Exercise and Intestinal Tumourigenesis in the Min Mouse

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Abstract

Colon cancer is the third most frequently diagnosed cancer in the UK and is often associated with a "Western" style of eating, high in fat and low in vegetables and fruits. There is strong epidemiological evidence that more physical activity is associated with reduced risk of colon cancer, but the amount or type of activity necessary to invoke this protection is disputed, and the mechanism responsible has not been elucidated.

In this project, Min mice were used in studies of the impact of physical activity on development of intestinal neoplasia. These mice have a mutation at codon 850 in the *Apc* gene and develop multiple intestinal polyps spontaneously. The numbers, sizes and anatomical distribution of these lesions can be altered by dietary and pharmacological agents. From 5 weeks of age, male and female Min mice were exercised by running on a treadmill at up to 21 m/min for 30-60 min on a 5% slope for 5d/week for 10 weeks (TR). Additional groups of mice were provided with an exercise wheel (WH) or with no exercise (CON). Throughout the study, mice had *ad libitum* access to a Western-style high fat diet. Interleukin (IL)-6, IL-10, caecal transit time, colonic short chain fatty acids and natural killer cell cytotoxicity were investigated as potential protective mechanisms. On average, WH mice ran 2.99km/d (maximum 18.35km/d) compared with a maximum 0.96km/d for TR mice. Female mice were more willing treadmill runners and ran further in the wheels than did males. There was no significant reduction in total number of tumours or tumour burden in TR or WH compared with CON mice. Molar proportion of butyrate was significantly greater in TR mice compared with CON ($P = 0.002$). None of the other investigated mechanisms were different between exercise groups, although sex differences were observed for transit time. Non-exercise physical activity (NEPA) undertaken by the TR and CON mice was quantified for 23h per day (i.e. excluding period associated with treadmill running) using an Inframot device. NEPA was significantly higher for TR compared with CON mice ($P=0.001$), and for females compared with males ($P<0.001$).

This study demonstrates that the tumour load in Min mice fed a high fat diet is not modulated by an exercise regime.

List of abbreviations

ACF	Aberrant crypt foci
ADH	Anti-diuretic hormone
AICR	American Institute for Cancer Research
AOM	Azoxymethane
APC	Adenomatous polyposis coli
BMI	Body mass index
CHRPE	Congenital hypertrophy of the retinal pigment epithelium
CIN	Chromosomal instability
COMA	Committee on Medical Aspects of Food Policy
CON	Control
COX/SDH	Cytochrome c oxidase/succinate dehydrogenase
COX-2	Cyclooxygenase-2
CRC	Colorectal cancer
CRUK	Cancer Research UK
DMH	1,2-dimethylhydrazine
EPIC	European Prospective Investigation into Cancer and Nutrition
FAP	Familial adenomatous polyposis
FFM	Fat free dry matter
HF	High fat
HNPCC	Hereditary non-polyposis colorectal cancer
IGFBP	Insulin-like growth factor binding protein
IL	Interleukin
LF	Low fat
MCR	Mutation cluster region
MET	Metabolic equivalents
Min	Multiple intestinal neoplasia
MMR	Mismatch repair
Mom	Modifier of Min
NEPA	Non-exercise physical activity
NK	Natural killer cell
NSAID	Non-steroidal anti-inflammatory drug
PAR	Physical activity ratio
PCR	Polymerase chain reaction
RMR	Resting metabolic rate
SCFA	Short chain fatty acids
SI	Small intestine
TDEE	Total daily energy expenditure
TNF	Tumour necrosis factor
TR	Treadmill
TT	Transit time
USCSWG	US Cancer Statistics Working Group
WCRF	World Cancer Research Fund
WH	Wheel
WHO	World Health Organisation

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Chapter 1

1 Introduction

In 2005, 655 000 people died from colorectal cancer (WHO, 2006). It has been estimated that as many as 91 700 of these deaths could have been prevented with increased physical activity (Slattery, 2004).

1.1 Incidence

In the year 2000, 128 874 people in the US and 34 539 people in the UK in 2001 were diagnosed with colorectal cancer (cancer of the colon and/or rectum). This represents 13% of UK total cancer cases for that year, and the third highest incidence, behind breast (15%) and lung cancers (14%) (Cancer Research UK, (CRUK) (2005)), and fourth most frequent in the US, behind breast, lung and bronchus and prostate (US Cancer Statistics Working Group, (USCSWG) (2003). Table 1.1 and Table 1.2 display specific figures relating to colon and colorectal cancers.

In 2003 there were 16 107 UK deaths from colorectal cancer, 10% of the total cancer mortality rate and second only to lung cancer (Cancer Research UK, 2005). In males, colorectal cancer accounted for 8 564 deaths (i.e.11% of all male cancer deaths, behind lung, 25%, and prostate, 13%). In females the figure is 7 543, i.e. 12% lower than males. This represents 10% of all female deaths due to cancer, behind lung (18%) and breast (17%).

Table 1.1 US* (2000) and UK (2001) cancer incidence data, (US Cancer Statistics Working Group, (2003) and Cancer Research UK, (2005))

Cancer site	US Male	US Female	UK Male	UK female
All sites	560 333	533 647	135 372	135 410
Colon and rectum	64 967	63 907	18 500	16 040
Colon only	45 362	48 738	10 921	10 689

***US data are for invasive cancer only**

Table 1.2 Age-standardised US*(2000) and UK (2001) cancer incidence per 100 000 population (US Cancer Statistics Working Group, (2003) and Cancer Research UK, (2005))

Cancer site	US Male	US Female	UK Male	UK female
All sites	546.9	409.4	404.2	339.6
Colon and rectum	65.0	47.0	54.9	35.5
Colon only	45.9	35.6	32.9	23.8

*** U.S. data are for invasive cancer only**

1.2 Pathology and prognosis

Intestinal tumours arise from unrepaired damage to proliferative cells within the epithelium, which provides them with a competitive advantage. The first visible signs of neoplasia are aberrant crypt foci (groups of enlarged crypts), some of which may acquire further genetic or epigenetic damage and develop into non-malignant adenomas (characterised by dysplasia) (Hamilton, 1996). Approximately one in ten adenomatous polyps will become malignant after 10 years (Scholefield, 2000). Further genetic or epigenetic changes allow the carcinoma to metastasise. The degree of malignancy of bowel tumours is graded using Dukes' staging A-D, or the TNM staging system:

- A - cancer is limited to the bowel wall with no spread to the lymph nodes
- B – cancer extends though the muscle wall, no spread to lymph nodes
- C – cancer has spread to nearby lymph nodes but not to other parts of the body
- D - the cancer has spread or grown into other organs or parts of the body

TNM staging system:

- T - depth the tumour has grown into the bowel wall
- N - the number of lymph nodes to which the cancer has spread
- M - indicates any metastases (American Cancer Society, (ACS) (2005))

Between 1986 and 1999 five-year age-standardised survival rates have increased, with survival at almost 50% (Cancer Research UK, 2005). The earlier tumours are diagnosed the more favourable the prognosis, with 90% of patients with Dukes' stage A cancer alive 5 years after diagnosis compared with 20% of Dukes' C patients where four or more lymph nodes have been affected (American Cancer Society, 2005).

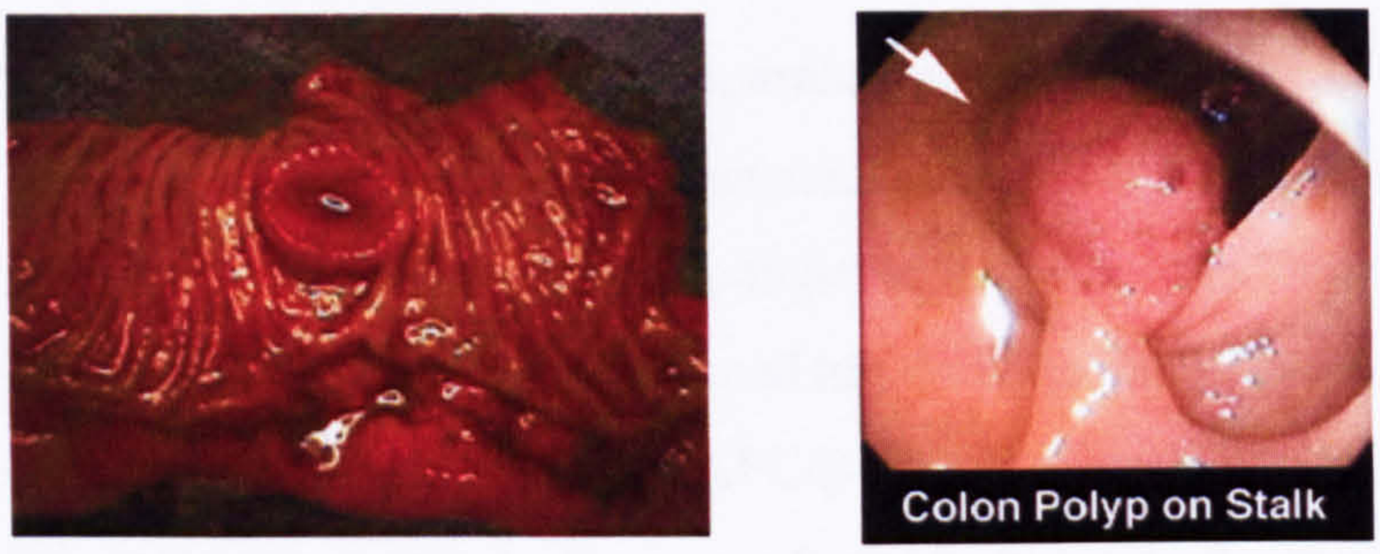

Figure 1.1 Example of a colonic cancer (left) and a polyp (right).

(Cancer picture from http://www.viahealth.org/body_surgical.cfm?id=473, polyp picture from http://www.gihealth.com/html/education/photo/colonPolypectomy.html)

1.3 Genetic basis of colorectal cancer

The majority (75%) of colon cancer cases are sporadic – due to the accumulation of multiple spontaneous somatic mutations which have not been repaired together with aberrant epigenetics e.g. hypermethylation of CpG islands in the promoter regions of tumour suppressor genes, causing gene silencing. The remaining proportions of cases are inherited or familial (Calvert & Frucht, 2002a). Specific inherited forms of colon cancer make up a very small percentage of total colon cancer cases; however, they provide valuable tools for studying the genetic basis for cancer. Mutations causing tumours usually occur in the genes controlling the cell cycle. Normal function is maintained with

one non-mutated allele – both alleles must be mutated to cause tumourigenesis. If one mutated allele is inherited (as with inherited forms of colon cancer such as Familial Adenomatous Polyposis (FAP) and Hereditary Non-Polyposis Colorectal Cancer (HNPCC)), only one mutation to the normal allele is required to produce the phenotype, hence the younger age at presentation than sporadic cases. In FAP, only one gene (*APC*) is affected, making it easily identifiable and easy to study. In most cases of FAP, mutations in the *APC* gene result in the expression of a truncated protein, causing uncontrolled cell proliferation. In HNPCC cases, mutation occurs in any of six mismatch-repair (MMR) genes, though usually *hMSH2* or *hMLH1* is affected (Calvert & Frucht, 2002a). This leads to an accumulation of errors in the genome and an increased risk of incorrect copying of genes involved in key cellular processes.

Recent research has examined apparently normal colon tissue from both colon cancer patients and a mouse model of intestinal cancer (the Min mouse) and discovered that gene expression is altered when compared to tissue from cancer-free patients (Chen et al., 2004). Cancer patients demonstrated highly variable expression levels compared to controls. Genes involved in the APC/β-catenin pathway, inflammation pathway, cell cycle and cell communication were found to be upregulated 50-200 times greater than control values. In some patients, certain proteins were downregulated by 50-100-fold compared with cancer-free controls (Chen et al., 2004). The same group has demonstrated altered gene expression in macroscopically normal colon tissue from patients who have a family history of sporadic cancer (Hao et al., 2005). These findings demonstrate that abnormalities to gene expression can occur before any lesion is visible and could be used as a screening tool for at-risk patients.

1.3.1 Familial Adenomatous Polyposis (FAP)

FAP is a condition where patients can develop several thousands of intestinal adenomas by the second decade of life. It is an autosomal dominant condition, caused by a germline mutation to *APC* (adenomatous polyposis coli) tumour suppressor gene on chromosome 5q21, usually resulting in frameshift mutations or stop codons (Shoemaker et al., 1997).

The most frequently detected somatic event is the mutation of the wild-type *APC* allele which allows easy detection by PCR (Reale & Fearon, 1996).

Extracolonic manifestations including osteomas and CHRPE (congenital hypertrophy of the retinal pigment epithelium) when combined with FAP are known as Gardners Syndrome (Reale & Fearon, 1996).

Patients show an increased incidence of desmoid tumours (a locally invasive fibrocytic growth), gastric cancers, thyroid cancers and hepatoblastoma, often demonstrating a mutation in the *CTNNb1* gene (which codes for β-catenin) (Polakis, 2000).

1.3.2 Hereditary Non-Polyposis Colorectal Cancer (HNPCC)

HNPCC is an autosomal dominant condition with colorectal cancer usually presenting at approximately 45 years of age. Extensive polyposis is absent and females with HNPCC are at increased risk of endometrial cancer (Burt & Peterson, 1996).

The condition is caused by a defect in DNA mismatch repair (MMR) mechanisms leading to microsatellite instability (Lengauer et al., 1998). Patients with HNPCC will usually have one normal allele, so MMR is normal. If the wild-type allele is inactivated, there is no MMR so mutations are allowed to accumulate, leading to tumourigenesis. MMR genes have been located to chromosomes 2p16 and 3p21, MMR genes hMSH2 and hMLH1 respectively.

An association is often found with mutated transforming growth factor β (TGFβ), which under normal conditions represses epithelial cell growth (Reale & Fearon, 1996).

1.3.3 Sporadic colorectal cancer

Somatic mutations in the tumour suppressor genes *APC, DCC* (deleted in colorectal cancer) and *p53* (which normally halts the cell cycle in G1 to allow DNA repair before replication or to induce apoptosis) are often found in sporadic colon cancers. Mutation in the *K-ras* gene (leading to decreased GTPase activity) is also frequently detected in colorectal cancers (Calvert & Frucht, 2002b).

Patients often demonstrate chromosome instability (CIN) (Lengauer et al., 1998), when whole chromosomes can be lost or gained, including those responsible for controlling the cell cycle. Cell growth is therefore not controlled leading to aneuploidy.

1.3.4 APC structure and function

APC (adenomatous polyposis coli) can be classed as the "gatekeeper" of the gastrointestinal tract, controlling the cell cycle. Mutations to APC lead to stimulated cell birth, which, when it exceeds cell death, leads to the formation of small benign tumours. To advance to adenoma/adenocarcinoma stage, other factors and pathways are required.

APC is a large multidomain protein of 8535 base pairs and 2843 amino acid residues (Fearnhead et al., 2001). The N-terminus contains multiple regions of heptad repeats including a stretch of arm repeats (Dikovskaya et al., 2001). Protein phosphatase 2A (PP2A) and APC-stimulated exchange factor (ASEF), a guanine nucleotide exchange factor (GEF) for Rac and Rho proteins, both bind to the arm region of APC (Dikovskaya et al., 2001). The central region of APC contains seven 20-amino acid repeat motifs which bind β-catenin, and also axin interaction sites. The C-terminal third interacts with structural proteins, including a basic region for microtubule binding.

1.3.4.1 APC Mutations

Nonsense and frameshift mutations account for 95% of germline APC mutations in FAP patients, with a third of all germline mutations occurring at either codon 1061 or 1309 (Fearnhead et al., 2001). Other mutations occur between codons 200 and 1600, but rarely past that. Somatic mutations are found concentrated in a mutation cluster region (MCR), between codons 1286 and 1513.

Cells lacking the C-terminus of APC display aneuploidy and errors in chromosome segregation. This is thought to be due to the inability of the mutated APC protein to bind to microtubules and therefore is unable to attach to kinetochores during mitosis.

1.3.5 β-catenin

β-catenin is one of a family of intracellular catenins that bind directly to cadherins. This provides a link between the actin cytoskeleton and cell-cell junctions (Henderson & Fagotto, 2002). β -catenin interacts with E-cadherin (responsible for cell-cell adhesion in epithelial cells) to link it to α-catenin, and from there to the actin network (Fearnhead et al., 2001). APC competes with E-cadherin for a binding site on β -catenin, and can regulate β -catenin by binding to it. Mutations in specific regions of the gene coding for β -catenin (*CTNNb1*) make it more resistant to APC regulation and to degradation (Polakis, 2000). These mutations occur in a variety of cancers, but are always mutually exclusive to APC inactivating mutations. Increased intracellular β -catenin can then bind to transcription factors which activate proliferative genes (Dikovskaya et al., 2001). β -catenin also participates in the wnt signalling pathway (see 1.3.6).

1.3.6 Wnt

The wnt proteins are secreted glycoproteins that are categorized according to their ability to promote neoplastic transformation (Polakis, 2000). Binding of a wnt to its receptor ("frizzled") causes phosphorylation of the "dishevelled" protein, which prevents glycogen synthase kinase 3β (GSK3β) phosphorylating substrates that include axin, APC and β-catenin. Unphosphorylated β-catenin avoids degradation by ubiquitin ligase and translocates to the nucleus where it is able to activate transcription factors such as T-cell specific transcription factor (TCF) and lymphoid enhancer factor (LEF) (Polakis, 2000).

There are at least 16 wnt ligands and at least 11 frizzled genes (Polakis, 2000). Function and ligand specificity differs between receptors and have not all been clarified.

1.4 Epigenetics

Epigenetics refers to the modulations of the genome which are copied from one cell generation to the next and which do not involve alterations to the DNA sequence. This includes histone modification by acetylation/deacetylation, chromatin remodelling and

methylation of CpG dinucleotides, which can alter the transcription rates of some genes (Nakao, 2001). Many CpG dinucleotides are found clustered together (CpG islands) in the promoter regions of genes. Methylation of these CpG islands silences gene expression and is a normal event in the cell (Davis & Uthus, 2004). Cancers display abnormal methylation patterns, with both hyper- and hypomethylation. The promoter regions of *hMLH1* and *E-cadherin* are hypermethylated in sporadic colon cancers (Wheeler, 2005) and tumours demonstrate global DNA hypomethylation (Davis & Uthus, 2004). Hypermethylation of promoter regions are thought to be at least as common as the more recognized cause of tumour suppressor gene activation, mutation (Davis & Uthus, 2004).

The mechanism behind the abnormal pattern of global hypomethylation is not fully understood, but is thought to include disruption to the action of DNA methyltransferases (Davis & Uthus, 2004).

Methylation status of colon tissue is currently measured from tissue biopsies obtained from colonoscopy/flexible sigmoidoscopy, with some discomfort for the patient. If methylation patterns could be reliably detected in more accessible specimens it would reduce both patient anxiety and cost of the procedure. Extracting patient DNA from faecal samples is a pain-free method of assessing methylation in colonocytes, and levels of methylation in faecal DNA samples correlate well with methylation of certain genes in the mucosa, e.g. *ESR1* (Belshaw et al., 2004). Another option is measuring methylation status in serum samples, and one group is having some success with this technique, looking at a range of genes that are frequently methylated in colorectal cancer (Leung et al., 2005), though the test needs further refinement to increase the sensitivity.

1.5 Diet and colorectal cancer

Diet is known to play an important role in the pathogenesis of colon cancer. Foods such as fruits, vegetables and whole grains have been promoted as reducing colon cancer risk, but recent studies suggest this association may be weaker than originally thought (McCullough et al., 2003).

Table 1.3 is taken from the 1997 World Cancer Research Fund (WCRF)/American Institute for Cancer Research (AICR) review of diet and cancer "Food, Nutrition and Prevention of Cancer: a global perspective" (WCRF/AICR, 1997) and summarises the strength of the evidence linking specific foods and food components with risk of colorectal cancer.

Table 1.3 WCRF summary regarding dietary components and risk of colorectal cancer (taken from (WCRF/AICR, 1997)

Level of evidence	Decreased risk	No relationship	Increased risk
Convincing	Vegetable intake		
Probable			Red meat Alcohol
Possible	Non-starch polysaccharide/fibre Starch Carotenoids	Calcium Selenium Fish	Frequent eating Sugar Total fat Saturated fat Processed meat Eggs Heavily cooked meat
Insufficient	Resistant starch Vitamins C/D/E Folate Methionine Cereals Coffee		Iron

1.5.1 Evidence for the recommendations

Fruit, vegetables, whole grains and dietary fibre

The Department of Health COMA report (1998) concluded that there was "moderately consistent" evidence for a reduced risk of colon cancer with increased fruit and vegetable intake from case-control studies, and that cohort studies presented weaker evidence. Since this report was published further studies have been completed, but the picture is still unclear. The Cancer Prevention Study II Nutrition Cohort of 62 609 males and 70 554 females found a borderline inverse association between vegetable intake and colon cancer risk in men (rate ratio (RR) 0.69, $P_{trend} = 0.10$). However, men with the lowest consumption of vegetables and dietary fibre were at increased risk of colon cancer compared with those in the highest four quintiles (RR = 1.79 $P_{trend} = 0.04$ and RR = 1.96 $P_{trend}<0.01$, respectively). Women in the lowest category of fruit consumption were at increased risk of colon cancer (RR = 1.86, $P_{trend} = 0.06$) (McCullough et al., 2003).

The evidence for non-starch polysaccharide (NSP), or dietary fibre, protecting from colon cancer is considered "moderately consistent" by COMA (1998). Bingham et al. (2003), as part of the European Prospective Investigation into Cancer and Nutrition (EPIC) a Europe-wide cohort study, measured the association between dietary fibre (non-starch polysaccharides) and colorectal cancer. They found that risk of colon cancer decreased as total fibre intake increased (P = 0.06). However, individual sources of fibre (fruits, cereals, vegetables and legumes) did not significantly affect risk. No association with rectal cancer was observed.

These studies suggest that although higher intakes of fruit and vegetables may not decrease the risk of developing colon cancer, low intakes may increase risk and the overall composition of the diet may be more important than the individual components.

Meat

The evidence of a positive association between increased consumption of red and processed meat and CRC is considered "moderately consistent" from cohort studies, although the relative risks in all studies were less than 2 (COMA, 1998). Since then, the relationship between meat intake and CRC risk has been examined as part of EPIC (Norat et al., 2005). CRC risk was positively associated with intake of red and processed meat ($P_{trend} = 0.03$, 0.08 and 0.02 for red and processed meats separately) and negatively associated with fish intake ($P_{trend} <0.001$), although when specific type of meat was

adjusted for intake of other meats, a significant risk was found only for increased pork intake ($P_{trend} = 0.03$). There was no association between intake of poultry and CRC risk.

Dietary fats

The evidence for increased risk of CRC with increased consumption of total fat intake is "weakly consistent" (COMA, 1998), and this may be due to this different types of fat in the diet and the foods that contain them.

Vitamin D and calcium

The WCRF/AICR table from 1997 considered there to be insufficient evidence of a protective effect of Vitamins C, D and E, and possible decreased risk from intake of carotenoids, although a case-control study from that year provided more evidence of protection (La Vecchia et al., 1997). They studied the diets of 1 953 CRC cases and 4 154 controls. A decreased risk was observed to occur with increased intake of carotenoids ($P_{trend}<0.001$), vitamin C ($P_{trend}<0.01$) and vitamin E ($P_{trend}<0.001$). When intake of antioxidants, vitamin D and calcium were in the upper tertile of consumption, risk was decreased by more than 50%. The protective effect was generally seen more in women than in men, with positive interactions with calcium and vitamin D (women) and carotene (men) (La Vecchia et al., 1997).

As part of EPIC, Kesse et al. (2005) examined the effect of vitamin D, calcium and dairy products on colorectal adenoma and cancer risk among a subset of French women. A trend towards decreased risk of both adenoma and cancer was observed with increasing calcium intake, and of dairy products on adenoma. No effect of Vitamin D intake on risk of adenoma or carcinoma was identified.

A more recent 3 year calcium and antioxidant intervention study found no association of nutrients on polyp growth (though several odds ratios were below 1.0), or adenoma recurrence (Almendingen et al., 2004).

1.5.2 Dietary patterns

Interest in the effect of diet on colon cancer has recently focussed on dietary patterns, to test the hypothesis that colon cancer risk is increased or lessened when certain foods are

combined in the diet. However, the evidence for a protective effect from a "prudent" diet as opposed to "western" is so far mixed, with some studies finding a positive but non-significant risk with a western diet in large cohorts (Wu *et al.*, 2004; Kim *et al.*, 2005), whilst a case-control study (Slattery et al., 2000) found an increased risk of developing colon cancer with increased consumption of a western style of eating. The effect of dietary combinations on CRC risk deserves further study.

1.5.3 Obesity

The increased prevalence of overweight and obesity has been well publicised, and is linked to readily available, energy dense, palatable foods and a decrease in habitual physical activity (Calle & Thun, 2004). The problems associated with being obese are many and varied, including increased risk of cardiovascular disease, stroke and type 2 diabetes. Cancer can be added to this list, as obese people are more at risk of cancer of the kidney and oesophagus, and women are more at risk of post-menopausal breast cancer and endometrial cancer (Calle & Thun, 2004). Indeed, one study has estimated that overweight and obesity could account for 14% of male and 20% of female deaths from cancer in the US (Calle et al., 2003).

Obesity has consistently been positively associated with colorectal cancer in both cohort (Giovannucci *et al.*, 1995; Giovannucci *et al.*, 1996; Terry *et al.*, 2002; Calle *et al.*, 2003; Lin *et al.*, 2004; Engeland *et al.*, 2005; Otani *et al.*, 2005) and case-control (Otake et al., 2005) studies, though not in a study of polyp recurrence (Sass et al., 2004). The mechanism linking obesity and CRC is likely to be multifactorial, as many candidates have been suggested, including insulin and insulin-like growth factor (IGF), elevated blood glucose levels and inflammatory proteins such as C-reactive peptide, tumour necrosis factor (TNF)-α and interleukin-6 (IL-6). Inflammatory markers are of interest as they are elevated with obesity (and decrease with weight loss (Esposito et al., 2003)), and sufferers of ulcerative colitis show an increased occurrence of colon cancer in both animal (Bertone et al., 1998) and human (Biasco et al., 1995) studies.

1.6 Epidemiology of links between physical activity and bowel cancer in humans

1.6.1 Exercise or physical activity?

The terms physical activity and exercise are often used inter-changeably but it is important to distinguish between the two. Physical activity encompasses all bodily movements requiring energy above the basal rate whilst exercise refers to structured, deliberate, often repeated movements.

1.6.2 Benefits of being active

The benefits conferred by physical activity have been well documented and include physical, mental and social aspects, all of which are equally important as individual needs will differ. Some of the positive aspects of being physically active follow:

- Lowered blood pressure, reduced risk of stroke and coronary heart disease
- Changed body shape – increased muscle tone, decreased fat storage, weight control
- Decreased diabetes risk
- Releases endorphins, improved mood, general psychological benefits
- Boosted circulatory and immune systems
- Increased fitness, mobility and flexibility
- Improved cardiovascular health
- Improved appetite
- Social aspects – team building, increased confidence, make new friends

In contrast to the benefits of being active are the problems induced by inactivity, which can contribute to obesity and the problems associated with it, such as diabetes, sleep

apnoea, joint pain and immobility. In addition to the physical detriment is the cost to health authorities when treating problems that could otherwise be solved by a lifestyle change. According to the Department of Health's Health and Social Care Information Centre (HSCIC) (2005), in 2004 just 35% of men and 24% of women in England were meeting the government recommendation of 30 minutes of moderate intensity activity on at least 5 days of the week, figures that decrease with age. A recent study of a health insurer in America attributed $35.3million to treating diseases caused by physical inactivity in a group of 1.5 million people in 2000 (Garrett et al., 2004) and this figure is thought to be an underestimate.

1.6.3 Measuring physical activity

There are three factors to consider when measuring physical activity. Total daily energy expenditure (TDEE) consists of:

- RMR (resting metabolic rate), basal and sleeping conditions plus the added cost of arousal
- Thermogenic effect of consumed food
- Energy expended during physical activity and recovery

These parts contribute approximately 60-75%, 10% and 15-30% to TDEE, respectively (McArdle et al., 2001).

There are several methods for measuring energy expenditure in humans. These include:

- **Direct calorimetry**

  This method utilizes the heat produced by the body as a result of metabolism. During **airflow calorimetry,** the subject is kept in a sealed room and energy intake (food, or "gross energy") is carefully measured using a bomb calorimeter. The walls of the room contain thermocouples to measure the change in room temperature as a result of body heat. The change in humidity of air leaving the room compared with air entering the room allows heat lost from the lungs and body surface as water to be quantified. Energy expended through excreta is also measured, and when subtracted from energy

intake provides the amount of energy available for use by the body ("metabolisable energy"). Other forms of direct calorimetry are available, all of which have several drawbacks: the subject is not free-living and therefore is not undertaking their normal daily tasks, so energy expenditure is not reflecting a real-life situation.

- **Indirect calorimetry**

This involves measuring the quantities of O_2 consumed and CO_2 produced by the subject as an estimate of energy expenditure. This method is based on the knowledge that combustion of different metabolic substrates produces a different proportion of CO_2 produced to O_2 consumed, or RQ (respiratory quotient), where RQ = moles CO_2 produced/moles O_2 consumed, e.g. for glucose, RQ = 1.0, for fat RQ = 0.7. A blend of lipid, protein and carbohydrate burned in one litre of oxygen produces approximately 4.82 kcal (20.20 kJ) energy, and varies only slightly with different metabolic mixtures (McArdle et al., 2001). Drawbacks to this method are similar to those of direct calorimetry, but the results produced are very similar to those as measured by direct calorimetry.

- **Doubly-labelled water** (Lifson & McClintock, 1966)

This technique is an example of indirect calorimetry, is relatively simple to carry out and has the major advantage of being a feasible way to study TDEE in free-living creatures. The subject is fed a known quantity of "labelled" water, containing ^2H and ^{18}O. This equilibrates readily with the H_2O already in the body. ^{18}O can leave the body in the form of $H_2{}^{18}O$ or $C^{18}O_2$. ^2H can only leave the body as ^2H_2O (sweat, urine and pulmonary water vapour). Urine samples are taken at specified intervals and the ratio of rates of loss of ^2H to ^{18}O provides an estimate of how much additional ^{18}O has been lost from the body via the lungs as CO_2, and therefore how much activity has been undertaken (assuming an RQ of 0.85). This method is suitable for measuring over long periods and during extreme activities (e.g. the Tour de France). The major drawback to this method is the cost of the isotopes and their subsequent quantification.

- **METs (Metabolic equivalents)**

METs attempt to quantify the amount of energy required to undertake specific activities in multiples of the resting metabolic rate. This is usually expressed as millilitres of oxygen per kilogram of body mass per minute ($ml \cdot kg^{-1} \cdot min^{-1}$) with 5 kcal approximating to 1 litre of oxygen consumption. 1 MET is equivalent to the resting oxygen consumption ($\approx 250\ ml \cdot min^{-1}$ for men and $200\ ml \cdot min^{-1}$ for women). Therefore, exercise at 2 METs requires twice the resting oxygen consumption, 3 METs requires 3 times the oxygen consumption, and so on. With this technique it is more accurate to consider variations in body size, with 1 MET = $3.5\ ml \cdot kg^{-1} \cdot min^{-1}$ (McArdle et al., 2001). Difficulties with this technique include duration and intensity of exercise and fitness differences between individuals. When assessing lifetime activity through the form of detailed exercise questionnaires, this technique has some value, although omission of light or daily activities or exaggeration can introduce recall bias and inaccuracy.

- **VO_2max**

This technique is the "gold standard" for **current fitness** levels, by measuring maximal oxygen uptake. It is possible to obtain an estimate of RQ from this technique, by measuring exhaled CO_2, but cannot be used to assess lifetime activity levels and would not be a suitable method of measuring total energy expenditure.

Table 1.4 shows comparisons between VO_2max and METs.

Table 1.4 Comparison of VO_{2max} and MET quantification (adapted from (McArdle et al., 2001))

		Absolute intensity (METs)			
Category	**Relative intensity (%VO_2max)**	**Young**	**Middle aged**	**Old**	**Very old**
Rest	<10	1.0	1.0	1.0	1.0
Moderate	<70	<9.0	<7.0	<5.0	<2.8
Heavy	>70	>9.0	>7.0	>5.0	>2.8
Maximal	100.0	13.0	10.0	7.0	4.0

- **Heart rate**

Increasing the intensity of aerobic exercise shows a linear relationship between heart rate and oxygen consumption. Heart rate readings can therefore be used to estimate oxygen consumption and therefore energy expenditure during exercise. Drawbacks to this otherwise simple technique are the specificity of the heart rate-consumption linearity to individuals, with different subjects requiring different amounts of oxygen at different heart rates, and also heart rate being affected by things other than exercise, such as emotional state, environmental temperature, etc.

- **Accelerometers/pedometers**

Uni-axial or tri-axial accelerometers are small, portable devices that measure velocity changes in 1 or 3 axes. Data is accumulated frequently and allows estimation not just of total activity and activity pattern but also intensity of activity (Cooper et al., 2000).

Pedometers count the number of steps taken, and are therefore more useful for less active subjects.

As many intervention trials are first performed on animals, several methods of assessing activity have been developed:

- **Biotelemetry**

Devices are available that monitor gross activity of individual animals via an implantable transmitter (e.g. Mini Mitter®). The strength of the signal alters depending on the distance of the transmitter from the receiver. Changes in signal are interpreted as activity counts. Additional physiological parameters such as body temperature and heart rate can be measured simultaneously.

- **Infrared (1)**

IR beams are set up around the cage in one, two or three dimensions and coupled to a computer. Every time a beam is broken the activity level can be measured, and depending on the number of beams broken at any given time, different activities such as rearing can be identified (e.g. Opto-Max from Columbus Instruments, Ohio, US).

- **Infrared (2)**

A single sensor placed on top of the home cage that detects infrared (body heat) movement greater than 5ms in length and records them in a spreadsheet. This gives information about general activity but does not distinguish between animals or types of activity (e.g. InfraMot from TSE Systems, Bad Homburg, Germany).

- **Doppler radar**

Low power electromagnetic waves are beamed through the cage and reflected by skin. The returning waves can be amplified and filtered allowing movement to be recorded (Kropveld & Chamuleau, 1993).

- **Video analysis**

This is the crudest method of monitoring activity, by constantly videoing the animals in their cages and scoring any movements. Problems occur when attempting to quantify different movements/activities, such as rearing, nest building, feeding etc, especially if there are several animals per cage.

1.6.4 Exercise/physical activity and cancer risk

There is substantial evidence (at least 50 studies (Lee, 2003)) to suggest that being physically active can reduce the risk of developing cancer, particularly of the breast and colon (McTiernan et al., 1998), indeed one report suggests that high levels of physical activity may reduce the risk of colon cancer by up to 50% (Stein & Colditz, 2004).

1.6.4.1 Cohort studies

The Framingham Study of 1906 men and 2308 women found an increased risk of large bowel cancer in men with low levels of activity, but no effect for women (Ballard-Barbash et al., 1990). However, few women reported intense activity, and fewer women than men reported moderate activity.

The Health Professionals Follow-up Study of 31 055 men in the USA found that men in the highest quintile of physical activity had half the incidence of colon cancer as men in

the lowest quintile (Giovannucci et al., 1995). The authors concluded that a moderate level of activity can reduce risk – the median activity level in this study was low, possibly due to the majority of the participants being retired and was equivalent to three hours walking per week.

A large study (over 80 000 participants) in Norway found that, for females, the equivalent of walking or cycling for at least four hours per week decreased risk of developing colon cancer, especially for the proximal colon, by almost 40% compared with a sedentary group (Thune & Lund, 1996). The same effect was observed in males aged 45 or older, and a protective effect of occupational activity was also observed in males. An inverse dose-response relationship was observed in both sexes (Thune & Lund, 1996).

The Nurses Health Study in 1997 found that increased leisure-time activity was associated with decreased colon cancer incidence, especially of the distal colon (Martinez et al., 1997). Moderate intensity activity for one hour per day reduced risk by 46%.

Chao et al. (2004) analysed the effect of recreational activity on colon and rectal cancer in older adults in the Cancer Prevention Study II Nutrition Cohort (70 403 men and 80 771 women) and observed a decrease in risk of colon cancer with increased time spent in activity (P_{trend} =0.007). This response was particularly due to walking plus other activities, as opposed to just walking. This may infer a requirement for more strenuous activity to offer protection, or an inability to accurately measure the pace of walking.

A recent Scandinavian study assessed the impact of leisure-time physical activity on cancer (Schnohr et al., 2005). The cohort of 13 216 women and 15 043 men generated 180 and 215 colon cancers respectively, and an inverse association between vigorous activity and colon cancer risk was observed for men only (P_{trend} = 0.06).

1.6.4.2 Case-control studies

Slattery et al.(1997a) conducted a large case-control study investigating more closely the effect of long-term physical activity. They found that decreased risk was associated with long-term vigorous activity (odds ratio 0.68), and that the length of each individual session was more important than the number of sessions per week. This inverse association was slightly weaker in those with a family history of colorectal cancer. A

family history of colon cancer or polyps is known to increase risk of colon cancer and may reduce the protective effects of activity (Kerber *et al.*, 1998; Slattery *et al.*, 2003).

A smaller case-control study of 177 cases and 228 controls (Hauret et al., 2004) confirmed the results of Slattery et al., with odds ratios of 0.63 when comparing the highest and lowest quintiles of physical activity.

Commuting physical activity was included in a study of physical activity and colon cancer risk in Shanghai (Hou et al., 2004). The high level of bicycle commuting among the subjects was unusual compared with other studies and led to high levels of activity even in the lowest tertile. This could have obscured any findings, but the authors still observed a trend of decreased risk of colon cancer with increased activity of all kinds, but particularly commuting for both sexes (P_{trend}= 0.007 in women and <0.001 in men) and occupational activity for women (P_{trend} = 0.009), particularly when continued over a lifetime.

Steindorf et al. reached similar conclusions in a Polish study of colon cancer (Steindorf et al., 2005), where lifelong high exercisers had an odds ratio of 0.28 compared with lifelong non-exercisers. No consistent association was observed for rectal cancers.

1.6.4.3 Polyp prevention

The Polyp Prevention Trial examined this association further by assessing polyp recurrence in a group of patients who had had at least one polyp which was removed before the start of this dietary intervention study (Colbert et al., 2002). Subjects were assigned to either a low-fat, high-fibre, fruits and vegetables diet or their normal diet. The study found that physical activity was not associated with polyp recurrence over the three years of the study, but note, however, that the intervention did not involve a change in physical activity and only activity in the preceding year was assessed. However, the Calcium Polyp Prevention Study (a trial of calcium carbonate supplementation on polyp recurrence) found an inverse association between level of physical activity and risk of polyp recurrence in men (Wallace et al., 2005) (P_{trend} = 0.005). No such association was observed in women, where the 95% CI was large for all variables (e.g. middle tertile of activity (MET-h/wk) 0.53-4.27 for women compared with 0.16-0.67 for men).

Project PREVENT used mail and phone contact to change risk factors in polyp patients to reduce the chances of recurrence (Emmons et al., 2005). Behaviours such as physical inactivity, smoking, fruit and vegetable and red meat consumption were targeted. The authors found that the intervention was effective in helping patients to change risky behaviour compared with patients who received usual care, although the recurrence of polyps was not reported. Future interventions of this nature may find a reduction in polyp recurrence in patients who change their behaviour.

1.6.4.4 Confounding factors

Several reviews have collated the many available data (Colditz *et al.*, 1997; McTiernan *et al.*, 1998; Giacosa *et al.*, 1999) and also considered other factors that may influence any physical activity-bowel cancer risk relationships. These confounders are important, and should be included in the statistical analyses. Several confounders have been described which can increase the risk of developing colon cancer, including diet (as discussed in 1.5), alcohol consumption (Sharpe et al., 2002) and smoking (Kuper et al., 2002). High total energy intake, increased body mass index (BMI) (Terry et al., 2002) and a high waist-to-hip ratio are all positively associated with colon cancer (Giacosa et al., 1999) as discussed in 1.5.3. People with these characteristics may also be less physically active (Boutelle et al., 2000). Given the implication of obesity in colon cancer, and the relationship between physical inactivity and colon cancer risk, this could be a causative or a confounding factor. However, a raised BMI and high energy intake had a nonsignificant effect on colon cancer risk in those who were physically active (Slattery et al., 1997b) suggesting that physical activity exerts its effects independently of BMI. Conversely, a recent study of caloric restriction in humans has found positive outcomes relating to the development of atherosclerosis, such as lowered serum triglycerides, fasting glucose, fasting insulin and BMI (Fontana et al., 2004). As these are important factors in colon cancer development, further study may reveal beneficial results of caloric restriction with respect to colon cancer prevention.

A recent study examined the effect of several potential confounders (Slattery & Potter, 2002), including total energy intake, red meat, fruit, vegetables, whole grains, calcium, alcohol, BMI and non-steroidal anti-inflammatory drug (NSAID) use. These were

considered both singly and collectively. The study revealed no confounding with physical activity and concluded that physical activity is an independent factor modifying colon cancer risk, with long term vigorous activity consistently producing a 40% reduction in risk compared with no vigorous activity (in accord with Thune and Lund (1996)).

1.6.5 Comparing studies

Studies to date have used various methods of data collection, different time points and different criteria to assess previous and current levels of physical activity (see Table 1.5). This inevitably leads to a lack of comparability between studies due to the limitations of observational epidemiology.

Table 1.5 Summary of the different methods of data collection and activity categories of 4 studies

Reference	Method of data collection	Categories
Giovannucci et al. (1995)	Questionnaire	8 moderate and vigorous activities. Report time/week spent on each. Converted to MET-hours/week
Thune and Lund et al. (1996)	Questionnaire	4 levels of activity for both occupation and recreation
Martinez et al.(1997)	Questionnaire	As Giovannucci et al. plus number of flights of stairs climbed daily
Slattery et al. (1997a)	Interview	Converted time and frequency spent on each activity to energy expenditure and ranked 1-4 (low expenditure-high expenditure)

1 MET (metabolic equivalent) = sitting quietly, 1 hr/week running = 10.2 MET-hours in Giovannucci et al. but 12 MET-hours in Martinez et al.

Differences in analyses include use of occupational or leisure-time activity; self-reported as opposed to interviewer-administered questionnaire; different time references, such as recent activity, activity in the past year, or lifetime activity; confounding factors such as BMI and family history; diet; and method of estimation of activity. This is possibly the

variable that is most different between studies, as mentioned in 1.6.3. Whilst the data need to as complete as possible, the procedure should not be an ordeal for the participant. Ideally, the type, duration and intensity of activity should be recorded, including occupational and leisure-time activities, as well as the time in life it occurred. Standardisation of these measures would be ideal, and help researchers and health officials to determine true exercise effects.

One current intervention study will attempt to quantify more precisely the amount of activity that is beneficial with respect to colon cancer markers (McTiernan, 2003). Few intervention studies of this type have been attempted as the imposition of a long-term exercise regime can be difficult for patients to adhere to. The Physical Activity for Total Health (PATH) study assessed the effect of a 1-year exercise intervention on sex hormone levels in post-menopausal women, and whilst excellent adherence to the exercise was achieved, there was variability between participants (Irwin et al., 2002). APPEAL (A Programme Promoting Exercise and an Active Lifestyle) is a study involving 100 men and 100 women aged 40-75 years who are at increased risk of developing colon cancer (McTiernan, 2003). The intervention group will be asked to complete 60 minutes of aerobic exercise 6 days per week for 12 months. Various activities are available for use by the participants, such as treadmills, stationary bicycles and elliptical machines, in addition to walking and outdoor jogging. These choices, combined with other incentives, offer variability to the programme and should help to retain participants. The study will measure the effect of exercise on colon and rectal cell proliferation, apoptosis and prostaglandins. Fat mass, weight, insulin, IGF-1 and IGFBP-3 will also be under observation. The results had not been published at the time of writing.

1.6.6 Mechanisms

The potential mechanisms involved in reducing the risk of colon cancer by being physically active were reviewed recently by Quadrilatero and Hoffman-Goetz (2003). Potential candidates include faster intestinal transit time, enhanced immune function,

lowered cholesterol and prostaglandins concentrations, increased levels of anti-inflammatory cytokines, and enhanced insulin sensitivity.

1.6.6.1 Intestinal transit time

The theory behind the protective effects of decreased transit time (time taken for food to travel through the gut) comes from the idea that increased physical activity speeds up transit as evidenced by the marathon runners' condition of runners' diarrhoea and from the converse observation of increased constipation in bed-bound patients. (Bingham & Cummings, 1989; Oettle, 1991; Liu *et al.*, 1993; Robertson *et al.*, 1993; Rao *et al.*, 1999; Dukas *et al.*, 2003; Sanjoaquin *et al.*, 2004). It is thought that by decreasing the amount of time that the gut contents are in contact with the mucosa, potential carcinogens and mutagens are less likely to cause damage, though no evidence appears to exist in support of this theory.

The problem with the transit time theory is the lack of consistent evidence.

Holdstock et al. (1970), whilst measuring the effect of a meal on propulsion in the colon, observed that activity of the subject led to the expulsion of markers in the stool that had otherwise been in a fixed position in the colon while the subject was stationary. This suggested that activity might speed up the passage of colon contents.

Bingham and Cummings (1989) assessed the effect of a 7-9 week physical training programme on total gut transit time and stool weight in subjects fed a constant diet. Transit time increased in 9 subjects but decreased in 6, so overall no change in transit time (55h v. 54h for sedentary v. exercise) or stool weight was observed. However, the exercise intervention was inconsistent and uncontrolled, with some subjects walking and some jogging, for different lengths of time.

Oettlé (1991) also investigated effects of exercise on gut transit time, in free-living subjects who followed a 3 week protocol of 1h/d of cycling/jogging or resting. Both jogging and cycling significantly decreased whole gut transit time, from 51.2 h at rest to 36.6h when cycling and 34.0 h when jogging ($P<0.01$). Stool weight and frequency were unchanged, and although diet was not controlled, fibre intake was unchanged.

Coenen et al. (1992) measured effect of moderate exercise on total transit time (oro-anal) in healthy young men given a specific diet. The 6km of jogging did not consistently alter transit time, as in one third of the volunteers it was unchanged, in one third it increased and in the remaining subjects transit time decreased. In contrast with the previous studies, stool weight increased, even though stool frequency and fibre intake were unchanged. This could have been explained by faster transit time, as there would have been less time for absorption of water, but no consistent effect on transit time was observed.

Robertson et al. (1993) studied the effect of moderate exercise on colon transit time in young males and did not find any consistent difference from resting, as in some patients it increased, in some there was a decrease and in some transit time was unchanged.

Liu et al. (1993) demonstrated that two weeks of inactivity in otherwise active elderly subjects almost doubled colonic transit time from 10.9±2.7 hours to 19.5±2.9 hours, while mouth-to-caecum transit time remained unaffected.

Dukas et al. (2003) used information from the 62 036 women aged 36-61 who took part in the Nurses Health Study 1980-1982. They observed that increased physical activity was associated with decreased prevalence of constipation. Sanjoaquin et al. (2004) published a study using data from the European Investigation into Cancer and Nutrition, Oxford Cohort (EPIC-Oxford) which involved 20 630 male and female participants aged 20-97 years. A positive association was found between the amount of vigorous exercise undertaken and the mean number of bowel movements per week. Significantly higher odds of a daily bowel movement were found in females who exercised vigorously for seven or more hours per week compared with females who took no vigorous activity. No significant association was observed in men (Sanjoaquin et al., 2004).

The highly individual responses of transit time to altered physical activity suggest that perhaps this is not an important factor in colon cancer prevention.

1.6.6.2 IGF-1 and insulin

Insulin and insulin-like growth factors (IGFs) are increasingly being examined for their potential involvement in CRC, due to the insensitivity of the body's tissues to insulin that occurs with physical inactivity and obesity. Insulin and IGFs are also recognised as stimulators of cell proliferation and inhibitors of apoptosis (Quadrilatero & Hoffman-Goetz, 2003). In addition, insulin and IGFs are also thought to promote angiogenesis, and taken together, these functions are important in the generation of tumours (Quadrilatero & Hoffman-Goetz, 2003). Therefore, reduction of these peptides by physical activity would reduce the risk of abnormal cell growth. IGFs in the circulation are usually bound to insulin-like growth factor binding proteins (IGFBPs) (Renehan et al., 2004), and so levels of binding proteins may also indicate the availability or otherwise of IGFs to promote cell proliferation.

Meta-regression analysis by Renehan et al., (2004) of five cohort studies of CRC found a positive association between increased IGF-I and risk of CRC, but not with IGFBP-3, whilst Renehan et al., (2000) observed an increased in serum IGF-II in patients with colorectal adenoma, although no association was found for serum IGF-I or IGFBP-3.

Chadan et al., (1999) found no change in IGF-1 concentration with physical activity in a group of healthy 62-67 year old women, though moderate exercise for 50 min resulted in decreased IGFBP1 and increased IGFBP2 and 3. Rosendal et al., (2002) also found no effect of previous vigorous physical activity on plasma IGF1 or IGFBP-3 concentrations in the Seattle Colon Cancer Family Registry, a population-based study of colorectal cancer cases, relatives and cancer-free controls.

Evidence for the role of IGFs and insulin in CRC is somewhat mixed, and further study in larger trials is warranted.

1.6.6.3 Cytokines

Cytokines are small polypeptides that are released by cells in response to infection or tissue injury and act with cytokine inhibitors and soluble cytokine receptors to regulate the immune response (Opal & DePalo, 2000). Whether a cytokine has a pro- or anti-

inflammatory action depends on many factors, including the timing of release, local milieu, presence of agonists/antagonists, receptor density and tissue responsiveness (Opal & DePalo, 2000), but most fall into either the pro- or anti- inflammatory category.

