HF diet (371,408), in the bone marrow we observed greater levels of Adipoq mRNA in the HF-SED group compared to the C-SED group consistent with a bone marrow specific response to HF feeding.

Crucially, despite the identical treatment they received between D60 and D120, the bone marrow micro environment retained a memory of early exercise carried out by the HF-EEX group such that their gene expression and adipocyte morphology were distinctly different from that of the HF-SED group at D120. While other studies reported an increase in WAT depots in rats following training cessation (366,367), we observed lower marrow adipocyte area in the HF-EEX group compared to the HF-SED group 60 days after ceasing exercise. In addition, 128 genes were differentially expressed between the HF-EEX and the HF-SED groups. Genes associated with mature adipocyte function were downregulated in the HF-EEX group indicating a reduction in adipocyte formation and maturation. The molecular mechanisms responsible for the retained gene expression memory remain unexplored and require future investigations.

6.2 Discussion
The procedures and work presented in Chapter 3 were primarily focused on evaluating the ability of mild to moderate exercise to influence body composition and bone parameters in a prepubertal male rat model. The rats in BPS and WEX groups exercised from D21 to D35. Even in such rapidly growing animals, 15 days of exercise was sufficient to increase the lean mass, decrease localised fat pad mass and modify femoral cortical bone parameters. Yao et al. (2000) first employed BPS as an exercise model to study the effects of exercise on the musculoskeletal system of six- and 10-month-old orchidectomized rats (427), and observed an increase in muscle mass, cortical bone volume and periosteal bone formation in
the tibiae of the exercised animals. The bone anabolic effects of exercise were observed after four weeks of exercise in the proximal tibial metaphysis and after eight weeks in the tibial shaft. Rosa et al. (2010) successfully utilised BPS to induce bone modelling in the tibiae of 100-day-old female rats following merely 21 days of exercise (281). In our study involving prepubertal animals, BPS activity did not result in statistically significant changes in any of the body composition and bone µCT parameters measured. It is possible that the duration of exercise in the study was not sufficient for the activity to have resulted in a difference in body composition and bone morphometry. Moreover, while efforts were made to detect and quantify the number and duration of the squats performed by the BPS group, a technical failure of the electronic apparatus meant we were unable to do so. As such, it remains a possibility that the rats, instead of extending on their hind legs, clung to the sides of the cage for easier access to the food. Finally, the skeletal site measured was different in our study (femur) compared to previous BPS studies, which utilised the tibia (280,281,336,427).

In Chapter 3, plasma corticosterone concentrations were used to assess the effects of physical activity on the hypothalamic-pituitary-adrenal (HPA) axis of the rats in the experimental groups (CON, BPS and WEX). The HPA axis is extremely sensitive to environmental disturbances. Traditionally, plasma glucocorticoid concentrations have been used to assess an animal’s neuroendocrine response to stress (428). However, the acts of handling, restraining or collecting a blood sample from the tail vein of small laboratory rodents can significantly elevate the glucocorticoid concentrations (429). The glucocorticoid concentrations in the body display a distinct circadian rhythm, and a given blood sample represents the glucocorticoid concentration at only the moment of sampling (430). Additionally, repeated blood sampling is not recommended due to limited blood volumes in small rodents. Faecal corticoid measurement offers an effective, non-invasive way of measuring neuroendocrine response to stress. In feces, diurnal fluctuations are attenuated for the most part (430). Thus faecal corticoid concentrations are an aggregation of glucocortoids and their metabolites over a period of time. Previous studies have utilised this method to assess stress response in several species (281,429,431–434).

Thus, in Chapter 3, we sought to determine faecal corticosterone concentrations in the experimental groups. Although several groups have successfully extracted and analysed faecal glucocorticoids (281,429,432,435), our results showed that neither corticosterone nor its metabolites could be detected in any of the samples tested. It has been reported previously that faecal steroids undergo bacterial degradation (436,437). One possible reason
for the absence of detectable glucocorticoid metabolites in our samples may be bacterial
degradation during the extraction procedure. In future experiments that require the
measurement of faecal glucocorticoid metabolites, optimisation of sample collection,
storage, extraction and analysis needs to be done prior to employing the technique. Thus,
plasma corticosterone concentrations were used to determine the stress response for the rats
in Chapter 3.

There have been studies in the past that aimed to identify the gene expression pattern of
bone that has been subjected to loading. Mantila Roosa et al. (2011) investigated the time
sequence of gene expression changes in the ulnae of adult female Lewis rats subjected to
axial loading three minutes per day for up to four days (153). Xing et al. (2005) used four
point bending to evaluate differential gene expression in the tibiae of C57BL/6J mice (438).
Zaman et al. (2009) employed dynamic loading to investigate gene expression behaviour in
the tibiae of WT, oestrogen deficient and sciatic neurectomised mice (439). The gene
expression studies mentioned above mainly employed extrinsic exercise models. As
mentioned in Chapter 1, external loading models are helpful for applying relatively well-
defined mechanical signals to bone and have advanced our knowledge regarding several
loading parameters such as amplitude, frequency, partitioning.