Anti-inflammatory cytokines are immunoregulatory molecules which control the response of the pro-inflammatory cytokines (Opal & DePalo, 2000). Anti-inflammatory cytokines include interleukin-1 receptor antagonist (IL-1ra), IL-4, IL-6, IL-10, IL-11, IL-13 and TGF-β. In a normal state, anti-inflammatory cytokines help to limit any injury caused by excessive inflammatory reactions, but a diseased state may demonstrate a lack of control over pro-inflammatory mechanisms or too much control, with over-suppression of the inflammatory response increasing the risk of systemic infection.

IL-6

IL-6 is considered to be primarily a pro-inflammatory cytokine and is the third cytokine released in the inflammatory cascade, mediating the acute-phase response. Persistent activity leads to chronic inflammation, which encourages further IL-6 release from lymphocytes and thus providing a boost in the generation of a chronic inflammatory state (Caruso *et al.*, 2004). However, it has also been suggested that IL-6 regulates concentrations of TNF-α and stimulates production of IL-1ra and IL-10 (both anti-inflammatory cytokines), thus acting in an anti-inflammatory manner (Petersen & Pedersen, 2005).

Strenuous exercise causes an acute increase in plasma levels of IL-6, which can increase 100-fold, though clearance from the circulation is rapid (Pedersen, 2000). However, resting IL-6 concentrations are decreased in those who undertake physical activity (Colbert *et al.*, 2004; Panagiotakos *et al.*, 2005a). IL-6 is often elevated in colon cancer patients, although the severity and prognosis appear to be related to genotype (Caruso *et al.*, 2004)

IL-10

IL-10 is a Th2 cytokine, so-called because it is secreted by $CD4^+$ T-helper subset 2 lymphocytes. It is also secreted by monocytes and B cells. Il-10 is considered to be the most important human anti-inflammatory cytokine (Opal & DePalo, 2000) and is

responsible for many actions, including i) inhibition of monocyte and macrophage derived cytokines, including IL-1, IL-6 and TNF-α, ii) inhibition of cytokines produced by neutrophils and natural killer cells, iii) promoting degradation of pro-inflammatory cytokine mRNA, and iv) inhibiting cell surface expression of MHC class II molecules and CD14 (LPS recognition and signalling molecule).

An IL-10 knockout mouse has been generated which demonstrates chronic enterocolitis with over-production of pro-inflammatory cytokines (TNF-α, IL-1, IFN-γ) suggesting that the primary defect of IL-10 knockout mice is a failure to control the normal intestinal immune response to enteric antigens (Kuhn et al., 1993). However, the effect of IL-10 on cancer development depends on the milieu of growth factors and cytokines in the tumour microenvironment, with some studies showing a protective effect and others showing a tumour-promoting effect of high IL-10 concentrations (Caruso *et al.*, 2004). This may be due to the dual function of IL-10 both limiting the inflammatory signal and also being able to stimulate B-cell activity (Caruso *et al.*, 2004). IL-10 is also produced by tumours, and studies have demonstrated an increase in serum IL-10 concentration in colon cancer patients compared with controls (Galizia *et al.*, 2002), and that IL-10 from stimulated blood cultures is positively correlated with both tumour proliferation and apoptosis (Evans *et al.*, 2006).

TNF- α

TNF-α is the first cytokine released in the pro-inflammatory cascade that occurs after injury (Petersen & Pedersen, 2005) and induces endothelial adhesion molecules which allow leucocytes to adhere to the endothelium before migrating into the tissue (Dinarello, 2000). TNF- α acts synergistically with IL-1. Both cytokines activate the same portfolio of genes, and result in actions including insulin release and β-islet cell death, insulin resistance and loss of lean body mass but only TNF- α induces apoptosis (Dinarello, 2000). The production of TNF- α is suppressed by IL-10, among others, and the outcome of the balance between pro- and anti-inflammatory cytokines will often determine the outcome of a disease (Dinarello, 2000).

TNF- α is increased with obesity, as the adipose tissue increases expression of the *TNF- α* gene (You et al., 2005), which may contribute to the chronic inflammatory state observed

in many obese individuals (as discussed in 1.5.3). Reduction of adipose tissue and the resulting decrease in inflammation may be a possible mechanism through which physical activity is protective, although TNF- α generally works locally and circulating levels of this cytokine may not be reduced correspondingly (Petersen & Pedersen, 2005). Greater leisure-time physical activity has, however, been linked to lower serum TNF- α (Colbert *et al.*, 2004; Panagiotakos *et al.*, 2005a) and TNF- α receptor (TNF-R) (Pischon et al., 2003) concentrations. The receptor is thought to act as a binding protein and/or a "slow release reservoir", prolonging the half-life of TNF- α in the circulation (Pischon et al., 2003).

Reduced inflammation has been postulated to be a mechanism by which greater physical activity is protective, as NSAID use masked any effect of physical activity on decreased risk of CRC in a case-control study (Hauret et al., 2004).

1.6.6.4 Immune function

Exercise in known to modulate the immune system, and in moderation these alterations are generally beneficial, however, exhaustive exercise can be detrimental, decreasing resting monocyte, T cell and natural killer (NK) cell count, and decreasing B cell function and the phagocytic function of nasal neutrophils (Nieman, 1997).

The beneficial effects of chronic exercise of moderate intensity include increased macrophage phagocytic and lysosomal enzyme activity (observed in mice), increased resting monocyte count, increased monocyte phagocytosis and increased NKC activity (Nieman, 1997).

Table 1.6 summarises several studies of exercise and immune function. The general outcome from the studies was an increase in cytotoxicity of the immune cells with moderate exercise, although none of the studies could give a definitive mechanism for this effect. These studies, however, have not investigated the effect of exercise on tumourigenesis. Zielinski et al., (2004) injected female BALB/c mice with EL-4

lymphoma cells and assigned the mice to either control or treadmill-exercise group. Changes in tumour mass and in the intra-tumoural cellular composition were then measured. The authors observed a delay in tumour growth in exercising mice and a faster time of rejection, compared with controls (Zielinski et al., 2004). Exercised mice also displayed a lower blood vessel density in the tumour, higher numbers of host lymphocytes and a lower number of inflammatory cells (neutrophils and macrophages) and mitotic cells. This apparent increase in effectiveness of immunosurveillance with increased exercise could help to explain the effect of exercise on other cancers, especially given the large immune network of the intestinal tract. However, while the data from animal studies is encouraging, there has been no systematic evaluation of immune function in reports of physical activity and colon cancer.

Studies into mechanisms by which exercise protects from colon cancer have produced conflicting findings. This suggests that decreasing risk of CRC through physical activity is likely to be multifactorial.

Table 1.6 Summary of animal studies which have investigated the effects of exercise on the immune system and cancer

Species/cell line	Treatment	Target	Effect	Reference
180 male inbred pathogen-free C3H/HeN mice 7-11 weeks old + SCA-1/p3 breast adenocarcinoma target cells	Injected with thioglycollate then moderate/exhaustive treadmill exercise for 3 days. Killed 0/3/8 h after exercise	Inflammatory MΦ*	↑ in vitro antitumour cytotoxicity	Woods et al., (1993)
120 male C3H/He mice 6-8 weeks old + CIRAS 3 and YAC-1 tumour cell lines	Treadmill or wheel for 9 weeks. Injected CIRAS 3 3h before killing	Natural killer cells	Treadmill & wheel both ↑ in vitro cytotoxicity, chronic exercise ↓ pulmonary tumour cell retention	MacNeil and Hoffman-Goetz (1993)
175 male C3H/HeN mice 5-7 weeks old and SCA-1/p3 adenocarcinoma target cells	Injected *P.acnes*** then moderate/exhaustive exercise on days 5-7, or injected 3h before exercise then exercised daily (7d)	Inflammatory MΦ	Daily moderate or exhaustive exercise during activation ↑ cytotoxicity. Only moderate exercise beneficial days 5-7 (when MΦ fully activated). ↑ plasma corticosterone but no correlation with cytotoxicity	Woods et al., (1994)
Pathogen free male C57BL/6 mice 6-8 weeks old + B16F1 melanoma cell line	Treadmill at different intensities - 1 acute bout then killed, or injected with tumour cells	MΦ	↑ cytotoxicity of alveolar MΦ immediately after 30 min exercise bout. Cytotoxicity still present 8 hours after fatiguing exercise (~3hrs)	Davis et al., (1998)
Specific pathogen free inbred male BALB/cByJNia mice, 2 months and 18 months	Treadmill exercise for 16 weeks	MΦ	↑ tumour cytolysis by peritoneal MΦ, ↑ NO production by MΦ in young mice	Lu et al., (1999)

*** MΦ – macrophage, **_Propionibacterium acnes_ bacterium, causes inflammation and activation of MΦ, peaking 5 days postinjection**

1.7 Animal studies

1.7.1 Mechanism of action of commonly-used carcinogens

Azoxymethane (as AOM per se or via endogenous metabolism of administered dimethylhydrazine (DMH)) has been used in all the rat studies included in this review. DMH is metabolised in the liver to AOM, which is converted to methylazoxymethanol (MAM), from which comes CH_3+ (methylcarbonium ion) which binds to the DNA of colonic stem cells (Corpet & Pierre, 2003). AOM induces tumours through this aberrant DNA methylation, which produces O^6-methylguanine (O^6MeG), a marker of exposure to methylating agents that can cause mutations in mismatch repair genes (Jackson et al., 1997). The mutations also reflect many characteristics of human tumours, such as mutated *K-ras* and β-catenin (*Ctnnb1*) genes (Takahashi & Wakabayashi, 2004). Mutated Kras activates the MAPK and PI3K pathways and upregulates expression of Cyclin D1 and cyclooxygenase-2 (COX-2). It also enhances inducible nitric oxide synthase (iNOS) expression in the presence of inflammatory stimuli (Takahashi & Wakabayashi, 2004). iNOS increases concentrations of nitric oxide (NO), which causes DNA damage and angiogenesis and activation of COX-2. Increased COX-2 leads to increased levels of prostaglandins which in turn lead to increased cellular proliferation and decreased apoptosis (Takahashi & Wakabayashi, 2004), both important factors for neoplastic transformation. In male F344 rates administered with AOM, Takahashi and Wakabayashi (2004) found G→A transitions in both *K-ras* and *β-catenin* genes which caused β-catenin to be found throughout the cell cytoplasm and in the nucleus, when it is normally localised to the cell membrane where it is involved in cell-cell adhesion (Takahashi & Wakabayashi, 2004). Mutation of β-catenin stabilises the protein in the cytoplasm and activates transcription of its signalling targets, which are thought to include the increase in expression of iNOS. However, AOM-induced tumours never induce *p53* gene mutations and rarely cause *Apc* gene mutations (Erdman *et al.*, 1997; Sohn *et al.*, 1999; Corpet & Pierre, 2003). Sohn et al., (1999) found only a small number of *Apc* mutations in male Sprague-Dawley rats treated with DMH. All 6 mutations were single base substitutions which did not truncate the protein, and 5/6 of the mutations were upstream

of the mutation cluster region (MCR), unlike human CRC where a large proportion of mutations are truncating mutations and occur in the MCR. No mutations were found in the *p53* gene, although rat *p53* has a different structure to human *p53* (Erdman et al., 1997), so mutations may have occurred in parts of the gene that were not investigated, or the altered structure may have been protective.

The disadvantages of chemical carcinogen models include i) they are genetically less site-specific than deliberately introduced genetic mutations and ii) they require injection into the animal, and may produce different effects if the quantity of carcinogen or site of injection varies (Hoffman-Goetz, 2003). In addition, mouse studies have demonstrated strain and dose-dependent variations in tumour generation in response to AOM administration (Bissahoyo et al., 2005).

1.7.2 Mouse models

Several mouse models of intestinal cancer are now available that do not require use of carcinogens. In 1990, Moser et al described the introduction of a germline mutation in B6 male mice by exposing them to ethylnitrosourea (Enu) (Moser et al., 1990). The treated males were then mated with AKR females and a circling trait was noticed in one of the progeny. This mouse was mated with a B6 male to see if the circling was hereditary. The offspring demonstrated progressive anaemia, which often led to death. On dissection, visible tumours were noticed in the intestine, but only in anaemic animals. It became clear that the primary phenotype of the animals was the formation of multiple adenomas with anaemia as the secondary phenotype. The gene responsible for the tumours was named "multiple intestinal neoplasia", or Min (Moser et al., 1990). The Min trait was transmitted to 50% of the progeny with an unbiased sex distribution, an autosomal dominant characteristic.

Min-/Min- pregnancies are embryonic lethal. They are able to form blastocysts and to implant but severe defects occur during primitive ectoderm development. Similar findings have been shown in mice homozygous for loss of β-catenin and E-cadherin, both important molecules in the process of cell adhesion (Shoemaker et al., 1997), and

demonstrate the requirement for functioning versions of these molecules, as discussed in 1.3.5.

In 1992, Su et al demonstrated using linkage analysis that the responsible gene was the murine homologue of *APC* (*mApc*), linked to the Min locus. A nonsense mutation was found at codon 850 of the *Apc* gene (Su et al., 1992), (TTG to TAG) which changed a leucine to a stop codon. This is the same gene that is mutated in FAP patients and in the majority of sporadic colorectal cancers (Calvert & Frucht, 2002b).

Oshima et al reported the production of a mouse that developed multiple intestinal neoplasias (Oshima et al., 1995). The *mApc* gene was truncated by inserting a DNA cassette at codon 716 ($Apc^{\Delta 716}$). All mice developed polyps by seven weeks of age and most (7 of 8) had polyps by 5 weeks of age. Polyps in the gut of 3 week old mice were detectable under a microscope, the authors postulate that initiation occurs before the third week. The number of polyps increased with age and varied in size and stage. By week 16 of post-natal life the mice had an average of 254 polyps. A dissecting microscope was used to score the tumours, counting all bigger than 0.2mm. The small intestinal polyps had a layer of normal villous epithelium covering the tumour. It was discovered that the microadenomas originated from single crypts – the proliferation zone of the crypt grew and formed outpockets which expanded into the inner (lacteal) side of neighbouring villi, but not into the lumen of the intestine. After dissecting out the intestinal microadenomas and performing PCR, it was discovered that the wild-type Apc allele had been lost but the mutant allele was unchanged. From this the authors postulated that loss of heterozygosity led to the formation of intravillous microadenomas (Oshima et al., 1995).

Further study of the mechanism of polyp formation in $Apc^{\Delta 716}$ mice suggests that the same mechanism is responsible for producing colon polyps (Oshima et al., 1997). The authors suggest that a functioning, normal Apc protein is required for cell migration along the crypt-villus axis of the small intestine, as the cells containing the mutation were not growing upwards towards the lumen as in normal tissue.

Mice with mutations at other locations in the *mApc* gene also exist, including *Apc*1309 (Corpet & Pierre, 2003), $Apc1638^{N}$ and $Apc1638^{T}$ (Fodde & Smits, 2001) and comparisons of the various models have established a genotype:phenotype relationship.

The length of mApc protein produced from the mutated allele of the *Apc* gene is inversely associated with the number of tumours produced in the intestine (see Table 1.7), i.e. mutations that result in larger amounts of the Apc protein cause the mildest phenotype. The exception to this is the *Apc*1638^{T} mouse, which does not produce any tumours, although the cells are predisposed to chromosomal instability (Fodde & Smits, 2001). The lack of tumour formation is due to the ability of the Apc protein to regulate β-catenin levels (see 1.3.5) despite being truncated (Fodde & Smits, 2001).

Table 1.7 Number of tumours produced by mice with different *mAPC* mutations

Mouse strain	Number of tumours produced in the gut (mean ± SD)
Apc$^{\Delta 716}$	250 ± 95
Min (mutation at codon 850)	40 ± 20
*Apc*1309	30
*Apc*1638^{N}	3
*Apc*1638^{T}	0

Data from Corpet and Pierre (2003)

1.7.2.1 Further details on the Min mouse

The characteristics of spontaneous multiple neoplasias make Min mice a useful choice as an animal model for human intestinal cancer. Each cell of the Min mouse contains only one copy of the *mApc* gene, so only one further mutation is required for aberrant cell growth. This is unlike "normal" mucosa in the human intestine where mutations to both alleles are required to cause disease. This leads to rapid formation of tumours in the Min mouse (within the first few weeks of life), compared with the normal human state, where intestinal tumours may not form until middle age or older. The growth of colorectal tumours is due to both increased proliferation (Wong *et al.*, 2002) and decreased apoptosis (Bedi *et al.*, 1995) of the epithelium. Spontaneous apoptosis in the crypts of normal tissue is approximately ten times more common in the small intestine than the colon, which may explain the difference in cancer distribution in humans between small

intestine and colon (Potten & Grant, 1998). Proliferation is greater in Min mouse small intestine than in the colon, reflecting the distribution of tumours found there (Bashir *et al.*, 2004).

Table 1.8 shows some of the pros and cons of the Min mouse model.

Table 1.8 Advantages and limitations of the Min mouse model

Advantages	Limitations
Polyp number can be altered by dietary and pharmacological agents, e.g. NSAIDs	Most Min polyps are in the small intestine, whilst being used to simulate colorectal tumours
Polyps develop rapidly	ACF are not observed
Share the same genetic mutation as many human colon cancers	Adenomas do not progress to carcinomas, nor metastasise
Tumours are easily located and measured	Mice have to be killed to allow localisation of tumours

1.7.3 Exercise and animal models

Animal models of colon cancer can provide insights into the impact of exercise (or other factors) on risk of human disease because they allow more controlled manipulation of the factor(s) under study, greater invasiveness of experimental approach for study of underlying mechanisms and a shorter timescale of disease progression. The majority of studies have used rats and have induced carcinogenesis through chemical means, as rates of spontaneous colon tumour formation in rats are low. Table 1.9 (rats) and Table 1.10 (Min mice) summarise the different rodent studies of exercise on intestinal tumourigenesis. The exercise interventions differed between studies, utilising both voluntary wheel-running (Andrianopoulos *et al.*, 1987; Reddy *et al.*, 1988) and forced treadmill running or walking (Klurfeld *et al.*, 1988; Thorling *et al.*, 1993; Thorling *et al.*, 1994; Colbert *et al.*, 2000).

A reduction in tumour number was achieved with exercise in all the carcinogen-treated rat studies, but not in the genetically altered Min mice (Figure 1.2). The greatest protection was seen in the Thorling et al. study (1994), where the corn oil-fed exercising rats had no colon carcinomas compared with 37% of the control animals, and no small bowel carcinomas compared with 5 carcinomas in control rats (Thorling et al., 1994). This group also reported a difference in tumour generation that was diet-dependent, as coconut oil-fed rats suffered fewer tumours than corn oil-fed rats, but no significant effect of exercise was seen with coconut oil-fed rats.

Table 1.9 Effects of exercise on large bowel neoplasia in azoxymethane (AOM)- or 1,2-dimethylhydrazine (DMH)-treated rats

Rat strain and sex	Type of exercise	Exercise protocol	Study duration	Timing of exercise treatment	Tumour incidence		Tumour multiplicity (tumours/rat)[1]		Reference
					Control	Exercise	Control	Exercise	
					(%)				
Sprague-Dawley male	Wheel	Free access to wheel 33 cm diameter	20 wk after last DMH injection	At same time as DMH injections	93 $P < 0.05$ n=20	55 n=11	2.0 ± 0.38 $P = 0.06$	0.9 ± 0.48	Andrianopoulos et al., (1987)
F344 male	Treadmill	60 min/d@ 24 m/min 7° gradient 5 d/wk	-	After last DMH injection	75	36	2.1 ± 0.4	1.3 ± 0.2	Klurfeld et al., (1988)
F344 male	Wheel	Free access to wheel 13.5 inch diameter	38 wk after last AOM injection	After last AOM injection	66 $P < 0.05$ n=27	41 n=27	1.22 ± 0.8 $P < 0.05$	0.44 ± 0.5	Reddy et al., (1988)
Fischer male	Treadmill	5 h/d@ 7 m/min 5 d/wk	38 wk after last AOM injection	After last AOM injection	78 n=32	53 n=32	1.12 $P < 0.05$	0.72	Thorling et al., (1993)
F344 male	Treadmill	5 h/d@ 7 m/min 5 d/wk	38 wk after last AOM Injection	After last AOM injection	37^2 $P < 0.001$	0^2	1.33^3	1.07^3	Thorling et al., (1994)[4]
					20^2 $P<0.3$ n=30	10^2 n=30	0.73^3	0.57^3	Thorling et al., (1994)[5]

[1] Numbers after ± refer to SD, [2] Carcinomas only; [3] Total neoplasms; [4] Corn oil fed group; [5] Coconut oil-fed group. Table adapted from Basterfield et al., (2005).

Table 1.10 Effects of exercise on intestinal neoplasia in *Apc*^{Min} mice

Type of exercise	Exercise protocol	Study duration	Total intestinal polyps (polyps/mouse)				Reference
			Males		Females		
			Control	Exercise	Control	Exercise	
Treadmill	60 min/d @ 18–21 m/min 5% gradient, 5 d/wk	7 wk (4wk old at start)	36.8 ± 4.9	24.8 ±3.6	38.0 ±6.3	40.9 ± 3.7	Colbert et al., (2000)
			No significant effect of exercise for either sex				

Adapted from Basterfield et al., (2005)

The other rat studies demonstrated reductions in tumour number with exercise of 20-64% (Table 1.9), with all sedentary rats generating at least 1 tumour (with the exception of the coconut oil-fed animals). In contrast, the Min mice generated far more tumours (< 41), but demonstrated a non-significant reduction in number for males only (Table 1.10). Indeed, exercising females generated slightly more tumours than control females.

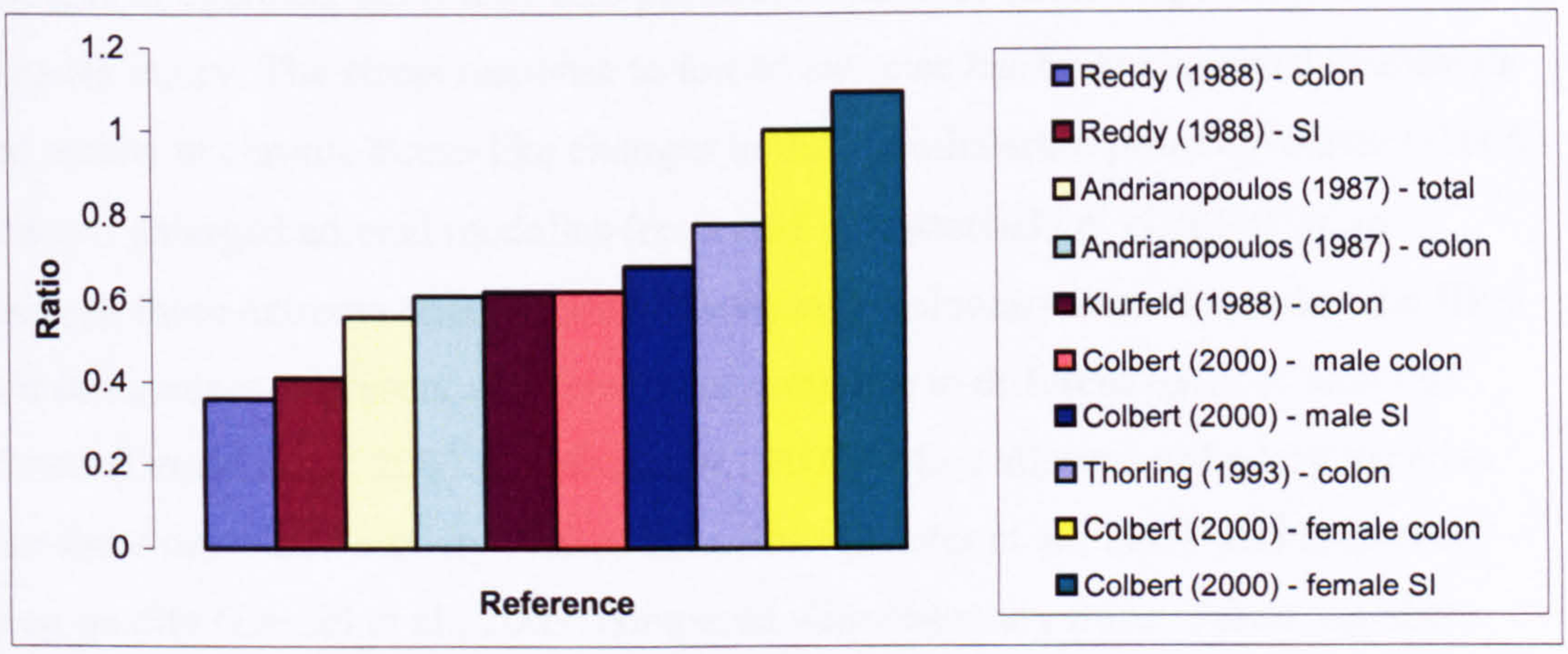

Figure 1.2. Ratios of tumour numbers between exercising and control animals, i.e. the number of tumours in control animals divided by the number of tumours in exercised animals. This allows studies generating different numbers of tumours to be compared easily. The references left to right correspond with the legend top to bottom.

1.7.4 Comparing animal studies

Several differences existed between the studies, including mode of exercise, duration, carcinogen used (or not) and rat strain.

Exercise mode

Mode of exercise can be separated into forced or voluntary, with 2 of the studies using the voluntary approach and 4 using the forced. The forced exercise varied from running for an hour to walking for 5 hours. This may be significant for several reasons – animals run great distances in wheels to which they have free access, up to 20km per day in mice in one study (Koteja et al., 1999) and may be more compliant with an unforced activity. A problem with the voluntary approach is that some animals will naturally run more than others and this inter-animal variability in exercise exposure may contribute to variability in tumour outcomes. For example, Reddy quotes highs of 10 000 and lows of 300 revolutions per day (Reddy et al., 1988) for rats allowed access to exercise wheels. In addition, the distance run per day may also decline with the duration of the study (Goodrick, 1980). This may, however, generate a "dose-response", with different running distances generating different amounts of tumours. The use of a treadmill is one way of ensuring that all animals are receiving equal amounts of exercise, but it may also produce an adverse stress response in the animals or cause injury. The stress response to forced exercise has been measured in animals and results in chronic stress-like changes to the hypothalamic-pituitary-adrenal (HPA) axis and enlarged adrenal medullas (reviewed in Basterfield et al., (2005)). In contrast, these extreme changes are not seen with voluntary exercise, rather the HPA axis reorganises to present a differentiated response to different types of stressful stimuli (Droste *et al.*, 2003; Penalva *et al.*, 2003). Mice allowed voluntary exercise also show decreased anxiety-related behaviour (Binder et al., 2004) and improved sleep quality (Lancel et al., 2003) compared with sedentary mice. Taken together, these findings present a case for the use of voluntary as opposed to forced exercise.

Length of study

The studies also varied in length, with the rat studies 20-38wk in length and the Min study 7 wk long. Long-term studies with Min mice are not feasible as the animals become noticeably ill by around four months of age and rapidly decline in health, whereas the rat studies must allow time for the carcinogen to work. To allow

comparison of the different strains, rat studies (after carcinogen administration) could be the length of the Min lifespan.

Strain differences

As mentioned in 1.7.1, strain and dose-dependent differences have been reported for the action of carcinogens (Bissahoyo et al., 2005), and differences in tumour number in rat studies may reflect this. Strain differences in metabolic adaptation to high fat diets have been reported in mice (Funkat et al., 2004), and therefore a species difference may also occur, accounting for differences in results between mice and rats. It must be noted that while the rats and Min mice produced tumours in both the colon and small intestine, rats have mostly colonic tumours and Min mice produce mostly SI tumours.

Sex balance

Another difference is the use of males only in the rat studies (Table 1.9), whilst the Min study used both males and females (Table 1.10). There was a tendency towards protection in the male Min mice (P = 0.06 overall, P = 0.08 for the SI and 0.06 for the colon). The possibility of a sex effect in the rat studies could not be examined as only males were used, and the presence of female rats may have negated the beneficial effects of exercise observed in those studies.

1.7.5 Dietary factors

A difference between the studies that may have an effect on the outcome is diet. Of the studies that mention diet, all but one used standard low fat rodent diets. However, to reflect the current human pattern of colon cancer incidence a Western-style high fat diet would be most suitable.

High fat diets

Fischer-344 rats fed a "high risk diet" (HRD) high in fat and low in fibre and calcium developed more AOM-induced ACF than rats fed a "low risk diet" (LRD, low in fat, high in fibre and calcium) (Shivapurkar et al., 1992), and 92% of the rats fed the HRD also developed colon tumours, whilst none of the LRD-fed rats did.

Wasan et al., (1997) reported that 10%- and 15%-fat diets increased polyp number and size in Min mice when compared with standard 3% fat diets. A LRD was found to suppress polyp formation in $Apc^{\Delta 716}$ mice by 36% in the small intestine and 56% in the colon compared with mice fed a HRD (Hioki et al., 1997).

Newmark et al., (2001) fed a western-style diet (high in fat, low in calcium, vitamin D and methyl donors) to C57BL/6 mice for 18 months and found that 42% of the mice generated intestinal tumours, compared with none in the control diet group. This demonstrated the ability of western-style diets to induce tumourigenesis in mice without the use of carcinogens or genetic modifications.

A high fat diet may cause an increase in body fatness, and obesity caused an increase in AOM-induced tumours in male Zucker rats (Weber et al., 2000). A leaner body composition (as achieved by physical activity) may be protective as increased adiposity is associated with increased risk of colon tumour occurrence (Calle & Thun, 2004). The reasons for this are believed to be due to increased circulating levels of insulin and free IGF-1 and decreased IGFBP-3 which all occur with increasing adiposity (see 1.6.6.2).

The extra energy expenditure of exercising animals may lead to a compensatory hyperphagia (Mehl et al., 2005b) which does not translate to increased body fat (Thorling et al., 1993), even when fed a high fat diet (Cohen et al., 1988), though others (Colbert et al., 2000) have not observed this effect.

1.7.6 Mechanisms

Several of the studies have examined the effect of lean body mass, body fat and food intake with respect to activity.

Body mass and food intake

Some animal studies of exercise and colon cancer have reported a decrease in body mass with exercise (Andrianopoulos *et al.*, 1987; Klurfeld *et al.*, 1988; Thorling *et al.*, 1993; Thorling *et al.*, 1994), but others have reported little (Reddy et al., 1988) or no (Colbert et al., 2000) difference.

The lower body weight of the exercised animals as reported by Andrianopoulos et al. (1987) was assumed to be due to increased energy expenditure alone, as the food intake of these animals was not measured. Klurfeld et al. (1988) also found decreased body weights in exercised animals, but noted that both exercised and sedentary animals consumed equal amounts of energy. The exercised animals had a similar percentage of body fat to sedentary animals (21.6 v. 21.8%), but half the tumour incidence. However, Reddy et al. (1988) found that body weights in both sedentary and wheel-running groups were comparable throughout the study with exercising rats weighing slightly more at death. The exercising rats consumed approximately 264kJ/day and the sedentary group 234kJ/day (Reddy et al., 1988).

Thorling et al. (1993) found a slower rate of weight gain in exercised compared with sedentary animals. Exercising animals weighed significantly less at the end of the experimental period even after consuming about 20% more food. The sedentary animals had considerably more body fat than exercising animals (96 ± 7g compared with 28 ± 5g or 22% v 8%). Thorling et al. (1994) reported that exercised rats ate 25% more than sedentary rats but still weighed less and had fewer tumours.

Colbert et al. (2000) found no difference in body weight or food intake between treatment groups in Min mice and also no difference in polyp number.

As 4/5 studies reported a decrease in body mass, the lower tumour incidence in exercising rats may therefore be due to the lower fat mass, and is probably not due to total energy intake but may be related to energy balance. A future Min mouse study that was able to show a reduction in tumour number may also demonstrate a difference in body mass, however, species or carcinogen effects may result in different mechanisms.

Other mechanisms

The only other potential mechanism examined was transit time. Klurfeld et al., (1988) used the indigestible marker chromic oxide and found a reduced intestinal transit time in exercised compared with sedentary animals ($P<0.05$), although no further details were given.

Investigations of other postulated mechanisms for tumour prevention in humans, such as enhanced immune surveillance (Armstrong & Mathers, 2000), prostaglandins and cytokines, have not been reported.

1.7.7 Non-exercise physical activity (NEPA)

One observation missing from all studies to date is the amount of activity undertaken by animals when not in the wheel/treadmill. Animals given/undertaking low levels of exercise may be more inclined to continue high levels of activity in the cage (nest building, rearing, climbing etc) which may obscure the effect of an imposed exercise regime. Animals receiving high/exhaustive levels of exercise may be much less inclined to undertake such activities than their sedentary counterparts. Golden hamsters housed with wheels show significantly less cage-bar climbing and stereotypic bar-mouthing than sedentary controls (Gebhardt-Henrich et al., 2005), and wheel-running mice show fewer but longer periods of sleep time (Lancel et al., 2003) both of which may indicate less NEPA.

1.7.8 Other modifiers of tumour number in animals

Diet and activity are not the only means by which tumourigenesis in animal models can be modified:

Caloric Restriction – Klurfeld et al., (1988) found a decrease in tumourigenesis in rats on a 25% calorie restricted diet, with tumour incidence reduced from 75% to 35%, and incidence was reduced to 21% with a 40% restricted diet. Reddy et al., (1987) also found a decrease in tumour number in rats with treated with AOM, with incidence decreasing from 83% to 33% with a 30% CR diet. A 40% calorie restricted diet in Min mice decreased intestinal polyp frequency by 57% (Mai et al., 2003), and Kakuni et al. (2002) found a significant decrease in polyps >2mm in mice with a 20% food restriction compared with control (similar decreases as those observed by Mai et al., 2003), though a rise in smaller (<2mm) polyps was observed.

Mom1 - Modifier of Min 1 (Dietrich *et al.*, 1993; Shoemaker *et al.*, 1997). Backcrossing Min with B6 mice produced mice with fewer tumours, suggesting that a genetic modifier was present. *Mom1* was located to mouse chromosome 4 and found to be a semi-dominant modifier of intestinal tumour size and multiplicity in Min. The gene responsible codes for secretary phospholipase A2 (Pla2g2a), involved in the synthesis of prostaglandins by the release of arachidonic acid from membrane lipids (Fodde & Smits, 2001).

Mom2 – Modifier of Min 2 (Silverman et al., 2002). Silverman et al. showed that one resistant allele at the Mom2 locus reduced intestinal polyps in Min by up to 95%, suggesting that it acts in a dominant fashion. The identity of Mom2 has yet to be proven.

Msh2 – mutS homolog 2 (Shoemaker et al., 1997). Msh2 is the murine equivalent of hMSH2 in humans, mentioned previously with respect to HNPCC (section 1.3.2). A homozygous mutation in Msh2 in mice produced skin tumours and lymphomas as well as intestinal tumours. When crossed with Min, the number of intestinal tumours increased by a factor of 3.4 (Shoemaker et al., 1997).

Dnmt – DNA methyltransferase (Shoemaker et al., 1997). Dnmt are knockout strain of mice. They display a 50% reduction in DNA methylation levels, with homozygotes dying *in utero*. When Min mice were also Dnmt heterozygotes the number of tumours generated was reduced by 2.5 fold. When Min mice were treated with 5-aza-2'-deoxycitidine (5-aza-dC – a potent DNA methyltransferase inhibitor) a 5.7 fold reduction in tumour number was seen. When 5-aza-dC treatment was given to Min that were heterozygous Dnmt, the tumour load was reduced by 57 times (Laird et al., 1995). The hypomethylation affects gene expression, which then alters expression of oncogenes or tumour suppressor genes and contributes to neoplastic transformations by affecting somatic mutation rates at 5-methylcytosine residues (see 1.4).

1.7.9 Relevance of rodent models to human bowel cancer

The relevance to humans of these studies depends on whether the exercise regimes can be translated into human equivalents. Colbert et al. used a value of 55-65% VO_{2max} for the mice (equal to speeds of 18-21 $m \cdot min^{-1}$), and studies using mice to study the immune effects of exercise also use speeds between 15 and 30 $m \cdot min^{-1}$ (MacNeil & Hoffman-Goetz, 1993; Woods *et al.*, 1993; Woods *et al.*, 1994; Lu *et al.*, 1999). Neither of the papers by Thorling gives a reason for the activity level chosen but it is of a low intensity over a long time period, which contrasts with the faster, shorter exercise of Klurfeld et al., (1988), which was estimated to be equivalent to 25% of the daily energy intake, performed 5d/wk.

25% of daily energy intake is equivalent to 2.7MJ in a 74kg man and 2.0MJ in a 60kg woman, given a daily energy intake of 10.6MJ and 8.1MJ, respectively (estimated average requirements for adults aged 19-50 with light energy requirements recommended by COMA (1991)). This equates to approximately 2 hours of walking[1]/1-1.5h jogging[2]/1.5h cycling[3], all at a moderate pace (McArdle et al., 2001). This compares favourably with recommendations from the World Health Organisation of 1h walking per day (WHO/FAO, 2003), with the findings of Thune and Lund of 4h cycling per week (1996), and with Martinez et al., (1997) of 4-5h/wk moderate intensity activity. As the intensity of the exercise increases, the time spent in activity will decrease accordingly. Each of these activities is at an intensity and duration that can be fitted into everyday life. As all of the rat studies showed a decrease in tumour number with exercise, these are potentially achievable levels for humans.

A reduction in tumour number in the Min mouse would be the most exciting find, given the genetic similarity with some human conditions. The lack of evidence found so far in this model might indicate the overwhelming strength of the genetic predisposition, or that by week 3/4 of age when the exercise was started the tumour initiation process is too advanced to be slowed or reversed by exercise. This would be in accordance with a study by M. L. Slattery and R. Burt who examined the effect of physical activity in a group of patients with attenuated familial adenomatous polyposis (AAPC; the phenotype is less severe than with classical FAP but retains an elevated risk of colon cancer (Spirio et al., 1993)). No effect was seen (unpublished data, M. L. Slattery, personal communication). No other studies examining the effect of physical activity on FAP patients appear to have been published, and this group of patients is generally omitted from epidemiological studies of physical activity due to the strong disposition to cancer.

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[1] 18.39kJ/min required for 59kg woman at 3mph, 22.00kJ for 74kg man
[2] 33.44kJ/min required for 59kg woman running 11.5 min/mile, 41.80kJ for 74kg man
[3] 24.66kJ/min required for 59kg woman cycling at 9.4mph, 30.93kJ for 74kg man

1.8 Objectives of thesis

The main objectives of this thesis are:

- To investigate the utility of the Min mouse model in studies of exercise on tumourigenesis
- To quantify the types and durations of exercise which are beneficial in reducing intestinal tumourigenesis
- To investigate the possible mechanism for the protective effects of exercise
- To examine the effect of exercise combined with diets of different fat content on body fatness and non-exercise physical activity (NEPA)

The remainder of this thesis is set out in the following order: Chapter 2 – General Methods and Materials. Chapter 3 – detailed reports of three experiments using the Min mouse. Each experiment is reported individually, with a summary of the results followed by a brief discussion of points specific to that experiment. The collated results are then presented and discussed in detail. Chapter 4 – describes a study of the effects of two diets differing in fat content and of exercise on body fatness and NEPA. Chapter 5 – contains the conclusions from these studies and suggestions of how this research area could be taken forward.

Chapter 2

2 General Methods and Materials

2.1 Animals

All mice used in this study were bred in the animal facility of Newcastle University, the Comparative Biology Centre. Female C57BL/6 either from the colony or purchased from Harlan (Oxon, UK) were mated with male colony Min mice. Parent animals were fed standard rodent chow (SDS, Essex, UK) ad libitum. Water was freely available.

Tail snips were taken from 4 week old mice and genotyped using an established PCR method (based on Su et al., (1992)) to distinguish those carrying the *Apc* mutation characteristic of Min mice (see Appendix 6.1).

2.2 Min PCR results

Min fragments were separated on an 8% polyacrylamide gel. Wild types are cut twice on both strands by the restriction enzyme Hind III and produce one band at 123 bp (see Figure 2.1 and Figure 2.2) and a digest product of 11bp. Min heterozygotes show a distinctive double band at 123 and 144bp as the mutated strand is cut only once, producing the longer fragment (144bp) as well as the shorter wild-type fragment (123bp).

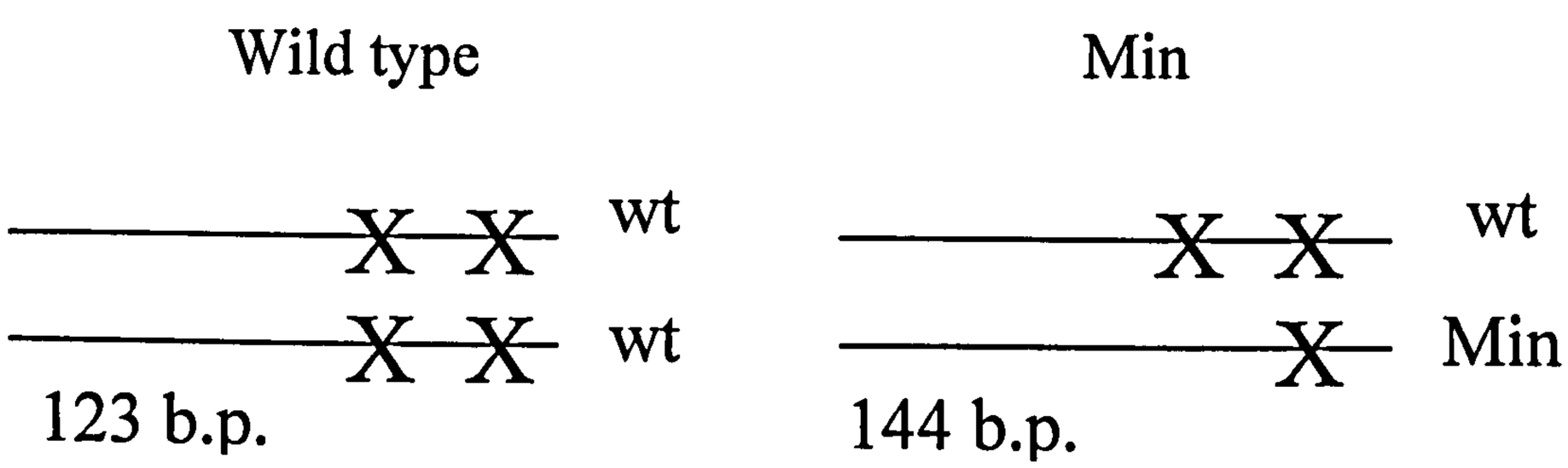


Figure 2.1 Diagram of fragments produced during enzyme digestion stage of genotyping

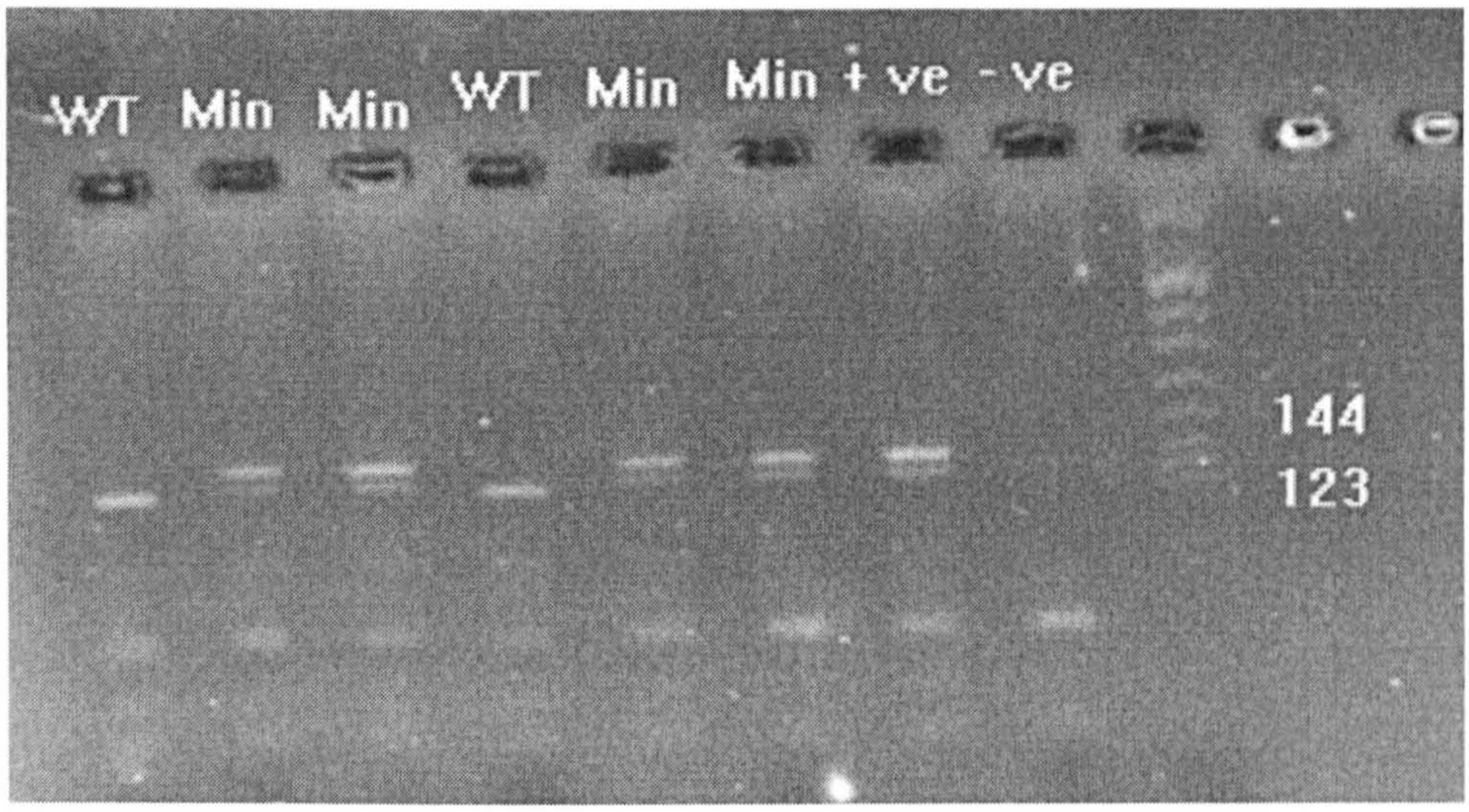


Figure 2.2 Example of genotyping results, showing Min and wild-type fragments

2.2.1 Housing

Mice were housed in cages containing 1-6 mice. A 12 hour light:dark cycle was maintained (with the light period 7am-7pm) in a room with 15 changes of air per hour. Room temperature was 20°C ± 2°C.

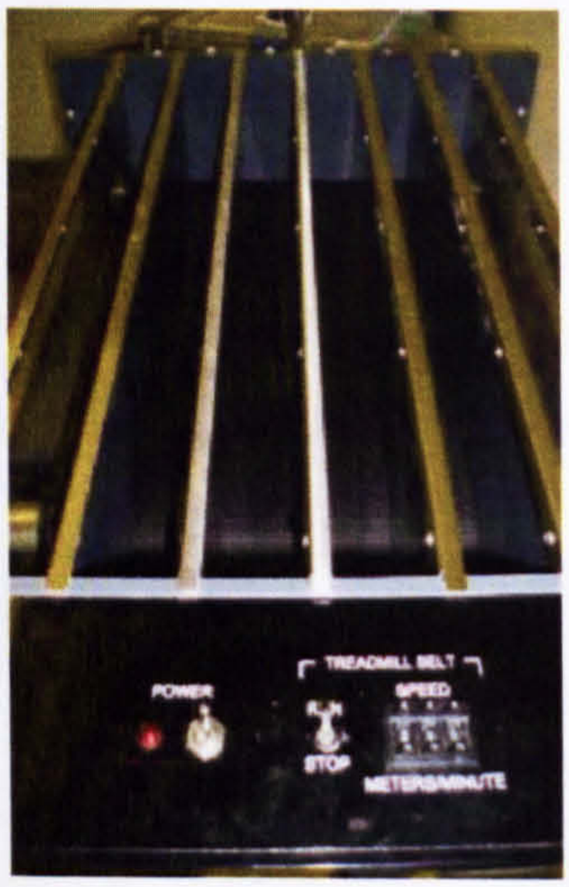
2.2.2 Experimental diet

Mice were weaned at four weeks of age and offered 6g of the "Western" diet each, daily (see Appendix 6.2 and Williamson et al.,(1999)). This diet was relatively rich in animal fat, animal protein and sucrose. Refusals were removed and weighed daily to obtain food intake, which was also calculated on a per gram body mass basis using the weight of each mouse at the beginning of each week. Water was freely available. Mice were weighed weekly.

2.2.3 Treadmill

Treadmill mice (TR) were run on an Exer-3/6 rodent treadmill (Columbus Instruments, Ohio, USA, supplied by Linton Instrumentation, Norfolk, UK). The

treadmill consisted of a rubberised running surface, connected to a motor whose speed was controllable in increments of 0.1 meters per minute up to a maximum of 99.9 meters per minute (Figure 2.3).