However, in light of evidence that bone metabolism is influenced by whole body energy
metabolism and vice versa, it is imperative to understand the gene expression changes in
bone in the context of whole body physiology. Thus, in the study described in Chapter 3,
two forms of non-invasive intrinsic exercise models were employed to investigate the gene
expression changes in growing bone. To our knowledge, this is the first study that has
utilised the capabilities of next generation sequencing to explore the gene expression
landscape of bone in response to voluntary physical activity in prepubertal rats.

In Chapter 3, we reported the observation of differential expression of several genes with
well known roles in muscle cell development and function. The differential expression was
seen in response to both BPS and WEX activities (Tables 4.2 and 4.3). All muscle tissue
was stripped from the femoral diaphysis and bone marrow was removed by centrifugation
prior to RNA extraction. Thus the observed response of muscle-related genes is unlikely to
be explained by contamination. Moreover, muscle-related genes have been identified by
several groups in gene expression studies concerning bone metabolism. Mantila Roosa et al.
(2011) reported that muscle genes were the largest group of downregulated genes during the
matrix formation phase in rat ulnae following axial loading (153). The group consisted of
genes that encoded muscle structural components such as actin, myosin and troponin
families of proteins. Paic et al. (2009) noted that genes related to muscle function, development and differentiation were upregulated in osteocytes compared to osteoblasts (350). Twenty one of the 47 muscle genes reported by Paic et al. (2009) were observed to be differentially expressed in the BPS group compared to the CON group in the gene expression study in Chapter 3. Zaman et al. (2010) reported that in mouse tibia, titin (a protein involved in contraction of muscle cells) was one of the genes that was upregulated in response to loading (439). The results from our study along with previous published reports strongly indicate that the traditionally muscle-associated genes may have previously unidentified roles to play in bone metabolism.

Bone marrow has long been considered to be a passive, metabolically inert tissue that expands and occupies the bone marrow cavity as seen in ageing, osteoporosis, anorexia nervosa, diabetes and skeletal unloading (440). However, it has now emerged that marrow fat is distinct from white and brown adipose tissues with a unique lipid profile (210,211), progenitor cell population (207,208), mitochondrial content (209), and response to metabolic disease states (441). There is now evidence that adipocytes and osteoblasts in the bone marrow share a common progenitor cell (223,224) and that there is a hormonal link between bone and fat (158,442–444). As such, MAT has become the focus of studies that aim to understand the functional relationship between MAT and bone metabolism (441,445,446).

However, global gene expression studies which help elucidate the molecular mechanisms that precede the phenotypic changes in bone in response to high-fat diet are rare. Xiao et al. (2010) fed four-week-old C57BL/6 mice a HF diet for 12 weeks to identify the effects of HF diet on gene expression in the proximal femur (256). Their study identified several genes with roles in adipogenesis, fatty acid oxidation, IGF-1 signalling and osteoclastogenesis. The gene expression changes described in their study reflect changes in both bone and marrow as the RNA used for the microarray procedure was obtained from the proximal one-third of the femur which included marrow. By contrast, we aimed to isolate the gene expression changes exclusive to the marrow. We found that the expression of several genes with prominent roles in both adipocyte and bone metabolism was modulated by a HF diet.

In humans, hypoadiponectinemia is a feature of obesity, insulin resistance and type 2 diabetes (170,447). However, studies using diet induced obese rodent models found that the observed decrease in mRNA levels of adiponectin in the epididymal fat pads did not translate into a decrease in serum adiponectin concentrations (408,448). This points to: (1) a
possible post-transcriptional mechanism that controls circulating adiponectin levels; (2) adiponectin expression by other adipose depots in the body contributing to the circulating adiponectin concentrations. Marrow adipose tissue is a source of circulating adiponectin (449) and the expression patterns of adiponectin in MAT differ from other adipose tissues in the body. For example, caloric restriction is associated with reduced WAT mass but not a decrease in serum adiponectin concentrations (450–453). This apparent inconsistency is explained by adiponectin secretion from MAT being greater than WAT during caloric restriction (217). Crucially, inhibiting MAT formation resulted in a decrease in circulating adiponectin concentrations. Our observations support the premise that MAT can be a significant contributor to circulating adiponectin concentrations in HF diet-fed rodents. Specifically, we observed a ~3 fold upregulation of Adipoq expression levels in response to a HF diet. This increase has the potential to not only affect systemic energy metabolism by its contribution to circulating adiponectin concentrations but also may have implications for local bone metabolism (172). Studies show that adiponectin, through its peripheral actions, has an augmenting effect on bone mass through inhibition of bone resorption while promoting osteoblast formation and function (444,454). Our observations support the premise that MAT can be a significant contributor to circulating adiponectin concentrations in HF diet-fed rodents. Gene expression of adiponectin in WAT depots in the body and the circulating adiponectin concentrations need to be determined in order to further our understanding on the contributing role of MAT to systemic energy metabolism through its secretion of adiponectin.