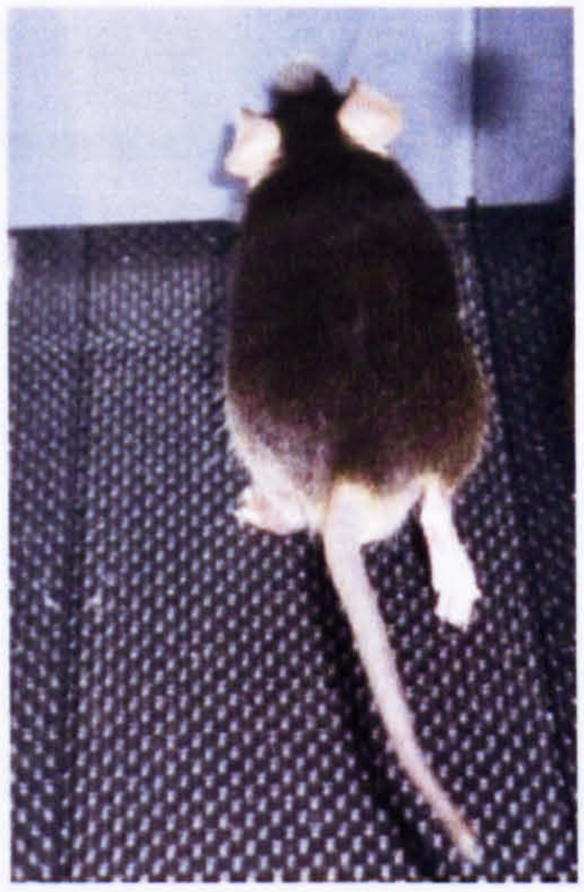


Figure 2.3 The treadmill and a male mouse running

The running surface was divided into 6 individual lanes measuring approximately 37cm long x 6cm wide by removable plastic slats and the incline could be increased from 0% to 20% in increments of 5%. There was a non-moving area at the rear of the running surface where an electric shock grid could be placed but this was not used in these studies. A brush-covered gap of approximately 1cm between the running surface and the non-moving rest area allowed faeces and urine to drop from the running surface to be collected on a tray under the treadmill. To prevent mice from stopping unnecessarily, a barrier was cut from 1cm thick foam rubber which slotted onto the lane dividers at the back of the running surface. This also prevented any tails or feet slipping into the gap between running surface and rest area. The barrier was not used for mice that ran competently. The treadmill was cleaned thoroughly after every session by scrubbing with hot water.

2.2.4 Wheel-running mice

Wheel-running mice (WH) were housed individually in cages containing a running wheel. Twelve wheels were borrowed from Barrie Sandells (Novartis Institute for Medical Sciences, London, UK) and 12 were purchased separately ("5½" Comfort Wheel", Pets International, Ltd., IL, USA). A magnet (Abel Magnetics, Sheffield, UK) was attached to the wheel which actuated a magnetic counter (RS Components Ltd, Northants, UK, product # 260-375) on every revolution. The counter was fastened to the top of the cage and was actuated by wheel movements in both directions. The wheels had internal diameters of 10cm (borrowed wheels, (Fig. 2.4)) and 13cm (purchased wheels) producing internal circumferences of 62.8cm and 81.7cm (i.e. 62.8/81.7cm travelled per wheel revolution).

Figure 2.4 Example of small running wheel showing the counter attached to the cage lid

Daily revolutions were recorded for all mice. Free access was allowed to the wheel which was cleaned weekly. Other housing and feeding conditions were identical to TR. WH were kept in the same room as the treadmill to subject them to similar environmental conditions as TR.

2.2.5 Control mice

Control mice (CON) were kept under the same conditions as TR and fed the same diet. While TR were on the treadmill, CON were placed in individual control lanes for the same period of time, to subject them to the same handling, noise and isolation

stresses as TR. Control lanes were constructed from laminated paper and placed in a large plastic box next to the treadmill. Mice were unable to climb out of the lanes. The lane dividers and box were cleaned with hot water after each use.

2.3 Necropsy

Mice were killed within one week of finishing the study (see specific chapters for details of study length). Total anaesthesia was obtained using 3% isofluorane in oxygen followed by cardiac exsanguination and cervical dislocation. Each mouse was weighed before a midline section was made and organs dissected out. Tumours were scored in millimetres diameter by the same person who was unaware of the exercise protocol of each mouse (A. Kitching, CBC, Newcastle University, UK). Tumour location and size were noted for each mouse and tumour burden calculated by summing the individual tumour diameters to produce total diameter of tumour present.

2.3.1 Blood

Blood was collected into an EDTA tube and mixed by inversion. A haematocrit was obtained from the whole blood and the remaining blood was centrifuged to obtain the plasma. To measure the haematocrit, blood was drawn up into two glass capillary tubes per mouse, sealed with plasticine and centrifuged for 10 minutes in a micro haematocrit-centrifuge (Hawksley, Sussex, UK). Haematocrits were read on a micro haematocrit reader (Hawksley, Sussex, UK). Both plasma and whole blood portions were stored at -80°C.

2.3.2 Stomach

The stomach was weighed with its contents, then opened and digesta was removed and discarded. The tissue was examined for tumours and then re-weighed. The tissue was snap frozen in liquid nitrogen and stored at -80°C.

2.3.3 Small intestine

The small intestine was weighed intact and the length measured. It was then cut into two equal parts – proximal and distal. Each part was opened longitudinally and the

contents removed and discarded. Each section was examined for the presence of tumours, with site and size being recorded. Two cm of normal tissue was taken from the top of the proximal section and placed in Carnoy's fixative for future crypt cell proliferation analysis (if a tumour was present the normal sample was taken from the nearest section of tissue free of tumours). The tissue was re-weighed, snap-frozen and stored at -80°C.

2.3.4 Caecum

The caecum was weighed intact and then opened. The contents were placed into an eppendorf and stored at -80°C for use in determination of transit time. The presence of tumours was noted and the tissue re-weighed, snap-frozen and stored at -80°C.

2.3.5 Colon

The colon was weighed and then opened longitudinally. The contents were placed in an eppendorf and deproteinising solution (DPS) (Mathers *et al.*, 1990; Mathers & Fotso Tagny, 1994) added half weight by volume and stored at -20°C for future short chain fatty acid (SCFA) analysis. The colon was examined for tumours, with size and site being recorded. The tissue was then reweighed, snap-frozen and stored at -80°C

2.3.6 Other organs

The liver, heart and spleen were removed and weighed. The spleen was placed in ice-cold PBS for Natural Killer (NK) cell assay (pilot study only), whilst the heart and liver were snap-frozen and stored at -80°C.

2.3.7 Carcass

The carcass was stored at -20°C until body composition analysis was performed.

2.4 Statistical analysis

Analyses were performed with SPSS for Windows v12.0 and Minitab v13.

Data were tested for normality (Anderson-Darling) and normally distributed data were tested using the General Linear Model (GLM) of analysis of variance (ANOVA),

Table 2.1. Means and standard errors (SEM) are given for these variables. Pooled SEM are presented where appropriate, calculated as √(mean square error/n). Non-normal data were transformed using a suitable transformation, usually Log10, natural log or square root. Data that have been transformed are indicated in the text thus: variable ^{Log10}, variable^{natlog}, variable^{Sqrt}. Means were back-transformed and are displayed with 95% confidence intervals (CI). Where a table contains both normal and transformed values, the CI is given for the normal values instead of the SEM, for clarity.

Any data that remained non-normal were tested using a suitable non-parametric test (Kruskal-Wallis) and are indicated as variable^{KW}. The data generated by this method are median values, and are displayed with the range of the variable.

Unique tests are indicated and described in the text.

P values less than 0.05 were taken to be significant.

Table 2.1 Skeleton ANOVA table from study 2

Source of variation	Degrees of freedom	Sum of squares	Adjusted sum of squares	Mean square	F	P
Sex	1					
Exercise	2					
Sex*Exercise interaction	2					
Error	56					
Total	61					

2.5 Analytical methods

2.5.1 Body composition analysis

2.5.1.1 Body fat

Mouse carcasses were transferred to a -80°C freezer for 24 hours and then placed in a vacuum freeze drier (Edward's Modulyo freeze drier). Weights were recorded every 3

days for 2 weeks until constant weight was achieved and all water assumed to have been removed. The proportion of fat in the freeze-dried carcasses was measured using the Soxhlet procedure (see Appendix 6.3).

2.5.1.2 Mineral content

A sample of fat-free freeze-dried carcass (typically1-2g) was ashed in a crucible at 450°C overnight in a Muffle furnace (Carbolite, Sheffield, UK) to remove all organic matter in the dry, fat-free carcass. After cooling in a dessicator, the ash was weighed. This represents the mineral portion of the carcass.

2.5.2 Crypt cell proliferation

Crypts were microdissected from macroscopically normal sections of proximal small intestine. The tissue was initially fixed in Carnoy's and then transferred to 70% ethanol for storage. The tissue was then rehydrated in 50% ethanol, hydrolysed in 0.1M hydrochloric acid, stained in Schiffs' reagent (Sigma) and groups of crypts separated for examination of mitotic bodies (see Appendix 6.4). Number of mitoses in the 3 compartments of the crypt (upper, middle, lower) were recorded, as well as width and length. 10 crypts were counted for each sample.

2.5.3 Transit time determination – caecal chromic oxide concentration

Caecal contents were freeze-dried before use and the micromethod for determination of chromic oxide was used (see Appendix 6.5) as described by Mathers and Fotso Tagny (1994). Briefly, the rate of Cr_2O_3 input is known (the amount of food eaten per day), and the concentration of Cr_2O_3 in the caecal contents is calculated. The amount of Cr_2O_3 in the caecum is then divided by the rate of input to give an estimation of the time taken for the digesta to travel through the caecum. For this method, the flow of digesta into the caecum is assumed to be constant, as mice have constant access to their food (Mathers & Fotso Tagny, 1994).

The calculation for the transit time (in days) of digesta through the caecum is as follows:

$$\frac{\text{Mass of dry matter in the caecum x } Cr_2O_3 \text{ concentration in the caecum}}{\text{Daily food dry matter intke x } Cr_2O_3 \text{ concentration in dietary dry matter}}$$

2.5.4 Short chain fatty acid (SCFA) determination

Short chain fatty acids in the colon contents were determined using gas chromatography (see Appendix 6.6). Only samples with complete duplicates with discernable peaks were analysed.

Concentrations of SCFA in samples were calculated relative to the standards and internal standard (3 methyl-valerate, 3MV), present in DPS in the samples.

e.g. Response factor (RF) for acetate in the standard:

$$\frac{\text{Peak area 3MV x [acetate]}}{\text{Peak area acetate x [3MV]}}$$

Concentration of acetate in the sample:

$$\frac{\text{RF for acetate (std) x [3MV] x peak area acetate (sample)}}{\text{Peak area 3MV (sample)}}$$

2.6 Non-exercise physical activity (NEPA)

An infrared-sensing device ("Inframot", TSE Systems, Germany) was used to quantify cage activity performed by singly housed mice (see Figure 2.5). The Inframot senses movements of 5milliseconds length and greater and stores the data accumulated in set timescales in a spreadsheet. The data can then be manipulated to display activity at different times of day. There were four sensors available for four simultaneous recordings. Further details are given in 6.7.

Figure 2.5 Example of an Inframot device in place on the home cage.

Chapter 3

3 Investigations into treadmill- and wheel-running exercise

3.1 Pilot study

3.1.1 Introduction

At the start of this project, there was only one published study on effects of exercise on intestinal polyp development in Min mice (Colbert et al., 2000). The use of a treadmill (to quantify the amount of exercise imposed and to ensure that equal amounts of exercise were undertaken by each mouse) was considered to be the best way to test for an effect in this study. In addition, this approach provided a means of replicating aspects of the Colbert study.

This pilot study was designed to learn the best ways of using the treadmill, to develop techniques to get the mice to run and to develop standardised exercise protocols.

3.1.2 Aims

- To understand the best way of getting mice to run on the treadmill
- To follow the treadmill protocol as used in published studies
- To establish assays which could be used to investigate possible mechanisms for any change in tumour number

3.1.3 Hypotheses

- The number of tumours in treadmill-exercised mice will be reduced compared with non-exercised mice as a result of decreased transit time
- Treadmill running will reduce body fat content, increase plasma Interleukin-10 concentrations, decrease plasma Interleukin-6 concentrations and produce fewer mitoses in cells in the top third of small intestinal crypts

- Decreased tumour number will be associated with increased butyrate in colon samples

3.1.4 Materials and Methods – Pilot Study

3.1.4.1 Treadmill regime

From 4-5 weeks of age, treadmill-running mice (TR) were run 5 $d \cdot wk^{-1}$ for 12 weeks. Males and females were initially run separately to prevent the males fighting when returned to their cages. When two different speed groups developed, the sexes were run together. Weeks 1-2 were used to train the mice, and started with relatively slow speeds (10 $m \cdot min^{-1}$) for short time periods (10 min) on the target gradient of 5%. Speeds and times were gradually increased over the two weeks until the mice could run at 16$m \cdot min^{-1}$ for 60 minutes (three periods of 20 minutes with five minutes rest in between). The target speed was 20 $m \cdot min^{-1}$, and whilst several mice were capable of achieving this speed, several suffered damaged hind toenails, or became morose and refused to run. Mice that refused to run in the training weeks were swapped with control mice. After the 2 weeks training period, mice that refused to run were not replaced and were removed from the study.

Due to the number of problems encountered attempting to achieve the target of 60 minutes non-stop running at 20$m \cdot min^{-1}$ on a 5% gradient (Colbert et al., 2000), modifications were made to the exercise protocol. Mice ran in 10 min bursts with five minutes rest in between. A speed of 16$m \cdot min^{-1}$ was maintained by the majority of mice initially but by week 5 mice were running in two separate groups: a "fast" group running at 16$m \cdot min^{-1}$ and a "slow" group running at 11-12 $m \cdot min^{-1}$. The slow group ran for 15 minutes at a time. This continued to the end of the study, with mice changing groups as necessary. The lid was not used on the treadmill when it was necessary to use the rubber barrier to prevent the mice stopping, to make it easier to move any mice that became trapped at the rear of the treadmill after refusing to run.

3.1.4.2 Control mice

Control mice (CON) were placed in control lanes for the same length of time that the TR mice spent on the treadmill, as described in the General Methods and Materials section (Chapter 2).

3.1.4.3 Necropsy - specific sample collection

Mice were on study for 12 weeks, and were killed when aged 16-17 weeks. Tissues were collected as detailed in General Methods and Materials (Chapter 2).

3.1.4.4 Colon tumour Scanning Electron Micrograph (SEM)

Two large colon tumours were chosen at random and placed in 2% gluteraldehyde to be processed for scanning electron microscopy (see Appendix 6.8).

3.1.4.5 Interleukin-6 and Interleukin-10 determinations

Interleukin-6 (IL-6) and Interleukin-10 (IL-10) concentrations in plasma were determined by Enzyme-Linked Immunosorbent Assay (ELISA, Insight Biotechnology Ltd, Wembley, UK) using plasma samples obtained at death (see Appendix 6.9 for details.) The minimum concentrations of IL-10 and IL-6 in mouse plasma which could be detected reliably were 15 and 4 pg/ml respectively. Standards were assayed in triplicate for each concentration. Samples were assayed once due to the small quantities of plasma available.

Absorbances for standards were averaged for each concentration and then plotted. Regression analysis was used to calculate the equation of the line of best fit and sample absorbances were converted to concentration of IL-6/IL-10 using the relevant equation.

3.1.4.6 Natural Killer (NK) cell assay

Cell culture

A K562 leukaemic cell line (donated by Matthew Henry, School of Surgery and Reproductive Sciences, Newcastle University) was used in all immunological assays. Cells were maintained in an incubator at 37°C 5% CO_2 and split twice a week.

Principle of isolation of NK cells

Spleen cells were labelled magnetically with Anti-NK cell (DX5) microbeads and passed through a separation column which was placed in the magnetic field of a MACS separator. The magnetically labelled NK cells were retained in the column while the unlabelled non-NK cells ran through (the unlabelled cells were depleted of NK cells). After removal of the column from the magnetic field, the magnetically retained NK cells were eluted as a positively selected cell fraction. For further details see 6.10 and Arase, Saito et al., (2001).

MACS Anti-NK (DX5) microbeads, MS Separation Columns, MiniMACS Magnetic Separator were all from Miltenyi Biotec Ltd, Surrey, UK.

3.1.5 Results - Pilot study

23 Min mice started the study (9 female 14 male), but only 18 completed the 12 weeks (see Table 3.1 for details). Of the 5 males that did not finish the study, 3 were treadmill mice that stopped running after the 2-week training period when there were sufficient animals in the control group and so removed from the study. The other 2 male treadmill mice lost weight and ate less from week 7, became moribund and were killed in week 11. One mouse had 28 tumours and a tumour burden of 95mm, the other had 16 tumours, a tumour burden of 60mm and an intussusception (prolapse of part of the intestine into an adjacent part), most probably caused by the tumours. These 2 mice are excluded from growth curves and analysis of tumours and organs but are included in exercise analysis.

Table 3.1 Distribution of mice completing the pilot study

Exercise protocol	Male	Female	Total
Treadmill	4	5	9
Control	5	4	9
Total	9	9	18

3.1.5.1 Growth curves

Weights of CON and TR mice were similar for weeks 1-5; thereafter CON mice were heavier (Figure 3.1a). Control mice were significantly heavier on weeks 9 and 11 (P = 0.049 and 0.046, see Table 3.2). Although the mice gained weight throughout the study (except for the final week), the amount of weight gained each week decreased with time (see Figure 3.1b). Body weight and weight gain both plateaued towards the end of the study and then decreased in the last 1-2 weeks.

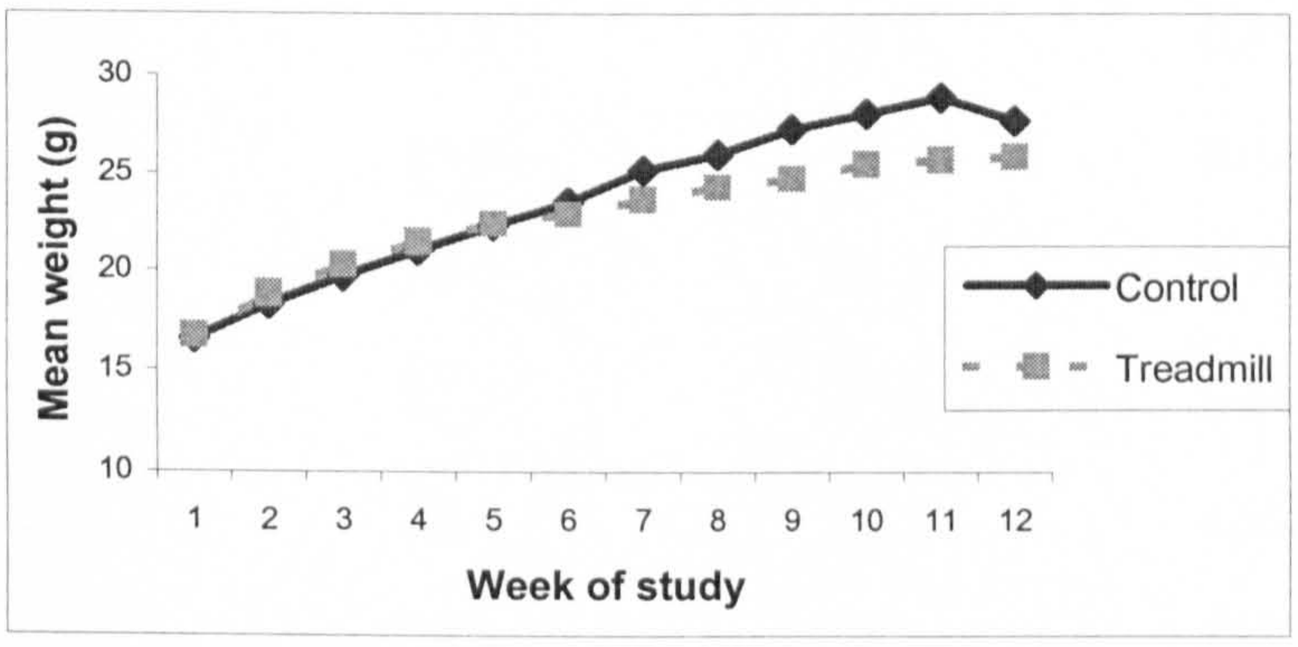
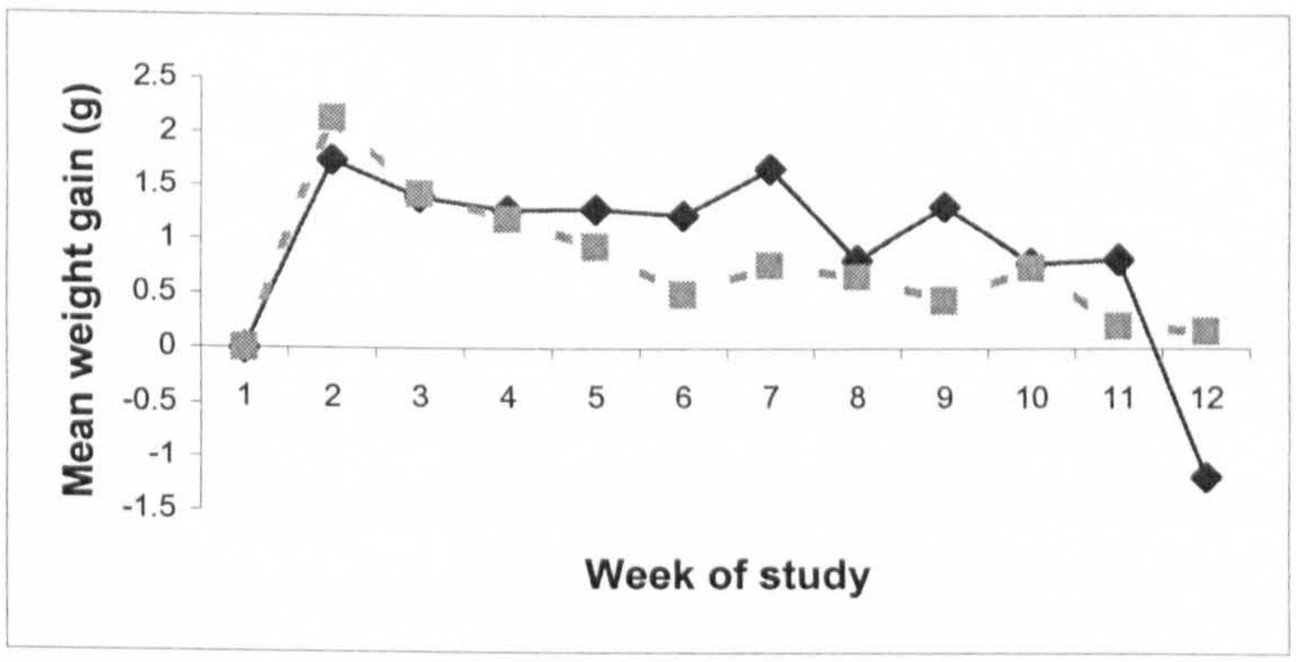


Figure 3.1a) Mean weekly weights of mice by exercise group



b) Mean weekly weight gain of mice by exercise group

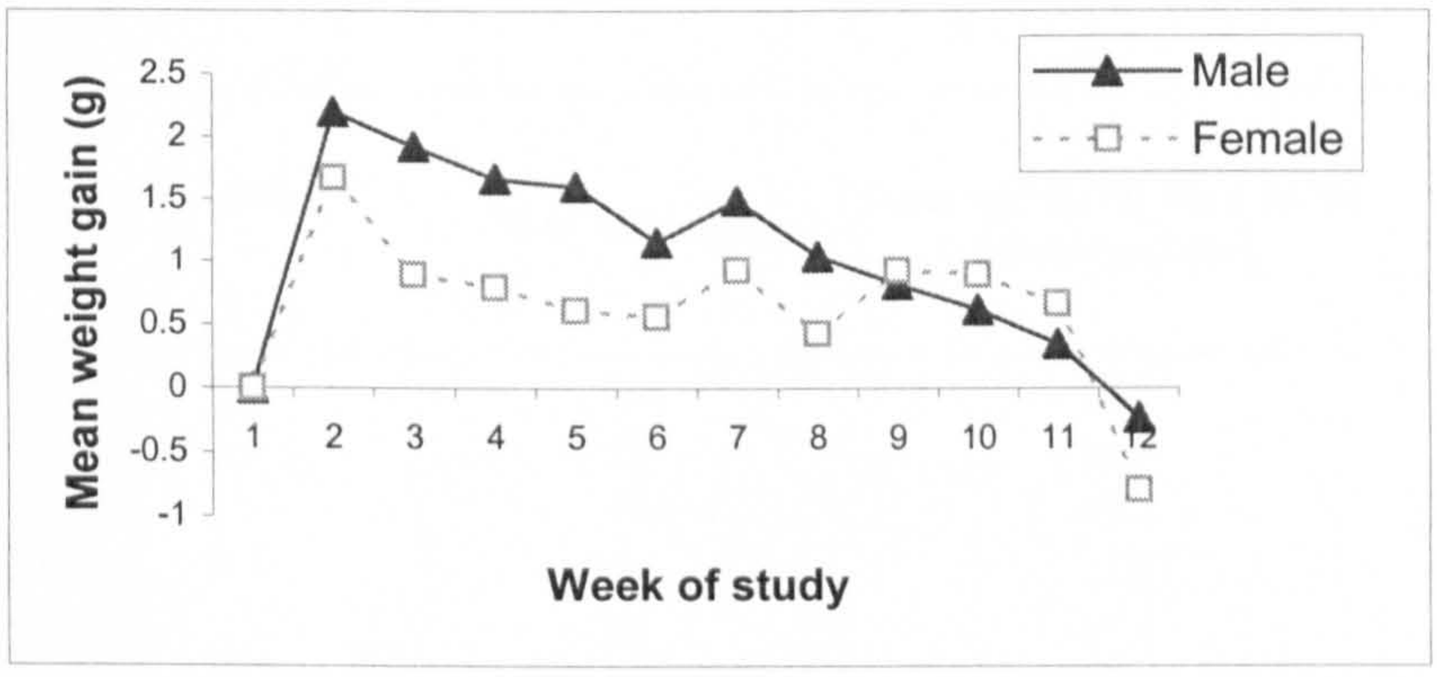
Table 3.2 Mean body weights (g) by week, of mice by exercise group

Week	CON	TR	SEM	Probability of effect
1	16.6	16.7	0.59	0.876
2	18.3	18.9	0.42	0.395
3	19.7	20.3	0.43	0.366
4	21.0	21.5	0.51	0.525
5	22.3	22.4	0.50	0.861
6	23.5	22.9	0.57	0.461
7	25.2	23.7	0.65	0.123
8	26.0	24.3	0.78	0.156
9	27.3	24.8	0.83	0.049
10	28.1	25.5	1.02	0.057
11	28.9	25.7	1.03	0.046
12	27.7	25.9	1.24	0.313

The pattern of weight gain was very similar for male CON and TR, but for females the rate of body weight gain begin to diverge at week 5, with the body weight of female TR mice remaining lower than CON for the remainder of the study. This difference in body mass was greatest in week 11, although there was no significant interaction between sex and exercise group.

At the start of the study (week 1), male mice were heavier than female mice (18.3 and 15.1g, respectively) and males remained significantly heavier than females throughout the study ($P < 0.002$, Figure 3.2a). Over the 12 weeks of the study, males gained on average 11.4g compared with 7.1g for the females, an increase in body weight of 62% for the male mice and 47% for the female mice. Again, body weight gain decreased with time and was negative in the final week (Figure 3.2b).

Figure 3.2a) Weekly weights of mice by sex



b) Mean weekly weight gain of mice by sex

3.1.5.2 Time and amount of running

The mice were given two weeks of training on the treadmill to get them used to this form of exercise and to get them up to the proposed speed. When the mice were proficient at running on the treadmill, the protocol required the mice to run for a total of 60 minutes a day, 5 days a week for 10 further weeks. Unfortunately, the mice were averse to this type of activity with many stopping frequently or refusing to run altogether (see supporting film on c.d.). The target speed was initially 18-21m·min^{-1} but was reduced to 16m·min^{-1} after seeing the difficulties the mice were having keeping pace with the treadmill. The initial target time of 60 minutes non-stop was also altered to 3 periods of 20 minutes with 5 minutes rest in between. Table 3.3 shows the percentages of possible days of running that were completed by the mice and their average fastest speed over the ten weeks of study (data from the two week initial training period are not included).

Only one mouse (female) ran on every day of the study, reaching the target speed of 16m·min^{-1}. The inability/unwillingness of the mice to keep up with the pace led to the formation of two running groups – one at 16m·min^{-1}, the other at 12m·min^{-1} to encourage the mice to do some running and, if possible, to coax them into running faster again. Despite these concessions, the average speed of the mice was 14.6 m·min-1 and the average daily time spent running was 43 minutes (see Table 3.3). There were no significant differences in running performance between the sexes.

Table 3.3 Treadmill performance data for individual mice averaged over weeks 3-12

Mouse number (sex)	% of days run	Average daily time spent running (min)	Average best speed (m·min^{-1})
3 (m)	92	48.8	14.8
4 (m)	90	45.3	14.5
15 (m)	78	41.2	13.6
16 (m)	64	29.8	13.9
17 (m)	85	47.2	14.6
18 (m)	76	37.4	14.4
1 (f)	84	41.6	13.5
2 (f)	86	44.8	14.6
19 (f)	93	46.9	15.8
20 (f)	87	37.1	14.7
21 (f)	100	57.8	16.0
Mean	**85**	**43.4**	**14.6**

3.1.5.3 Food intake

The amount of food eaten each day by individual mice was relatively constant throughout the study (Figure 3.3). CON mice ate significantly more than TR mice during weeks 5, 9 and 10 ($P < 0.035$) whilst the reverse was true during week 12 ($P=0.031$, Figure 3.4a).

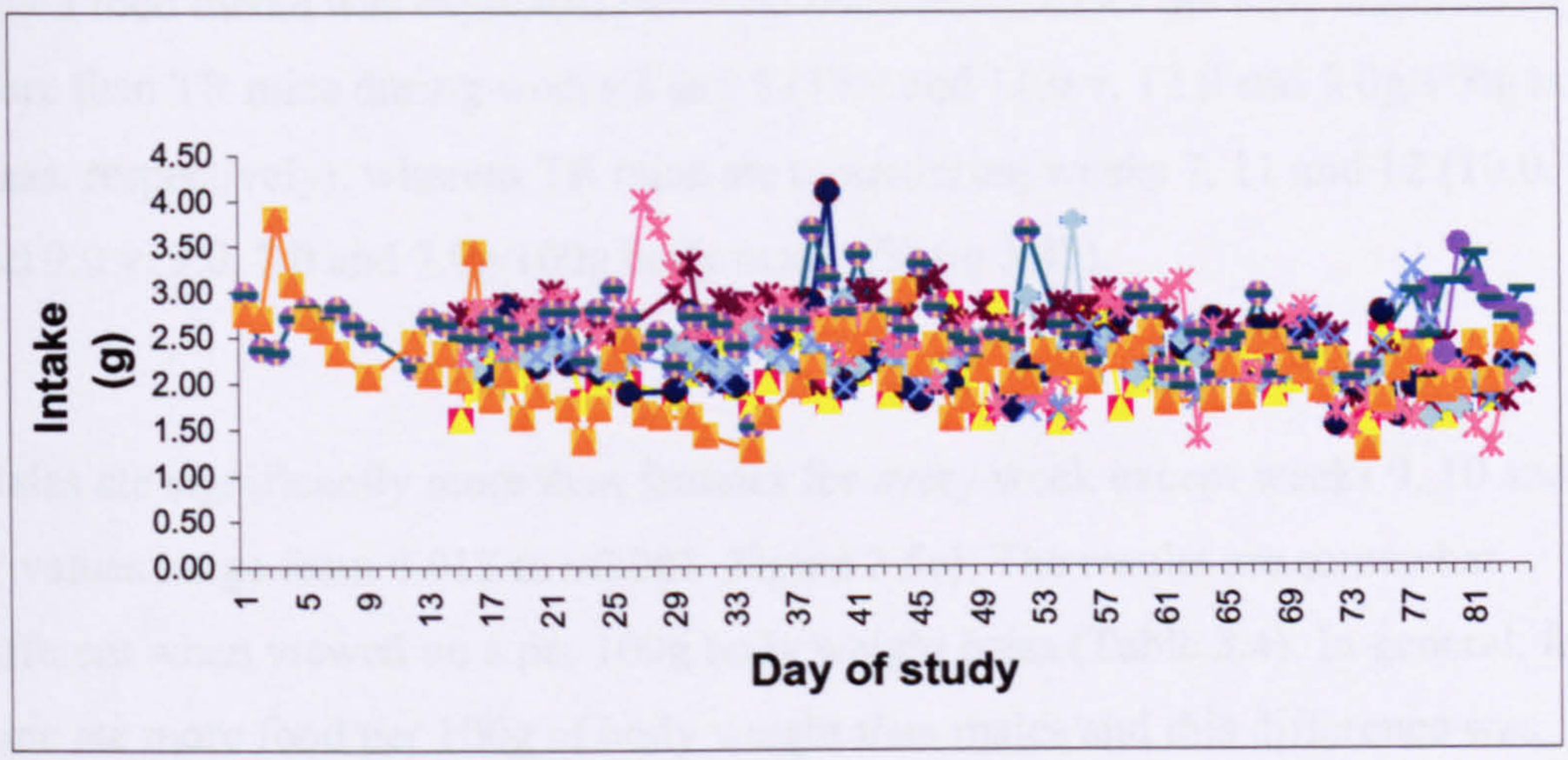


Figure 3.3 Daily food intake for each mouse throughout the study

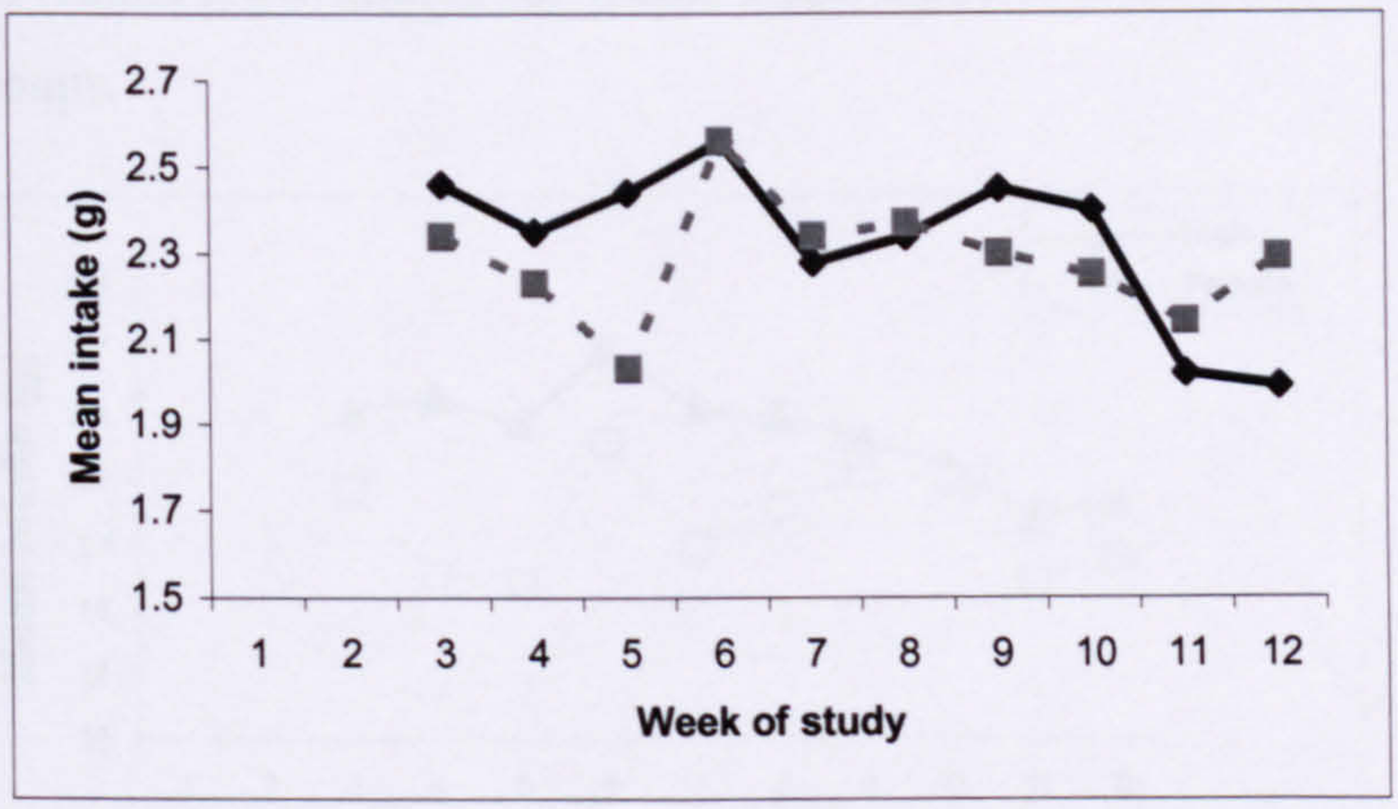
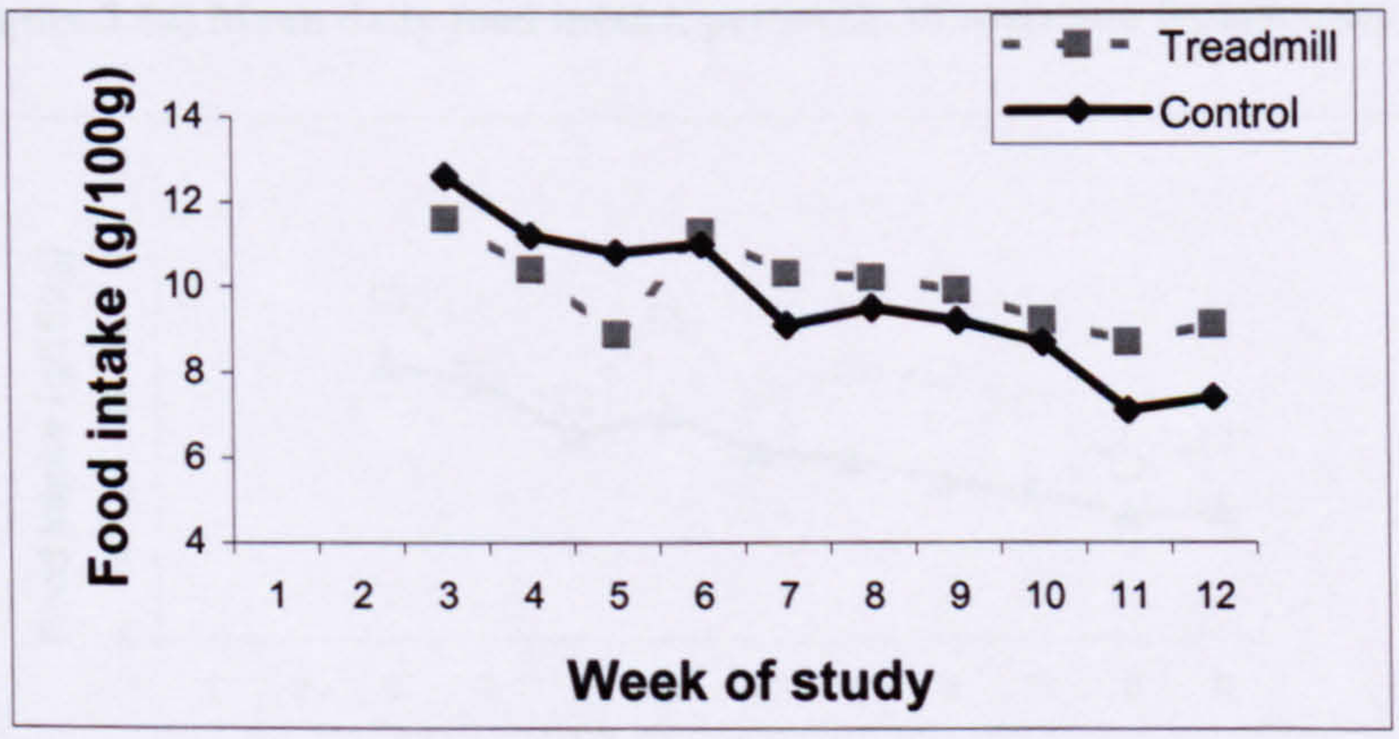


Figure 3.4a) Mean daily food intake per week by exercise group



b) Mean daily food intake per 100 g body weight, per week, by exercise group

When food intake was expressed per 100g body mass, CON mice ate significantly more than TR mice during weeks 3 and 5 (13.0 and 11.0 v. 12.0 and 9.0g/100g body mass, respectively), whereas TR mice ate more during weeks 7, 11 and 12 (10.0, 9.0 and 9.0 v. 9.0, 7.0 and 7.0g/100g body mass, Figure 3.4b).

Males ate significantly more than females for every week except weeks 9, 10 and 12 (P values range from 0.015 to <0.001, Figure 3.5a). The results are somewhat different when viewed on a per 100g body weight basis (Table 3.4). In general, female mice ate more food per 100g of body weight than males and this difference was significant during weeks 3 and 6-12 (with females averaging 10.0 g/100g and males averaging 9.0g/100g body mass across these weeks (Figure 3.5b).

No results are available for weeks 1 and 2 due to some mice changing exercise groups.

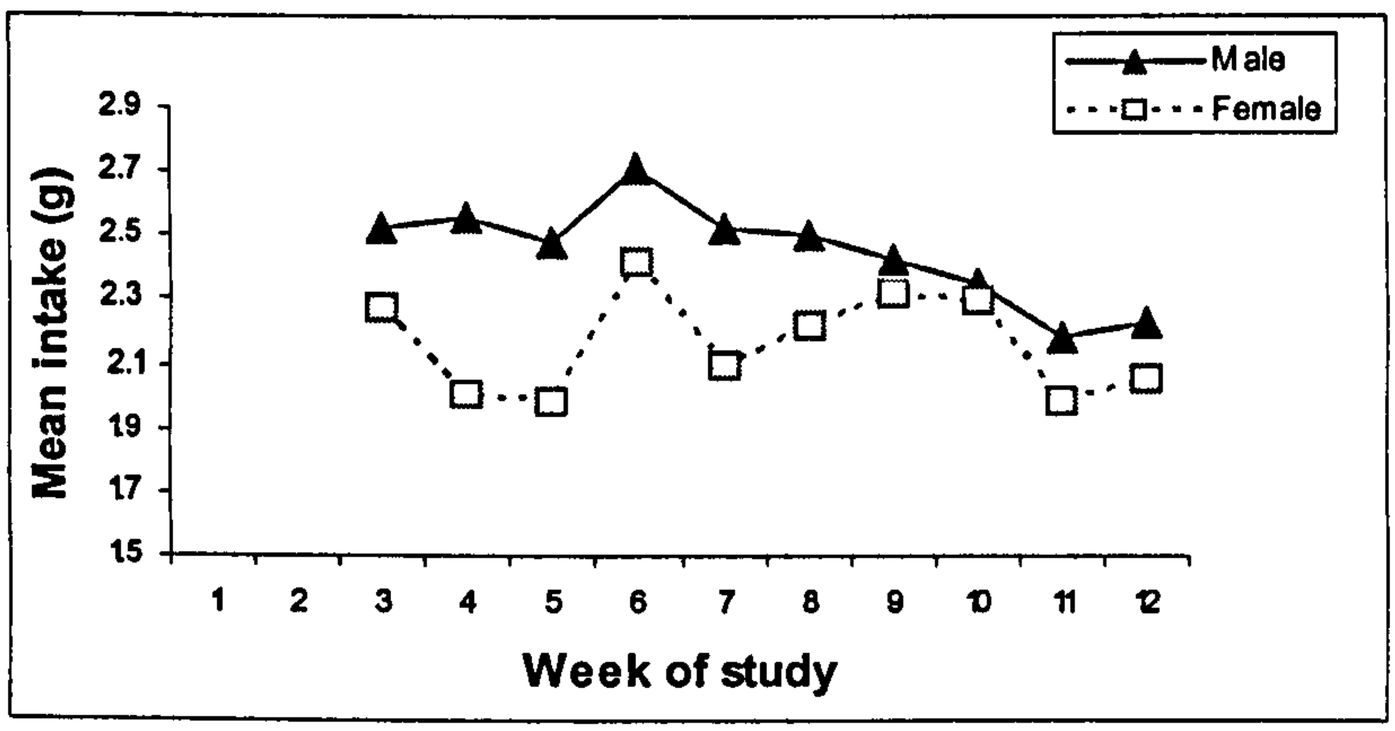
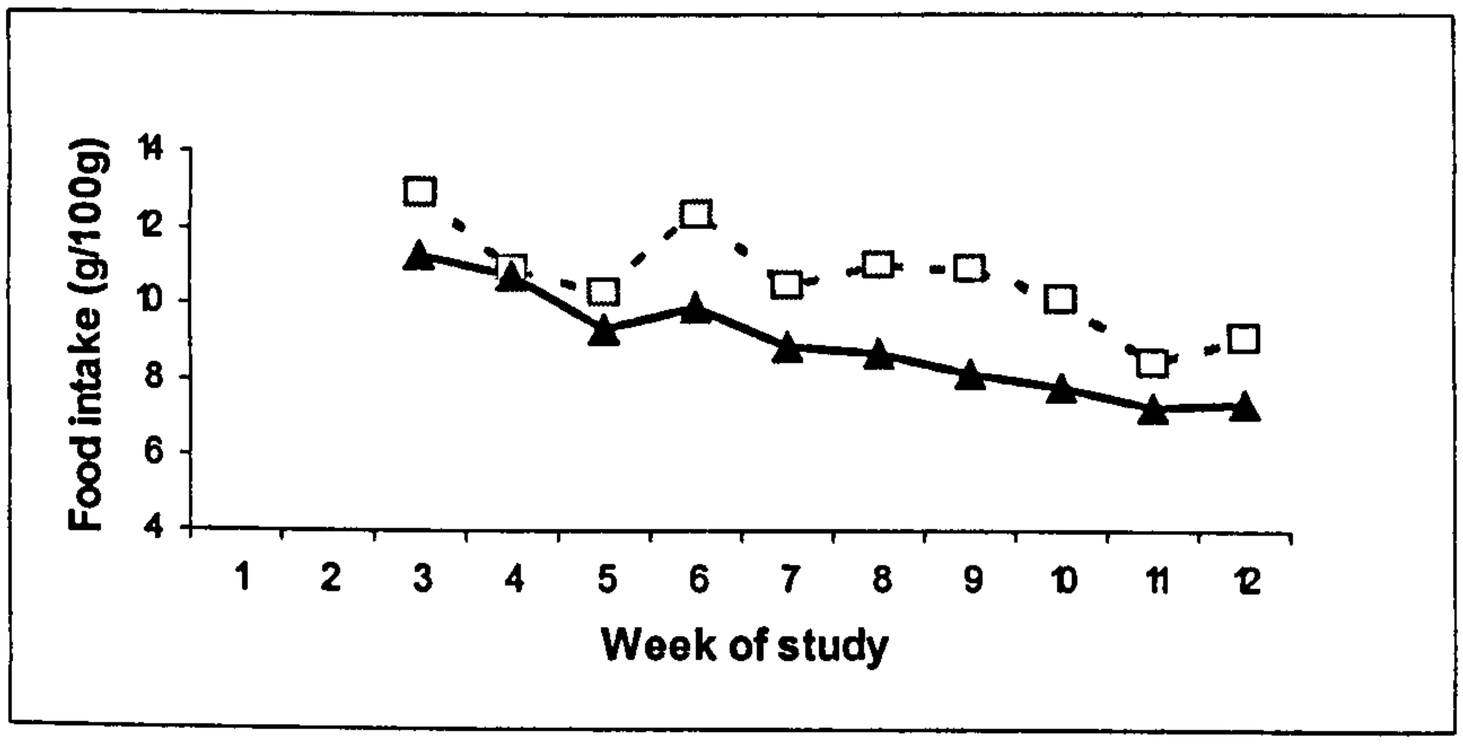


Figure 3.5a) Mean daily food intake, per week, of male and female mice



b) Mean daily food intake per 100 g body weight, per week, by sex

Table 3.4 Summary of statistical analyses of the effect of exercise protocol and sex on food intake

	Daily food intake		Food intake per 100g of body weight	
Week of study	**Sex effect**	**Treadmill exercise effect**	**Sex effect**	**Treadmill exercise effect**
3	Male*	NS	Female**	Control*
4	Male**	NS	NS	NS
5	Male**	Control**	NS	Control**
6	Male*	NS	Female**	NS
7	Male**	NS	Female*	Treadmill*
8	Male*	NS	Female**	NS
9	NS	Control*	Female**	NS
10	NS	Control*	Female**	NS
11	Male*	NS	Female*	Treadmill**
12	NS	Treadmill*	Female*	Treadmill*

The table shows the group with the greater food intake and the level of significance. NS, non-significant ($P \geq 0.05$); * , ($P<0.05$); ** , ($P \leq 0.001$).

3.1.5.4 Tumours

Tumour numbers, sizes (estimated as tumour diameter in mm) and location were recorded and analysed according to sex and exercise protocol. As anticipated, the large majority (over 97%) of tumours were found in the small intestine, with approximately equal proportions in the proximal and distal halves (100 and 108 tumours, respectively).

The exercise protocol (Table 3.5) did not alter the number of tumours at any location at the $P < 0.05$ level.

Table 3.5 Tumour multiplicity, tumour burden and distribution of tumours of TR and CON mice

Variable	CON (n=9)	TR (n=9)	Pooled SEM	Probability of exercise effect
Proximal SI	6.0	5.1	1.02	0.512
Distal SI	6.5	5.5	1.06	0.502
Total SI	12.5	10.6	1.64	0.351
Colon^{KW}	0.0 0-1	0.0 0-1	-	0.638
Total tumours	12.9	11.0	1.68	0.401
Tumour burden (mm diameter)	40.2	30.5	4.41	0.135

Male mice had significantly more tumours in the distal portion of the small intestine (P=0.006), a greater total number of tumours (P=0.047) and a greater tumour burden (total diameter of tumours in mm, P=0.048) than female mice (Table 3.6). The difference in the number of colon tumours was not statistically significant. The total number of small intestinal tumours was lower in females compared with males (9.3 v. 13.8) and this difference approached significance (P = 0.057).

Table 3.6 Means and SEM of tumour parameters by sex

Variable	Female (n=9)	Male (n=9)	Pooled SEM	Probability of sex effect
Proximal SI	5.8	5.3	1.02	0.703
Distal SI	3.6	8.4	1.06	0.006
Total SI	9.3	13.8	1.64	0.057
Colon^{KW}	0.0 0-1	1.0 0-1	-	0.159
Total tumours	9.7	14.2	1.68	0.047
Tumour burden (mm)	28.8	41.9	4.41	0.048

Table 3.7 shows the tumour data by sex and exercise group. There was no interaction between sex and exercise for any of the measured variables, with males generally having more tumours than females.