In the study described in Chapter 5, several genes were downregulated in HF-EEX group compared to HF-SED group 60 days after exercise was ceased. This observation suggests that there may be possible epigenetic mechanisms at play that may be activated due to the altered cellular mechanical environment due to the exercise that was carried out by the HF-EEX groups between D_{22} and D_{60}. Exercise has been identified as one of the environmental factors that can alter the sub-cutaneous adipose tissue epigenome (455). Ronn et al. (2013) aimed to identify the differences in the adipose tissue DNA methylation pattern in 23 healthy men before and after six months of exercise intervention (455). Seventeen thousand nine hundred and seventy five CpG sites in 7663 genes showed differential methylation in response to exercise. Altered DNA methylation is one possible epigenetic mechanism that could explain the differential gene expression observed in the study. Aqp7, Gpd1, Plin1, Fabp4, Pck1 and Adipoq are all genes that are expressed predominantly in mature adipocytes and that were all downregulated in the HF-EEX group compared to the
HF-SED group. There is evidence in current literature that all the above genes may be subject to epigenetic regulation (456–460). In a study that evaluated the genome-wide DNA methylation profiles in the subcutaneous tissue of 106 men and women, Aqp7 DNA methylation was associated with adiposity phenotypes (457). Boque et al. (2013) found that an increase in Aqp7 mRNA levels was associated with different methylation patterns in the promoter region of Aqp7 in a study aimed to assess the ability of apple polyphenols to prevent diet induced obesity in Wistar rats (456). In HF-fed mice, the promoter of Pckl was found to be hypermethylated compared to that of low-fat-fed mice (461). A bioinformatic exploration of CpG islands in the promoter regions of obesity-related genes identified that Fabp4 promoter regions contained a high density of CpG islands (459). Functional enrichment analysis of differentially methylated regions between obese and lean pigs revealed that genes involved in lipid transport and localisation were enriched for regions that were hypermethylated in obese pigs (458). Plin1 was among the genes that contained the hypermethylated promoter sites. It remains to be seen whether the marrow adipocytes undergo epigenetic modification in response to changes in cellular mechanical environment.

6.3 Strengths

One of the strengths of the experimental studies presented in this thesis is the use of voluntary or encouraged mild-moderate physical activity in young rats. As mentioned earlier, forced forms of exercise such as swimming and treadmill running cause a physiological stress response through activation of the HPA axis in rodents (462). Stress has the ability to affect several body systems and must be carefully considered or avoided as a potential confounding factor when choosing an exercise model. Chronic stress can increase visceral adiposity, decrease lean body mass and suppress osteoblast activity (463). Moreover, glucocorticoids have a suppressive effect on immune and inflammatory responses and also contribute to the development of insulin resistance by inducing hepatic gluconeogenesis (15). As the central theme of this thesis involved the investigation of changes in body composition and bone metabolism due to physical activity, it was imperative to minimise the confounding effects of the HPA-induced stress responses to the chosen exercise model. As evidenced by results in Chapter 3, the adapted wheel and squat exercises did not trigger a detectable stress response in the animals used in the study. The use of high-intensity forced exercise models also makes it difficult to extrapolate the findings to a human population as it is not feasible to apply such forced physical activity regimens in human subjects. When physical activity is prescribed as a lifestyle intervention
to combat obesity, it is often moderate physical activity that is suggested and not the intense exercise used in some animal models of exercise.

In our ‘effects of prepubertal exercise’ study described in Chapters 3 and 4, we employed a short-term mild-moderate voluntary exercise model that started a day after weaning and ended before the rats reached puberty. Randomised controlled trials and intervention studies in humans have identified that the prepubertal period offers a window of opportunity during which dietary and physical activity interventions have the maximum effect on improving bone mass, density, geometry and strength (6,7,139,140,464–466). In light of evidence that bone mass gained during childhood may be an important determining factor of bone mass in later life (138,467,468) and as exercise has been shown to be successful in increasing bone mass during growth, studies have been carried out in both humans (312,469–471) and animals (14) to determine whether bone mass gain through exercise is preserved following exercise cessation. However, results from these studies have been equivocal with some reporting the retention of bone mass gains (14,312) while others (314,469–471) observed bone loss following exercise cessation. Critically, the exercise protocols in these studies, which aimed to explore the effects of deconditioning on bone growth in young animals, extended well into the pubertal period, and in humans have lasted years after puberty. A strength of our study described in Chapters 3 and 4 is that we have examined the short-term and long-term effects of exercise, where the duration of the activity protocol is restricted to the prepubertal period. As described in Chapter 3, the short-term exercise induced mild but significant changes in the cortical thickness and porosity in the group that undertook wheel exercise (Chapter 3, table 3.1 and figure 3.6).