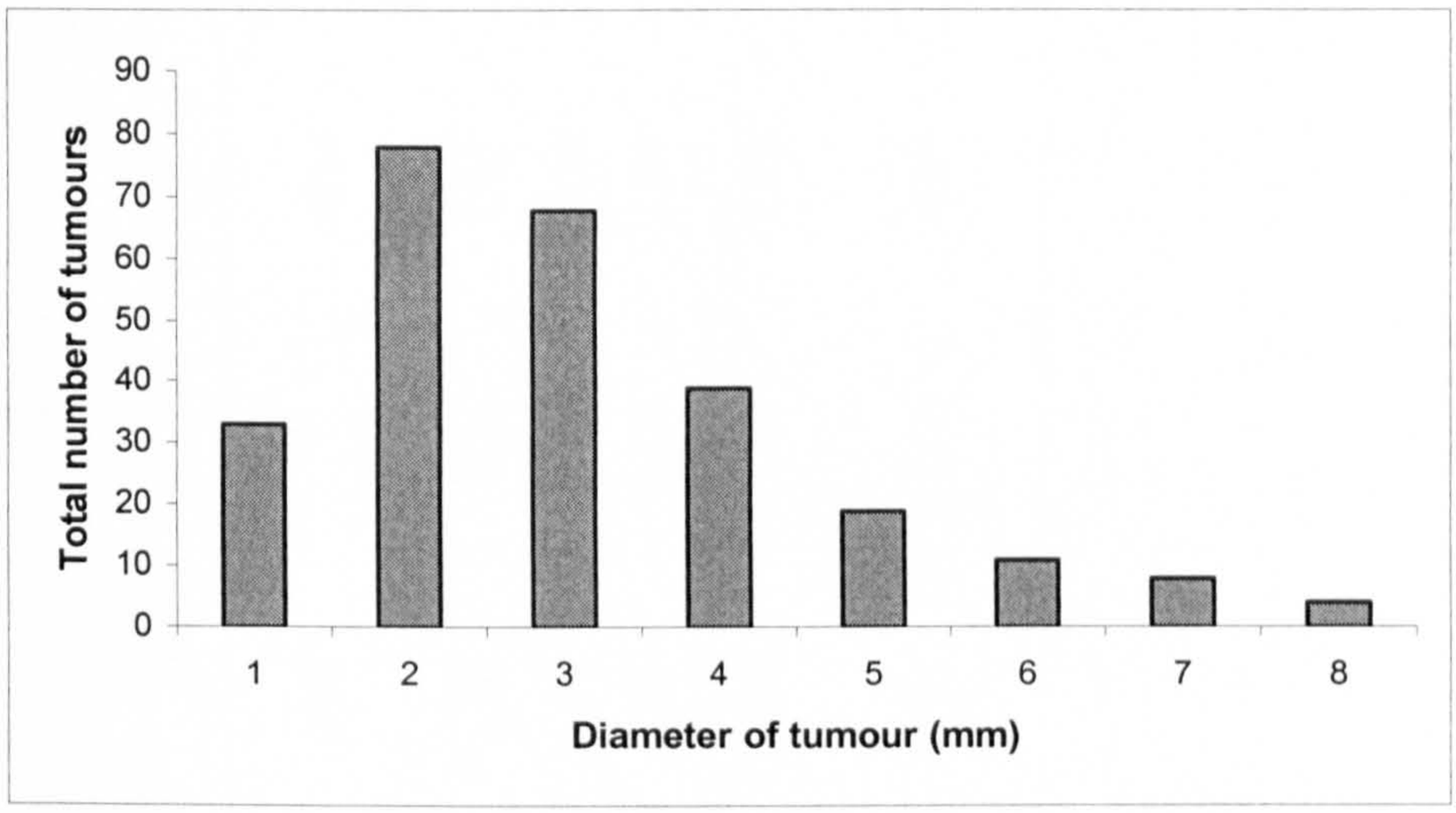


Figure 3.6 Distribution of tumours sizes

Figure 3.6 shows the distribution of tumour sizes in this study with the majority (72%) being 1-3mm diameter. There were no significant differences between sexes or exercise groups with regards to tumour size.

There were some negative correlations between treadmill running variables and tumour variables, when data from all TR mice were included (Table 3.8, Figure 3.7). Decreased tumour numbers and burden occurred with increased speed and % of days run. However, when the outlier was removed, so was the significance ($P>0.4$).

Table 3.7 Intestinal distribution of tumour numbers and tumour burden by sex and exercise group (n)

	Female				Male				
Tumour variable	**CON (4)**	**SEM**	**TR (5)**	**SEM**	**CON (5)**	**SEM**	**TR (4)**	**SEM**	**Probability of effects (interaction)**
Proximal SI	5.2	1.45	6.2	1.30	6.6	1.30	3.8	1.45	0.188
Distal SI	5	1.56	2.4	1.40	8.2	1.40	8.8	1.56	0.306
Total SI	10.3	2.24	8.6	2.00	14.8	2.00	12.5	2.24	0.880
Total tumours	10.5	2.30	8.8	2.06	15.2	2.06	13.3	2.30	0.955
Tumour burden (mm)	32.3	6.23	25.0	5.92	47.8	5.92	35.8	6.23	0.708

Table 3.8 Correlation between treadmill running and tumour variables

Treadmill variable	Tumour variable	Pearson's R	Probability
% days run	Total number of tumours	-0.576	0.064
% days run	Tumour burden	-0.666	0.025
Mean best speed	Total number of tumours	-0.562	0.072
Mean best speed	Tumour burden	-0.556	0.076

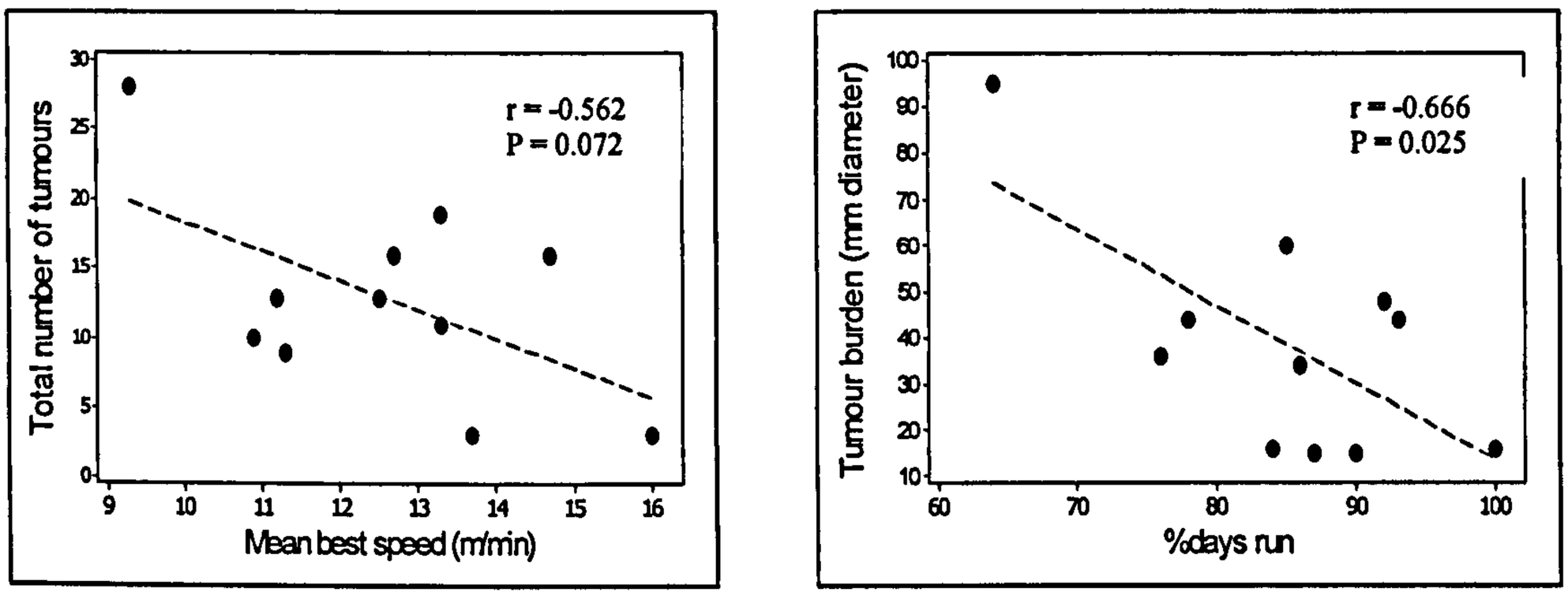


Figure 3.7 Relationship between treadmill running and tumour variables, with fitted regression line

3.1.5.5 Body organs

Weights of intestinal organs with their contents, tissues and content weights were not different between the two treatment groups (Table 3.9), with the exception of the weight of the caecal contents, which was greater for TR mice compared with CON mice (0.183 v. 0.134g, P = 0.044).

Table 3.9 Mean intestinal organ dimensions of treadmill and control mice

Organ dimension	CON	TR	Pooled SEM	Probability of effect
Slaughter weight (g)	25.3	26.5	1.51	0.572
Length (cm)				
Small intestine	28.3	29.7	1.065	0.354
Colon[KW]	5.5	5.0		
	4.5-6	4.5-6.5	-	0.546
Weight (g)				
Stomach (full)	0.42	0.363	0.043	0.334
Stomach (tissue)	0.153	0.146	0.012	0.609
Stomach (contents)	0.272	0.218	0.037	0.318
SI (full)	1.097	1.066	0.049	0.665
SI (tissue)	0.983	0.963	0.043	0.748
SI (contents)	0.113	0.102	0.024	0.749
Caecum (full)	0.235	0.280	0.020	0.143
Caecum (tissue)	0.100	0.098	0.007	0.773
Caecum (contents)	0.134	0.183	0.016	0.044
Colon (full)	0.262	0.227	0.023	0.308
Colon (tissue)	0.172	0.158	0.011	0.359
Colon (contents)	0.090	0.063	0.016	0.253

When expressed as g/100g final body mass, there were no statistically significant effects of exercise regime on organ mass (Table 3.10).

Table 3.10 Contribution of intestinal organs (without contents) to final body weight in CON and TR mice

Organ (g/100g body mass)	CON	TR	Pooled SEM	Probability of effect
Stomach	0.71	0.68	0.057	0.650
SI	4.48	4.46	0.217	0.946
Caecum	0.45	0.45	0.032	0.955
Colon	0.78	0.72	0.050	0.431

Both absolute weight and proportions (g/100g body mass) of liver and heart were not significantly different between CON and TR mice (Table 3.11). Spleen weight in CON mice was heavier than in TR mice, and the difference approached significance (0.383 v. 0.267g, P = 0.073). However, when spleen weight was expressed as g/100g final body mass, the effect of exercise was not significant (P = 0.213, Table 3.11). Haematocrit was not significantly affected by exercise, 23.1% and 27.7 % in CON and TR mice respectively (pooled SEM = 2.48, P = 0.215).

Table 3.11 Contribution of liver, heart and spleen to final body mass of CON and TR mice

Organ	CON	TR	Pooled SEM	Probability of effect
Absolute weight (g/mouse)				
Liver	1.109	1.078	0.049	0.663
Heart	0.171	0.167	0.009	0.750
Spleen	0.383	0.267	0.042	0.073
As g/100g body mass				
Liver	5.00	4.96	0.192	0.867
Heart	0.81	0.77	0.066	0.678
Spleen	1.72	1.26	0.249	0.213

Male colons were longer by 14% than those of female mice (5.7 v. 5.0cm, median values, P = 0.036, (Table 3.12).), though there was no significant difference in SI length. The weights of the full stomach (0.327 v. 0.461g) and full caecum (0.211 v. 0.304g) were significantly lower in females than in males (P = 0.006 and P = 0.046 respectively), whilst the weight of the full SI approached significance (1.011 v. 1.152g in females and males respectively, P = 0.063).

Table 3.12 Mean intestinal organ dimensions of male and female mice

Organ dimension	Female	Male	Pooled SEM	Probability of effect
Slaughter weight (g)	22.1	29.7	1.48	0.003
Length (cm)				
Small intestine	28.5	29.5	1.065	0.499
Colon[KW]	5.0 4.5-6	5.7 5-6.5	-	0.036
Weight (g)				
Stomach (full)	0.327	0.461	0.043	0.046
Stomach (tissue)	0.145	0.154	0.012	0.708
Stomach (contents)	0.183	0.307	0.037	0.032
SI (full)	1.011	1.152	0.049	0.063
SI (tissue)	0.923	1.023	0.043	0.123
SI (contents)	0.087	0.128	0.024	0.244
Caecum (full)	0.211	0.304	0.020	0.006
Caecum (tissue)	0.091	0.107	0.007	0.125
Caecum (contents)	0.118	0.200	0.016	0.002
Colon (full)	0.224	0.265	0.023	0.245
Colon (tissue)	0.154	0.176	0.011	0.169
Colon (contents)	0.069	0.084	0.016	0.530

Table 3.13 Contribution of intestinal organs to final body mass in male and female mice

Organ (g/100g body mass)	Female	Male	Pooled SEM	Probability of effect
Stomach	0.77	0.62	0.057	0.075
SI	4.93	4.01	0.217	0.010
Caecum	0.49	0.41	0.032	0.117
Colon	0.81	0.69	0.050	0.108

The contents of the organs were heavier in males compared with females, significantly so for the stomach (P = 0.032) and caecum (P = 0.002), but not the SI or colon (Table 3.12).

When the difference in body mass was taken into account and organ dimensions were expressed as a proportion of the final body mass, female intestinal organs were heavier than those of males (Table 3.13). The empty small intestine was significantly heavier in females than in males (4.93 v. 4.01 g/100g, P = 0.010), and the difference in stomach weight approached significance (0.77 v. 0.62 g/100g, P = 0.075). Neither the difference in SI weight nor colon weight was statistically significant.

The weights of the other organs (liver, heart and spleen) were heavier in male mice compared with female mice (Table 3.14), with statistically significant differences for liver weight (1.179 v. 1.007g, P = 0.027) and heart weight (0.186 v. 0.152g, P = 0.014). When measured as contribution to final body mass, females had significantly heavier livers (5.37% v. 4.60%, P = 0.013). Heart and spleen weights were not significantly different between the sexes (Table 3.14). Haematocrits in female and male mice were also not different, 26.6% and 24.2% respectively (pooled SEM = 2.53, P = 0.613).

There were no interactions between exercise and sex on any of the organ proportions i.e. mass of organ per 100g body mass (Table 3.15).

Table 3.14 Liver, heart and spleen weights of male and female mice

Organ	Female	Male	Pooled SEM	Probability of effect
Absolute weight (g/mouse)				
Liver	1.008	1.179	0.049	0.027
Heart	0.152	0.186	0.009	0.014
Spleen	0.291	0.359	0.042	0.273
As g/100g body mass				
Liver	5.37	4.60	0.192	0.013
Heart	0.83	0.74	0.066	0.375
Spleen	1.72	1.26	0.249	0.769

Table 3.15 Organ mass (g/100g body mass) by sex and exercise group

	Female				Male				
Organ (g/100g body mass)	**CON**	**SEM**	**TR**	**SEM**	**CON**	**SEM**	**TR**	**SEM**	**Probability of effects (interaction)**
Stomach	0.79	0.088	0.75	0.079	0.63	0.079	0.60	0.088	0.940
SI	4.85	0.334	4.99	0.299	4.10	0.299	3.91	0.334	0.620
Colon	0.86	0.077	0.77	0.069	0.70	0.069	0.68	0.077	0.670
Caecum	0.48	0.049	0.50	0.044	0.42	0.044	0.40	0.049	0.663
Liver	5.32	0.294	5.41	0.264	4.69	0.264	4.50	0.294	0.615
Heart	0.83	0.102	0.83	0.091	0.78	0.091	0.70	0.102	0.679
Spleen	1.56	0.372	1.48	0.333	1.83	0.333	1.00	0.372	0.308

A positive correlation was observed between spleen weight and the total number of tumours present (r = 0.608, P = 0.007, Figure 3.8a, and between spleen weight and tumour burden (r = 0.448, P = 0.062, Figure 3.8b). Linear regression equations were also calculated, as follows:

Spleen weight = 0.12 + 0.0171(total number of tumours) ($R^2 = 0.33$)

Spleen weight = 0.178 +0.00417(tumour burden) ($R^2 = 0.15$)

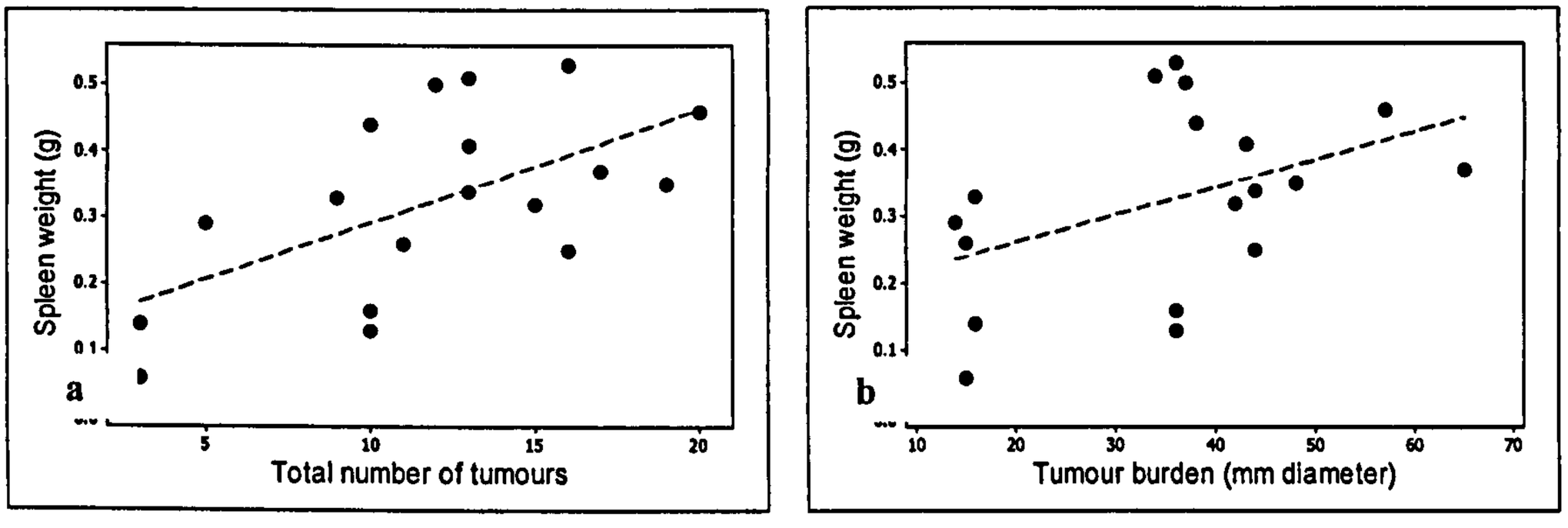


Figure 3.8 Relationship between spleen weight and (a) the total number of tumours and (b) tumour burden, with fitted regression line

3.1.5.6 Short chain fatty acids (SCFA)

The data obtained for concentration of SCFA in the large bowel were normally distributed only for acetate. Therefore, data for propionate, isobutyrate, butyrate, isovalerate and valerate were log-transformed before analysis. When expressed as molar proportion of total SCFA, butyrate was the only SCFA requiring log-transformation.

The concentrations of acetate, propionate, isobutyrate, isovalerate and valerate in colonic contents were not significantly different betweenr CON and TR mice (Table 3.16, P>0.05). The concentration of butyrate was significantly higher in colonic contents from CON mice compared with colonic contents from TR mice (3.72 v. 1.40

μmol·g colon contents, P = 0.039). When the concentrations of SCFA were converted to molar proportions of total SCFA, the effect of exercise on butyrate remained significant (P = 0.041, Table 3.17). The isobutyrate proportion was higher in TR compared with CON mice (21.5 v. 15.3 mmol/mol, P = 0.033). TR mice also had a greater proportion of isovalerate compared with CON mice (P = 0.027, 18.0 v. 13.3 mmol/mol total SCFA). There were no significant differences in the proportions of acetate, propionate and valerate.

Table 3.16 SCFA concentrations (mean and 95% CI) (μmol·g colon contents) by exercise protocol

Individual SCFA (μmol·g colon contents)*	CON	TR	Probability of effect
Acetate	21.80	21.06	0.834
	16.83-27.27	16.86-25.76	
Propionate	3.18	3.61	0.541
	2.35-4.31	2.79-4.68	
Isobutyrate	0.43	0.51	0.403
	0.32-0.58	0.40-0.66	
Butyrate	3.72	1.40	0.039
	1.95-7.08	0.81-2.43	
Isovalerate	0.33	0.42	0.193
	0.26-0.43	0.34-0.52	
Valerate	0.11	0.13	0.558
	0.07-0.17	0.09-0.20	

*** data have been log-transformed**

Table 3.17 Molar proportions (mmol/mol total SCFA) of colonic SCFA by exercise group

SCFA	CON	SEM/CI	TR	SEM/CI	Probability of effect
Acetate	702	27.7	738	23.6	0.345
Propionate	111	13.4	141	11.4	0.115
Isobutyrate	15	2.1	22	1.7	0.033
Butyrate ^{Log10}	126	73-216	56	35-89	0.041
Isovalerate	13	1.5	18	1.3	0.027
Valerate	4	0.9	6	0.8	0.157

The concentrations of SCFA were similar in colonic contents from male and female mice (Table 3.18). A significant difference existed only in the concentration of acetate (P = 0.015), with the concentration in samples from females being, on average, 64% of that in samples from males (16.7 v. 26.1 μmol·g colon contents). However, when expressed as molar proportions of total SCFA, this difference became non-significant (P = 0.195, Table 3.19), and values for other SCFA were also not significantly different in samples from both males and females. The exception to this was the proportion of isovalerate, which was greater in samples from females compared with samples from male mice (18.3 v. 14.0 mmol/mol total SCFA, P = 0.032).

Table 3.18 SCFA concentrations (mean and 95% CI) (μmol·g colon contents) by sex

Individual SCFA*	Female	Male	Probability of effect
Acetate	16.71	26.14	0.015
	11.89-21.45	21.54-30.60	
Propionate	2.90	3.98	0.140
	2.19-3.84	3.05-5.20	
Isobutyrate	0.41	0.55	0.184
	0.31-0.54	0.42-0.71	
Butyrate	1.86	2.38	0.508
	0.94-3.65	1.25-4.51	
Isovalerate	0.33	0.43	0.193
	0.26-0.43	0.34-0.54	
Valerate	0.11	0.14	0.508
	0.07-0.16	0.09-0.20	

***log-transformed data**

Table 3.19 Molar proportions (mmol/mol total SCFA) of colonic SCFA by sex

SCFA	Female	SEM/CI	Male	SEM/CI	Probability of effect
Acetate	697	26.1	747	24.7	0.195
Propionate	130	13.3	127	12.6	0.840
Isobutyrate	20	2.2	18	2.1	0.360
Butyrate^{Log10}	85	48-150	72	42-125	0.742
Isovalerate	18	1.6	14	1.5	0.032
Valerate	6	0.9	5	0.8	0.495

There were no interactions between exercise and sex for the molar proportions of any SCFA ($P \geq 0.224$).

One colonic digesta sample (male control) did not produce discernable peaks on the chromatography output and so concentrations of SCFA could not be measured, therefore n = 11 for treadmill and 8 for control mice.

3.1.5.7 Crypt cell proliferation (CCP)

The numbers of cells in mitosis in each third of the length of the crypts from the proximal small intestine were analysed according to sex and exercise protocol.

Few mitotic cells were detected, and there were no significant differences between CON and TR mice in any region of the crypt, nor in crypt dimensions (Table 3.20).

Table 3.20 Small intestinal crypt variables by exercise group

Variable	CON	SEM	TR	SEM	Probability of effect
Mitoses per crypt region					
Lower	0.76	0.084	0.88	0.080	0.297
Middle	1.44	0.128	1.38	0.121	0.719
Upper[KW]	0.70		0.90		
	0.20-2.60	-	0.70-1.10	-	0.173
Total	3.02	0.278	3.12	0.264	0.809
Crypt dimensions (μm)					
Length	113.3	3.6	112.9	3.4	0.933
Width	60.3	2.2	63.1	2.1	0.376

There were no significant differences between the sexes in either number of mitoses per crypt compartment or crypt dimensions (Table 3.21).

Table 3.21 Small intestinal crypt variables by sex

Variable	Female	SEM	Male	SEM	Probability of effect
Mitoses per crypt region					
Lower	0.83	0.089	0.81	0.076	0.916
Middle	1.26	0.135	1.51	0.116	0.181
Upper[KW]	0.70		0.90		
	0.20-1.10	-	0.50-2.60	-	0.226
Total	2.75	0.295	3.31	0.252	0.172
Crypt dimensions (μm)					
Length	114.8	3.8	111.8	3.3	0.561
Width	63.5	2.3	60.4	2.0	0.327

3.1.5.8 Body composition

The body composition data reported below refer to the whole carcass minus the intestine, liver, spleen, heart and approximately 0.5ml blood.

As expected, the major component of the mouse carcasses was water, accounting for >55%, with fat making up a further 28-32% by weight (Table 3.22). Other non-fat organic matter contributed 11-13% of carcass weight and minerals less than 3%. There were no significant ($P>0.05$) effects of exercise on these aspects of body composition (Table 3.22).

The two treadmill males who did not complete the study were not included in the analysis, and two control males were excluded due to experimental error.

There was no significant difference in body composition between the sexes (Table 3.23), and no interaction between sex and exercise ($P<0.60$).

Table 3.22 Body composition of mice by exercise protocol

Body compartment (%)	CON (7)	SEM	TR (11)	SEM	Probability of effect
Water	55.5	2.03	56.9	1.62	0.610
Fat	31.5	3.76	27.7	3.00	0.441
Non-fat organic matter	10.5	1.77	12.5	1.41	0.389
Mineral	2.5	0.42	2.9	0.34	0.423

Table 3.23 Body composition of mice by sex

Body compartment (%)	Female (9)	Male (9)	Pooled SEM	Probability of effect
Water	55.2	57.6	1.75	0.384
Fat	31.7	26.7	3.27	0.338
Non-fat organic matter	10.4	13.0	1.55	0.305
Mineral	2.7	2.8	0.37	0.970

3.1.5.9 Interleukin-6 (IL-6) and Interleukin-10 (IL-10)

Standard curves for the IL-6 and IL-10 ELISA are shown in Figure 3.9.

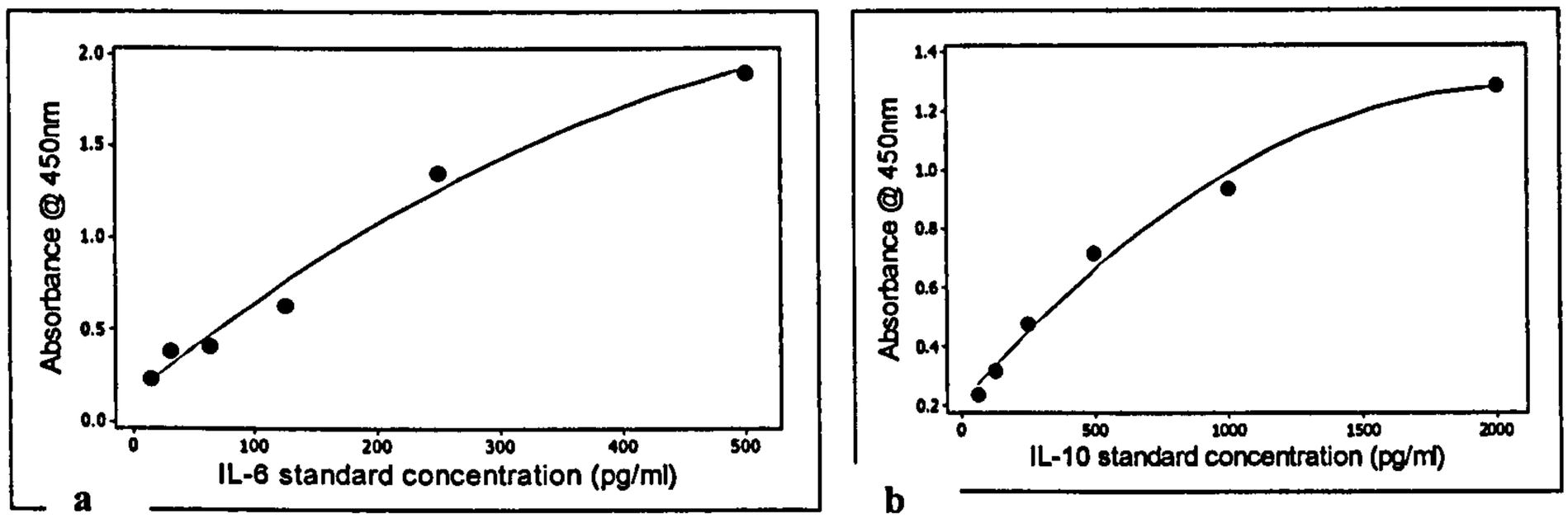


Figure 3.9 Standard curves for (a) IL-6, $R^2 = 0.97$, and (b) IL-10 = $R^2 = 0.98$

Plasma levels of both cytokines were close to the limits of the sensitivity of the assays, and unaffected by the exercise protocol (Table 3.24).

Table 3.24 Plasma concentrations (pg/ml) of IL-6 and IL-10 by exercise group (n)

Interleukin	CON (8)	SEM	TR (11)	SEM	Probability of effect
IL-6	25.1	2.33	22.1	1.98	0.354
IL-10	83.8	1.57	80.9	1.34	0.181

Female mice had a tendency towards lower concentrations of IL-6 compared with males (P = 0.081, Table 3.25), but IL-10 concentrations were identical.

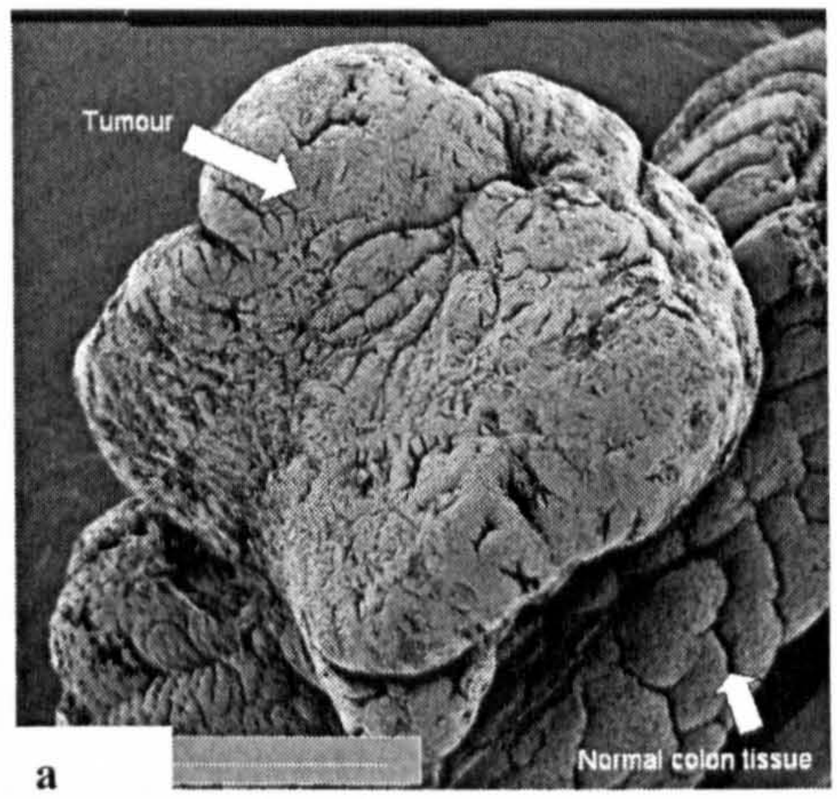
Table 3.25 Plasma concentrations (pg/ml) of IL-6 and IL-10 by sex (n)

Interleukin	Female (8)	SEM	Male (11)	SEM	Probability of effect
IL-6	19.9	2.32	25.9	1.98	0.081
IL-10	82.2	1.62	82.0	1.38	0.824

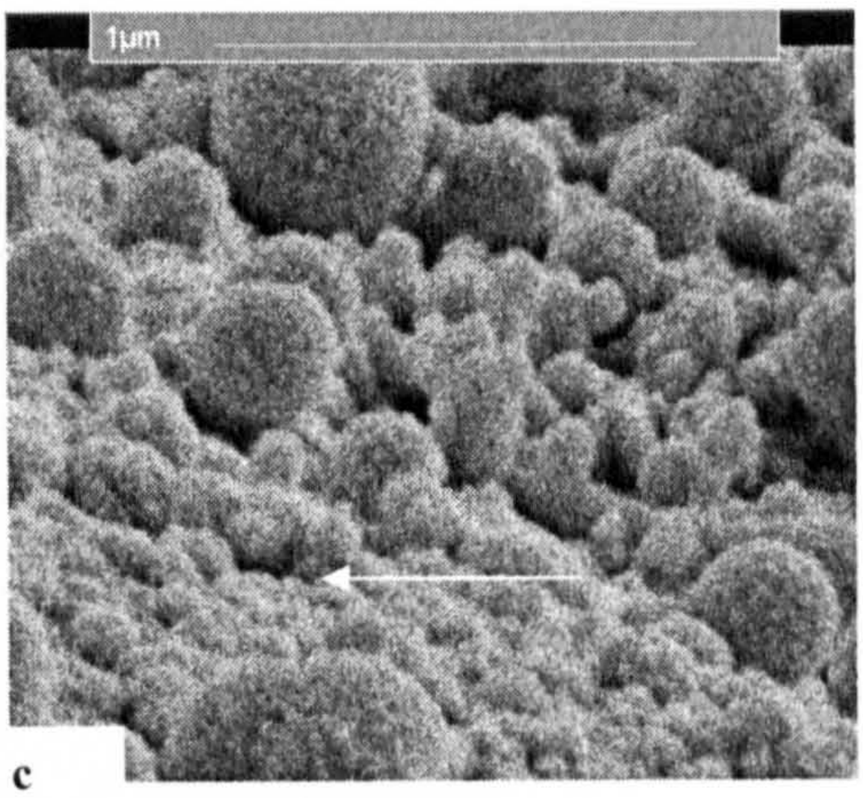
No plasma was available for mouse 5.

3.1.5.10 Scanning electron micrograph (SEM)

Two polypoid colon tumours, both 7mm diameter, from a female CON and a female TR mouse were taken for SEM. Some shrinkage occurred during the dehydration stage (Figure 3.10a) but the saddle-shape of the tumour is apparent. The SEM revealed surface details of the tissue, such as the microvilli on the normal colonocytes (Figure 3.10b) and the lack of such features on the tumour surface (Figure 3.10c).



a



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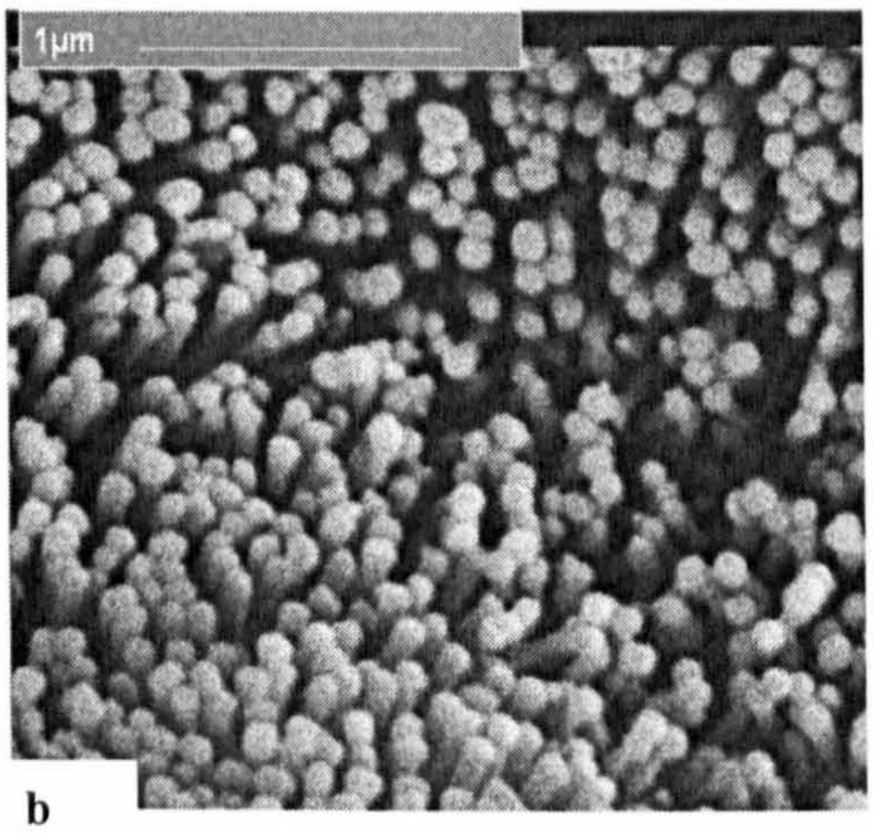


b

Figure 3.10

a) Polypoid colon tumour with surrounding normal tissue (folds in the mucosa are just visible)

b) Microvilli on the surface of the normal mucsoa

c) Surface of the tumour, lacking microvilli and other identifiable structures, but possibly showing globules of mucus from goblet cells (arrows)

3.1.5.11 Evidence of stress

Mice were observed carefully for "normal" behaviour and for any signs of stress. Whilst running on the treadmill, mice were still interested in their surroundings, attempting to look over and under the lane dividers or jump over the front barrier. During rest periods on the treadmill, mice showed the same behaviours and most spent the time grooming. Control mice initially explored their surroundings, attempted to escape and then groomed or slept. When returned to their cages, mice again displayed "normal" behaviours including climbing on the roof bars.

3.1.6 Summary of results

Table 3.26 Summary of pilot study results (P<0.05)

Variable	Effect of treadmill exercise regime (compared with Control)
Body weight gain	No difference
Food intake	Increased
Proximal SI tumours	No difference
Distal SI tumours	No difference
Total SI tumours	No difference
Colon tumours	No difference
Total number of tumours	No difference
Tumour burden	No difference
Intestinal organ weights (%)	No difference
Non-intestinal organ weights (%)	No difference
SCFA concentration - butyrate	Decreased
Acetate/propionate/isobutyrate/ isovalerate/valerate	No difference
SCFA molar proportion	
-butyrate	Decreased
-isobutyrate/ isovalerate	Increased
Acetate/propionate/valerate	No difference
CCP	No difference
Body composition	No difference
IL-6	No difference
IL-10	No difference

3.1.7 Specific discussion

3.1.7.1 Treadmill performance

The average times and speeds run by mice in this study (Figure 3.11) were less than those of Colbert et al. (2000). Mice *could* run at the target speed (18-21m·min^{-1}), but most would not. This is more surprising when considering the young age (21d) and

small size (~12g) of mice in the Colbert (2000) study. Without resorting to noxious stimuli, such as electric shocks, no way of encouraging the mice to run faster was discovered. Whether this was due to the early formation of tumours, individual preference, or the "late" age of mice when commencing the exercise treatment remains to be elucidated. Naturally, this created heterogeneity in exercise exposure, which the use of the treadmill was designed to prevent, and the results should be viewed in that light.

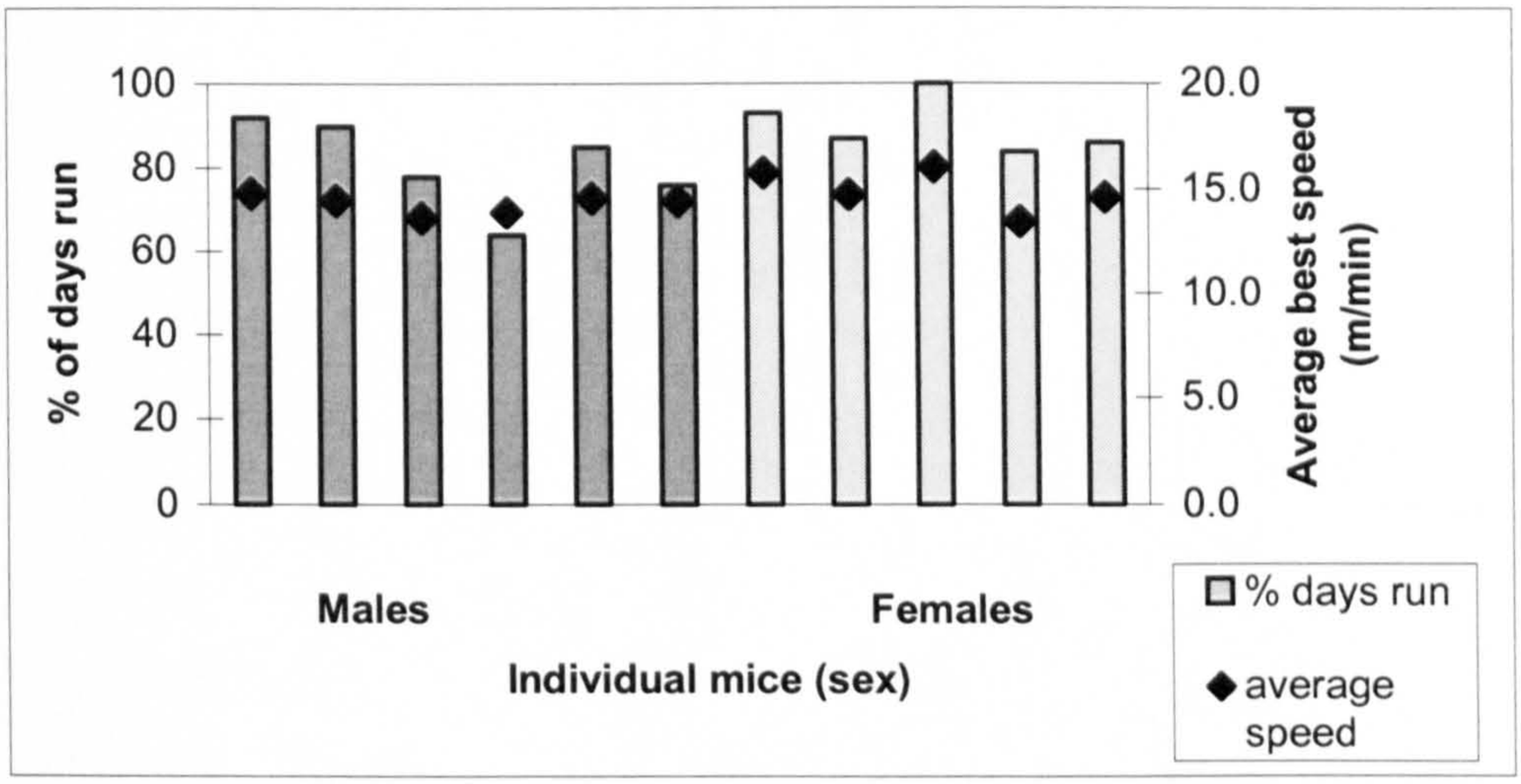


Figure 3.11 The percentage of days run and the average speed attained by mice over weeks 3-12 of the study

3.1.7.2 Organ dimensions

Weights of non-intestinal organs were unaffected by the treatment, although the weight of the spleen tended to be heavier in CON mice compared with TR mice ($p = 0.073$). This possible splenomegaly may reflect the greater number of tumours in CON mice (as shown by the correlation between spleen weight and tumour burden) or the greater extent of anaemia shown by the slightly lower haematocrit of the CON mice and which is a characteristic of the Min mouse (Moser et al., 1990). It is also possible that the TR mice had reduced levels of systemic inflammation, a condition that causes spleen enlargement as lymphocytes are generated (Mehl et al., 2005b).

3.1.7.3 IL-6 and IL-10

IL-6 and IL-10 levels were measured to assess the involvement of the pro- and anti-inflammatory systems on tumour development. IL-6 is often elevated in colon cancer patients (Chung & Chang, 2003) and acute exercise is known to increase IL-6 concentrations (Petersen & Pedersen, 2005). However, the effects of chronic, non-endurance exercise on IL-6 concentration are so far unknown. Reduction of this pro-inflammatory cytokine may be particularly useful in patients with colitis, where inflammation of the bowel leads to tumour formation.

IL-6 should normally be undetectable unless the mice are suffering from trauma, stress or infection (Mehl et al., 2005b) and the concentrations in plasma in this study were low, and not altered by exercise. Using a more sensitive assay, Mehl and colleagues (2005b) detected IL-6 concentrations of 1.2 pg/ml in treadmill-running mice, a reduction of 96% compared with their control animals at 27.9pg/ml. The control value is in accord with that from this study (25.1pg/ml), and the lack of reduction of IL-6 in the plasma of TR mice may simply reflect the need for a more sensitive test. Alternatively, it is possible that the treadmill treatment caused stress to the animals, which may have negated any reduction in IL-6 due to the exercise. However, urinary corticosterone was measured by Colbert et al. (2003) as a marker of stress and no difference was observed between treadmill-running and control mice. A 2001 study by Colbert at al. used C57BL/6 mice and found that in mice given a fatiguing exercise bout, IL-6 plasma concentration was significantly higher than that of control mice. However, IL-6 was undetectable in mice given a moderate amount of exercise (Colbert et al., 2001). As the authors killed the animals in that study 0, 1.5 and 3h following exercise, the lack of detection is probably not due to a rapid half-life of the cytokine. However, these results demonstrate the acute effects of exercise, which may be very different from its chronic effects.

IL-10 is an anti-inflammatory cytokine that is released during exercise (Petersen & Pedersen, 2005) and although levels in this study were measurable, there was no increase due to the treadmill protocol. No other animal study has investigated effects on this cytokine so direct comparisons are not possible at this stage.

A small sex difference existed for IL-6, with female mice having lower concentrations than males. The difference tended towards significance ($P = 0.081$) and may reflect

the lesser tumour burden of the female mice. Mehl et al., (2005b) did not report a sex difference in IL-6 concentrations, although female control mice and those with access to a running wheel had lower IL-6 concentrations than their male counterparts, whilst female treadmill mice had slightly greater IL-6 concentrations than male treadmill runners. Other researchers report decreased concentrations of IL-6 in females, a phenomenon that is believed to be linked to oestrogen levels, with physiological levels of oestrogen suppressing IL-6 production (Kovacs et al., 2002).

3.1.7.4 NK cell assay

As exercise is known to modulate immune function (Nieman, 1997) it was hypothesised that beneficial effects of the treadmill regime on tumour number could have been due to improved immune defence (Armstrong & Mathers, 2000). The spleen provides a good supply of immune cells, but in this study insufficient NK cells were harvested for the assay. A recent paper has discovered that Min mice produce progressively fewer NK cells as they age, with losses of 58% at 110 d old and 72% at 120 d old (Coletta et al., 2004), approximately the age of mice when killed in this study. In addition to NK cell depletion, the authors observed a general lymphodepletion after 80d of age and speculate that the stress of the tumour burden may contribute to this change (Nieman, 1997; Coletta *et al.*, 2004).

3.1.8 Conclusions from pilot study

This study was undertaken to develop protocols for treadmill running by Min mice and focussed on attempts to follow published treadmill protocols (Colbert et al., 2000). Reasons for any reduction in tumour number were investigated. There was no effect of exercise on any of the measured tumour variables, though there was evidence of reduced tumour burden with increased treadmill performance. Some differences in food intake, organ dimension and SCFA concentration were observed which deserve further investigation.

3.2 Treadmill exercise study 2 (ES2)

3.2.1 Introduction

The exercise protocol for this study was modified after reviewing the results of the pilot study, and also the publication of a study on the effect of treadmill running on older Min mice (Colbert et al., 2003). The length of the study was shortened to 10 weeks to prevent the loss of mice before the end of the study. The target speed, time and gradient remained the same. A possible reason for the mice not running on the treadmill could have been the forced nature of the exercise, so a voluntary option was needed. A wheel-running group (WH) was introduced to determine whether the mice would run further when they were given free access to a wheel with no compulsion or reward for using it. Numbers of mice were increased to give greater statistical power to detect effects of the exercise interventions.

3.2.2 Aims

- To develop a protocol for enabling and quantifying voluntary exercise in mice using the wheel-running paradigm
- To compare distances run voluntarily and on the treadmill
- To assess the effects of the exercise regimes on tumour multiplicity and total tumour burden
- To investigate possible mechanisms for differences in tumourigenesis

3.2.3 ES2 Specific methods

67 Min mice, 41 females and 26 males, started the study and were randomised to one of the three groups: Treadmill-running (TR), Wheel-running (WH) or Control (CON).

3.2.3.1 Treadmill regime

17 Min mice started the treadmill protocol (10 females, 7 males). Those that did not drop out were run on the treadmill for a total of 10 weeks. In the first two weeks the speed and time were gradually increased, as described in the General Methods and

Materials (2.2.3). The target was the same as in the pilot study (ES1) i.e. for the mice to run at 18-21mmin^{-1} on a 5% gradient for 60 min. Mice ran for four 15 minute sessions with 5 minutes rest between each session.

3.2.3.2 Wheel mice

29 Min mice (17 female, 12 male) randomised to the wheel group were singly housed for the first 5 weeks with a small wheel (10cm internal diameter). For the last 5 weeks, WH mice were singly housed with a larger wheel (13cm internal diameter).

3.2.3.3 Control mice

21 Min mice were initially assigned to the control group (14 female, 7 male), with conditions as stated in General Methods and Materials (2.2.5).

3.2.3.4 Necropsy

Mice were killed after 10 weeks of study, (see General Methods and Materials 2.3 for details). The same tissues were sampled as in the pilot study (ES1), but this time the spleen was removed to record its weight only and the blood was used only for the haematocrit measurement.

3.2.3.5 Mitochondrial mutation analysis

Two cm of tumour-free tissue from the end of the distal small intestine and the beginning of the colon were removed. Each sample was then cut into two and mounted on filter paper using OCT cryo-embedding medium (BDH). One sample was mounted directly onto the OCT to allow transverse sections through the crypts/villi, whilst the other was sandwiched between pieces of agarose gel containing OCT and mounted to allow longitudinal sections through the crypts.

The samples were then frozen using an iso-pentane freezing bath and stored at –80°C. Samples were sectioned using a cryostat (Microm HM5060). Briefly, a sample was frozen to a metal chuck and fixed to the cryostat. 8µm sections of the frozen tissue were placed onto a microscope slide. Sections were then stained using a Cytochrome c oxidase/succinate dehydrogenase (COX/SDH) stain to determine the presence or absence of cytochrome C oxidase -deficient cells (see Appendix 6.11 for details).

Deficient cells stained blue, normal cells stained brown. The number of deficient cells was counted in at least five samples and the mean was calculated.

3.2.3.6 Statistical analysis

Data were analysed by the General Linear Model of ANOVA, with sex included as a covariate, and comparisons between treatment groups investigated using Dunnett's test. This allowed both WH mice and TR mice to be compared with CON separately.

3.2.4 ES2 Results

Sixty two Min mice completed the study and were distributed among the exercise protocols as shown in Table 3.27.

Table 3.27 Numbers of mice completing the second study according to sex and exercise protocol

Exercise Protocol	Male	Female	Total
Treadmill	3	6	9
Wheel	11	14	25
Control	10	18	28
Total	24	38	62

Five further mice were killed before the end of the study because of deteriorating health. Two females had 74 and 100 tumours respectively, 1 male had 57 tumours, 1 female had 2 polypoid colon tumours of over 9mm and 1 male was hydrocephalic. The data from these mice were not included in the analysis. The average time between finishing the exercise protocol and being killed was 3 days (range 1-7days, CON and TR 4 days, WH 3 days, P = 0.194).

3.2.4.1 Body weight

Most mice continued to increase in weight throughout the study, but some lost weight in the later stages. There were no significant differences between exercise protocols in body mass at death (P = 0.733, Figure 3.12) though TR mice were the heaviest at 27.6g, followed by WH mice (26.7g) and CON were the lightest at 26.4g.

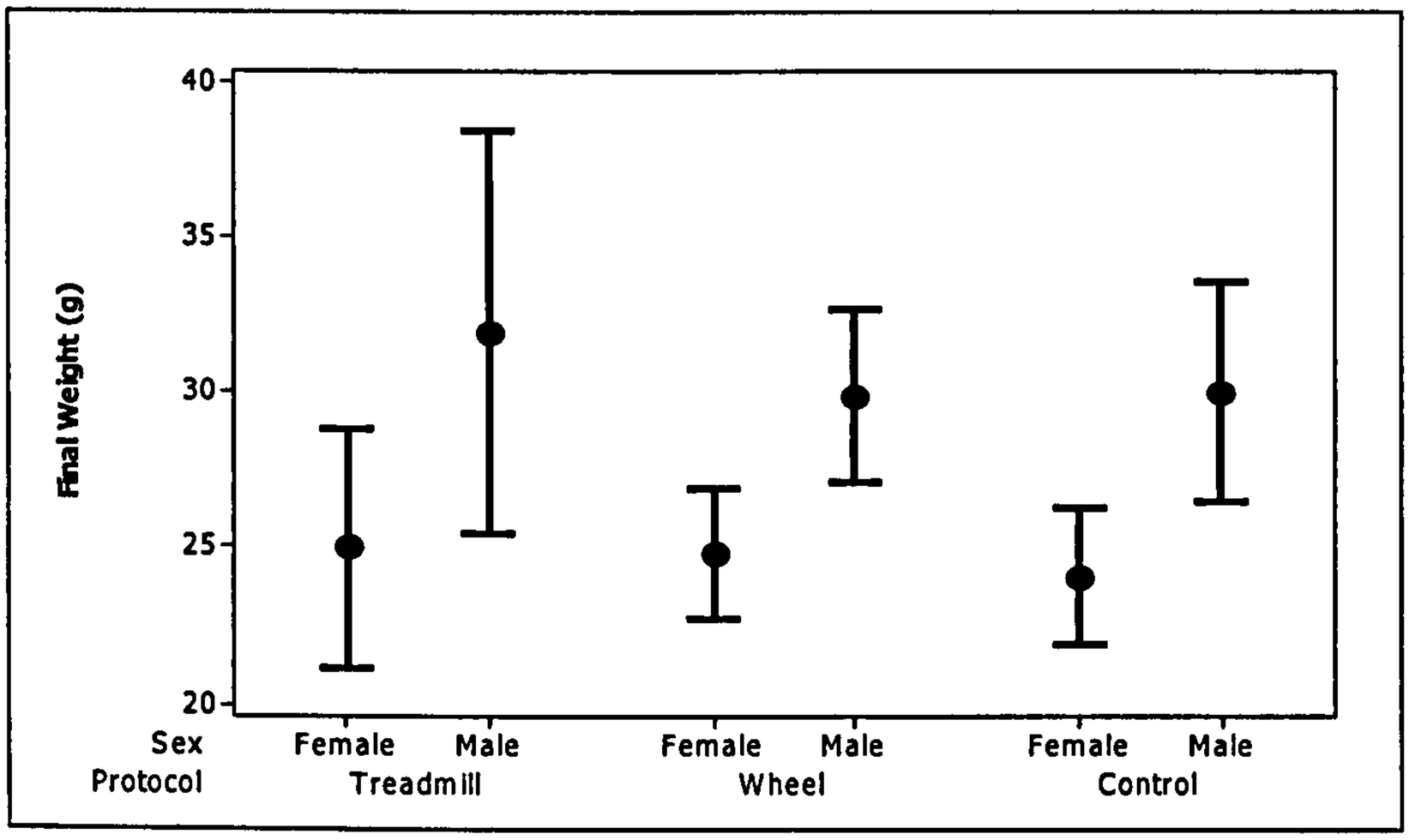


Figure 3.12 Final (slaughter) body weights by exercise group and sex, 95% CI for the mean

Weekly weights of mice by treatment group were similar, with no significant differences between them at any week, though CON mice were slightly heavier on weeks 7, 8 and 9 (Figure 3.13).

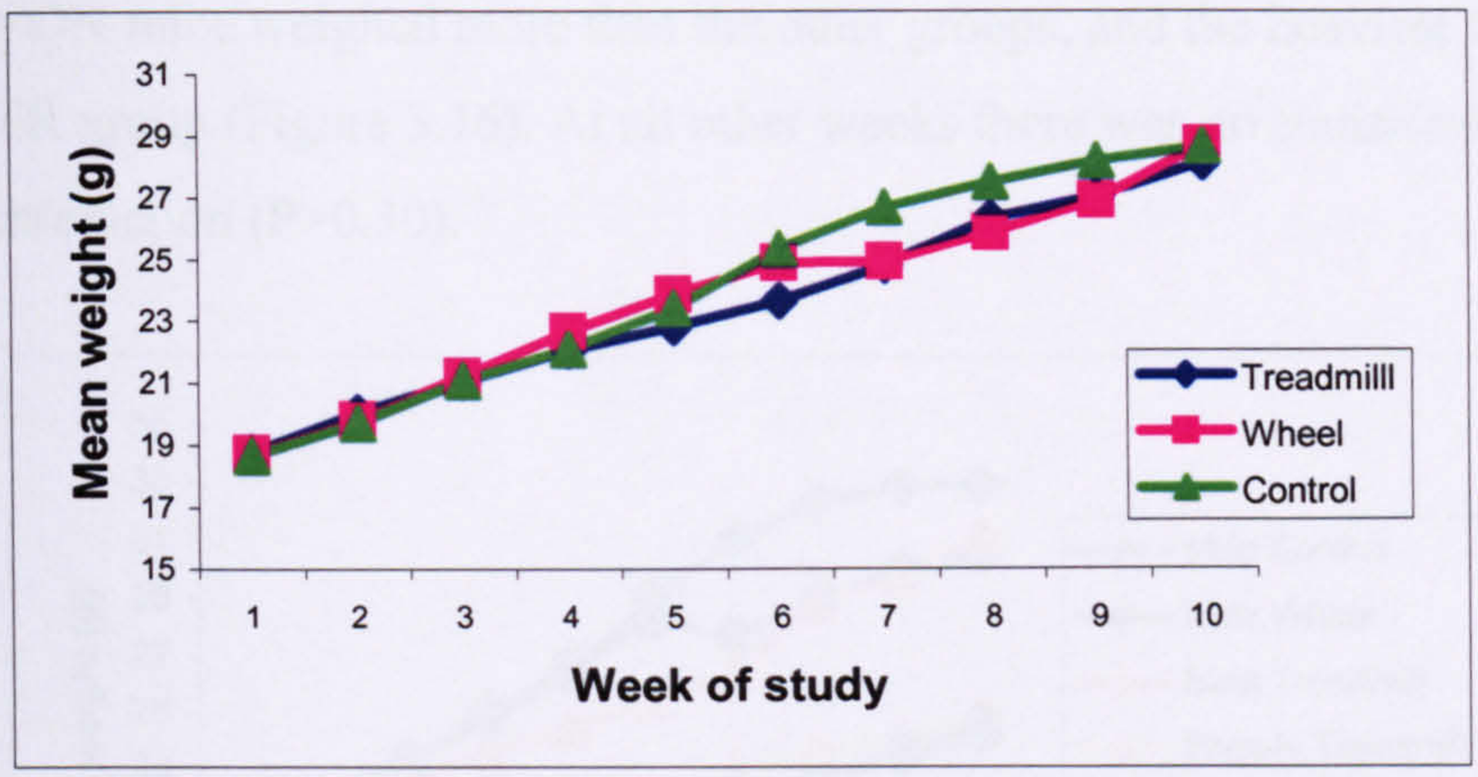


Figure 3.13 Weekly weights of mice by exercise protocol

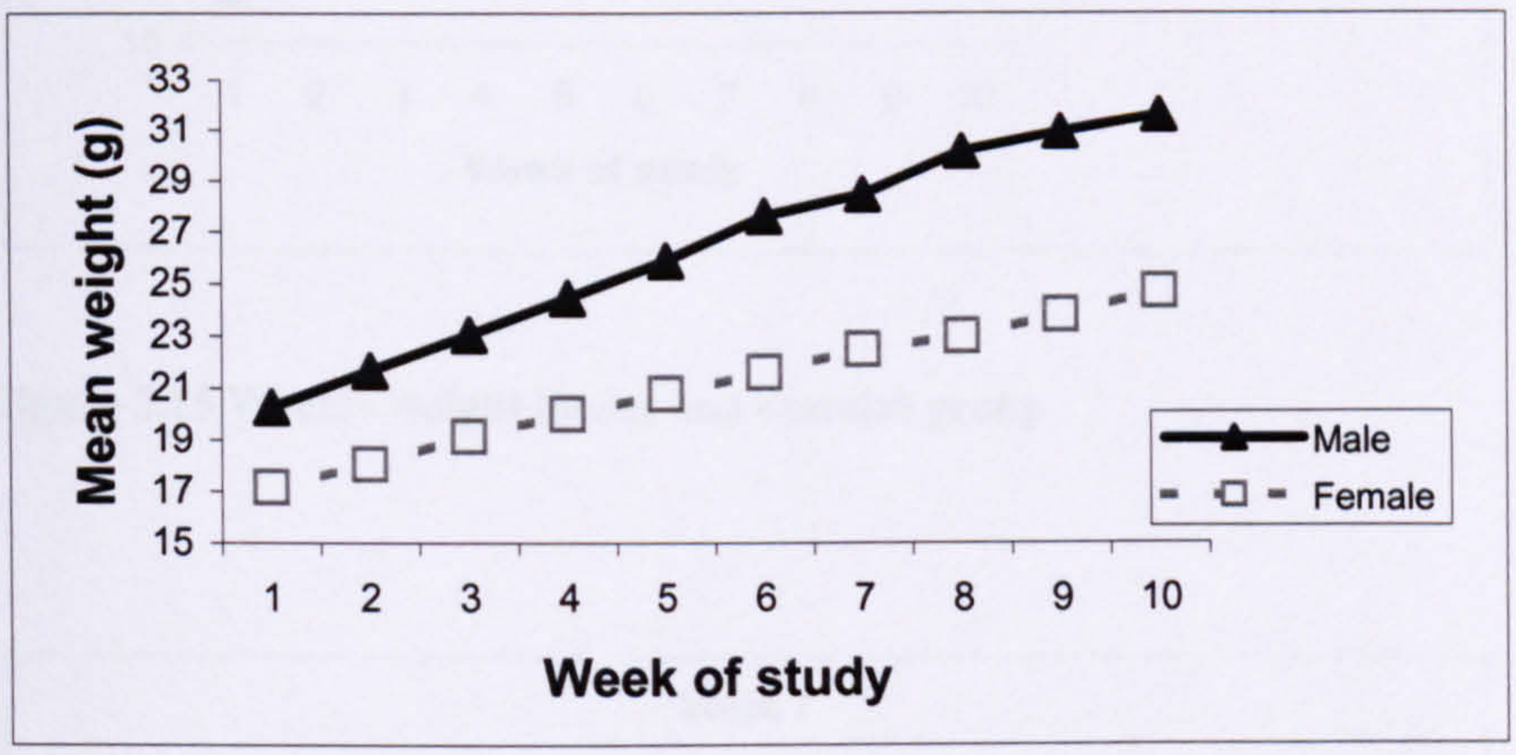


Figure 3.14 Weekly weights of female and male mice

Females were significantly ($P < 0.001$) lighter at every stage of the study (Figure 3.14) and also at death (Figure 3.12).

Figure 3.15 shows the weekly weights by sex and treatment group. The pattern of weight gain was very similar for the mice of the different exercise groups. In contrast, TR males were the lightest male group from week 2-9 and CON males were the heaviest, particularly increasing in weight at week 5 before reaching a plateau. Weights of male WH mice were similar to those of CON until week 6, when a decrease in weight occurred, followed by an increase at week7 and body masses thereafter tracked the growth of male TR mice.

A sex*exercise interaction existed at week 7 ($P = 0.033$, Figure 3.16) and at week 8 the interaction approached significance ($P = 0.054$). On both of these weeks, male

CON mice weighed more than the other groups, and the heaviest females were in the TR group (Figure 3.16). At all other weeks there was no statistically significant interaction (P>0.30).

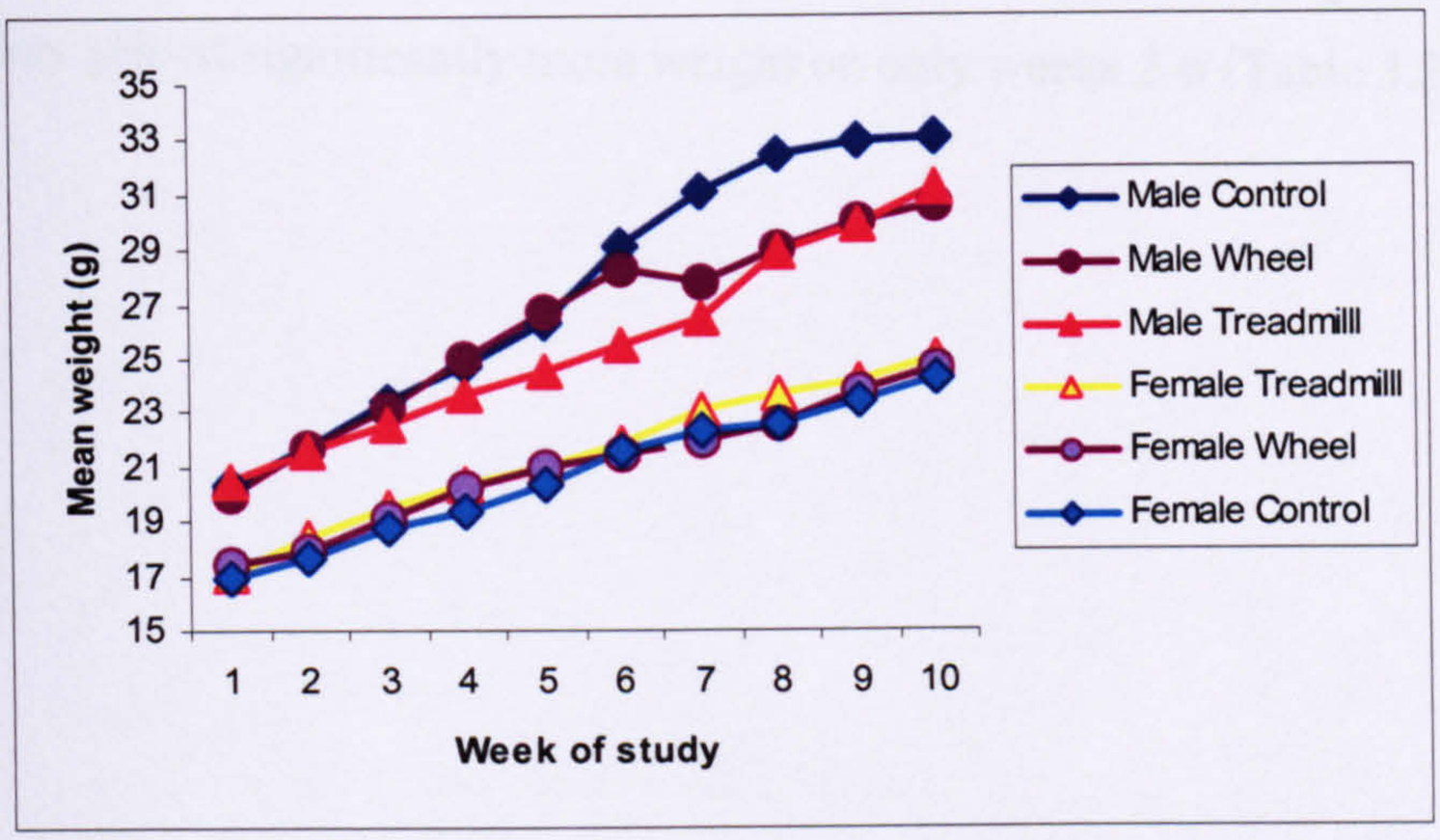


Figure 3.15 Weekly weight by sex and exercise group

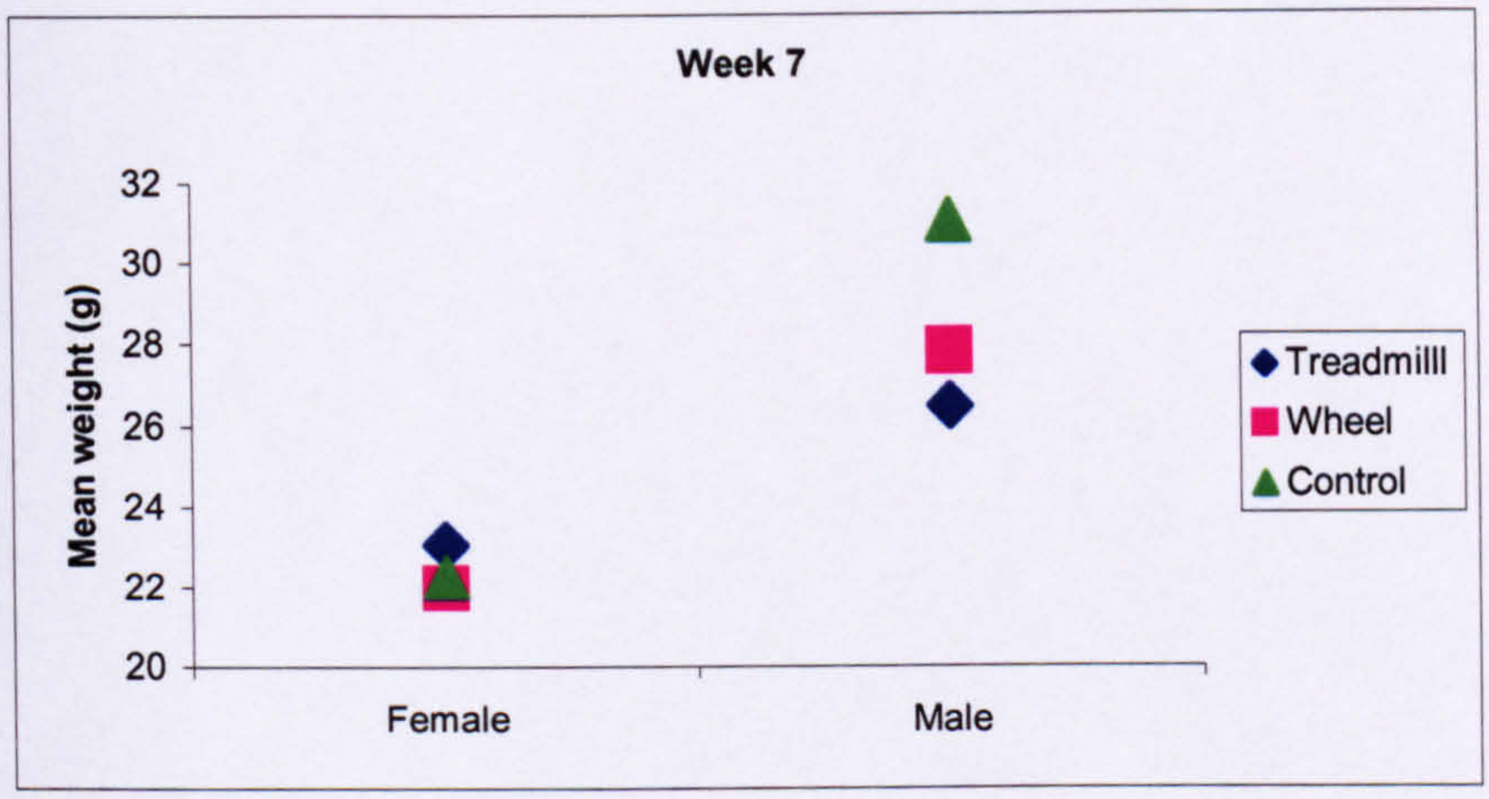


Figure 3.16 Interaction between sex and exercise on weight at week 7 (P = 0.033)

Examination of weekly weight gain reveals more variation between the groups (Table 3.28). There were no significant differences between treatment groups in total weight gain over the study, (TR 9.2g v. WH 8.3g v. CON 8.1g, P = 0.734). However, there were significant differences in weekly weight gain between exercise groups on weeks

4, 6 and 7, with WH gaining more weight on week 4 compared with CON, but CON gaining more weight on week 6 and again at week 7 (Table 3.28).

Male mice gained significantly more weight than female mice over the length of the study (10.3g v. 7.4g, P = 0.008). While males were consistently heavier than females, they gained significantly more weight on only weeks 2-6 (Table 3.29).

Table 3.28 Weekly weight gain (g) by exercise protocol

Group		Weekly weight gain									
		1	2	3	4	5	6	7	8	9	10
Treadmill	Mean	-	1.28	1.00	0.99	0.73	0.84	1.16	1.53	0.70	1.10
	SEM	-	0.32	0.22	0.20	0.26	0.28	0.29	0.29	0.37	0.35
Wheel	Mean	-	1.08	1.33	1.45	1.22	1.02	0.04	0.68	1.05	0.79
	SEM	-	0.18	0.13	0.12	0.15	0.16	0.17	0.17	0.22	0.22
Control	Mean	-	1.06	1.37	1.00	1.31	1.90	1.33	1.00	0.67	0.47
	SEM	-	0.18	0.12	0.11	0.14	0.16	0.17	0.16	0.22	0.21
Probability	TR v CON	-	0.506	0.557	0.931	0.125	0.021	0.996	0.714	0.952	0.411
	WH v CON		0.981	0.997	0.012	0.830	0.001	<0.001	0.274	0.343	0.641

N.B. Weight gain is calculated thus: weight at week 2 – weight at week 1 = gain week 2 etc, hence week 1 is empty

Table 3.29 Weekly weight gain (g) of male and female mice

Sex		Weekly weight gain									
		1	2	3	4	5	6	7	8	9	10
Female	Mean	-	0.89	1.08	0.88	0.76	0.77	0.85	0.73	0.82	0.90
	SEM	-	0.16	0.11	0.10	0.14	0.14	0.15	0.15	0.19	0.18
Male	Mean	-	1.39	1.38	1.41	1.41	1.73	0.84	1.42	0.79	0.67
	SEM	-	0.22	0.15	0.14	0.18	0.19	0.20	0.20	0.26	0.25
	Probability	-	0.004	0.011	<0.001	<0.001	<0.001	0.776	0.137	0.501	0.166

An interaction existed between sex and exercise on week 6 (P = 0.030), week 7 (P<0.001) and week 8 (P = 0.021) (Figure 3.17).

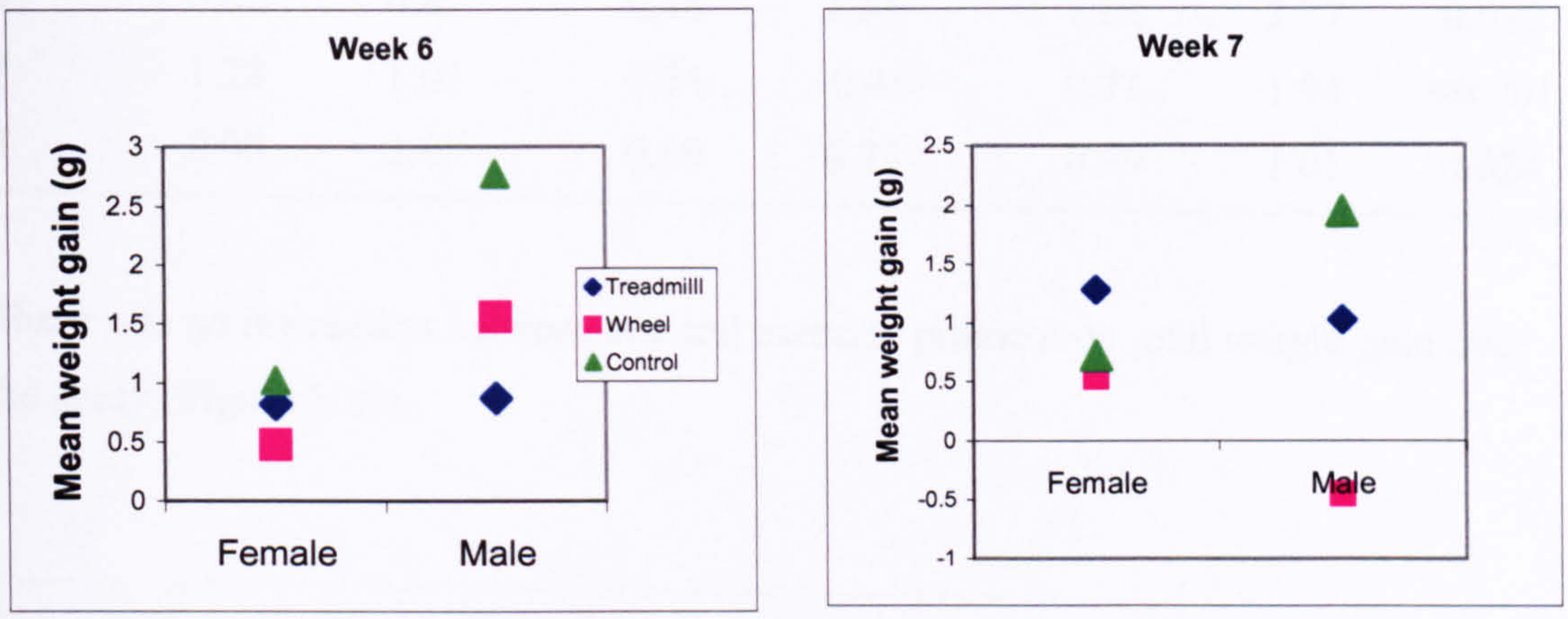


Figure 3.17 Interaction between sex and exercise protocol on weight gain in week 6, 7 and 8

In week 6 male mice gained 2-3 times more weight than females with the exception of male TR, which gained a similar amount to female TR (0.87g and 0.82g respectively, Figure 3.17, Table 3.30). In week 7 females gained more weight than males, except for male CON which gained on average 1.9g compared with 0.7g for female CON (Figure 3.17). In week 8, male TR gained more weight than female TR (2.5 v. 0.6g), while both sexes in CON and WH groups gained similar amounts (Figure 3.17 and Table 3.30).

Table 3.30 Mean body weight gain (g) of mice by sex and exercise group at weeks 6, 7 and 8

	Treadmill		Wheel		Control		
Week	**Female**	**Male**	**Female**	**Male**	**Female**	**Male**	**P**
6	0.82	0.87	0.48	1.56	1.02	2.77	0.030
7	1.28	1.03	0.54	-0.45	0.72	1.94	<0.001
8	0.60	2.47	0.59	0.77	0.99	1.01	0.024

There was no interaction between sex and exercise protocol on total weight gain over the study (Figure 3.18).

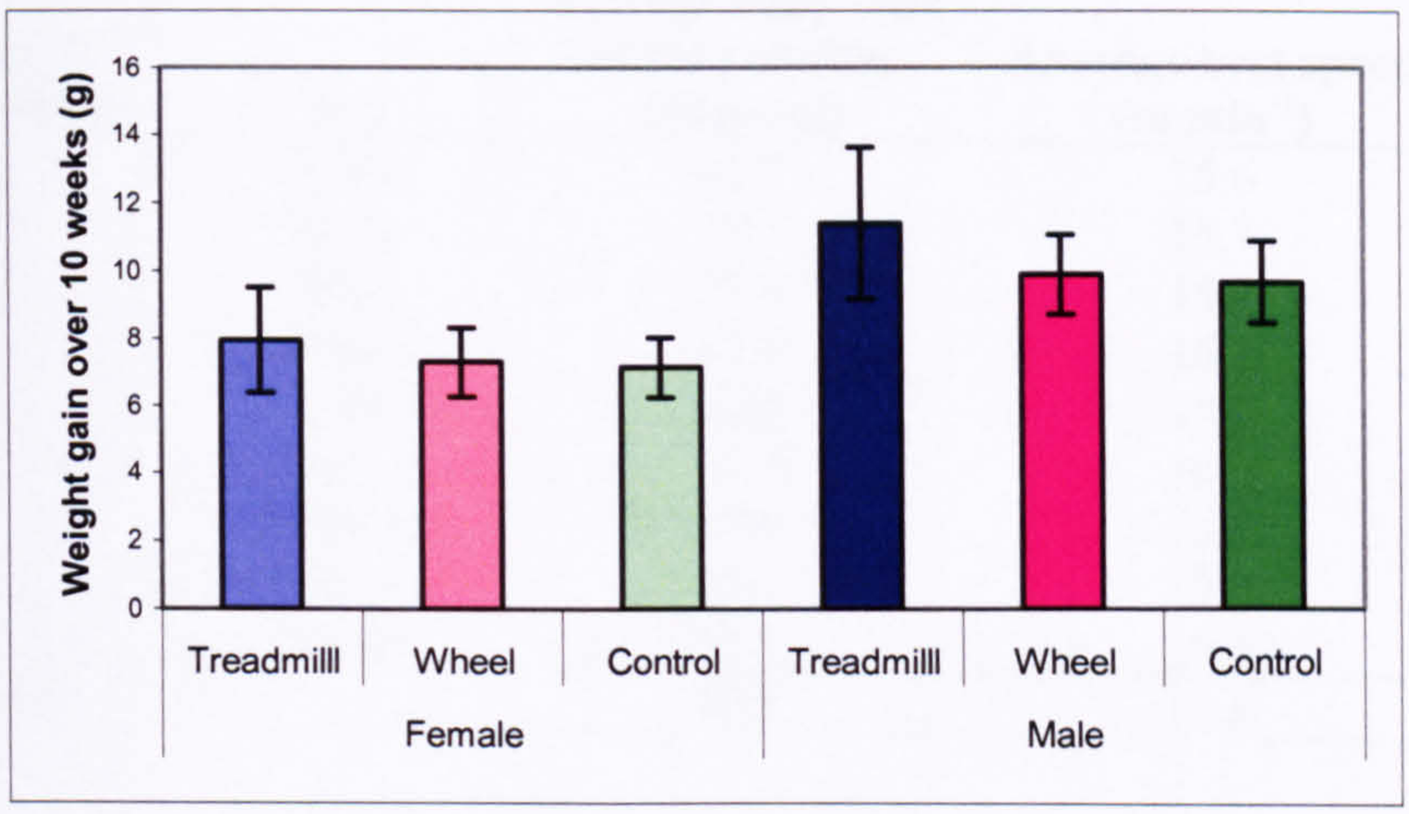


Figure 3.18 Total weight gain between the groups (slaughter weight minus initial weight)

3.2.4.2 Time and amount of running – treadmill

The treadmill protocol was altered slightly from the first study (ES1), including more breaks and running for shorter times. This resulted in a greater number of animals running on every day of the study, a faster average speed and longer daily time spent running. There was still a problem with drop-outs - 7 males and 10 females started on the treadmill. The study ended with 3 males and 6 females. All dropouts but 1 were transferred to the CON group, the other was added to the wheel group but killed early due to a large burden of tumours.

Data analysed were means of the total time run daily by each mouse, the percentage of days on which the mice ran and the fastest speed achieved each day. Individual mouse data for the running parameters are summarised in Table 3.31. There was no significant difference between males and females in the average fastest speed run daily (15.6 $m \cdot min^{-1}$ v. 15.9 $m \cdot min^{-1}$), but differences occurred in the percentage of days run throughout the study (80% v. 99%, P = 0.003) and the average time spent running daily (37 min v. 56 min, P = 0.001), with females running on more days and also running for longer. No male mice ran on every day of the study.

Table 3.31 Time and speed data for individual mice on the treadmill

Mouse number	Sex	Average daily time spent running (minutes)	Average best speed ($m \cdot min^{-1}$)	% of days run
26	Male	44.7	15.6	91.7
24	Male	39.1	15.7	79.2
25	Male	28.4	15.4	68.8
33	Female	60.0	16.1	100.0
28	Female	60.0	16.1	100.0
30	Female	56.4	16.0	100.0
47	Female	55.6	15.8	100.0
49	Female	55.2	15.8	100.0
50	Female	51.3	15.4	95.8
Mean		**50.1**	**15.8**	**92.8**

Mean daily running time was positively correlated with running speed (r = 0.75, Figure 3.19), indicating that mice choosing to run for longer also ran faster. The regression equation connecting mean daily fastest running speed and mean daily running time is:

best speed = 14.8 + 0.0191*running time ($R^2 = 0.49$, P = 0.021).

N.B. "best speed" represents the fastest speed run by each mouse in each session, as the speed was changed if mice could not keep up/dropped out. Therefore different mice may have different maximum speeds depending on how long they ran for.

Figure 3.20 shows that six of the nine mice ran for shorter periods towards the end of the study.

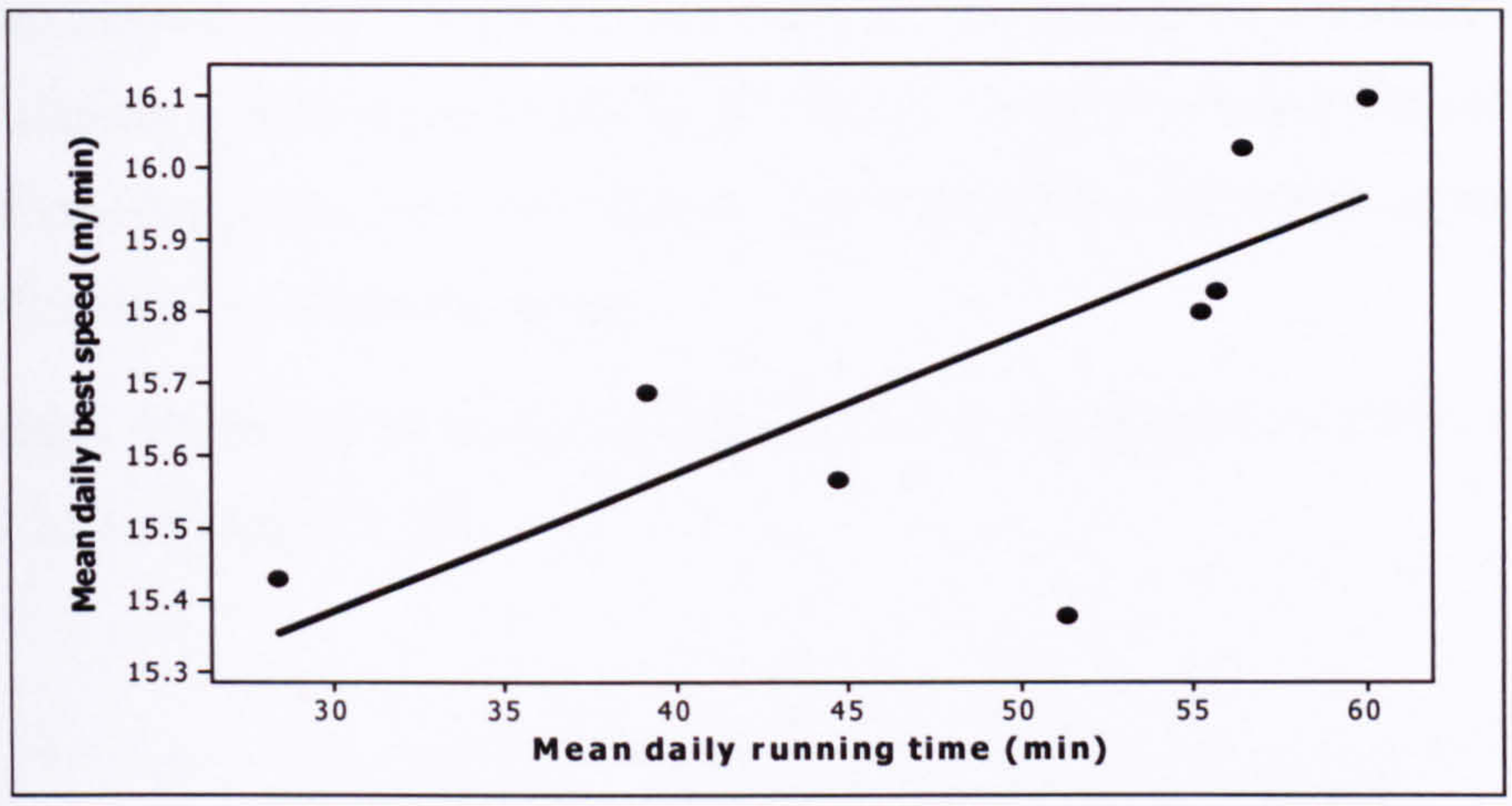


Figure 3.19 Plot of maximum daily running speed against mean daily running time for individual treadmill mice with fitted regression line

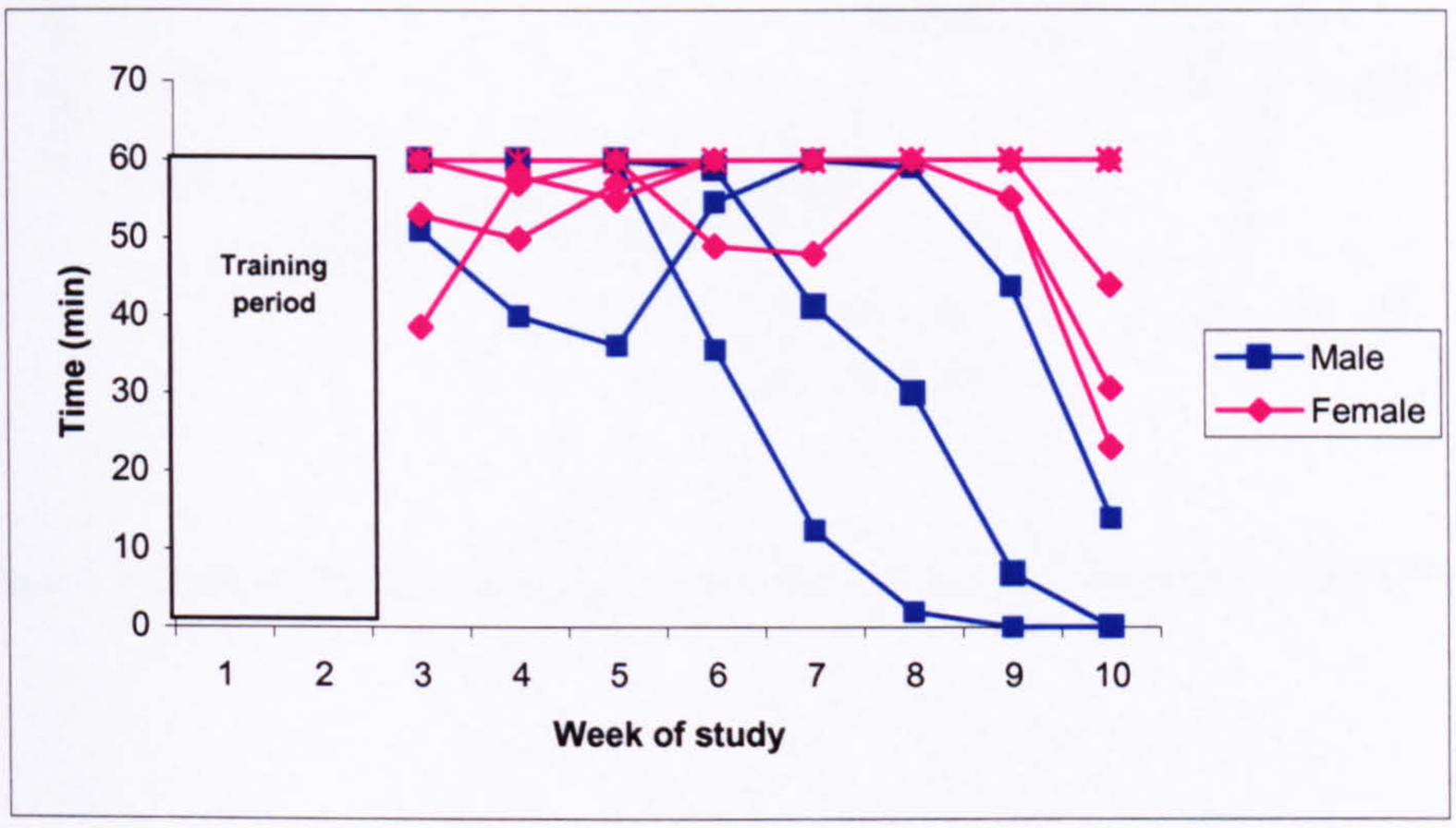


Figure 3.20 Average daily running time for each mouse during the final 8 weeks of the study (3 female mice ran for 60min·d and therefore appear as one line)

3.2.4.3 Wheel running

The response of the mice to the running wheels was highly individual. Some ran on the first day and continued to run for the length of the study (Figure 3.23), others ran considerable distances at the beginning of the study and much less towards the end, or ran very little at the beginning of the study and more towards the end (Figure 3.21) and the distance covered varied widely between mice (e.g. Figure 3.22 and Figure 3.24). There was a marked difference between males and females, with females running significantly ($P = 0.004$) further after 10 weeks, an average of 314.4 v. 147.0

km (Figure 3.26). Most mice continued to run throughout the study (Figure 3.25). Running speeds were estimated by filming the mice running and counting the number of revolutions in a set time period. This method revealed running speeds of 30-40m·min^{-1} in a female mouse.

Many females also demonstrated a 3-5 day cyclical pattern in their running (Figure 3.21 and Figure 3.22).