Another strength of the work is the use of RNA-seq to determine the differences in the transcriptomes of the experimental groups (Chapters 4 and 5). Previous studies that aimed to determine gene expression changes in the bone due to mechanical loading and/or high-fat diet have mostly relied on microarrays or quantitative reverse transcriptase polymerase chain reaction (RT-qPCR) (152,153,155,256,364,472). Microarray-based approaches have several limitations associated with them which include: (1) reliance on existing knowledge about the genome sequence under investigation; (2) high background levels due to cross-hybridisation; (3) limited dynamic range of detection due to background and signal saturation; and (4) cross-comparisons between different experiments are difficult and involve complex normalisation procedures (473). RNA-seq has several advantages over microarrays: (1) it requires less RNA sample; (2) de novo transcripts can be discovered as the cDNA sequence is directly determined without relying on probes; (3) very low background signal as the sequences can be mapped to the genome unambiguously; (4) large
Another notable strength of the RNA-seq experiments described in Chapters 4 and 5 is the number of biological replicates included in the studies. In Chapter 4, four biological replicates for the control group and five replicates for each of the exercise groups were used for sequencing and differential gene expression analysis. In Chapter 5, seven biological replicates for the C-SED group and 10 replicates for each of the HF-fed groups were used for sequencing and differential gene expression analysis. In an RNA-seq experiment, the tradeoff between sequencing depth and the number of replicates is an important consideration. In order to achieve the maximum power for differential gene expression detection on a given budget, a compromise between sequencing depth and replicate number must be reached. Liu et al. (2014) reported that increasing the number of biological replicates at less depth is an effective strategy to increase the power and accuracy in large-scale RNA-seq studies. In an experiment designed to empirically address the question of whether to sequence more biological replicates with less depth or vice versa, the authors reported that the number of differentially expressed genes between E2-treated MCF7 cells and control cells increased by 6% when the sequencing depth was increased from 10M reads to 15M reads. Instead, when the number of biological replicates was increased from two to three, although the total number of reads for a given experimental group stayed the same, there was a 35% increase in the number of differentially expressed genes detected. An increase in sequencing depth and biological replication both can increase the statistical power of a sequencing experiment. However, while read depth increase gave diminishing returns on increasing statistical power, adding more biological replicates resulted in significant increase in statistical power. Moreover, biological replicates also improve the accuracy of expression level estimation for all genes while adding sequencing depth improves estimation accuracy for low expression genes. Thus the number of biological replicates along with the sequencing depth render the RNA-seq experiments described in this thesis robust with high statistical power.

6.4 Limitations and future directions

One of the limitations of the studies presented in this thesis is the use of only male animals due to resource constraints. This limitation means that sexually dimorphic responses to dietary and exercise protocols are missed. The rationale behind employing male SD rats exclusively was to eliminate the confounding effects of the oestrous cycle on the body composition and bone metabolism. Also, the earlier onset of puberty in female rats...
would have significantly shortened the duration of physical activity able to be undertaken in the prepubertal exercise study (Chapters 3 and 4) (479).

Sexually dimorphic response to exercise is not limited to the direct effects of the oestrous cycle on puberty and the duration of exercise before puberty. Indeed, there is evidence that running wheel behaviour in female rats is different from that of males (326,480–482) with gender-specific changes to food intake, weight gain and body composition in response to the introduction of a running wheel (326,327). Moreover, Lange et al. (2012) showed that in B(io) B(reeding) rats (BB/OK) fed a high-fat diet, the gene expression profile in bone exhibited a distinct gender dimorphic response (364). In their study, of the 53 genes studied between rats fed HF diet and rats fed standard chow, 20 genes were differentially regulated. However, only two of these 20 genes were common between males and females. Future experiments should thus incorporate the use of both male and female animals so that direct gender dimorphic responses to diet and exercise can be determined.

In Chapter 5, we report the identification of 128 genes that retained a memory of early exercise in a HF diet environment. Gene expression is not a simple product of linear DNA sequence but is affected by epigenetic mechanisms such as DNA methylation, histone modification (acetylation, methylation and phosphorylation) and the synthesis of non-coding RNAs (e.g., micro RNAs). Environmental factors such as diet and physical activity can induce gene expression changes through heritable yet potentially reversible epigenetic modifications (483). In fact, there is ample evidence that physical activity modifies the expression of genes in several body systems through epigenetic mechanisms (425). For instance, Barres et al. (2012) found that whole genome methylation was reduced in the skeletal muscle biopsies of both sedentary men and women after acute exercise in a dose-dependent manner (484). Hypomethylation of peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α), pyruvate dehydrogenase (PDK4) and PPARγ correlated with upregulation of the genes. Exercise has also been shown to induce epigenetic regulation of inflammatory processes (485), CNS (486) and affect aging process (487–489). Chen et al. (2015) showed that mechanical stimulation in the form of fluid shear stress decreased DNA methylation of both adipogenic and osteogenic markers in the C3H10T1/2 progenitor cell line (490). This was associated with an increase in the expression of late osteogenic markers. The observed gene expression changes in HF-EEX group compared to HF-SED group in our study may potentially be due to epigenetic alteration of gene expression. Analysis of DNA extracted from the marrow of the rats in the experimental
groups for epigenetic modifications would reveal if epigenetic modifications (e.g., CpG methylation) underlie the preserved gene expression changes observed in the study.