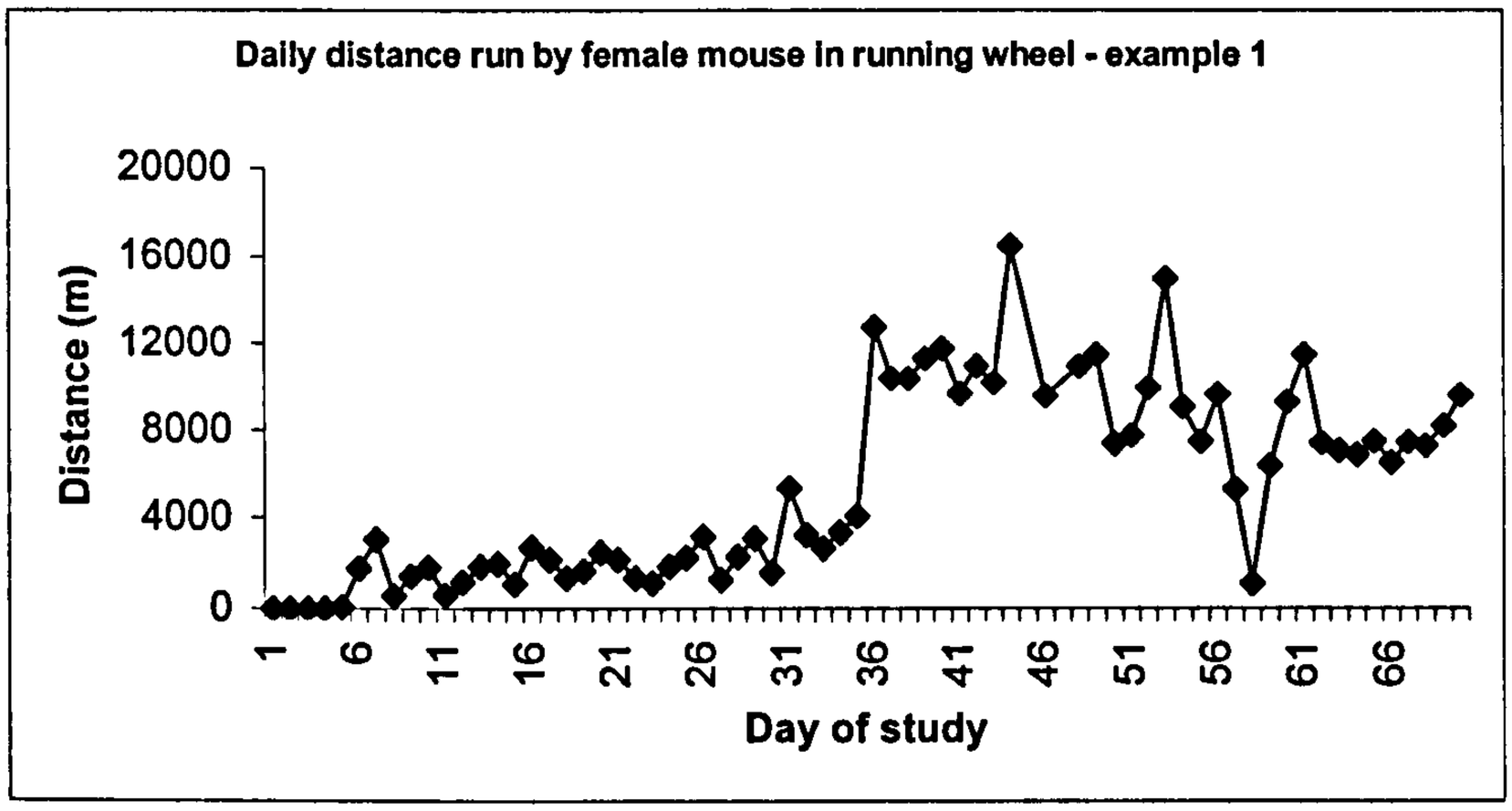


Figure 3.21 Daily distance run by a mouse that ran more in the larger wheel (day 36 onwards)

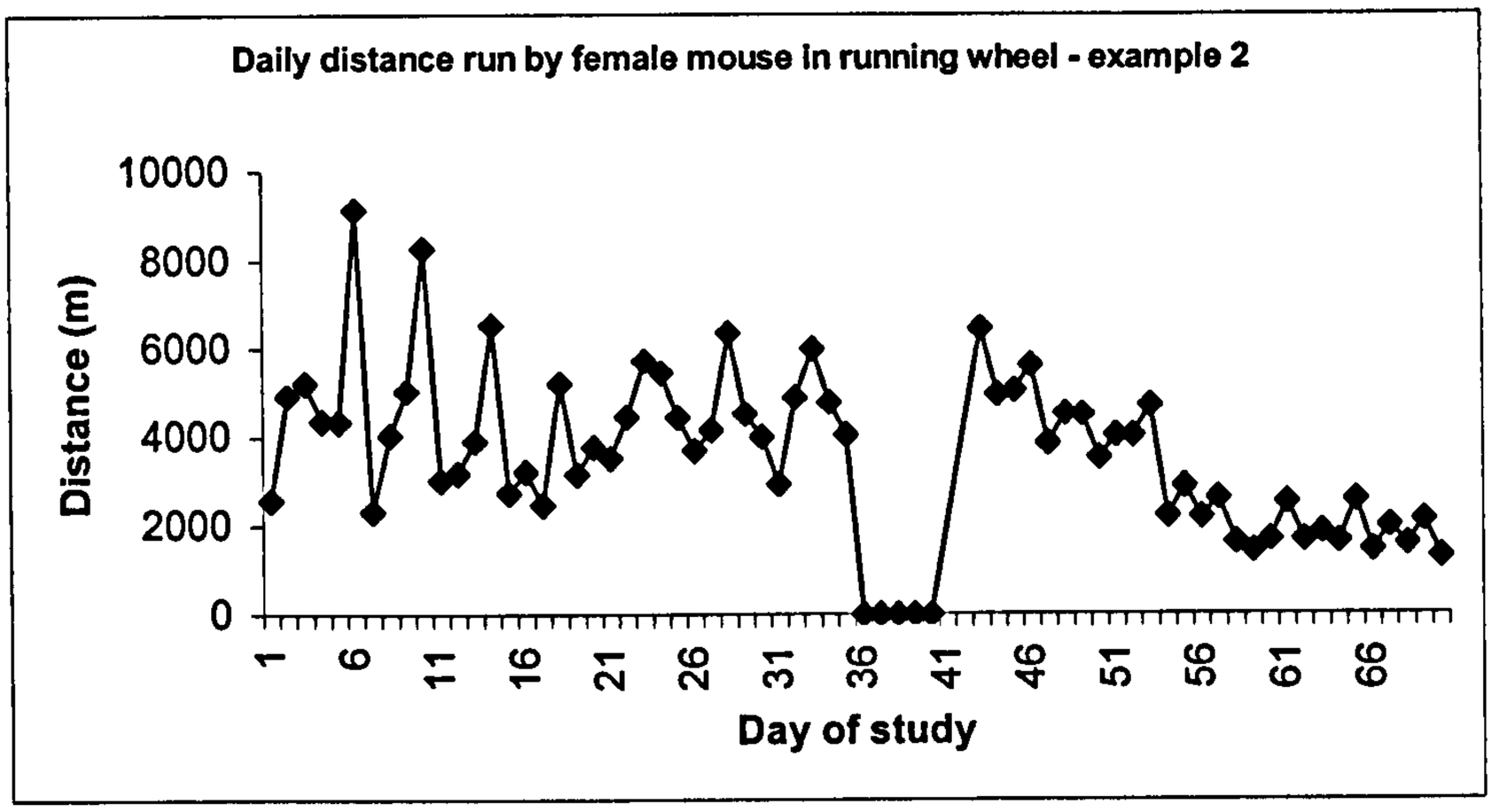


Figure 3.22 Daily distance run by a female mouse which ran more in the small wheel (up to day 35) than in the larger wheel. Days of zero distance correspond with the time period immediately after the wheel change

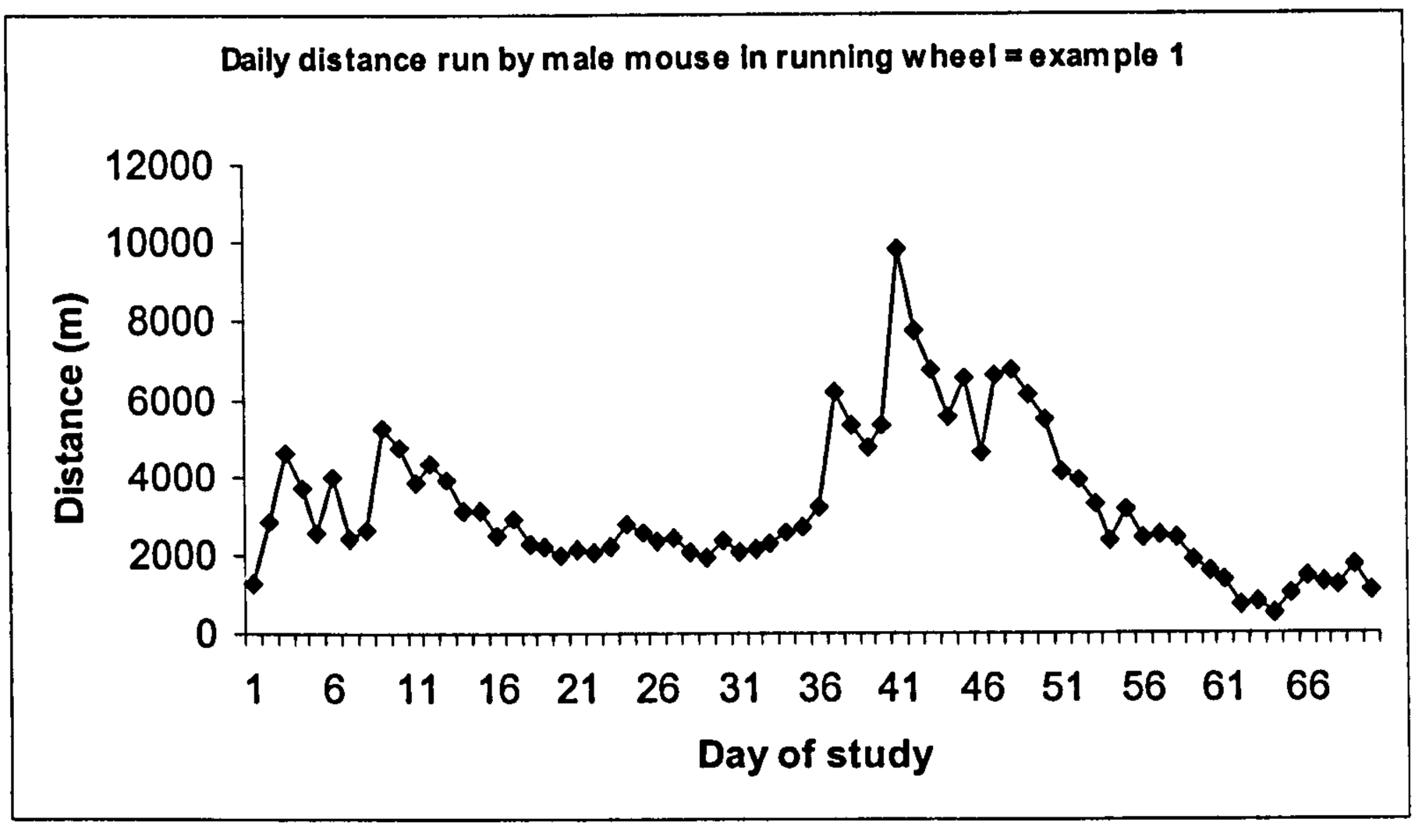


Figure 3.23 Typical example of the daily distance run by a male mouse – daily distances varied less than in females. The large increase in distance follows access to the larger wheel, although distances returned to levels similar to those run in the small wheel

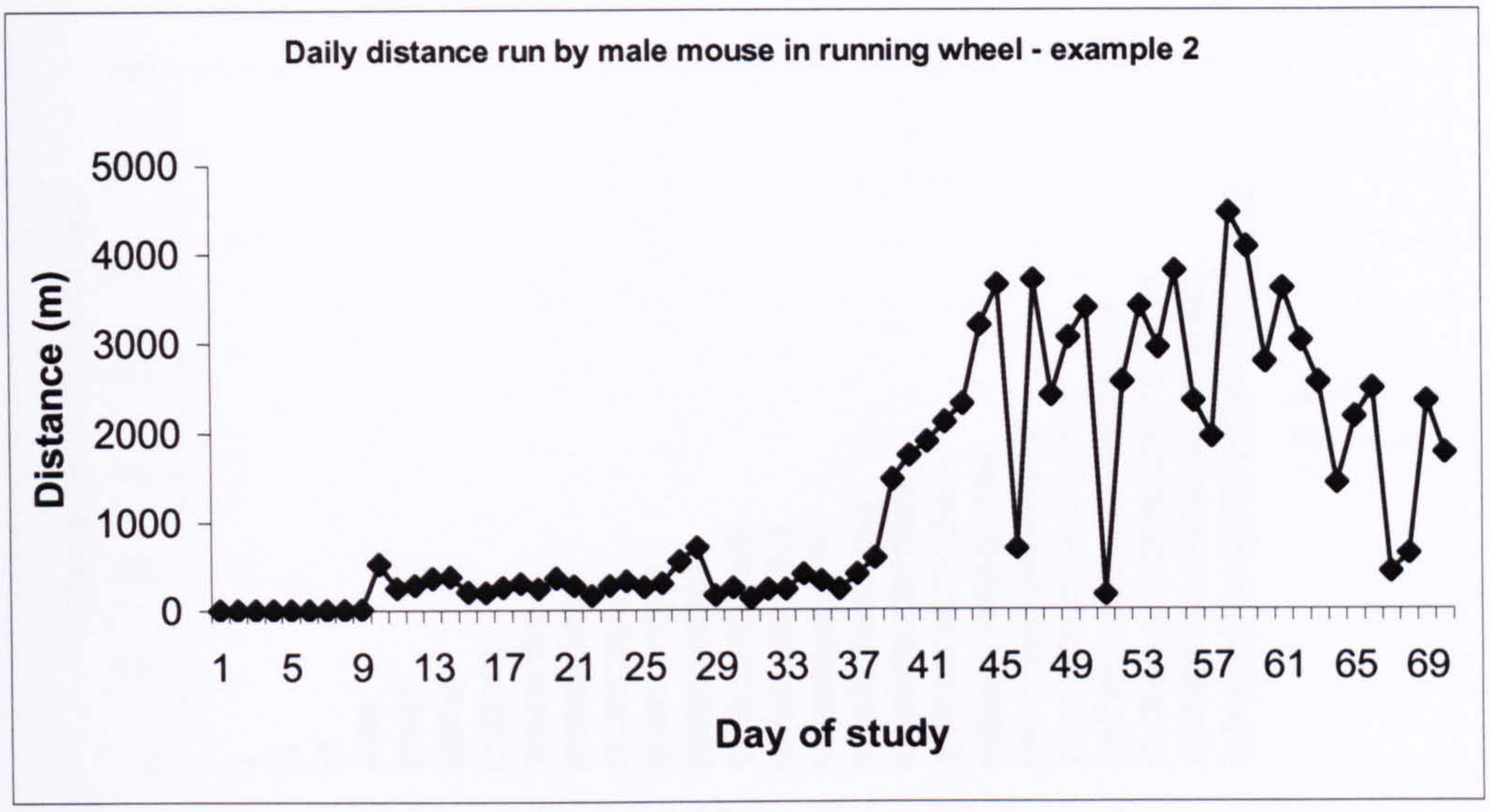


Figure 3.24 Daily distance run by a mouse that ran relatively little in the small wheel (<400m/day), but regularly ran over 2km a day when given access to a larger wheel, showing that individual animals varied in their response to wheels

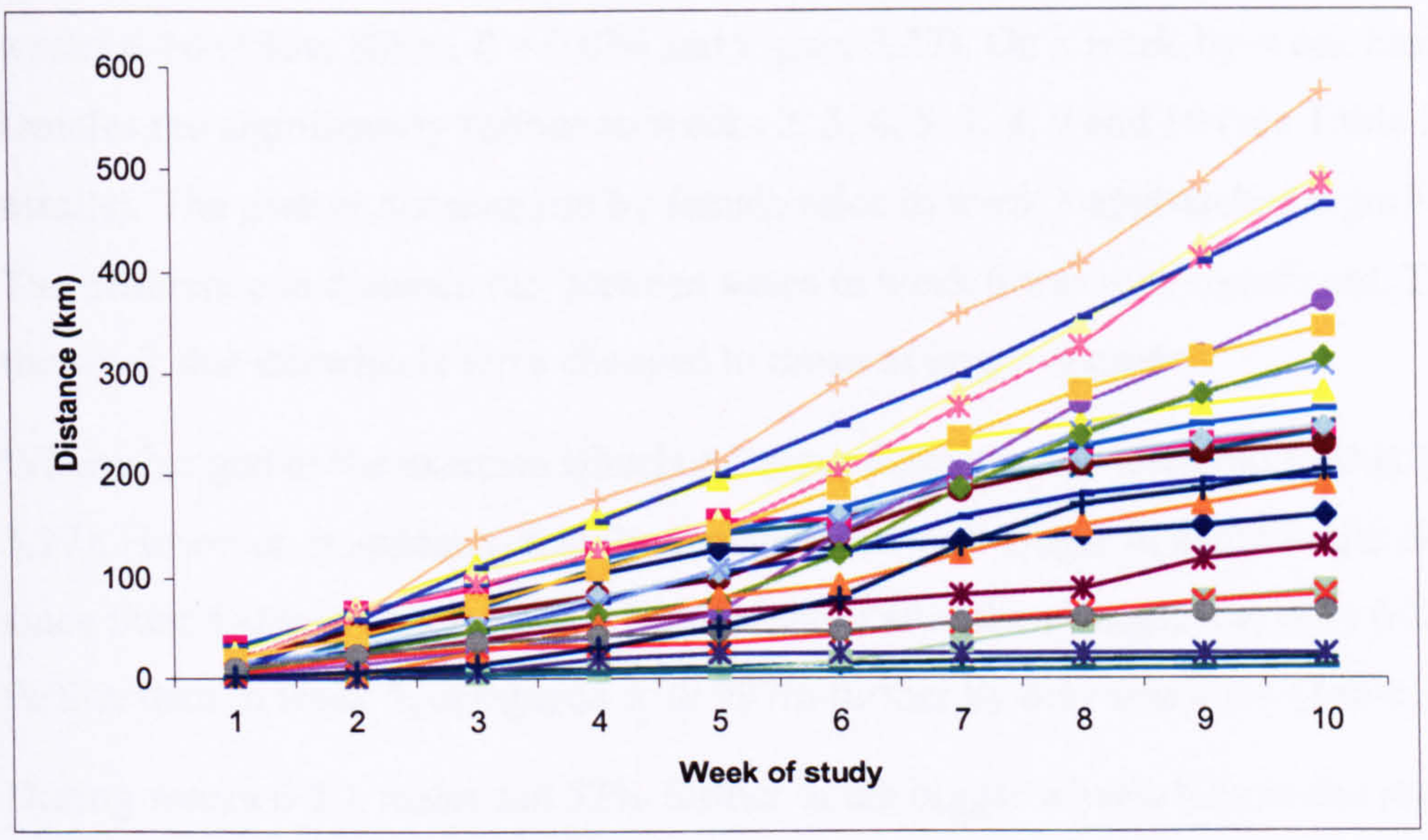


Figure 3.25 Cumulative distances run by individual mice in wheels

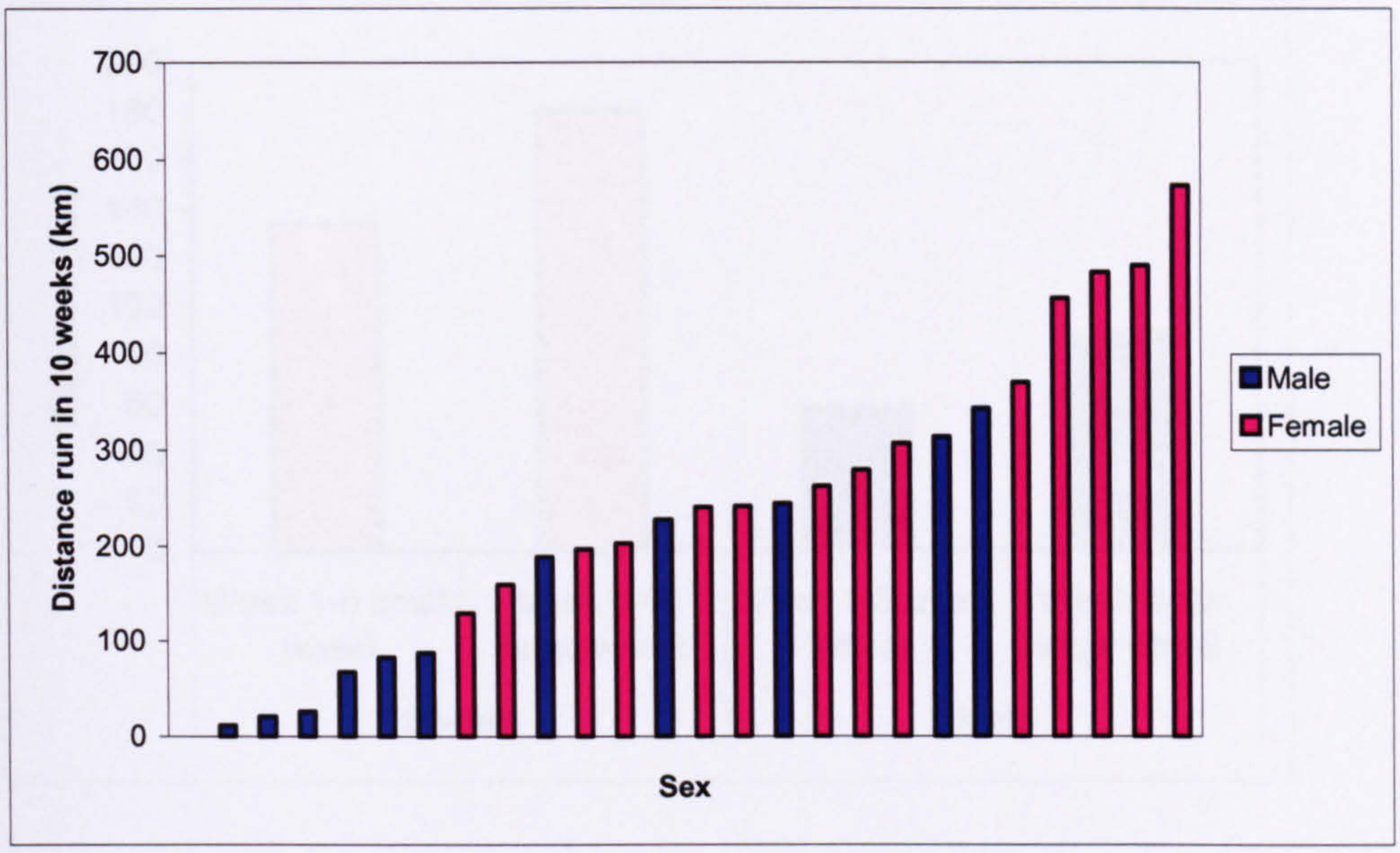


Figure 3.26 Total distances run in 10 weeks by male and female mice

Females ran further than males in weeks 1-5 (134 v. 58km, P = 0.001) and again in weeks 6-10 (180v. 88km, P = 0.036 and Figure 3.27). On a week-by-week basis, females ran significantly further on weeks 2, 3, 4, 5, 7, 8, 9 and 10 (see Table 3.32 for details). The greater distance run by female mice in week 1 approached significance. The difference in distance run between sexes in week 6 was non-significant. This was the week that the wheels were changed to those of larger diameter.

When changed to the exercise wheels of larger diameter, the mice ran further (Figure 3.27). However, it appeared that the female mice took longer to adapt to the change since their distance run in week 6 (immediately after the change) was only 642m further than in week 5, compared with 997m further by the male mice (Table 3.32).

During weeks 6-10, males ran 52% further in the bigger wheels than in the smaller wheels (88 v. 58km), compared with a 35% increase by the females (180 v. 134km), and this difference was significant (P = 0.041) for both sexes (Figure 3.27 and Figure 3.28).

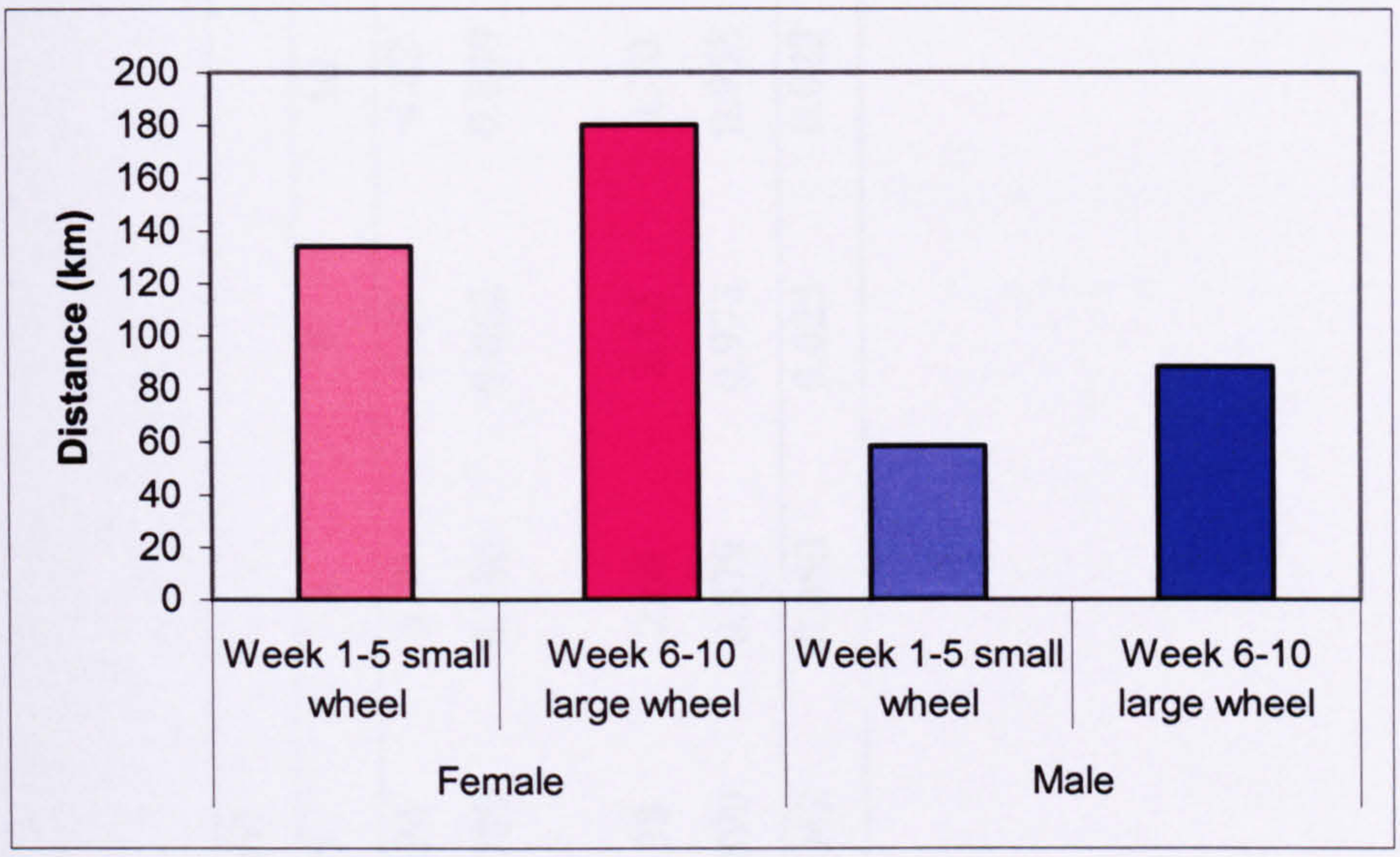


Figure 3.27 Distances run in small and large wheels, by sex

Table 3.32 Mean weekly distance (km) run in wheels by the sexes

	Distance run in each week of study (km)									
Sex (n)	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Female (14)	2.19	4.79	4.35	4.1	4.03	4.68	5.94	5.09	5.24	4.82
SEM	0.378	0.627	0.578	0.413	0.396	0.921	0.789	0.780	0.862	0.849
Male (11)	1.03	1.70	1.99	1.85	1.78	2.78	3.38	2.66	2.12	1.70
SEM	0.426	0.708	0.652	0.466	0.447	1.039	0.890	0.879	0.973	0.958
Probability	0.054	0.003	0.013	0.001	0.001	0.185	0.042	0.050	0.025	0.023

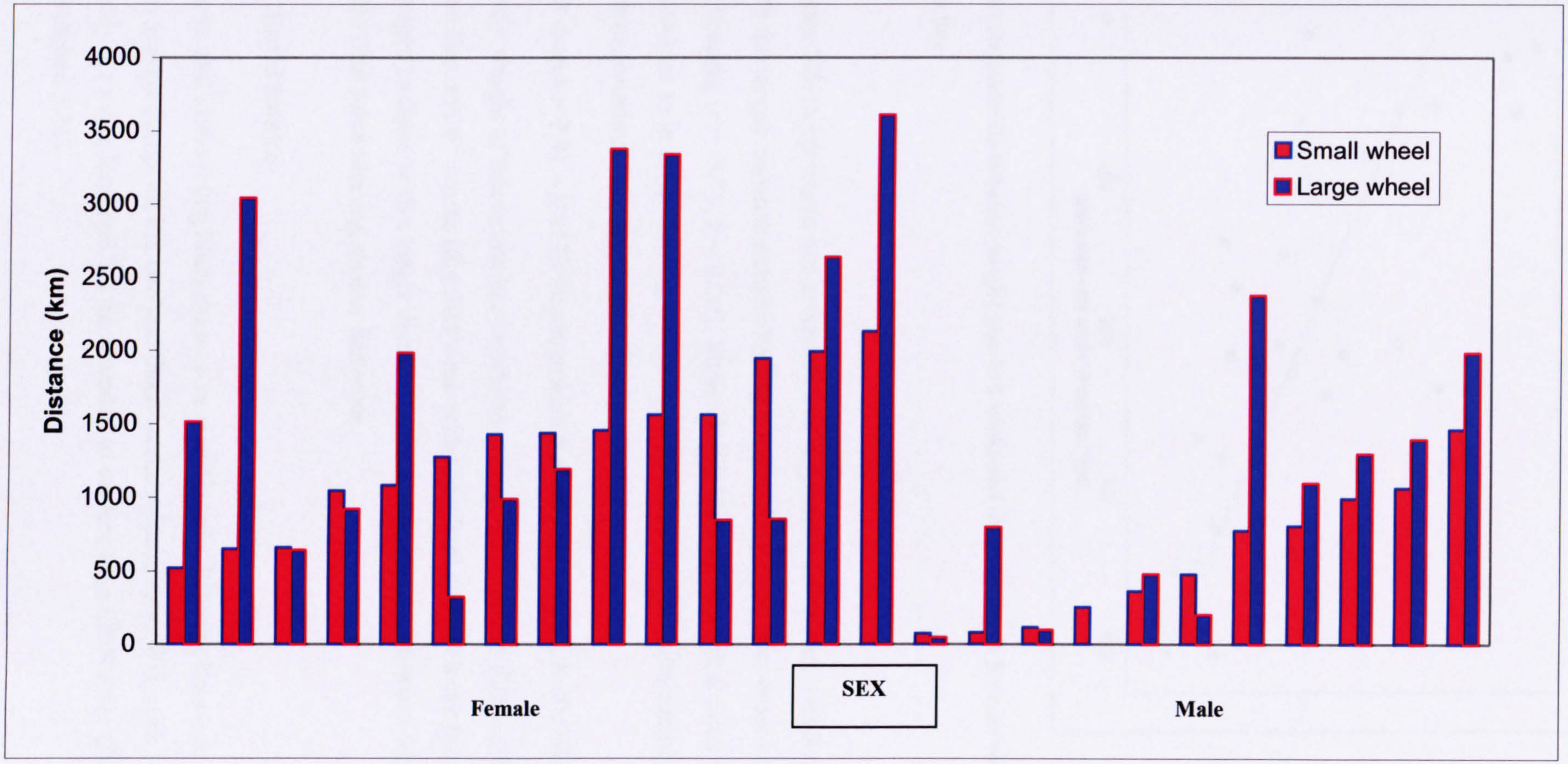


Figure 3.28 Distances run in small and large wheels in 5 weeks by individual mice

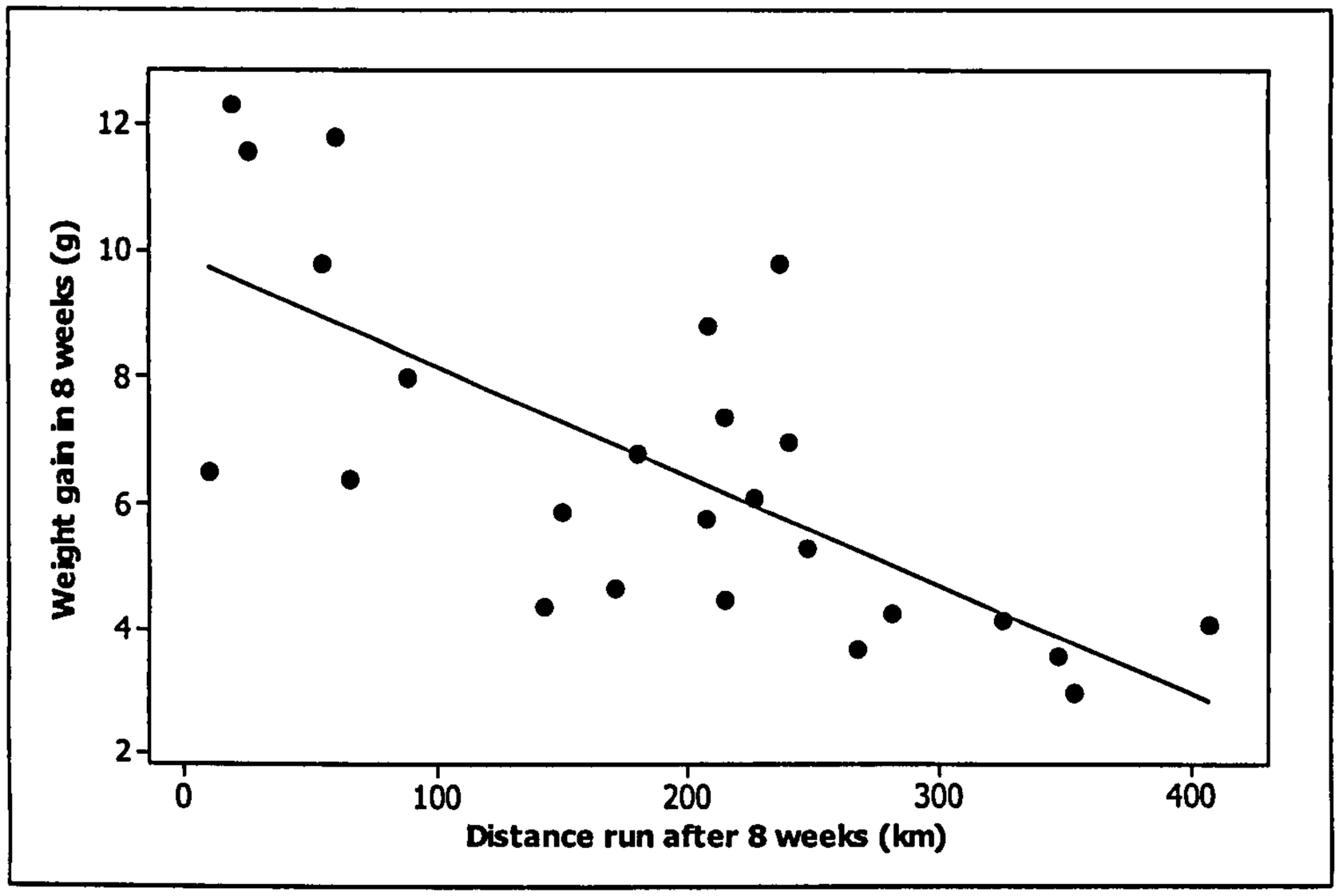


Figure 3.29 Relationship between weight gain in 8 weeks and distance run in 8 weeks with fitted regression line

Over weeks 1-8, the distance run in wheels was negatively correlated with body mass gain, with the longer distance runners having a lower body mass than those covering shorter distances ($r = -0.71$, $P < 0.001$, Figure 3.29). Data up to week 8 were chosen for this analysis as in the following weeks some mice lost weight (presumably due to tumour manifestation). The regression equation was:

Gain in 8 weeks = 9.91 – 0.0173*distance after 8 weeks ($R^2 = 0.50$, $P < 0.001$).

The weekly weight of mice correlated with the average distance run that week in all except the first and 6th weeks (the first week with the wheel and the week the wheels were changed to those with a larger diameter). Keen runners had a lower body mass ($P < 0.025$) than mice running shorter distances.

3.2.4.4 Food intake

Food intake and intake/100g body mass were significantly different between treatment groups during all but two and three weeks respectively. WH mice consistently ate more food and TR mice tended to eat less than CON mice (Figure 3.30 and Figure 3.31).

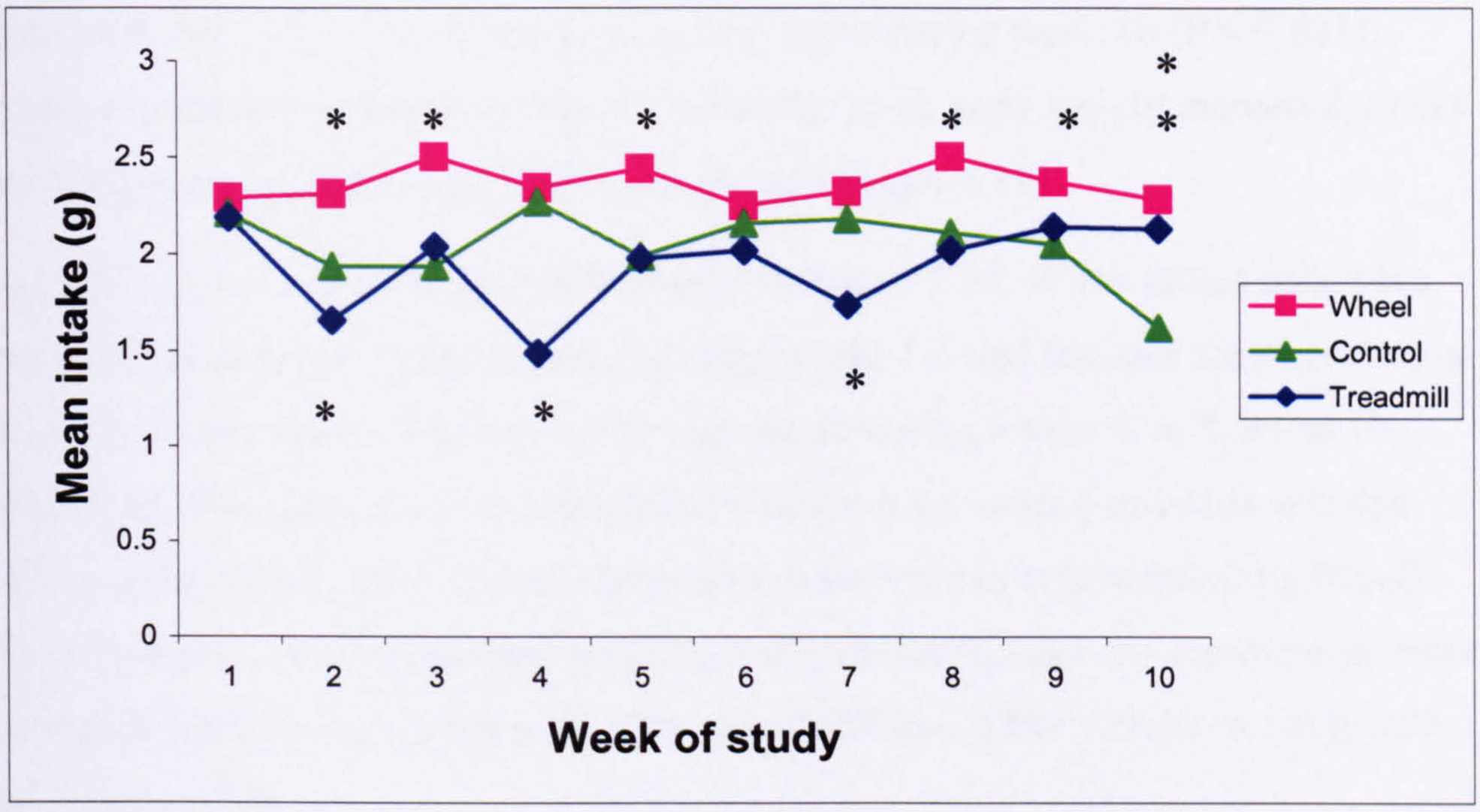


Figure 3.30 Daily food intake per week for each treatment group (* represent values significantly different from Control)

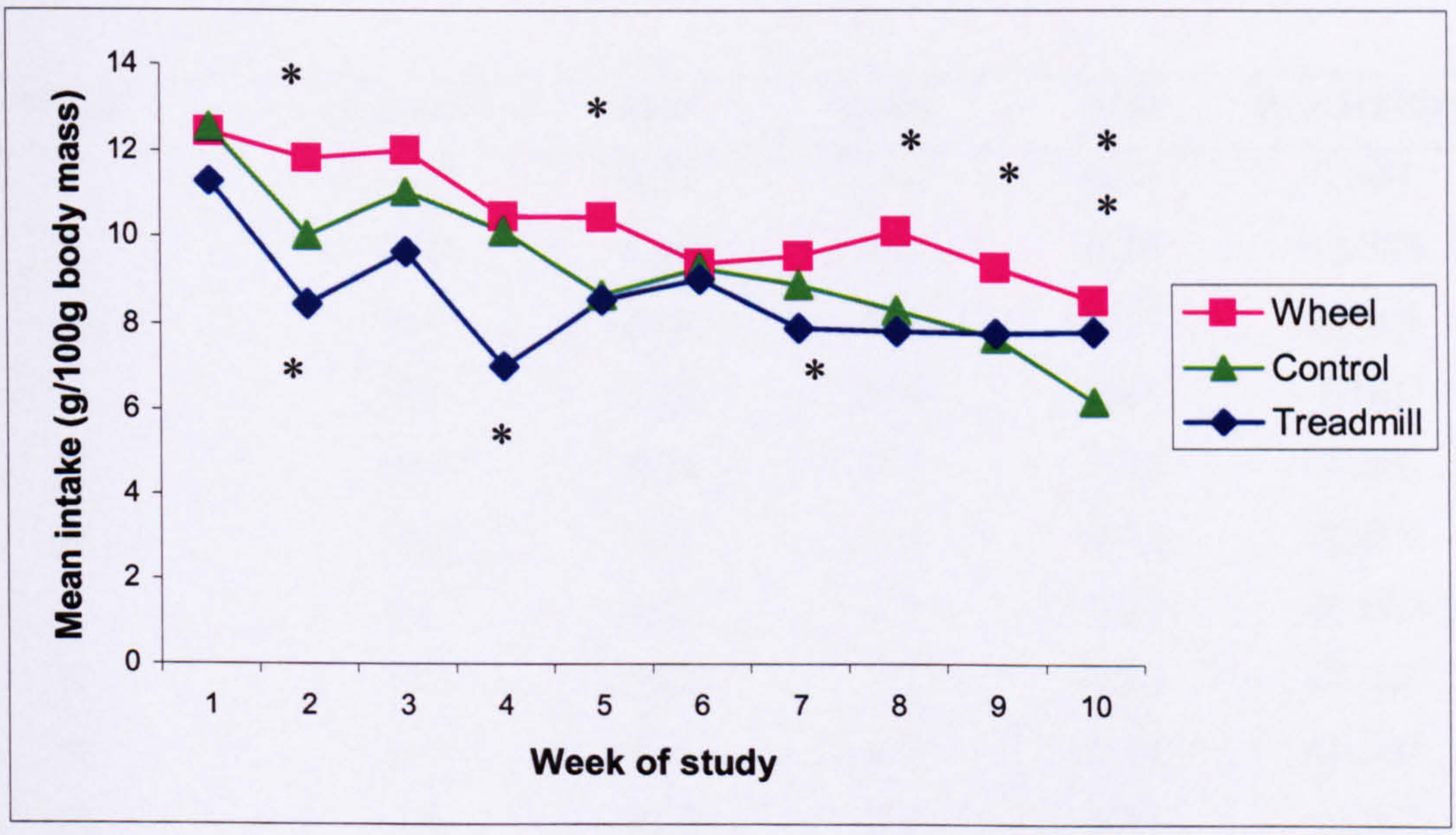


Figure 3.31 Daily food intake per week per 100 grams body mass for each exercise protocol (* significantly different from Control)

WH ate significantly more during weeks 2 (P<0.001), 5 (P = 0.004), 8 (P<0.001), 9 (P = 0.007) and 10 (P<0.001). TR ate significantly less during weeks 2 (P = 0.008), 4 (P<0.001) and 7 (P = 0.027) and significantly more during week 10 (P = 0.011). Intake was relatively stable throughout the study, so as body weight increased, intake per 100g body mass decreased correspondingly (Figure 3.31).

Sex differences in food intake are displayed in Figure 3.32. When intake data were expressed in g/mouse, males ate more during weeks 1-5 and females ate more during weeks 6-10 and these differences were significant during weeks 4, 6, 8, 9 and 10 (P≤0.025). This changeover in relative food intake from week 6 onwards was due both to a decreased intake by male mice and an increase in consumption by female mice. However, when expressed per 100g body weight females ate significantly more during all weeks except week 4, (P between <0.001 and 0.004, details in Table 3.33 and Figure 3.33).

Table 3.33 Mean food intake (g per 100g body mass) of males and females

Week	Female	SEM	Male	SEM	Probability
1	12.8	0.32	11.4	0.38	0.004
2	10.7	0.26	9.5	0.26	<0.001
3	12.0	0.36	9.7	0.39	<0.001
4	9.4	0.32	9.0	0.41	0.161
5	10.1	0.34	8.4	0.34	<0.001
6	10.8	0.31	7.6	0.34	<0.001
7	9.8	0.29	7.8	0.38	<0.001
8	10.3	0.27	7.2	0.33	<0.001
9	9.6	0.41	6.8	0.44	<0.001
10	8.8	0.23	6.2	0.31	<0.001

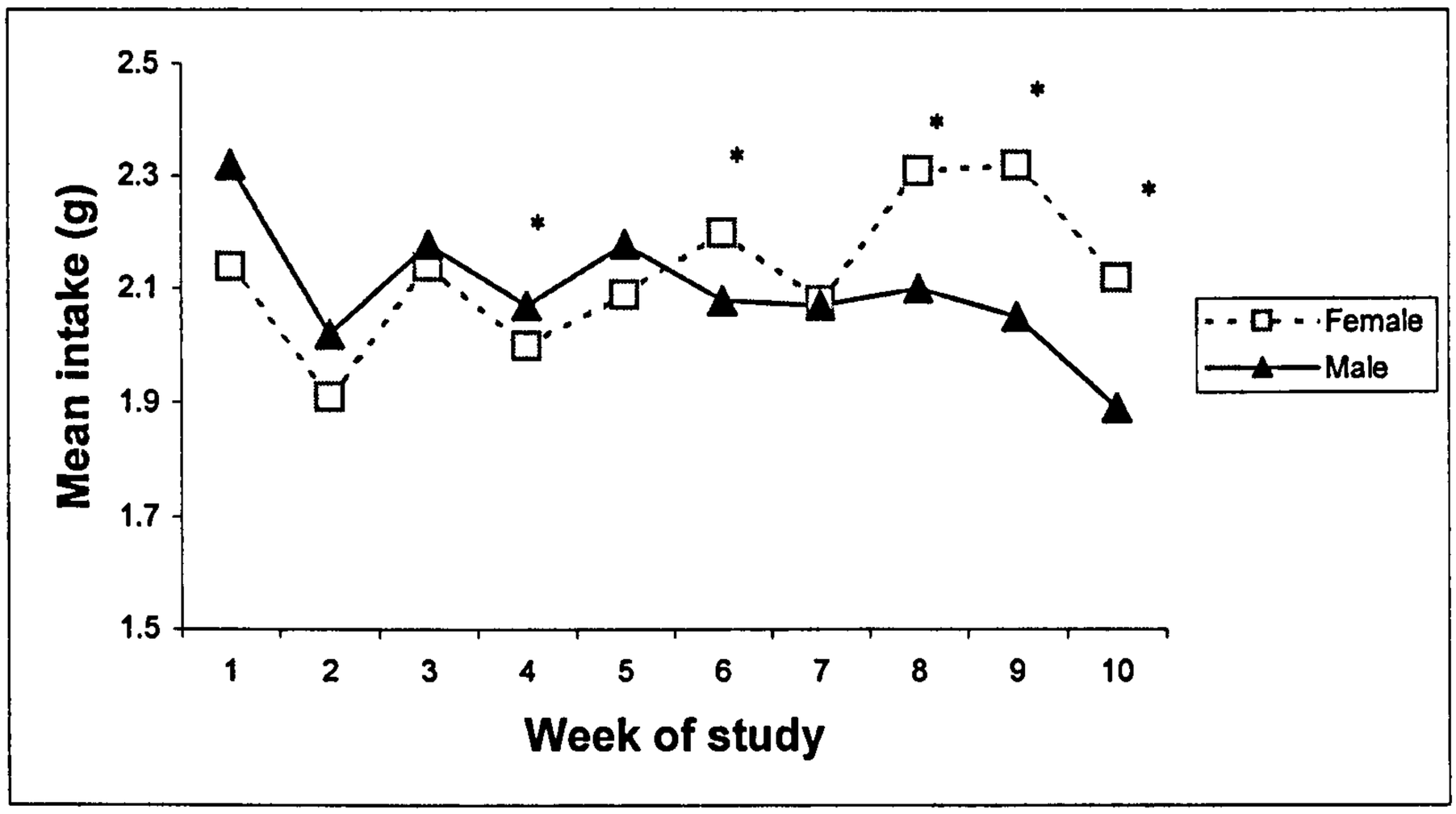


Figure 3.32 Mean daily food intake per week of male and female mice (* denote significant differences)

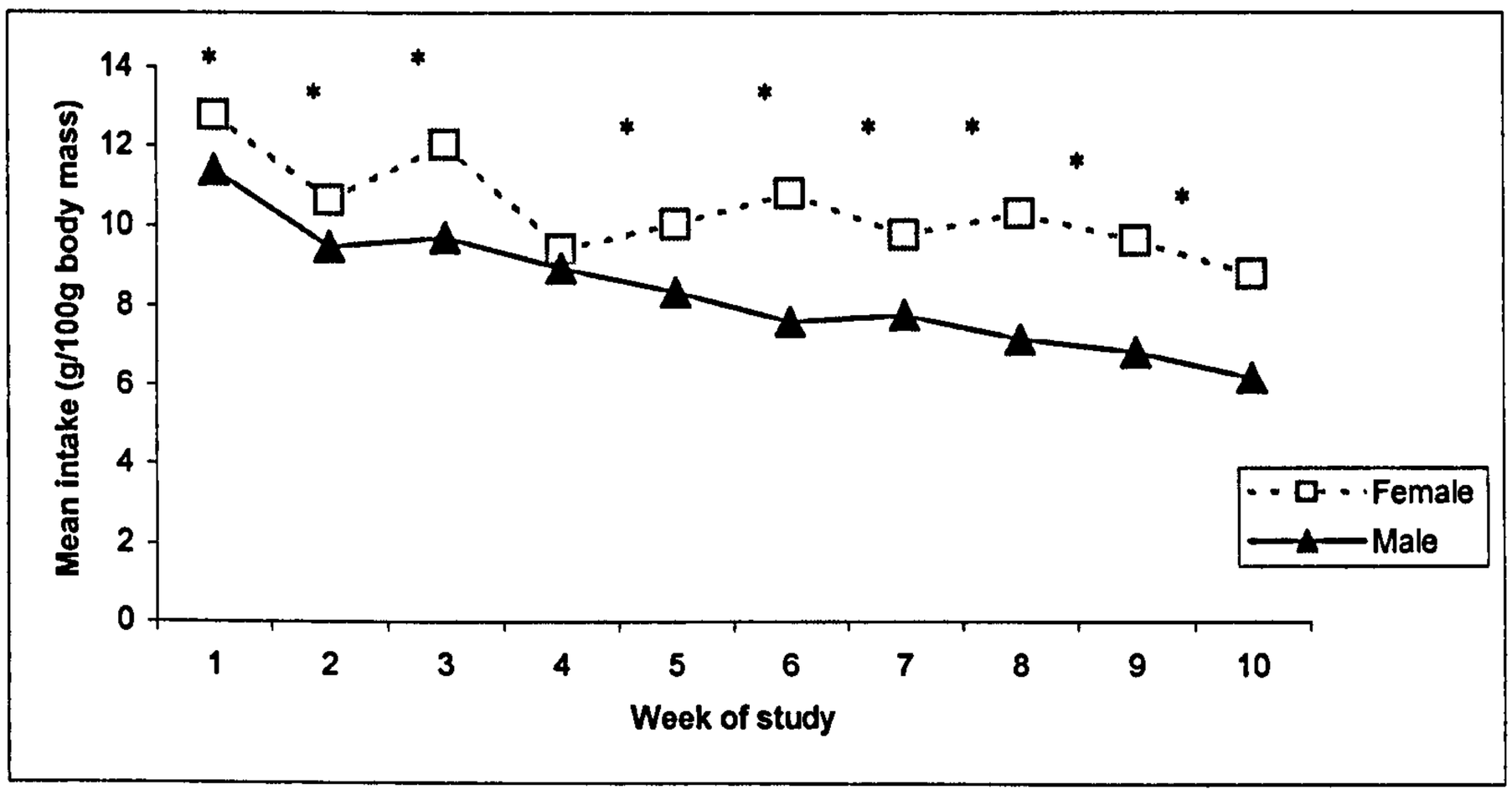


Figure 3.33 Mean food intake per 100g body mass for male and female mice (* denote significant (P<0.05) differences)

Variability in intake within separate sex-exercise groups (female TR, male TR etc) was quite pronounced (Figure 3.34), especially for female TR and male and female CON. When displayed as intake per 100g body mass, the pattern became more stable,

with intake decreasing gradually as the study progressed. The exceptions to this were the female TR mice where food intake remained relatively constant throughout the study (Figure 3.35).