Previous studies investigating the consequences of exercise cessation on white adipocyte cellularity in rats found that detraining was associated with stimulation of adipocyte hypertrophy (366,367,491). Adipocyte cross-sectional area is routinely used as a measure of adipocyte hypertrophy (492,493). Sertie et al. (2013) found that in six-week-old Wistar rats trained on a treadmill for eight weeks and detrained for four weeks, the periepididymal adipocyte cross-sectional area was significantly larger than that in sedentary and in trained groups (367). Similar findings were reported by Kump et al. (2005) who sought to determine the effect of cessation of voluntary wheel running on the mass and cellularity of epididymal adipose tissue (491). In contrast, our results in Chapter 5 (figure 5.2) showed that the adipocyte area in the bone marrow of HF-EEX group was identical to that of C-SED group. Moreover, genes involved in adipocyte function were downregulated in the bone marrow of the HF-EEX group (compared to HF-SED group) at D_{120}, showing that the bone marrow adipocyte function was not the same to that in the HF-SED group. However, the total body fat percentage was not significantly different between the HF-EEX group and HF-SED group. These results prompt us to hypothesize that the adipose tissue in the bone marrow may differ in its response to physical activity cessation compared to WAT depots in the body. To address this hypothesis, it is imperative to determine the morphology and gene expression of WAT depots (e.g., epididymal and perirenal adipose tissues) in HF-EEX rats. This will help us to identify the differences, if any, between the white adipose and bone marrow adipose tissues with respect to adipocyte morphology, cellularity and gene expression. This would lead us a step closer to understanding the distinct nature of the two adipose tissue types, and the possible role in the relationship between whole body metabolism and bone metabolism and their responses to early life environmental influences.

6.5 Concluding comments

The results of the research presented in this thesis provide the first evidence that:

- Prepubertal short-term mild-moderate voluntary physical activity affects body composition, bone mass indices and cortical bone gene expression in a modality dependent manner;
- Early life HF diet influences body composition and bone mass;
- Early life HF diet results in a pro-inflammatory and pro-adipogenic gene expression profiles in the bone marrow;
- Voluntary physical activity modulates the effects of HF diet on body composition and bone mass;
- The effects of short-term voluntary physical activity are not retained when the activity is ceased;
- In the bone marrow, gene expression changes retain a memory of the early life physical activity 60 days after ceasing exercise.
Appendix I: Genes that were showed two-fold or greater differential expression in (A) HF-SED group compared to the C-SED group and (B) HF-LEX group compared to HF-SED group

(A)