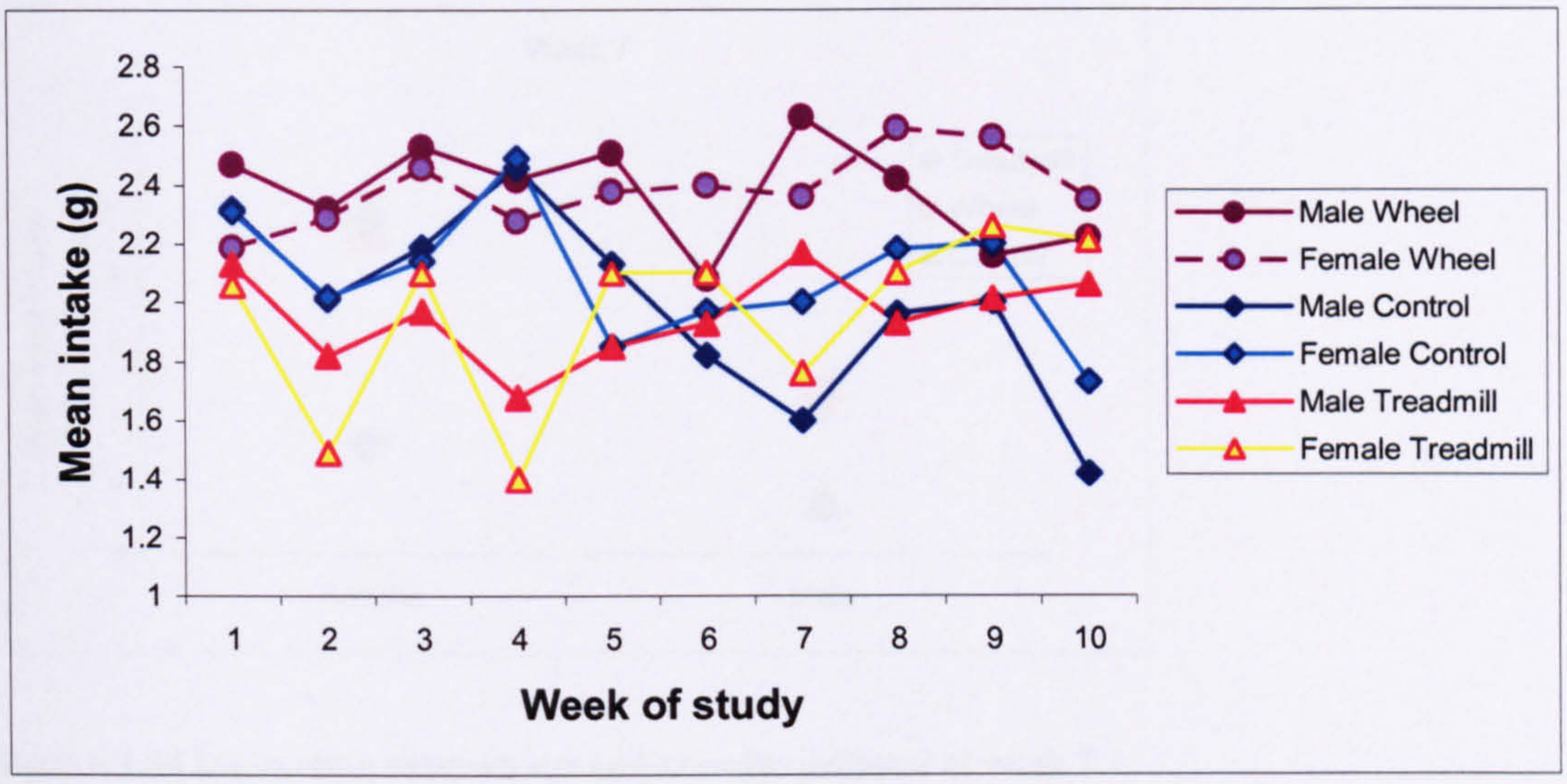


Figure 3.34 Daily food intake during each week of the study by sex and exercise group

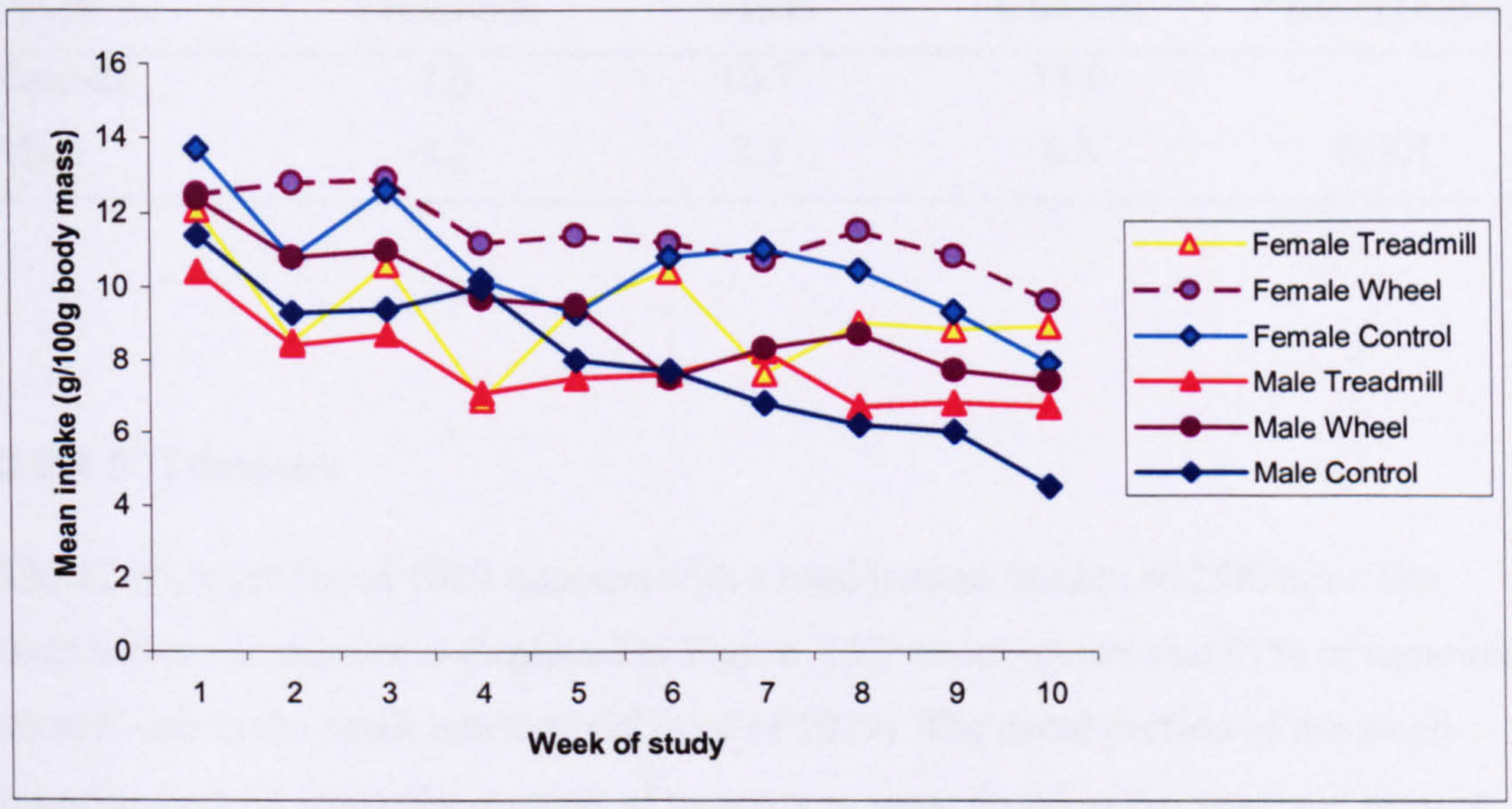


Figure 3.35 Daily food intake per 100g body mass for each sex and exercise group

There were significant interactions between intake/100g body mass and sex during week 7 (P = 0.001, Figure 3.36 and Table 3.34) with females eating more than their male counterparts, with the exception of male TR who ate more than female TR.

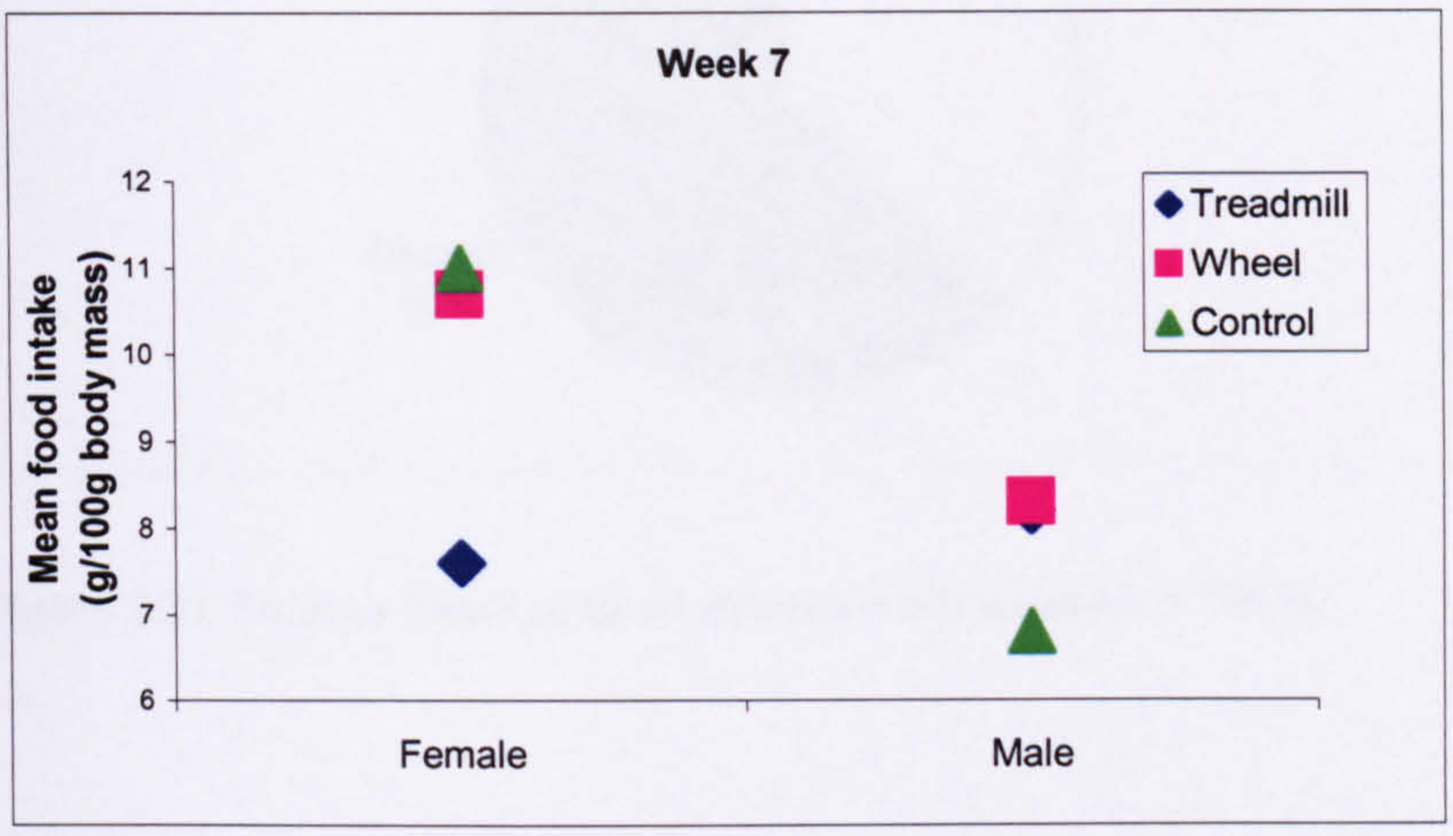


Figure 3.36 Interaction between sex and exercise protocol at week 7

Table 3.34 Mean food intake (g/100g body mass) by sex and exercise group at week 7

Week 7	Treadmill	Wheel	Control	P (interaction)
Female	7.6	10.7	11.0	
Male	8.2	8.3	6.8	0.001

3.2.4.5 Tumours

The 62 mice produced 1019 tumours with a total tumour burden of 2587mm. The distribution of tumours is displayed in Figure 3.37, which shows that 97% of tumours were found in the small intestine (987 out of 1019). The distal portion of the small intestine had 1.6 times the number of tumours as were found in the proximal portion (608 v. 379).

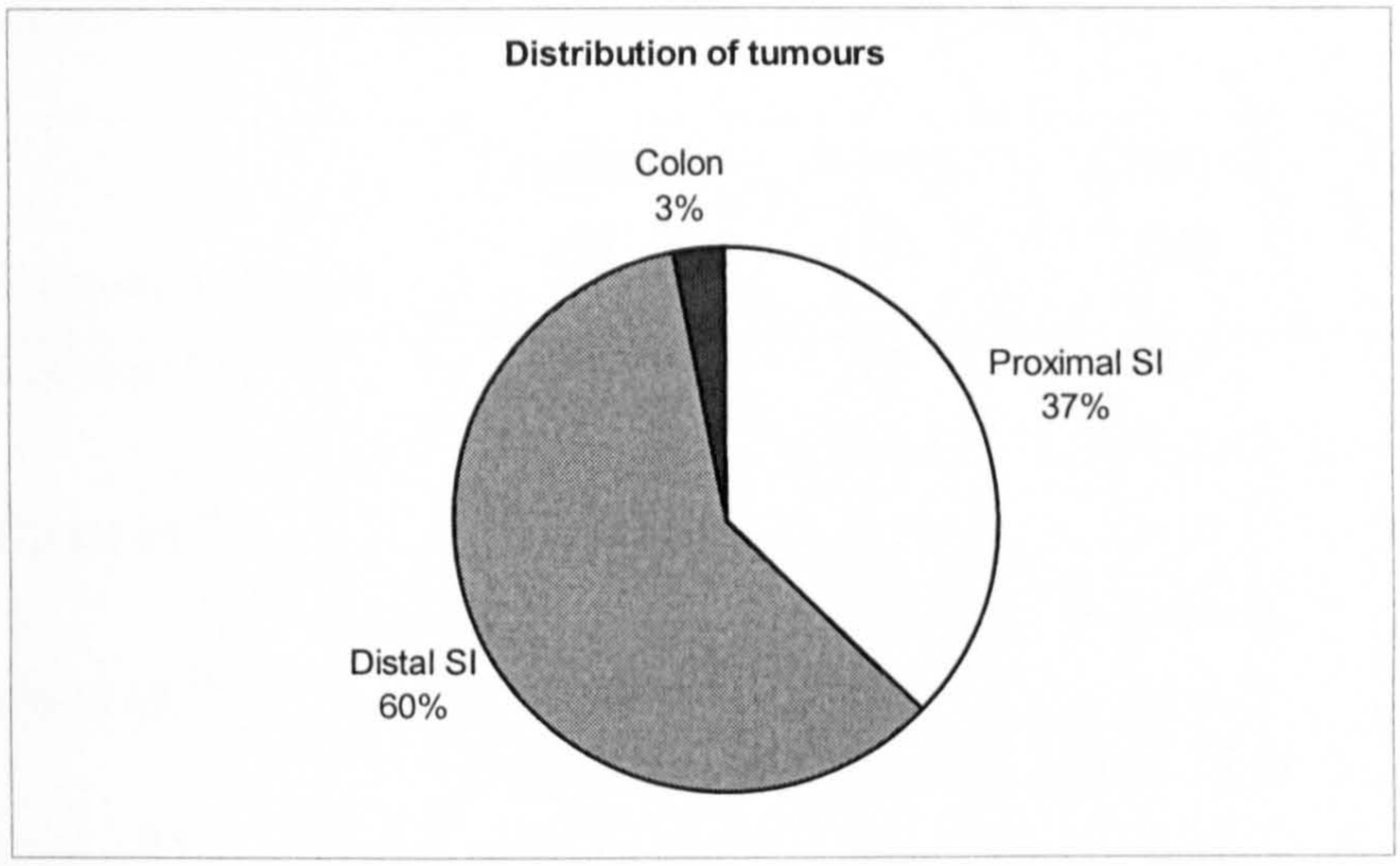


Figure 3.37 Tumour location in all mice (n = 62, tumours = 1019)

There were no statistically ($P<0.05$) significant effects of the exercise protocols on tumour variables, although several approached significance (Table 3.35). The mean number of distal tumours was reduced in WH mice compared with CON (6.9 v. 10.9, $P = 0.053$), and TR mice had fewer tumours of 1-3mm diameter (7.9 v. 13.3, $P = 0.056$) as well as fewer tumours in total (10.6 v. 15.8 $P = 0.068$). For both of these variables, WH mice had mean values between those of CON and TR mice (data in Table 3.35). WH mice had slightly fewer 4-6mm diameter tumours than CON ($P = 0.067$). For all the measured variables, CON mice suffered greater tumourigenesis than either WH or TR mice, even though the effects were not statistically significant.

Table 3.35 Details of tumour variables by exercise protocol (n)

Tumour variable	Treadmill (9)	Wheel (25)	Control (28)	Probability of effect	
				TR v CON	WH v CON
Proximal SI^{Sqrt}	3.4	5.9	6.0	0.081	0.996
	1.83-5.47	4.55-7.43	4.69-7.42		
Distal SI ^{Sqrt}	6.9	6.9	10.9	0.189	0.053
	3.68-11.04	4.87-9.31	8.42-13.68		
Total SI ^{Log10}	10.3	12.5	15.8	0.102	0.247
	7.10-14.95	10.00-15.64	12.80-19.53		
Colon ^{KW}	0.0	0.0	0.5	0.190	0.089
	0.0-2.0	0.0-3.0	0.0-2.0		
Total number ^{Log10}	10.6	13.0	15.8	0.068	0.204
	7.41-15.14	10.47-16.22	12.88-19.50		
Burden (mmdiameter)^{Log10}	29.8	33.8	42.7	0.124	0.181
	21.38-41.69	27.54-40.74	35.48-51.29		
1-3mm ^{Log10}	7.9	11.1	13.3	0.056	0.477
	5.37-1.75	8.71-14.13	10.72-16.60		
4-6mm ^{KW}	2.0	2.0	2.0	0.900	0.067
	0.0-6.0	0.0-4.0	0.0-7.0		
7+mm ^{KW}	0.0	0.0	0.0	0.136	0.860
	0.0-0.0	0.0-2.0	0.0-2.0		

There was no effect of sex on tumour variable (see Table 3.36), with the exception of colon tumours, with males having more than female mice (0 v. 1, median values, P = 0.012) .

There were no significant interactions between exercise and sex on any of the measured tumour variables.

Table 3.36 Tumour variables for males and females (n)

Tumour variable	Female (38)	Male (24)	Probability of effect
Proximal SI^{Sqrt}	5.9	5.0	0.212
	4.73-7.10	3.75-6.51	
Distal SI^{Sqrt}	9.3	7.5	0.271
	7.30-11.61	5.24-10.10	
Total SI ^{Log10}	14.6	11.9	0.193
	12.15-17.59	9.46-15.07	
Colon^{KW}	0.0	1.0	0.012
	0.0-2.0	0.0-3.0	
Total number^{Log10}	15.0	12.8	0.300
	12.59-17.78	10.12-16.22	
Tumour burden (mm diameter)^{Log10}	39.2	33.5	0.270
	33.11-45.71	27.54-40.74	
1-3mm ^{Log10}	12.1	10.6	0.425
	10.00-14.79	8.32-13.80	
4-6mm^{KW}	2.0	2.0	0.352
	0.0-7.0	0.0-5.0	
7+mm^{KW}	0.0	0.0	0.736
	0.0-2.0	0.0-1.0	

3.2.4.6 Body organs

Organs were removed and analysed as described in ES1. The weights of most intestinal organs were not different between exercise groups, however WH mice had a heavier full small intestine and also a heavier proximal section of small intestine than did CON mice (P= 0.007 and 0.005 respectively, Table 3.37). The colon of TR mice was significantly longer than that of CON mice (5.5 v. 5.0 cm, P= 0.026).

When the weights of organs were expressed as a percentage of the final body mass there were no significant differences between the treatment groups (Table 3.38)

Table 3.37 Intestinal organ dimensions of exercise groups

Organ dimension		Treadmill	SEM	Wheel	SEM	Control	SEM	Probability of effect	
								TR v. CON	WH v. CON
Slaughter weight (g)		27.6	1.36	26.7	0.82	26.4	0.77	0.664	0.940
Length (cm)	SI^{KW}	29.0 24.0-31.0	-	30.0 23.0-34.0	-	29.0 23.0-32.0	-	0.681	0.383
	$Colon^{KW}$	5.5 5.0-6.0	-	5.0 4.0-6.0	-	5.0 4.0-6.0	-	0.026	0.728
Weight (g)	Full stomach ^{Log10}	0.365 0.294-0.452	-	0.373 0.328-0.425	-	0.356 0.315-0.403	-	0.979	0.844
	Stomach tissue	0.185	0.012	0.174	0.007	0.186	0.007	0.996	0.413
	Stomach contents	0.185	0.039	0.223	0.023	0.196	0.022	0.962	0.622
	Full SI	1.063	0.046	1.098	0.027	0.982	0.026	0.232	0.007
	Proximal SI	0.638	0.029	0.717	0.018	0.639	0.017	0.998	0.005
	Distal SI	0.356	0.029	0.361	0.018	0.362	0.016	0.978	0.999
	Full caecum	0.256	0.022	0.275	0.013	0.244	0.012	0.873	0.166
	Caecal tissue	0.127	0.010	0.128	0.006	0.121	0.006	0.848	0.588
	Caecal contents	0.129	0.017	0.147	0.010	0.123	0.010	0.948	0.194
	Full colon	0.257	0.018	0.227	0.011	0.232	0.020	0.394	0.905
	Colon tissue	0.197	0.014	0.184	0.009	0.188	0.008	0.817	0.939
	Colon contents	0.060	0.012	0.043	0.007	0.045	0.007	0.409	0.976

Table 3.38 Intestinal organs (tissue) as % final body mass by treatment group (mean and 95% CI)

Organ as %final body mass	Treadmill	Wheel	Control	Probability of effect	
				TR v. CON	WH v. CON
Stomach	0.674	0.661	0.721	0.618	0.247
	0.579-0.768	0.604-0.718	0.668-0.775		
Proximal SI	2.335	2.745	2.498	0.565	0.107
	2.038-2.632	2.563-2.927	2.329-2.667		
Distal SI ^{Log10}	1.283	1.324	1.359	0.745	0.891
	1.107-1.487	1.209-1.450	1.250-1.478		
Caecum ^{Log10}	0.455	0.476	0.454	0.999	0.716
	0.387-0.534	0.432-0.524	0.414-0.497		
Colon ^{Log10}	0.718	0.679	0.703	0.958	0.791
	0.626-0.823	0.625-0.739	0.651-0.760		

Several statistically significant differences in organ dimensions existed between the sexes, with males having heavier full stomach, stomach tissue, full colon, colon contents, full caecum and caecal contents compared with females (Table 3.39). Males also tended to have a slightly longer small intestine. However, when these data were re-expressed as a percentage of final body weight the differences between male and female mice altered somewhat. Females had a heavier proximal SI (2.50 v. 2.30%, $P < 0.001$) than males and also a heavier distal portion (1.41 v. 1.23%, $P = 0.028$). Female mice also had heavier stomach, caecum and colon tissue than males, and these differences approached significance (P=0.062, 0.070 and 0.071 respectively, Table 3.40).

Table 3.39 Organ dimensions of male and female mice

Organ dimension	Female	SEM	Male	SEM	Probability of effect
Slaughter weight (g)	20.5	0.65	30.2	0.82	<0.001
Length (cm)					
SI[KW]	28.5 23.0-32.0	-	30.0 25.0-34.0	-	0.074
Colon[KW]	5.0 4.0-6.0	-	5.0 4.0-6.0	-	0.160
Weight (g)					
Full stomach[Log10]	0.336 0.303-0.373	-	0.414 0.363-0.471	-	0.022
Stomach tissue	0.173	0.006	0.193	0.007	0.036
Stomach contents	0.183	0.019	0.239	0.024	0.085
Full SI	1.013	0.024	1.085	0.030	0.077
Proximal SI tissue	0.661	0.016	0.684	0.019	0.534
Distal SI tissue	0.349	0.014	0.378	0.017	0.207
Full caecum	0.236	0.011	0.294	0.013	0.002
Caecal tissue	0.120	0.005	0.132	0.006	0.146
Caecal contents	0.116	0.009	0.162	0.011	0.002
Full colon	0.218	0.009	0.259	0.011	0.004
Colon tissue	0.180	0.007	0.199	0.009	0.092
Colon contents	0.038	0.006	0.060	0.007	0.015

Table 3.40 Intestinal organs (tissue) of male and female mice as % final body mass (mean and 95% CI)

Organ as % final body mass	Female	Male	Probability of effect
Stomach	0.72	0.64	0.062
	0.673-0.766	0.585-0.701	
Proximal SI	2.75	2.30	<0.001
	2.596-2.900	2.109-2.487	
Distal SI ^{Log10}	1.41	1.23	0.028
	1.309-1.511	1.124-1.344	
Caecum ^{Log10}	0.48	0.43	0.070
	0.448-0.523	0.391-0.475	
Colon ^{Log10}	0.73	0.65	0.071
	0.679-0.775	0.601-0.709	

The weights of extra-intestinal organs are displayed in Table 3.41 and show that, compared with CON mice, WH had significantly heavier livers (1.163 v. 1.098g) and both WH and TR mice had lighter spleens than CON mice (0.173, 0.121 and 0.246g respectively). These differences persisted when the organs were expressed as % final body mass (Table 3.41).

The weights of liver, spleen and heart were all heavier in males compared with female mice, and were significantly so for liver and heart (Table 3.42), but this sex difference was reversed when the organs were expressed as % final body mass, although significantly so for the liver only (4.52 v. 4.12%, P<0.001).

Table 3.41 Liver, heart and spleen weights of mice by treatment group (mean and 95% CI)

Organ weight (g)		Treadmill	Wheel	Control	Probability of effect	
					TR v. CON	WH v. CON
	Liver	1.163	1.222	1.098	0.478	0.011
		1.060-1.266	1.160-1.284	1.040-1.156		
	Spleen ^{Log10}	0.121	0.173	0.246	<0.001	0.017
		0.089-0.165	0.143-0.208	0.206-0.293		
	Heart	0.191	0.203	0.207	0.313	0.827
		0.169-0.213	0.191-0.215	0.191-0.215		
As % final body mass	**Liver** ^{Log10}	4.234	4.594	4.200	0.957	<0.001
		4.011-4.469	4.447-4.746	4.073-4.330		
	Spleen ^{Log10}	0.444	0.655	0.952	<0.001	0.025
		0.315-0.627	0.532-0.806	0.783-1.157		
	Heart	0.706	0.779	0.812	0.214	0.734
		0.592-0.820	0.710-0.848	0.747-0.877		

Table 3.42 Liver, heart and spleen weights of mice by sex (mean and 95% CI)

Organ weight (g)	Female	Male	Probability of effect
Liver	1.102	1.245	0.002
	1.049-1.155	1.178-1.312	
Heart	0.194	0.218	0.004
	0.184-0.204	0.206-0.230	
Spleen [Log10]	0.181	0.213	0.172
	0.153-0.214	0.172-0.263	
As % final body mass			
Liver [Log10]	4.516	4.122	<0.001
	4.386-4.651	3.972-4.277	
Spleen [Log10]	0.747	0.711	0.770
	0.620-0.900	0.563-0.899	
Heart	0.811	0.740	0.128
	0.755-0.866	0.682-0.798	

3.2.4.7 SCFA

Short chain fatty acids in the colon contents were measured as described in ES1. Several of the variables were not normally distributed and these are indicated in the text.

Table 3.43 displays the concentrations of SCFA in colon contents by exercise group, with acetate present in the greatest concentration. There were no statistically significant differences between the groups. However, when the concentrations were converted to molar proportions (Table 3.44), TR mice had a significantly ($P = 0.006$) smaller proportion of isovalerate than CON mice, with 18 compared with 25mmol/mol (Table 3.44).

Table 3.43 SCFA concentrations (mean and 95% CI) (µmol·g colon contents) by exercise protocol

Individual SCFA (µmol·g colon contents)*	TR (n = 9)	WH (n = 22)	CON (n = 26)	Probability of effects	
				TR v CON	WH v CON
Acetate	25.47	21.73	22.54	0.544	0.898
	20.60-31.50	18.96-24.90	19.90-25.54		
Propionate	3.15	3.15	3.33	0.895	0.824
	2.50-3.96	2.72-3.65	2.91-3.80		
Isobutyrate	6.53	7.00	7.47	0.669	0.846
	4.60-8.72	5.82-8.42	6.30-8.85		
Butyrate	2.00	1.17	1.11	0.099	0.974
	1.21-3.31	0.85-1.60	0.83-1.50		
Isovalerate	0.565	0.573	0.649	0.385	0.679
	0.40-0.83	0.41-1.23	0.32-2.27		
Valerate	0.51	0.55	0.49	0.983	0.741
	0.36-0.73	0.44-0.69	0.40-0.61		
Total concentration	32.60	27.61	28.91	0.588	0.864
	26.15-40.04	24.08-31.65	25.51-32.76		

*** all SCFA Log10-trasnformed, except isovalerate which was tested using Kruskal-Wallis**

Table 3.44 Molar proportions (mmol/mol total SCFA) of colonic SCFA by exercise group, mean and SEM/95% CI

Molar proportion	TR	WH	CON	Probability of effects	
				TR v CON	WH v CON
Acetate	789	788	783	0.961	0.952
	18.5	11.8	10.9		
Propionate	100	117	118	0.126	0.992
	8.4	5.4	4.9		
Isobutyrate KW	14	13	14	0.070	0.273
	7.4-14.5	8.8-23.0	0.0-28.7		
Butyrate	66	42	44	0.161	0.972
	10.7	6.9	6.3		
Isovalerate Log10	18	24	25	0.006	0.782
	15.5-21.7	21.4-26.5	22.6-27.5		
Valerate	16	15	14	0.775	0.869
	2.6	1.6	1.5		

Table 3.45 SCFA concentrations (mean and 95% CI) (µmol·g colon contents) by sex

Individual SCFA (µmol·g colon contents)*	Female (n = 33)	Male (24)	Probability
Acetate	21.88	23.88	0.261
	19.55-24.37	20.98-27.17	
Propionate	3.16	3.33	0.543
	2.81-3.55	2.90-3.82	
Isobutyrate	7.00	7.33	0.699
	6.03-8.12	6.15-8.72	
Butyrate	1.19	1.32	0.528
	0.91-1.55	0.97-1.81	
Isovalerate	0.606	0.576	0.974
	0.404-1.857	0.320-2.271	
Valerate	0.48	0.58	0.218
	0.40-0.57	0.47-0.72	
Total concentration	28.05	30.13	0.352
	25.12-31.33	26.47-34.30	

***all SCFA Log10-trasnformed, except isovalerate which was tested using Kruskal-Wallis**

Table 3.46 Molar proportions (mmol/mol total SCFA) of colonic SCFA by sex

Molar proportion	Female	Male	Probability
Acetate	780	794	0.377
	9.5	11.1	
Propionate	115	113	0.649
	4.4	5.2	
Isobutyrate [KW]	14	13	0.639
	0.0-23.0	7.4-28.7	
Butyrate	50	43	0.538
	5.7	6.6	
Isovalerate [Log10]	24	22	0.219
	22.0-26.5	20.1-25.0	
Valerate	16	13	0.155
	1.3	1.5	

Both concentrations and molar proportions of SCFA in samples from male and female mice were not statistically significantly different (P>0.05) as shown in Table 3.45 and Table 3.46.

There was no interaction between sex and exercise protocol for any of the measured variables (P>0.05).

3.2.4.8 CCP

Of the three variables used in the assessment of proximal SI crypt cell proliferation, one was significantly different with regards to exercise protocol. WH mice had significantly more mitoses in the upper third of the crypt compared with CON (0.79 v. 0.53, P = 0.024, see Table 3.47). Crypt width was almost significantly greater in TR mice compared with CON (63.5μm v. 55.7μm, P = 0.052, see Table 3.48).

Table 3.47 Mean mitotic cells per crypt section by exercise group

Region of crypt	Treadmill	SEM	Wheel	SEM	Control	SEM	P	
							TR	WH
Upper	0.53	0.12	0.79	0.07	0.53	0.07	0.998	0.024
Middle	0.65	0.13	0.85	0.08	0.83	0.07	0.391	0.985
Lower	0.50	0.08	0.55	0.05	0.57	0.04	0.731	0.971
Total	1.68	0.25	2.19	0.16	1.93	0.14	0.613	0.398

Table 3.48 Mean crypt dimensions between exercise groups

Dimension (μm)	Treadmill	SEM	Wheel	SEM	Control	SEM	P	
							TR	WH
Length	128.6	5.22	131.5	3.20	130.3	2.96	0.942	0.956
Width	63.5	3.00	52.9	1.84	55.7	1.70	0.052	0.451

Table 3.49 Mean crypt mitoses and dimension of male and female mice

Mitoses	Female	SEM	Male	SEM	P
Lower	0.53	0.04	0.59	0.05	0.312
Middle	0.77	0.06	0.87	0.08	0.388
Upper	0.58	0.06	0.71	0.08	0.233
Total	1.88	0.124	2.17	0.16	0.191
Dimension (μm)					
Length	131.8	2.50	128.4	3.22	0.388
Width	56.4	1.54	54.7	1.98	0.586

Mitoses and crypt dimensions were not significantly different between the sexes (P>0.05, Table 3.49).

3.2.4.9 Body composition

Body composition was measured on the carcass of the animal after the intestinal and related organs had been removed for analysis.

The proportion of non-fat organic matter (principally carbohydrate and protein) was significantly different between treatment groups (Table 3.50), with TR mice having a smaller proportion of non-fat organic matter than CON mice (P = 0.050). There was no significant difference between the proportion of organic matter in CON and WH mice. There was no significant difference for any of the other variables.Further details are displayed in Table 3.50.

Table 3.50 Body composition (g/100g carcass) of mice in each exercise groups

Component (%)	Treadmill	SEM	Wheel	SEM	Control	SEM	Probability TR	Probability WH
Water	49.6	1.98	55.0	1.31	51.8	1.17	0.545	0.152
Fat	31.2	2.84	21.6	1.87	25.2	1.7	0.138	0.276
Non-fat organic matter[KW]	16.4		19.0		17.8		0.050	0.467
	6.04-20.03		15.18-28.25		14.68-33.98			
Mineral[Log10]	3.3		4.0		3.9		0.161	0.807
	2.79-3.84		3.62-4.47		3.51-4.24			

Table 3.51 Body composition (g/100g carcass) of male and female mice

Component (%)	Female	SEM	Male	SEM	P
Water	53.8	1.06	50.8	1.31	0.036
Fat	23.6	1.54	26.5	1.91	0.130
Non-fat organic matter[KW]	18.0 6.04-33.58		17.8 15.18-28.25		0.987
Mineral[Log10]	4.0 3.72-4.40		3.5 3.15-3.88		0.023

Sex differences in body composition existed for percentage of water and for mineral content (Table 3.51). Females had a greater proportion of mineral than males (4.0 v. 3.5%, P = 0.023) and also a higher water content (53.8 v. 50.8%, P = 0.036). The proportion of non-fat organic matter was not different between the sexes (17.8% in females and 18.3% in males, p = 0.987).

3.2.4.10 Caecal transit time

The difference in caecal transit time between exercise groups were not statistically significant (P=0.414 for TR v. CON and 0.531 for WH v. CON, data in Table 3.52). The difference between males and females was significant (P = 0.008), with males having a caecal transit time 30% slower than females (3.10 v. 4.50 hours). There was no interaction between sex and exercise protocol.

Samples from 7 mice were excluded from the analysis due to experimental problems (broken tubes, balance errors).

Table 3.52 Caecal transit times of treatment groups (h) (median and range)

Variable	Treadmill	Wheel	Control	Probability	
				TR v CON	WH v CON
Transit time[KW]	3.10 1.36-5.29	4.12 1.83-34.87	4.28 0.08-21.93	0.414	0.531

N = 9 (Treadmill), 23 (Wheel), 23 (Control)

Table 3.53 Caecal transit time of male and female mice (h) (median and range)

Variable	Female	Male	Probability
Transit time[KW]	3.10	4.50	0.008
	0.08-21.93	1.91-34.87	

3.2.4.11 Mitochondrial mutation analysis

Tissue samples from the distal end of the small intestine and the proximal colon were processed as outlined in the Specific Methods section of this chapter (3.2.3.5). Example of samples are shown in Figure 3.38.

10 random samples from each treatment group were selected and examined for the presence of blue-stained cells which indicate cytochrome c oxidase (COX) deficiency. After 38 slides had been examined only 1 blue cell had been found, so tissue samples from two older mice (from another study) were examined as the lack of COX-deficient cells could be due to the young age of the mice. Neither the 26 nor the 18 month-old mouse had any blue-stained cells, so it was decided that the remainder of the samples were unlikely to yield any greater numbers and were not examined.

Tissue samples from both sites were not available from all study mice due to the fragility of the tissues, particularly the distal small intestine.

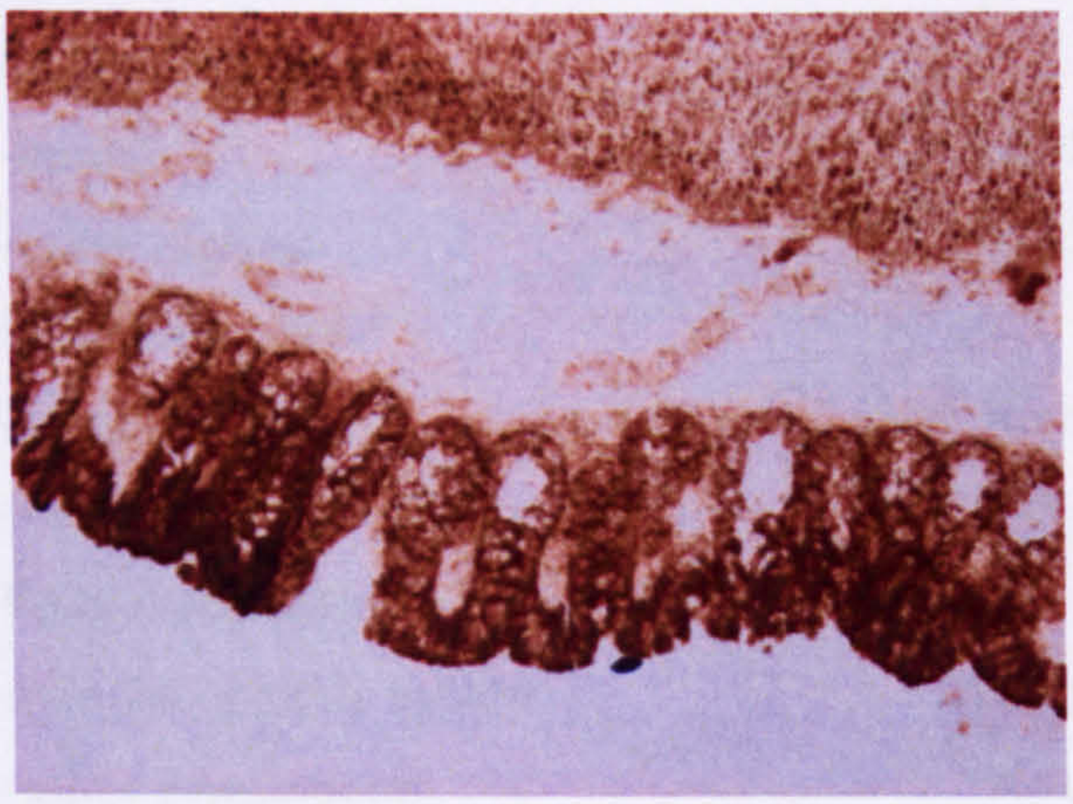
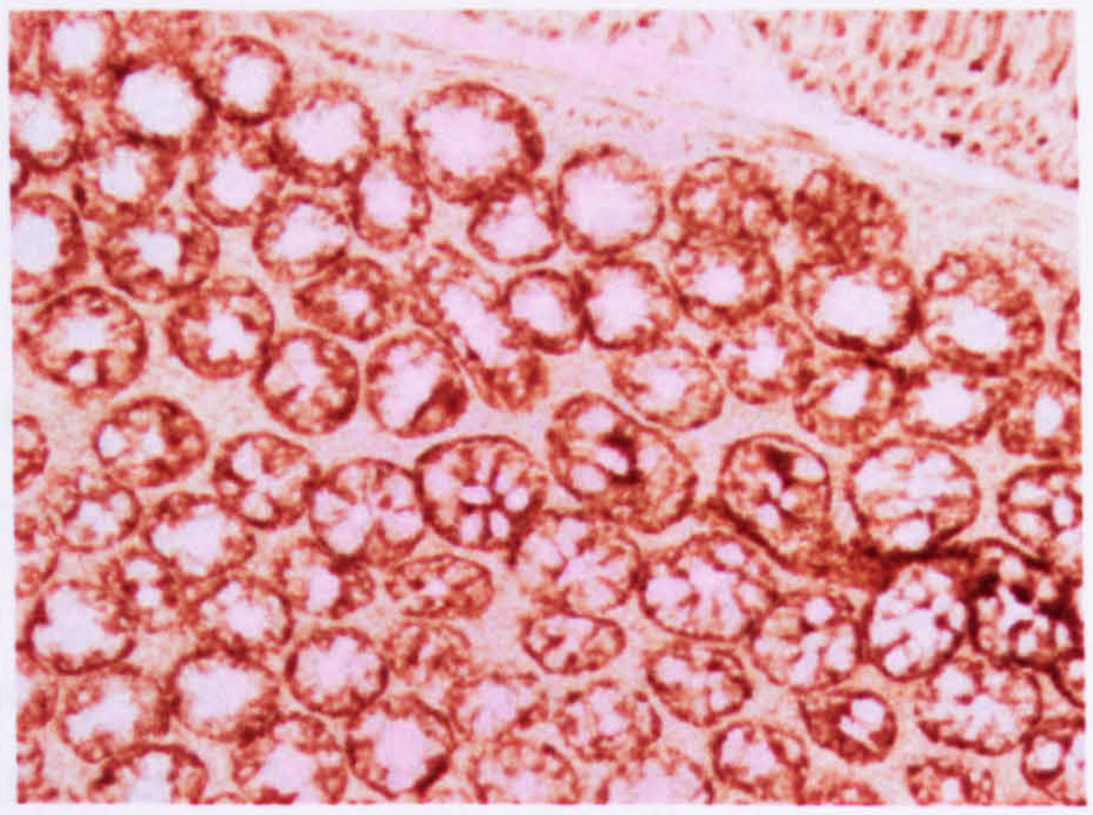


Figure 3.38 (a) Longitudinal section through colon crypts from a 26 month-old mouse



(b) Transverse section through colon crypts from an 18 month old mouse

3.2.5 Summary of results

Table 3.54 Summary of results from second experiment (p<0.05)

	Effect of exercise regime (compared with Control)	
Variable	**Treadmill**	**Wheel**
Body weight gain	No difference	No difference
Food intake	Decreased	Increased
Proximal SI tumours	Decreased (0.081)	No difference
Distal SI tumours	No difference	Decreased (0.053)
Total SI tumours	No difference	No difference
Colon tumours	No difference	No difference
Total number of tumours	Decreased (0.068)	No difference
Tumour burden	No difference	No difference
Intestinal organ tissue weights (%)	No difference	No difference
Non-intestinal organ weights (%)		
-liver	No difference	Increased
-spleen	Decreased	Decreased
-heart	No difference	No difference
SCFA concentration	No difference	No difference
SCFA molar proportion		
-Isovalerate	Decreased	No difference
-Acetate/Propionate/Isobutyrate/ butyrate/valerate	No difference	No difference
CCP mitoses		
-upper	No difference	Increased
-other regions	No difference	No difference
CCP dimensions		
-length	No difference	No difference
-width	Increased	No difference
Body composition		
-non-fat organic matter	Decreased (0.050)	No difference
-other components	No difference	No difference
Caecal transit time	No difference	No difference

3.2.6 ES2-specific discussion

3.2.6.1 Treadmill performance

The desired number of TR in mice ES2 was 12, i.e. 6 of each sex. This number was not higher due to the time taken to run the mice and the extra disturbance this would have caused. The treadmill only allowed 6 mice to be run simultaneously.

Nine mice finished the study, as in ES1, but compliance with the protocol was much improved, with the percentage of days when the mice ran increasing from 85 to 93%, and daily time run increasing from 43 to 50 min, and the fastest speed increasing from 14.6 to 15.8m·min^{-1}. The decrease in time spent running towards the end of the study reflects the declining health status of the Min mice and an increased unwillingness to run.

The lack of difference between the sexes with regards best speed was probably due to the best speed being run first. As running performance/inclination declined, so did the speed. For example, the speed ran by all the mice at the beginning of each session was the target speed of around 16m·min^{-1}, but as the session continued and the mice struggled to keep up with the treadmill the speed was decreased accordingly. Therefore, a mouse that ran for just 20 minutes will have run as fast as one that ran for the whole hour. For this reason, time and % days run are better indicators of performance (endurance), both of which were significantly better in females.

3.2.6.2 Wheel-running

The wheel running treatment was added to this study design in response to the mixed reaction to the treadmill in ES1. The idea was that a voluntary running option may be more attractive than forced treadmill running. Female mice initially took to the wheels more readily than males, although the performance of the males increased when the wheels were changed to those with the larger diameter. This difference could have been due to the male mice being slightly larger than the females, and the larger wheel gave them more comfort. The larger wheel also revolved slightly more freely than the small wheel, which may also have been a factor. The halted response of the females to the larger wheels suggests that either they did not like change, or were experiencing

the "hotel effect" in their new cage (i.e. the excitement of being in a new environment, with new smells), and were simply exploring the new cage and ignoring the wheel.

Whatever the reason, the distances run in the wheels were generally far in excess of those run on the treadmill. The TR mice ran a maximum of 930m/day (and on most days ran less), corresponding to a 10-week maximum of 46.5km (ignoring the fact that the first two weeks were for training). This is in stark contrast to the average distance run in wheels after 10 weeks of 314km by female mice and 147km by the males.

The speed of running also far exceeded that on the treadmill (approximately 30-40m·min^{-1} v. 16m·min^{-1}), and the pattern of running was also different. Wheel running was characterized by frequent short bursts of fast running by many mice, an observation also reported by De Bono et al., (2006), although other mice would run steadily for longer periods.

The average daily distances of 4.53km for females and 2.10km for male mice compare with those obtained by Colbert (2003) of 3.59km and 3.07km run by the mice during three weeks of quarantine. Results from other groups (Koteja et al., 1999) have shown that female mice run further than males, a combination of both increased speed and time on the wheel (De Bono et al., 2006).

Although there was a lack of difference in body mass gain between treatment groups, a negative correlation between body mass gain and increased running was observed. Other studies have not reported this type of analysis.

From this perspective, the wheels achieved the objective of increased running.

3.2.6.3 Crypt cell proliferation

Intestinal neoplasia is caused by uncontrolled crypt cell proliferation. Mitosis usually occurs in the bottom of the crypt, and cells migrate upwards towards the lumen. Mitotic cells in the upper part of the crypt may provide evidence of abnormal cell proliferation. Few mitotic cells were observed in this study, where vincristine was not used. Vincristine halts cells in mitosis so more cells would have been visibly mitotic than observed in this study, where only cells that were mitotic at death are stained.

The effect of exercise on CCP has not been examined previously. There was no increase or decrease in CCP with exercise detected by this study, with the exception of the upper third of the crypt, where WH mice had more mitotic cells, as analysed by non-parametric methods. The tissue that was examined for CCP came from the proximal intestine, and WH mice had slightly more proximal tumours than CON mice (6.7 v. 6.0), which may be connected to the increase in mitosis.

TR mice had wider crypts than CON or WH though the significance of this is unclear. Male mice had more upper mitoses and tended to have more mitotic cells in total. These discoveries deserve further exploration.

3.2.6.4 Mitochondrial mutation analysis

The observation of only one COX-deficient cell in mucosa from the mice in this study, and the absence of deficient cells in older mice suggests than either the exercise was not sufficient to produce a response or that deficiencies do not occur in the intestinal tissue. Navarro et al. (2004), treadmill–exercised CD-1 mice from 28wk to 78wk of age and measured the activity of various mitochondrial enzymes and found that whilst cytochrome oxidase activity decreased with aging this effect could be modulated by moderate exercise. Cytochrome oxidase activity was measured by isolating mitochondrial membranes from homogenized samples of brain, heart, liver and kidney. This suggests that a different method and organ may have produced different results in this study, or that deficiencies in the electron transport chain are not responsible for tumourigenesis in Min mice.

3.2.7 Conclusions

The wheel-running paradigm proved to be successful and deserves further exploration. Although there was no significant effect of the exercise treatments on tumourigenesis, there was a suggestion of reduced proximal tumours in TR mice, which may be uncovered if numbers of mice are increased.

3.3 Treadmill exercise study 3 (ES3)

3.3.1 Introduction

The results from the previous study (ES2) suggested that the voluntary aspect of wheel running was worth pursuing further. In addition, the effects of a treadmill exercise protocol may become clearer with greater numbers of mice undertaking the treadmill protocol. In essence, more mice were needed, with the aim being to achieve approximately equal numbers of animals in each exercise group. It is possible that the current protocols were confounded by the amount of activity undertaken by mice in their home cage, i.e. CON may make up for being confined for 60 minutes by increasing their activity levels in the cage, while TR may decrease the amount of activity they undertake in their cage after being forced to run for an hour. This non-exercise physical activity (NEPA) has not been measured in any previously published study and should be measured to understand how an exercise regime influences the total activity patterns of mice and to address any issues of possible confounding.

3.3.2 Aims

- To increase the number of mice completing the treadmill protocol
- To examine NEPA in TR and CON mice
- To further investigate the effects of the voluntary wheel-running protocol on tumourigenesis
- To examine tissues for evidence of stress

3.3.3 ES3 Specific methods

78 Min mice, 41 females and 37 males were randomised to Treadmill, Wheel or Control groups. Conditions were as stated in General Methods and Materials (Chapter 2).

3.3.3.1 Treadmill mice

31 mice (14 female, 17 male) started the treadmill protocol. The length of the study was kept at 10 weeks, which included 2 weeks of initial training. The target speed was 18-21m·min^{-1}, and to help achieve this, the duration of running each day was reduced to 30 minutes (3x10 minutes sessions with 3 minutes rest between bouts of running). Any mice that dropped out during the training period were placed in the wheel-running (WH) group if a wheel was available, or the control (CON) group if not.

3.3.3.2 Wheel mice

20 mice (13 female, 7 male) were randomised to WH, and singly housed with the smaller wheels (10cm internal diameter) for the first 5 weeks and the larger wheels (13cm internal diameter) for the second 5 weeks.

3.3.3.3 Control mice

27 mice (14 female, 13 male) were initially assigned to CON, with conditions as stated in General Methods and Materials. Time in the control lanes was reduced to 30 minutes daily, in line with the altered treadmill protocol.

3.3.3.4 Non-exercise physical activity (NEPA)

Non-exercise physical activity (NEPA) by singly housed TR and CON mice was recorded as often as was possible using the Inframot device (see General Methods and Materials, 2.6). Some WH mice were also recorded although it was recognised from the outset that it would not be possible to distinguish wheel-running from other activities using this device.

3.3.3.5 Necropsy

After 10 weeks of study mice were killed as described in General Methods and Materials. The tissues collected were: small intestine, caecum and contents, colon and contents, liver, adrenal glands, heart (weight only), thymus gland (weight only), brain and blood.

3.3.3.6 Storage of new tissue

The brain was used for in situ hybridisation of anti-diuretic hormone (ADH) mRNA in the hypothalamic paraventricular nucleus (work performed by Dr J.M.H.M Reul, Bristol University). The brain was removed within 2 minutes of death in a manner that protected the hypothalamic area as follows. The head was cut from the body and the ears and any excess skin and fur removed. An incision was made through the eye socket and cut through to the other socket. The skull was carefully cut from the base along the midline to the incision between the eyes to split the skull in two. A small cut was made from the base of the skull to each auditory opening and the skull peeled away from the brain using fine forceps. The brain was then gently loosened from the front of the skull and optic nerves and dropped into a freezing bath of iso-pentane in dry ice. When frozen, the brain was wrapped in foil, weighed and buried in dry ice until storage at –80°C. The adrenal glands were placed into separate eppendorfs and weighed. They were placed on ice until storage at –20°C.