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Experimental log ratio</th>
<th>FDR adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Pcp4l1</em></td>
<td>2.95294</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Myoz1</em></td>
<td>2.82382</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Thrsp</em></td>
<td>2.29544</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Tmnt3</em></td>
<td>2.27474</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Des</em></td>
<td>2.27303</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Ankrd23</em></td>
<td>2.24795</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Pck1</em></td>
<td>1.91433</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Mylpf</em></td>
<td>1.81328</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Car3</em></td>
<td>1.7058</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Pgum2</em></td>
<td>1.69895</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Tpsb2</em></td>
<td>1.68913</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Cidec</em></td>
<td>1.55295</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Fxyd2</em></td>
<td>1.5191</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC100364500</td>
<td>1.51049</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Plin1</em></td>
<td>1.50633</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Tpsab1</em></td>
<td>1.45991</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Adipoq</em></td>
<td>1.43484</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Eno3</em></td>
<td>1.40004</td>
<td>0.003437</td>
</tr>
<tr>
<td>S100b</td>
<td>1.39956</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Atpov1e2</em></td>
<td>1.3831</td>
<td>0.01471</td>
</tr>
<tr>
<td>LOC102548268</td>
<td>1.3123</td>
<td>0.003437</td>
</tr>
<tr>
<td>C4b</td>
<td>1.28286</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC102556004</td>
<td>1.28024</td>
<td>0.003437</td>
</tr>
<tr>
<td>Sod3</td>
<td>1.27367</td>
<td>0.003437</td>
</tr>
<tr>
<td><em>Aqp7</em></td>
<td>1.26826</td>
<td>0.003437</td>
</tr>
<tr>
<td>RT1-DOa</td>
<td>1.24062</td>
<td>0.003437</td>
</tr>
<tr>
<td>Calml3</td>
<td>1.22602</td>
<td>0.003437</td>
</tr>
<tr>
<td>Cd3g</td>
<td>1.20643</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC100359922</td>
<td>1.17789</td>
<td>0.003437</td>
</tr>
<tr>
<td>Cpa3</td>
<td>1.16239</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC102553282</td>
<td>1.13237</td>
<td>0.003437</td>
</tr>
<tr>
<td>Rmrp</td>
<td>1.12861</td>
<td>0.003437</td>
</tr>
<tr>
<td>Mclnt1I</td>
<td>1.1088</td>
<td>0.003437</td>
</tr>
<tr>
<td>Pdlim3</td>
<td>1.10081</td>
<td>0.038104</td>
</tr>
<tr>
<td>LOC102550063</td>
<td>1.09121</td>
<td>0.003437</td>
</tr>
<tr>
<td>Asb2</td>
<td>1.06334</td>
<td>0.003437</td>
</tr>
<tr>
<td>Stac</td>
<td>1.05898</td>
<td>0.0086</td>
</tr>
<tr>
<td>Sifa2II</td>
<td>1.05259</td>
<td>0.016439</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>Experimental log ratio</th>
<th>FDR adjusted P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LOC102548964</td>
<td>-1.10578</td>
<td>0.003436</td>
</tr>
<tr>
<td>LOC688932</td>
<td>-1.10743</td>
<td>0.003436</td>
</tr>
<tr>
<td>Sh2d2a</td>
<td>-1.22312</td>
<td>0.003436</td>
</tr>
<tr>
<td>LOC100909466</td>
<td>-1.32927</td>
<td>0.003436</td>
</tr>
<tr>
<td>Pdzk1ip1</td>
<td>-1.39973</td>
<td>0.003436</td>
</tr>
<tr>
<td>Rgs1</td>
<td>-1.51681</td>
<td>0.0128102</td>
</tr>
<tr>
<td>Ndufaq6</td>
<td>-1.51865</td>
<td>0.003436</td>
</tr>
<tr>
<td>Eve2</td>
<td>-1.54769</td>
<td>0.003436</td>
</tr>
<tr>
<td>RGD1564801</td>
<td>-1.57684</td>
<td>0.003436</td>
</tr>
<tr>
<td>Rps9</td>
<td>-1.74234</td>
<td>0.003436</td>
</tr>
<tr>
<td>Gene ID</td>
<td>Experimental log ratio</td>
<td>FDR adjusted P-value</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td><strong>Upregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOC100909466</td>
<td>2.11708</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC102553290</td>
<td>1.7099</td>
<td>0.003437</td>
</tr>
<tr>
<td>Ndufa6f</td>
<td>1.32884</td>
<td>0.003437</td>
</tr>
<tr>
<td>Cd209f</td>
<td>1.31802</td>
<td>0.003437</td>
</tr>
<tr>
<td>Sh2d2a</td>
<td>1.16792</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC691846</td>
<td>1.0926</td>
<td>0.024363</td>
</tr>
<tr>
<td>Actr3b</td>
<td>1.00545</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC102546345</td>
<td>1.00148</td>
<td>0.045246</td>
</tr>
<tr>
<td><strong>Downregulated genes</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOC102546992</td>
<td>-1.17841</td>
<td>0.028883</td>
</tr>
<tr>
<td>Tesb</td>
<td>-1.2263</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC100364500</td>
<td>-1.30164</td>
<td>0.003437</td>
</tr>
<tr>
<td>LOC102546487</td>
<td>-1.30313</td>
<td>0.003437</td>
</tr>
<tr>
<td>Acta1</td>
<td>-1.35384</td>
<td>0.003437</td>
</tr>
<tr>
<td>Ckm</td>
<td>-1.49122</td>
<td>0.003437</td>
</tr>
<tr>
<td>Retn</td>
<td>-1.49774</td>
<td>0.003437</td>
</tr>
<tr>
<td>RT1-CE16</td>
<td>-1.53332</td>
<td>0.006142</td>
</tr>
<tr>
<td>RT1-Ba</td>
<td>-1.59697</td>
<td>0.003437</td>
</tr>
<tr>
<td>Pcp4l1</td>
<td>-1.80191</td>
<td>0.003437</td>
</tr>
</tbody>
</table>
Appendix II

Research Article

Different Short-Term Mild Exercise Modalities Lead to Differential Effects on Body Composition in Healthy Prepubertal Male Rats

D. M. Sontam, 1,2 M. H. Vickers, 1,2 J. M. O’Sullivan, 1,2 M. Watson, 3 and E. C. Firth 1,2,4

1The Liggins Institute, University of Auckland, Auckland 1142, New Zealand
2Gravida: National Centre for Growth and Development, University of Auckland, Auckland 1142, New Zealand
3Department of Medicine, University of Auckland, Auckland 1142, New Zealand
4Department of Sports and Exercise Science, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand

Correspondence should be addressed to E. C. Firth; e.firth@auckland.ac.nz

Received 16 September 2014; Accepted 8 December 2014

Copyright © 2015 D. M. Sontam et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Physical activity has a vital role in regulating and improving bone strength. Responsiveness of bone mass to exercise is age dependent with the prepubertal period suggested to be the most effective stage for interventions. There is a paucity of data on the effects of exercise on bone architecture and body composition when studied within the prepubertal period. We examined the effect of two forms of low-impact exercise on prepubertal changes in body composition and bone architecture. Weanling male rats were assigned to control (CON), bipedal stance (BPS), or wheel exercise (WEX) groups for 15 days until the onset of puberty. Distance travelled via WEX was recorded, food intake measured, and body composition quantified. Trabecular and cortical microarchitecture of the femur were determined by microcomputed tomography. WEX led to a higher lean mass and reduced fat mass compared to CON. WEX animals had greater femoral cortical cross-sectional thickness and closed porosity compared to CON. The different exercise modalities had no effect on body weight or food intake, but WEX significantly altered body composition and femoral microarchitecture. These data suggest that short-term mild voluntary exercise in normal prepubertal rats can alter body composition dependent upon the exercise modality.

1. Introduction

Bone is sensitive to the mechanical stimuli imposed on it and responds to the loads applied largely by changing its mass and morphology [1, 2]. Physical activity thus has a vital role in regulating and improving bone strength. The strength of the response is age dependent with the prepubertal period being the most effective stage for exercise to have maximal effect on bone mass [3–7].

Evidence is accumulating that supports the hypothesis that changes in bone mass and architecture as a result of exercise at a young age are retained even after the cessation of exercise. For instance, in a school-based impact exercise intervention program that spanned seven months and involved children who were 10.2 ± 0.6 years old, the bone mineral content (BMC) in the proximal femur of the group which participated in the exercise program was 3.6% higher than in the control group. Of note, the BMC of the intervention group remained 1.4% higher than that of the control group eight years after the cessation of the exercise program [8]. Likewise, bone health parameters in 75-year-old Swedish men were significantly positively correlated with the amount of competitive sport they participated in between the ages of 10 and 35 years [9]. Similar observations have been made in studies of bone health in gymnasts [5], tennis players [10], and weight lifters [11, 12]. Thus exercise not only has immediate positive effects on improving bone health in the young (reduction in child fracture risk) [13] but also appears to have the potential to mitigate bone loss in later life.

Consistent with studies in humans, work in animal models has shown that the effects of physical activity on bone are retained after exercise cessation. Four-week-old male
Appendix III

Sontam DM, Firth EC, Tsai P, Vickers MH, O’Sullivan JM. Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca$^{2+}$ signalling pathways in the male rat bone. Physiol Rep. 2015;3(10):e12568
Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca\(^{2+}\) signaling pathways in the male rat bone

Dharani M. Sontam\(^1,2\), Elwyn C. Firth\(^1,2,3\), Peter Tsai\(^4\), Mark H. Vickers\(^1,2\) & Justin M. O’Sullivan\(^1,2\)

1 The Liggins Institute, University of Auckland, Auckland, New Zealand
2 Gravida: National Centre for Growth and Development, University of Auckland, Auckland, New Zealand
3 Department of Sport and Exercise Science, University of Auckland, Auckland, New Zealand
4 School of Biological Sciences, University of Auckland, Auckland, New Zealand

Keywords
Cortical bone, exercise, gene expression, mechanotransduction, RNA-Seq.

Abstract
Mechanical loading is essential to maintain optimal skeletal health. Despite the fact that early-life exercise has positive, long-lasting effects on the musculo-skeletal system, the response of the musculo-skeletal system to spontaneous low-impact exercise has been poorly studied. Previously, we identified subtle morphological changes in the femoral diaphysis of exercised animals compared to nonexercised controls. We hypothesized that significant changes in gene expression of cells should precede significant measurable phenotypic changes in the tissues of which they are part. Here, we employed RNA-Seq to analyse the transcriptome of the cortical bone from the femoral mid-diaphysis of prepubertal male Sprague-Dawley rats that were assigned to control (CON); bipedal stance (BPS); or wheel exercise (WEX) groups for 15 days. We identified 808 and 324 differentially expressed transcripts in the BPS and WEX animals respectively. While a number of transcripts change their levels in an exercise-specific manner, we identified 191 transcripts that were differentially expressed in both BPS and WEX. Importantly, we observed that the exercise mode had diametrically opposite effects on transcripts for multiple genes within the integrin-linked kinase (ILK) and Ca\(^{2+}\) signaling pathways such that they were up-regulated in BPS and down-regulated in WEX. The findings are important for our understanding of possible ways in which different exercise regimens might affect bone when normal activities apply mechanical stimuli during postnatal growth and development.

Introduction
Associational studies have established positive and long-lasting effects of early-life exercise in people who engaged in competitive sport as youths or adolescents (Kannus et al. 1995; Karlsson et al. 1995). Due to the intense level(s) of training required to achieve this competitive rank, such activity is unsuitable as a health recommendation...
Appendix IV

A Memory of Early Life Physical Activity Is Retained in Bone Marrow of Male Rats Fed a High-Fat Diet

Dharani M. Sontam, Mark H. Vickers, Elwyn C. Firth, Justin M. O'Sullivan

Studies have reported opposing effects of high-fat (HF) diet and mechanical stimulation on lineage commitment of the bone marrow stem cells. Yet, how bone marrow modulates its gene expression in response to the combined effects of mechanical loading and a HF diet has not been addressed. We investigated whether early-life (before onset of sexual maturity at 6 weeks of age) voluntary physical activity can modulate the effects of a HF diet on male Sprague Dawley rats. In the bone marrow, early-life HF diet resulted in adipocyte hypertrophy and a pro-inflammatory and pro-adirogenic gene expression profile. The bone marrow of the rats that undertook wheel exercise while on a HF diet retained a memory of the early-life exercise. This memory lasted at least 60 days after the cessation of the voluntary exercise. Our results are consistent with the marrow adipose tissue having a unique response to HF feeding in the presence or absence of exercise.

Keywords: exercise, bone marrow, gene expression, memory

INTRODUCTION

Childhood obesity has reached epidemic levels worldwide (WHO, 2016). The metabolic dysfunction induced by obesity in childhood persists into adulthood (Mattsson et al., 2008; Juonala et al., 2011; Schmidt et al., 2011; Lloyd et al., 2012). There is a genetic component to this complex phenotype. However, environmental factors, including excess energy intake and reduced physical activity, make a measurable contribution to the increasing obesity prevalence. As such, physical activity has long been considered as a non-pharmacological strategy to combat obesity and its associated co-morbidities (Tuomilehto et al., 2001; Misra et al., 2008).

Achieving optimal bone mass and strength during growth has biologically relevant effects on skeletal competence in both early and later life (Baxter-Jones et al., 2011). Childhood and adolescence are periods of rapid bone growth and attaining optimal bone mass during early life reduces the prevalence of orthopedic diseases such as fracture during childhood, adolescence, and later life (Ducher and Bass, 2007; Rizzoli et al., 2010). The opinion that obesity has a protective effect on bone due to the increased mechanical demand imposed by the excessive body weight on the skeleton has been challenged in recent times (Goulding et al., 2001, 2008; Skaggs et al., 2001; Sabhaney et al., 2014). Evidence from animal studies heavily favors the hypothesis that obesity may be detrimental to bone health during growth with several studies reporting an inverse relationship between excess adiposity, and the mechanical and micro-structural properties of bone (Cao et al., 2009; Woo et al., 2009; Lorincz et al., 2010; Ionova-Martín et al., 2011; Zhao, 2013; Yan et al., 2015). Understanding the effects of excessive adiposity on the growing skeleton is essential given the rising rates of childhood obesity.
References


40. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/β-Catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell. 2005;8(5):739–50.


47. Kini U, Nandeesh BN. Radionuclide and Hybrid Bone Imaging. 2012


56. Arai F, Miyamoto T, Ohneda O, Inada T, Sudo T, Brasel K, et al. Commitment and


91. Liedert A, Kaspar D, Blakytny R, Claes L, Ignatius A. Signal transduction pathways


159. Waki H, Tontonoz P. Endocrine Functions of Adipose Tissue. Annu Rev Pathol


184. Xu Haiyan, Barnes GT, Yang Qing GT. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Endocrinol Metab. 2003;1821–30.


249. Ducher G, Bass SL, Naughton GA, Eser P, Telford RD, Daly RM. Overweight children have a greater proportion of fat mass relative to muscle mass in the upper limbs than in the lower limbs: Implications for bone strength at the distal forearm.


303. Ingenuity Systems. IPA® network generation algorithm. 2005

304. Ingenuity Systems. Calculating and interpreting the p-values for functions, pathways and lists in IPA®.
305. Ingenuity Systems. Regulator Effects in IPA®. 2015


365. Sontam DM, Firth EC, Tsai P, Vickers MH, O’Sullivan JM. Different exercise modalities have distinct effects on the integrin-linked kinase (ILK) and Ca\textsuperscript{2+} signaling pathways in the male rat bone. Physiol Rep. 2015;3(10):e12568.


402. Uysal KT, Scheja L, Wiesbrock SM, Bonner-Weir S, Hotamisligil GS. Improved


416. Lecka-Czernik B, Stechschulte LA, Czernik PJ, Dowling AR. High bone mass in adult mice with diet-induced obesity results from a combination of initial increase in bone mass followed by attenuation in bone formation; implications for high bone mass and decreased bone quality in obesity. Mol Cell Endocrinol. 2015;410:35–41.


422. Marędziak M, Tmieszek A, Chrząstek K, Basinska K, Marycz K. Physical activity increases the total number of bone-marrow-derived mesenchymal stem cells, enhances their osteogenic potential, and inhibits their adipogenic properties. Stem Cells Int. 2015;2015.


466. Johnston CC, Miller JZ, Slemenda, CW, Reister TK, Hui S, Christian JC, Peacock M.