3.3.4 ES3 Results

75 mice finished the study and provided data that were suitable for statistical analysis. These mice were distributed between exercise groups as shown in Table 3.55.

Table 3.55 Distribution of mice completing the study

Exercise protocol	Male	Female	Total
Treadmill	10	13	**23**
Wheel	12	12	**24**
Control	13	15	**28**
Total	**35**	**40**	**75**

2 mice were killed before the end of the study, a female WH with 3 polypoid tumours in the colon and many small tumours throughout the small intestine, and a male WH with hydrocephaly. One male treadmill mouse finished the study but was excluded from analyses as he lost a large amount of weight in the last 2 weeks of the study but

appeared to have only one small tumour, suggesting that he had other health complications which were not identified. The data from these three mice were not included in the statistical analyses.

3.3.4.1 Body weight

The exercise protocol did not have a significant effect on body weights at any week, although the difference at week 7 approached statistical significance (P = 0.052 for TR, the lightest group at 22.76 v. 23.18 (WH) v. 24.07g (CON), Figure 3.39). Weight gain between exercise groups was significantly different at weeks 2, 7 and 8 of study (Table 3.56). At week 2, TR and WH gained significantly less weight compared with CON mice (P = 0.038 and 0.024 respectively). At week 7, WH mice gained significantly less weight than CON (P= 0.006), and at week 8, TR and WH gained more weight than CON mice (P = 0.019 for TR and 0.025 for WH). There was no significant effect of exercise on total weight gain (P = 0.544 for TR v. CON, 7.2g v. 8.1g, and p = 0.439 for WH v. CON, 7.1g v. 8.1g).

Males were again significantly heavier than females at every stage of the study including death (p <0.001, Figure 3.40). When comparing weekly weight gain, males gained significantly more on weeks 2-6 and females gained significantly more on week 10 (Table 3.57). There was no significant difference in total weight gain of males and females over the length of the study, with means of 6.9g for females and 8.2g for male mice (P = 0.104).

An interaction between sex and protocol occurred for weight gain on week 7 of the study, (P = 0.009, Figure 3.42). No interaction occurred between sex and exercise on total weight gain (P = 0.294, Figure 3.43).

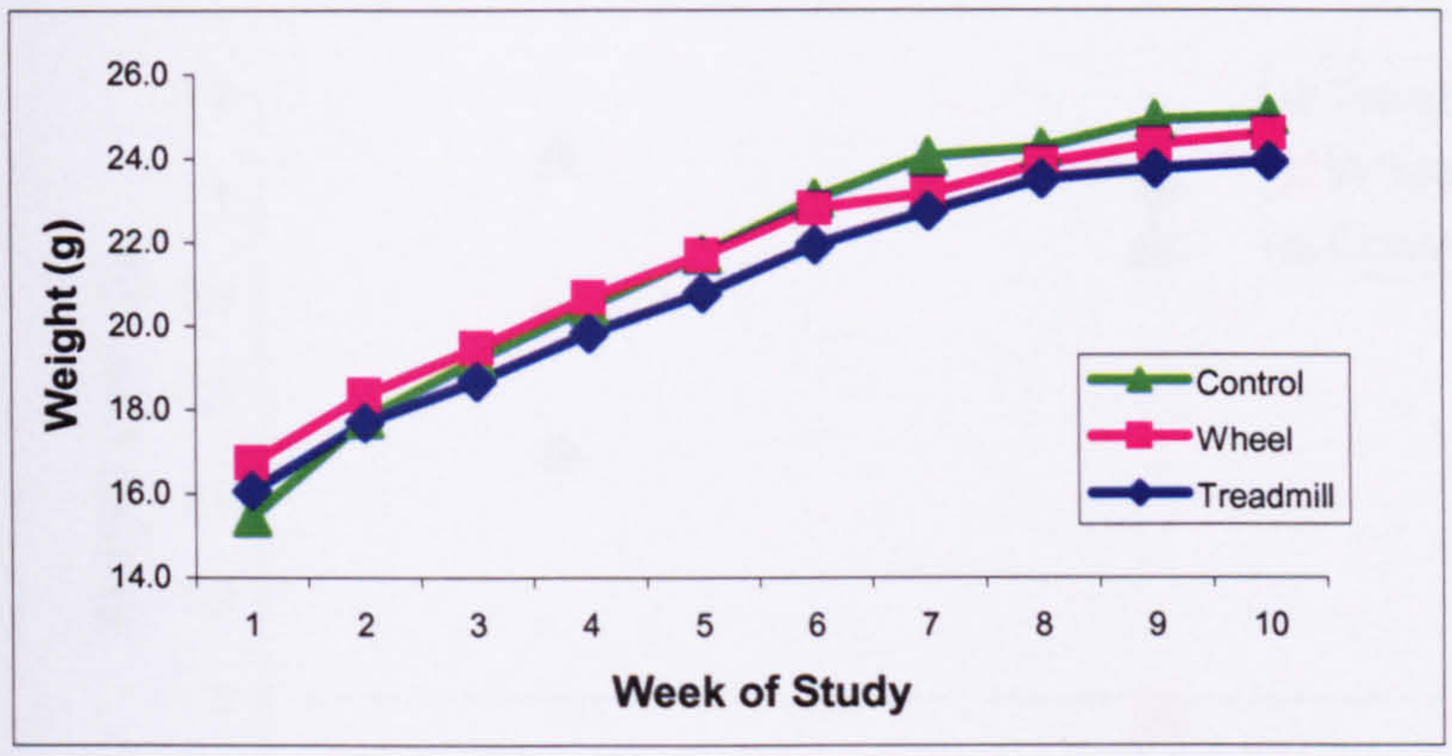


Figure 3.39 Mean weekly body weights, by treatment group

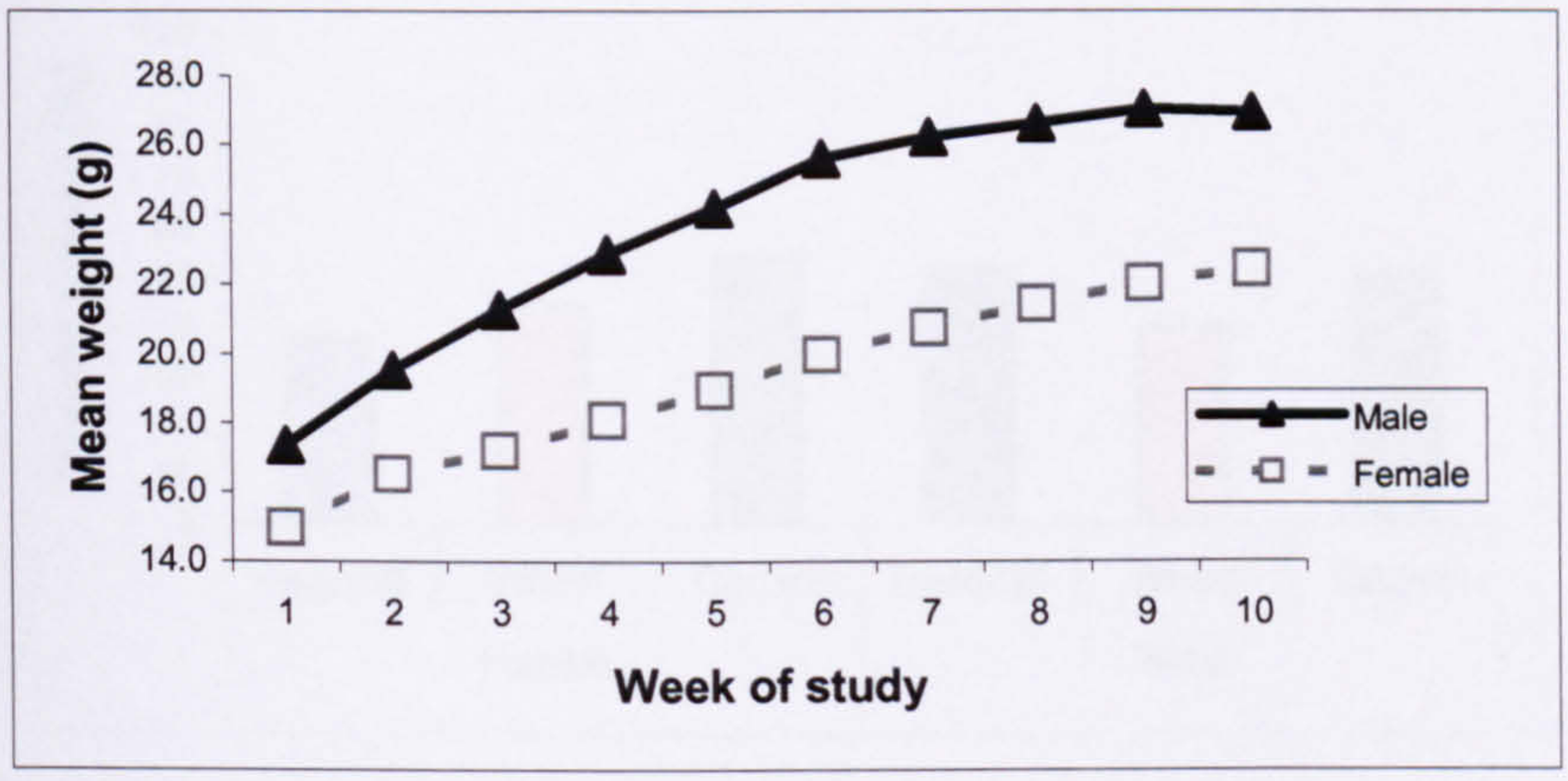


Figure 3.40 Weekly body weights of male and female mice

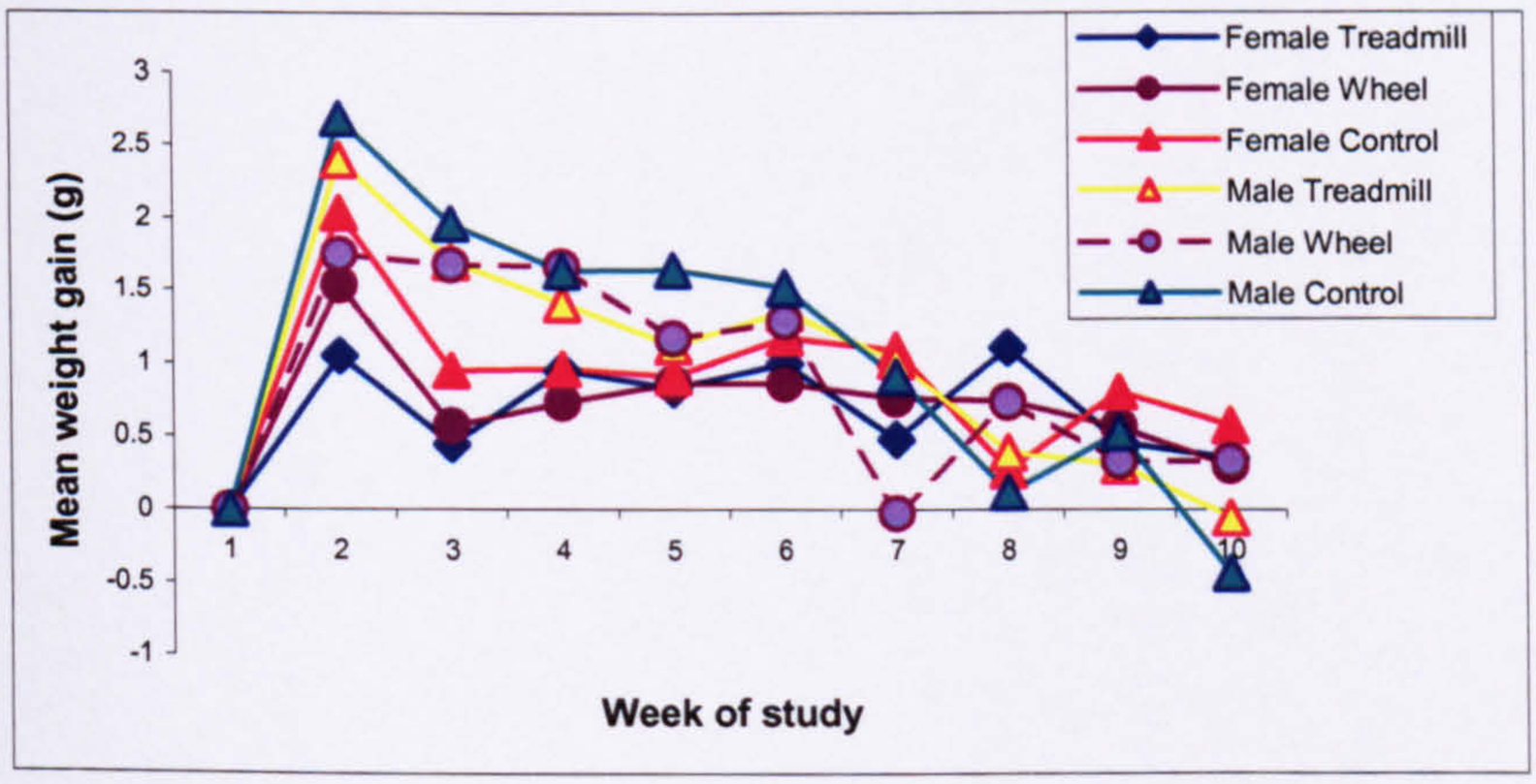


Figure 3.41 Mean weekly weight gain of mice by sex and exercise protocol

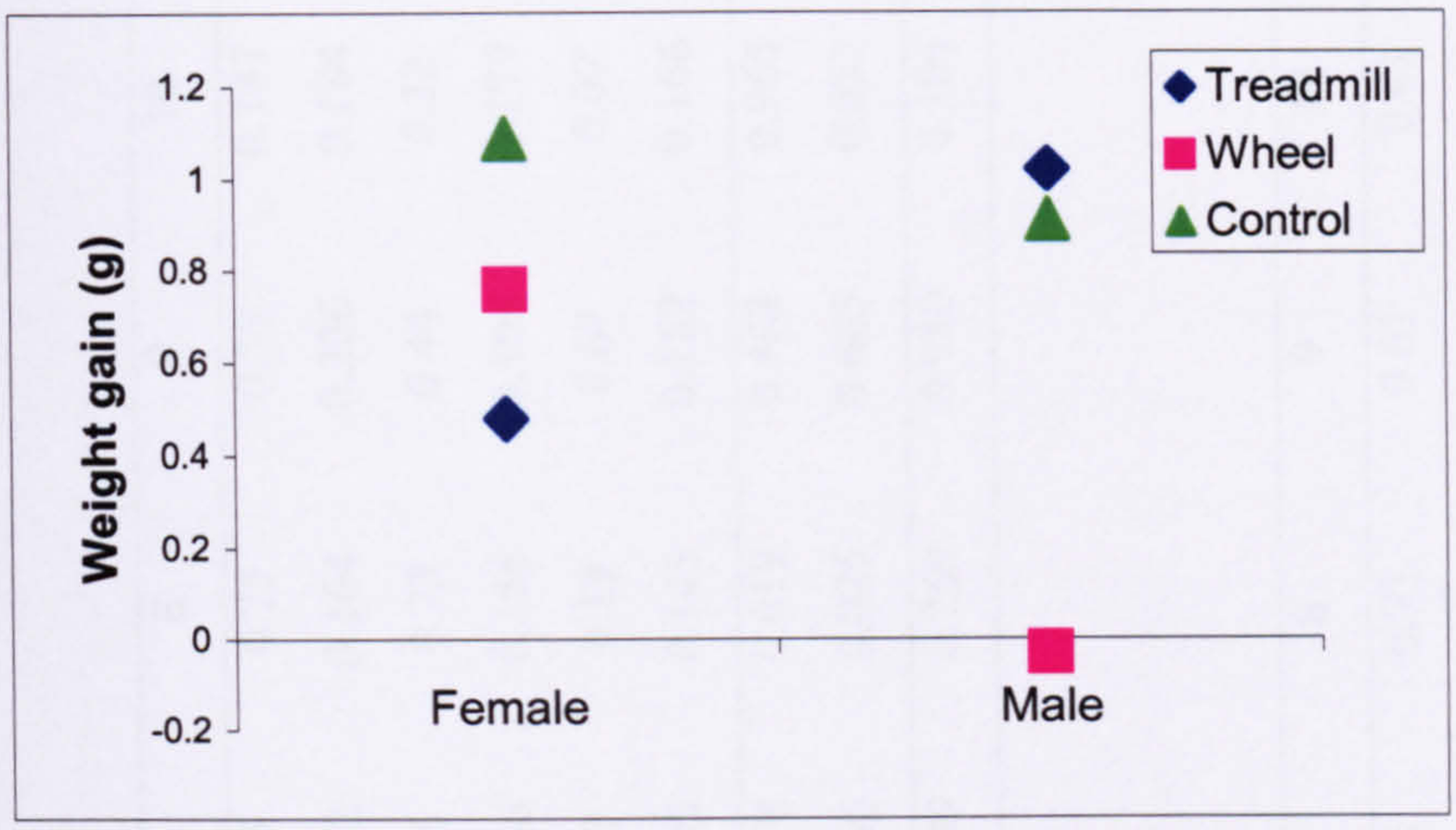


Figure 3.42 Interaction between sex and exercise on weight gain at week 7

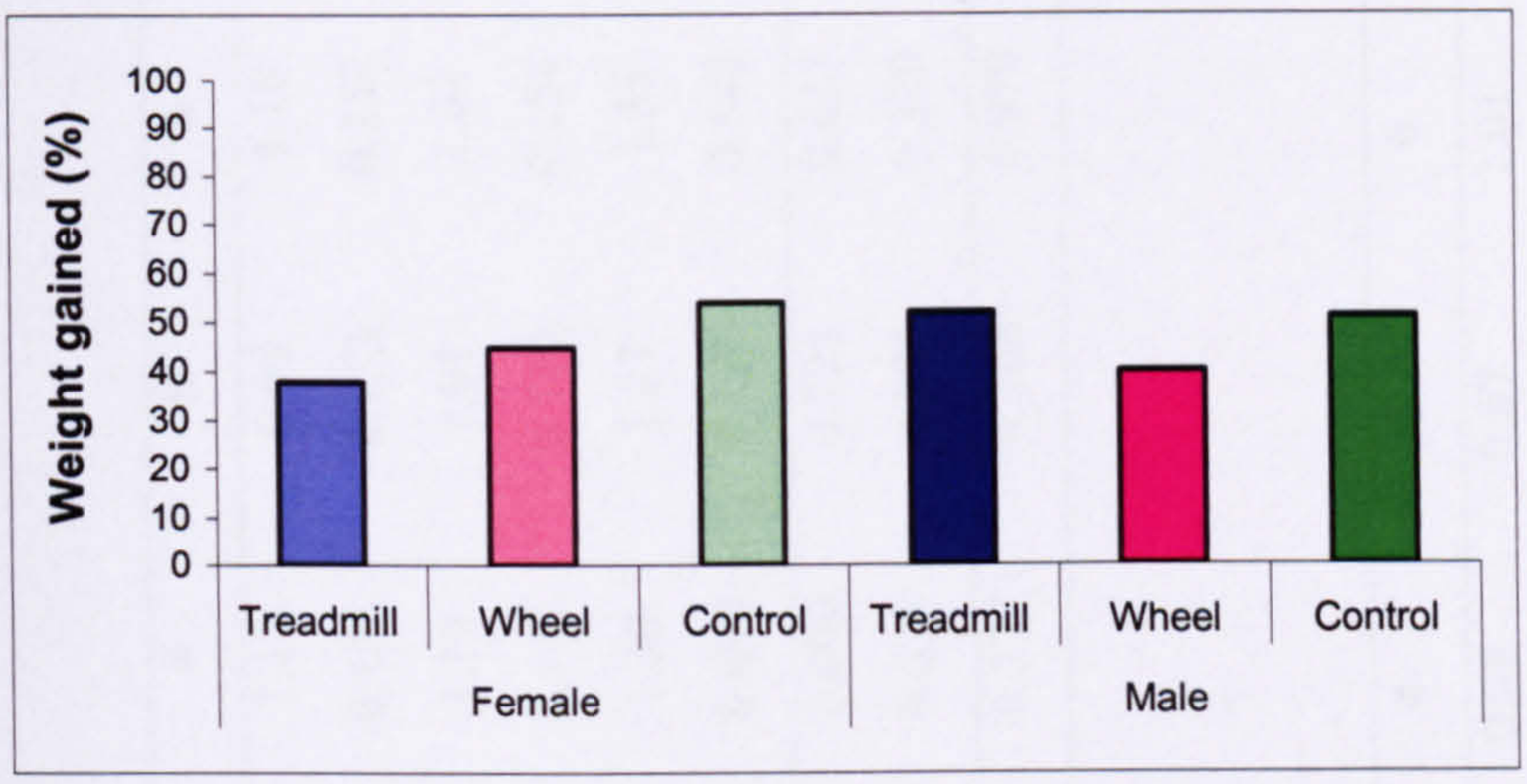


Figure 3.43 Weight gain of mice by sex and treatment group (% increase from initial weight)

Table 3.56 Weekly weight gain (g) for each exercise group

Group	Week	1	2	3	4	5	6	7	8	9	10
Treadmill	**Mean**	-	1.72	1.07	1.18	0.98	1.18	0.75	0.75	0.38	0.147
	SEM	-	0.210	0.146	0.105	0.133	0.158	0.153	0.164	0.206	0.184
Wheel	**Mean**	-	1.63	1.12	1.19	1.01	1.08	0.36	0.73	0.44	0.32
	SEM	-	0.204	0.142	0.102	0.130	0.154	0.146	0.156	0.196	0.179
Control	**Mean**	-	2.35	1.46	1.30	1.27	1.35	1.00	0.19	0.67	0.07
	SEM	-	0.19	0.132	0.094	0.120	0.143	0.135	0.145	0.182	0.166
Probability	**TR v CON**	-	0.038	0.077	0.690	0.221	0.627	0.318	0.019	0.463	0.965
	WH v CON	-	0.024	0.137	0.678	0.276	0.320	0.006	0.025	0.605	0.557
Sex*exercise interaction		-	0.161	0.836	0.277	0.368	0.970	0.009	0.269	0.980	0.105

Table 3.57 Weekly weight gain (g) for males and females

Group	Week	1	2	3	4	5	6	7	8	9	10
Female	**Mean**	-	1.53	0.66	0.88	0.87	1.01	0.78	0.71	0.61	0.41
	SEM	-	0.159	0.110	0.079	0.101	0.120	0.115	0.123	0.155	0.139
Male	**Mean**	-	2.27	1.77	1.57	1.31	1.39	0.63	0.41	0.39	-0.06
	SEM	-	0.170	0.118	0.085	0.108	0.128	0.121	0.130	0.164	0.149
Probability of sex effect		-	0.003	<0.001	<0.001	0.003	0.034	0.346	0.125	0.313	0.020

3.3.4.2 Treadmill running

The treadmill protocol was altered from the previous studies to aid compliance and to reduce the likelihood of mice dropping out of the study. The duration was reduced by half to 30 minutes, though the target speed of 18-21m·min^{-1} and the 5% gradient remained the same. The altered running protocol seems to have suited the mice. Many more mice kept up with the regime with a marked improvement especially for the males compared with previous studies. The males who dropped out early were added to the Wheel group (n = 6), the female who dropped out became a Control mouse as a wheel was not available. Most mice ran every day and also achieved the target speeds and times (displayed in Figure 3.44 and Table 3.58). There were no significant (P>0.05) differences in performance between the sexes and the drop-off in performance demonstrated in the previous study (ES2) was not observed.

Table 3.58 Treadmill-running data for females and males

Sex	Mean daily time spent running (minutes)	Mean best speed (m·min^{-1})	% of days run
Target	**30**	**18-21**	**100**
Female	28.4	20.5	98
Male	28.6	20.6	96.9
Mean	**28.5**	**20.5**	**97.5**

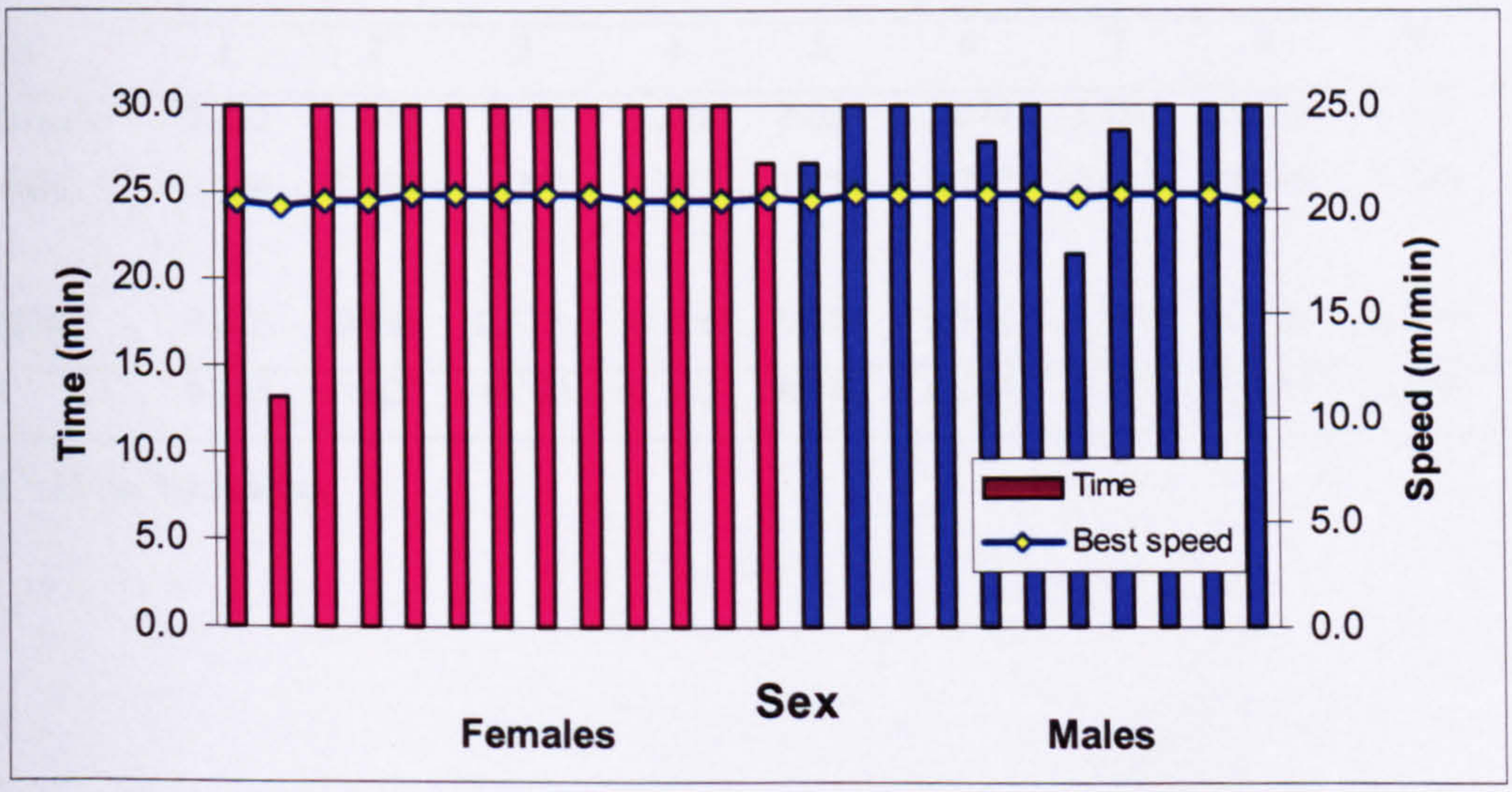


Figure 3.44 Daily average running time and best running speed by individual mice

3.3.4.3 Wheel running

The wheel protocol was the same as the previous study, with a smaller wheel being used for the first five weeks and a larger wheel for the final five weeks. Switching mice that would not run on the treadmill to wheels worked well, with the 6 males who were moved using their wheels and running between 33km and 332km over the remainder of the study.

Females ran further at all stages of the study, and the difference in distance after 10 week of study approached significance (231.8 v. 129.9km, P = 0.060, Figure 3.45). On a weekly basis, the difference in running distance was statistically significant only at week 8 (P = 0.046, Figure 3.46 and Table 3.59). A large inter-animal variation in distances run by the mice was observed, displayed in Figure 3.48.

The maximum daily distance run was highly significantly different between the sexes, with females having a greater average maximum compared with males (9.78 v. 5.15km, P = 0.009). The maximum daily distance run by an individual mouse was 18.35km for a female, whilst the corresponding maximum distance by a male mouse was 11.76km.

Table 3.59 Mean weekly distances run in wheels by each sex (km)

	Week of study									
Sex	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Female	0.722	1.617	2.192	2.328	2.034	5.048	5.703	5.500	4.515	3.868
Male	0.424	1.099	1.253	1.273	1.128	2.787	3.013	2.635	2.546	2.463
SEM	0.243	0.445	0.425	0.436	0.429	0.998	1.063	0.956	0.949	0.915
P	0.395	0.420	0.133	0.101	0.149	0.123	0.087	0.046	0.156	0.289

n = 12 for both sexes

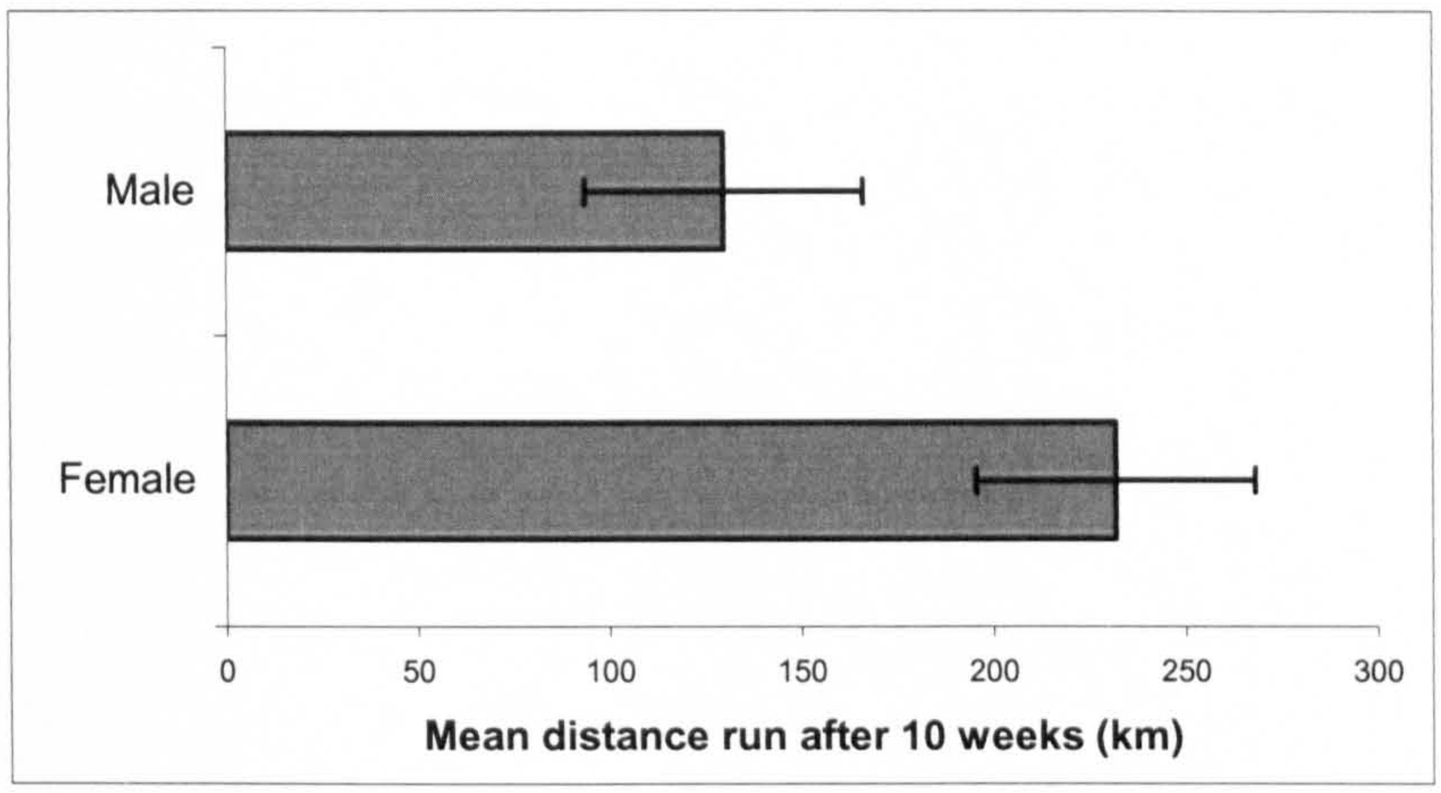


Figure 3.45 Total distance run after 10 weeks of study

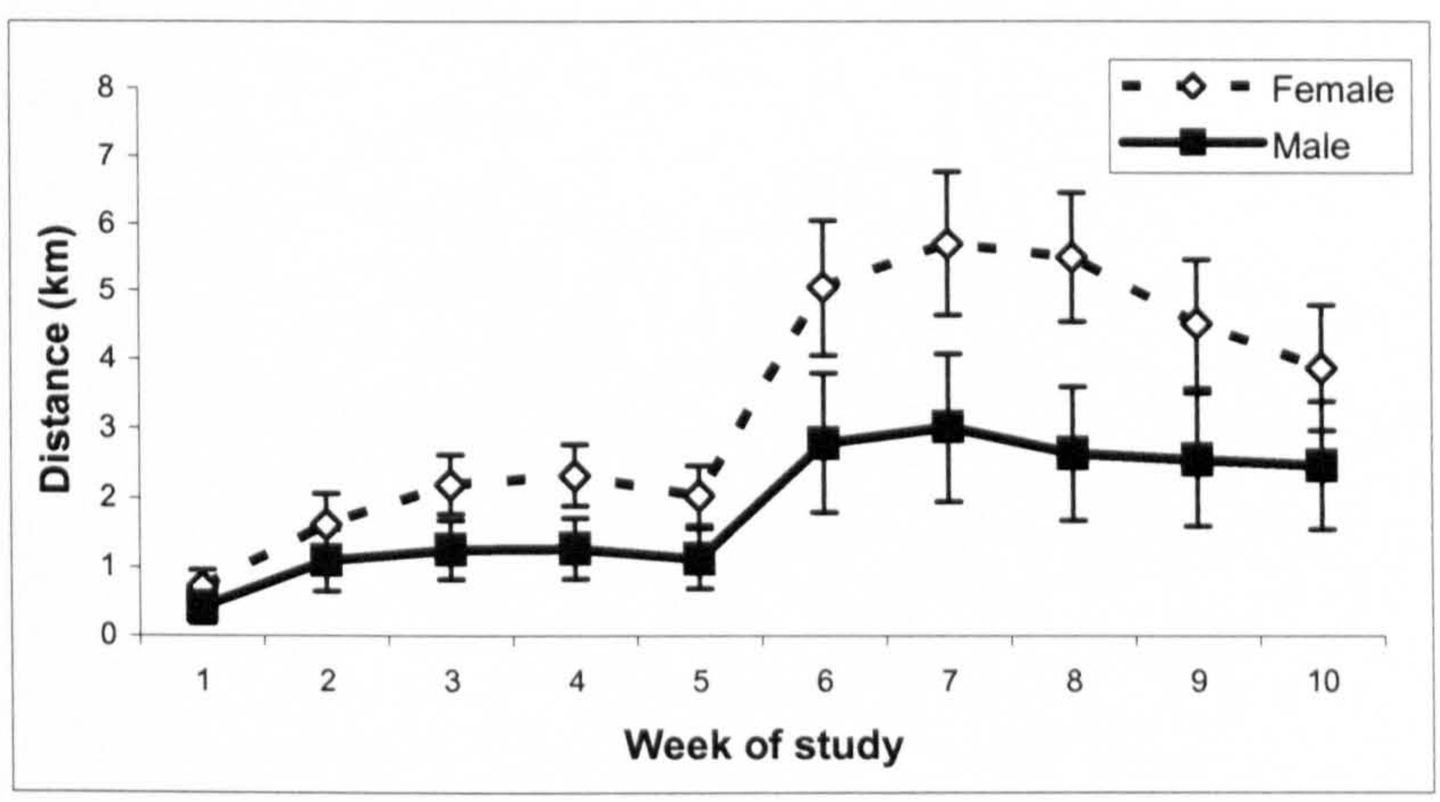


Figure 3.46 Mean daily running distance for each week of the study by male and female mice

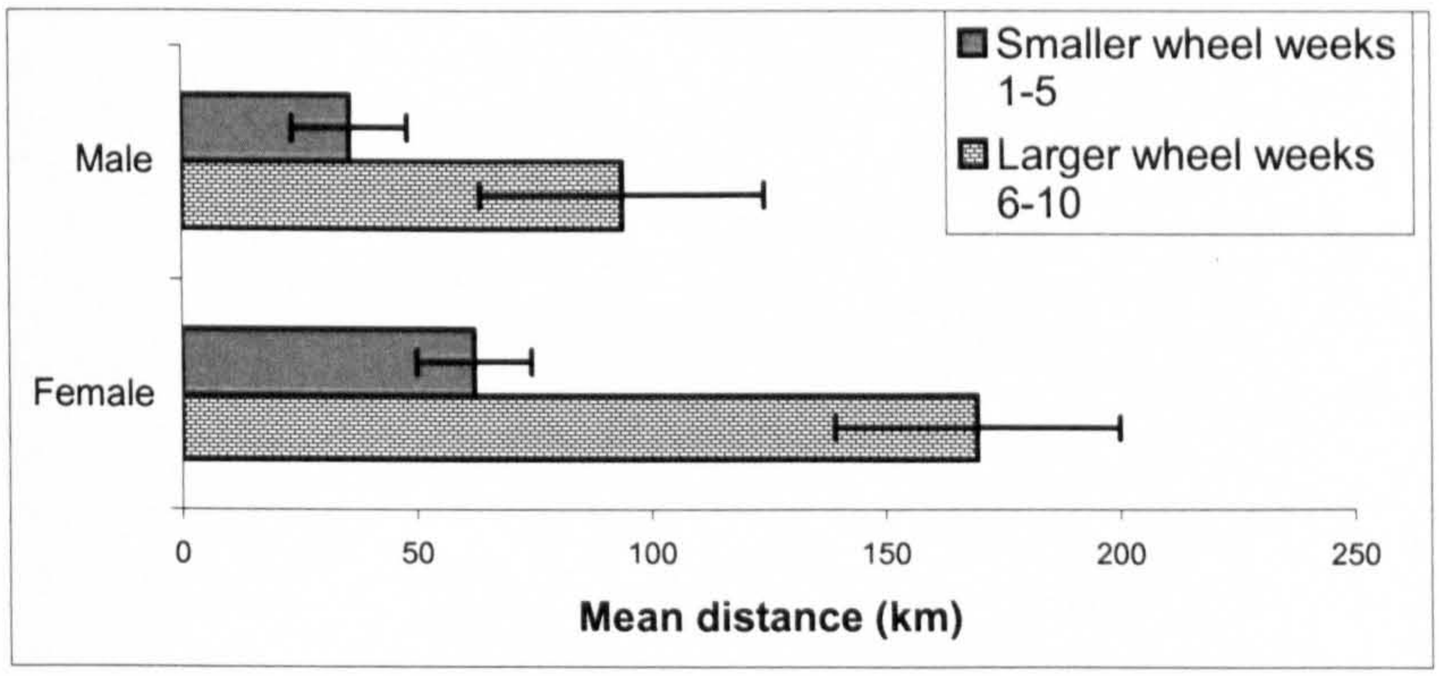


Figure 3.47 Total distances run in each type of wheel by male and female mice

As in the previous study, mice ran further in the larger wheels than the smaller wheels 49.05 v. 131.75 km average ($P = 0.001$, Figure 3.48). The total distance run in each size of wheel is displayed in Figure 3.47, although there was no significant difference in distance run between males and females in either type of wheel. In the small wheels

(weeks 1-5), female mice ran on average a total of 62.3km, compared with 35.9km by males (P = 0.144). In the larger wheels (weeks 6-10) females ran on average a total of 169.5km compared with 94.0km by the male mice (P= 0.092).

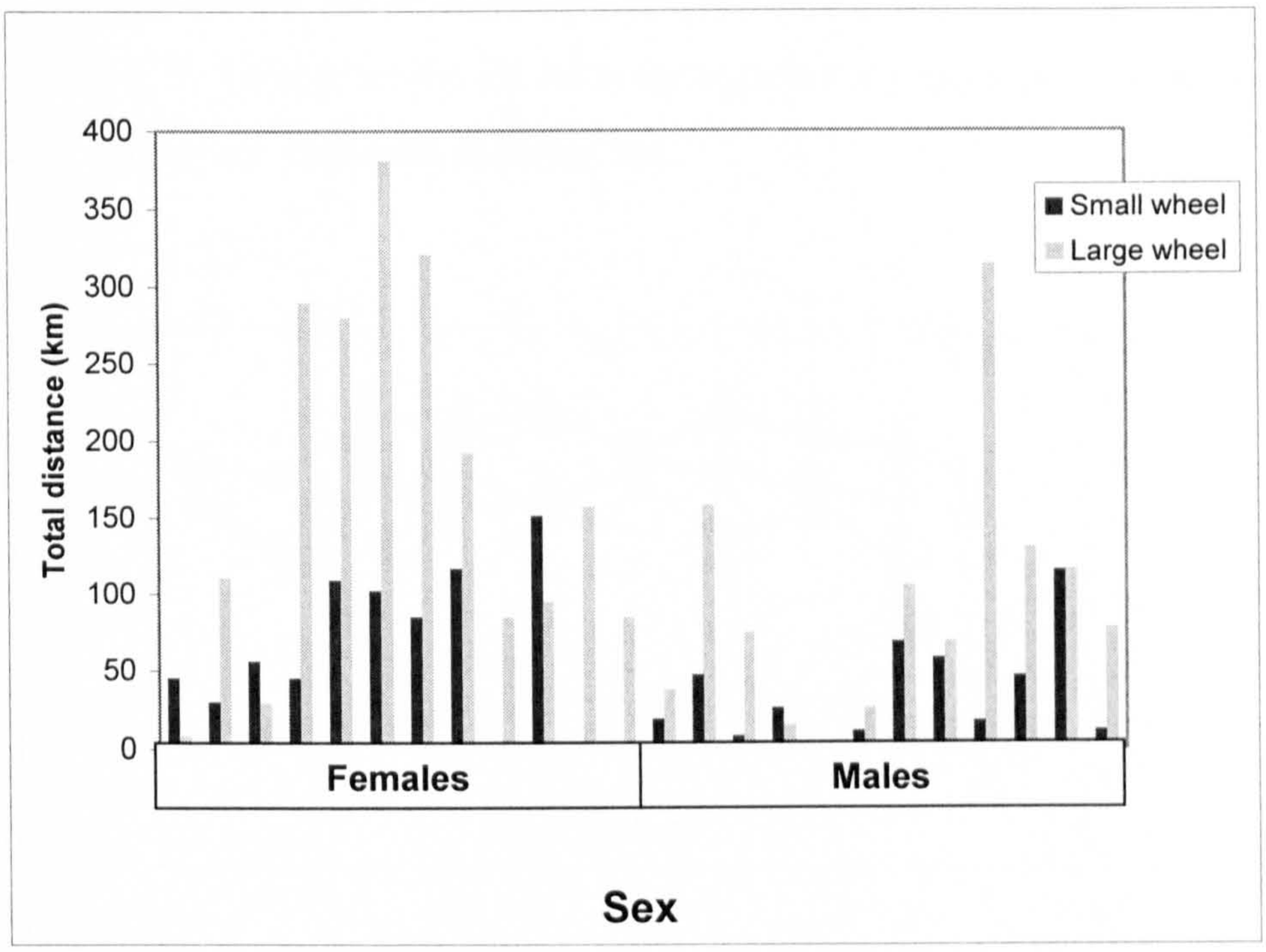


Figure 3.48 Total distances run by individual female and male mice in both small and larger wheels

There was no significant (P>0.05) correlation between distance run on any week and the body weight or weight gain during that week, or between total distance run and final body mass or total weight gain.

3.3.4.4 Food intake

Food intake was measured daily and also calculated on a per 100gram body weight basis. Measurement of food intake was not always possible for all mice because of the problems of food scattering, where mice would chew the food into small balls and spread it through the sawdust

There was a significant effect of exercise protocol on food intake during weeks 3, 5, 7 and 9, with WH eating more than CON on all weeks and TR eating more than CON during weeks 3 and 7. Figure 3.49 shows absolute food intake (g/d), whilst Table 3.60 shows relative food intake (g/100g body mass) for each exercise group. Overall, CON mice ate less than the other groups. WH mice ate significantly more than CON during weeks 3, 5, 7 and 9, whilst TR mice ate significantly more than CON during weeks 3 and 7. Data are displayed in Table 3.60.

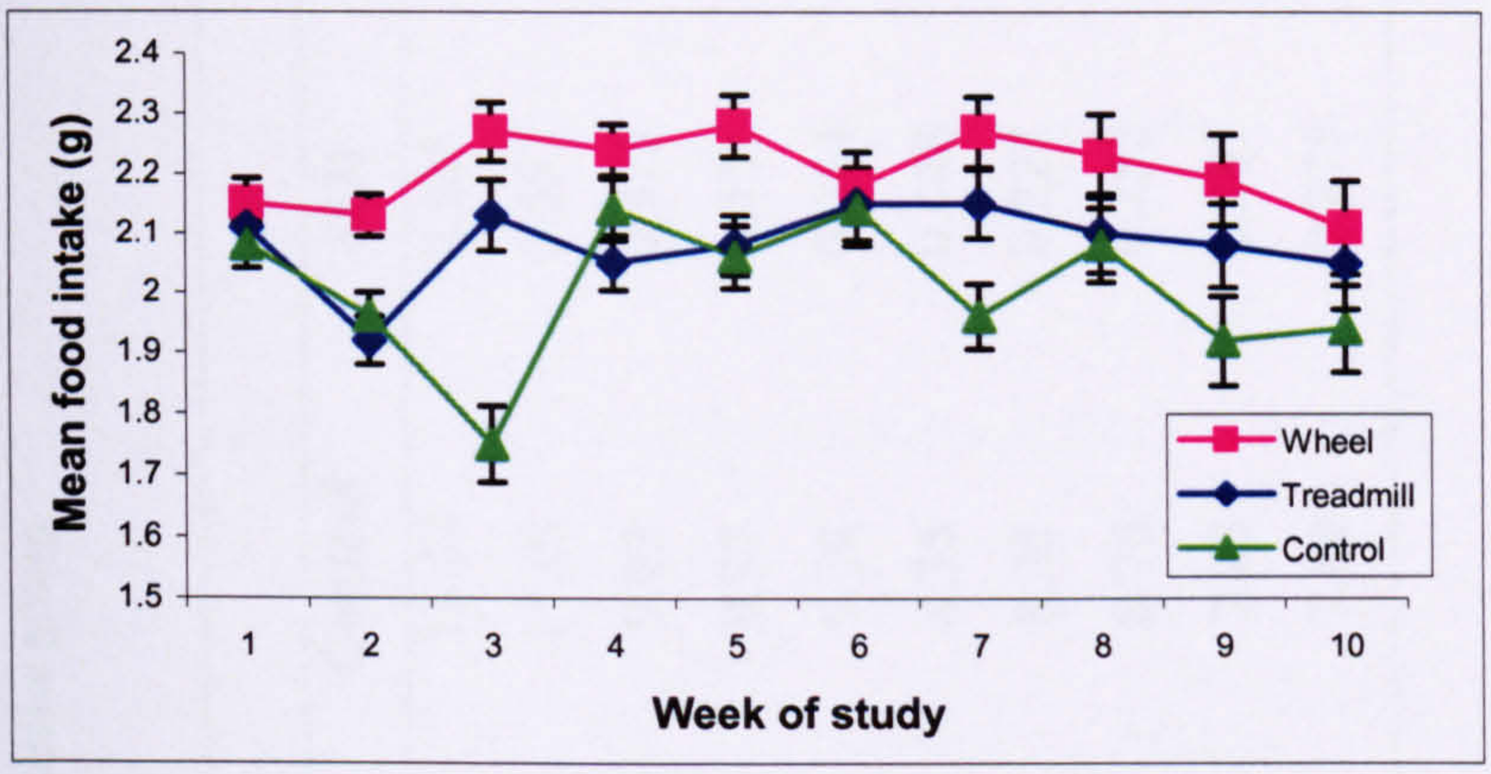


Figure 3.49 Food intake(g/d) by treatment group

Table 3.60 Mean daily food intake (g/100g body mass) for each week of study, by exercise group

							Probability	
Week	**Treadmill**	**SEM**	**Wheel**	**SEM**	**Control**	**SEM**	**TR v CON**	**WH v CON**
1	13.33	0.368	12.98	0.352	13.54	0.325	0.887	0.406
2	11.73	0.552	11.71	0.488	11.01	0.552	0.550	0.530
3	11.46	0.367	11.69	0.301	9.03	0.375	0.0001	<0.0001
4	10.33	0.500	10.94	0.466	9.37	0.527	0.316	0.055
5	9.93	0.354	10.62	0.361	9.08	0.354	0.164	0.006
6	9.78	0.469	9.67	0.438	8.35	0.448	0.058	0.073
7	9.56	0.241	10.06	0.246	8.25	0.227	0.0004	<0.0001
8	8.95	0.267	9.54	0.273	8.76	0.252	0.822	0.073
9	8.79	0.286	9.10	0.292	7.93	0.287	0.069	0.011
10	8.70	0.297	8.71	0.303	7.89	0.275	0.094	0.093

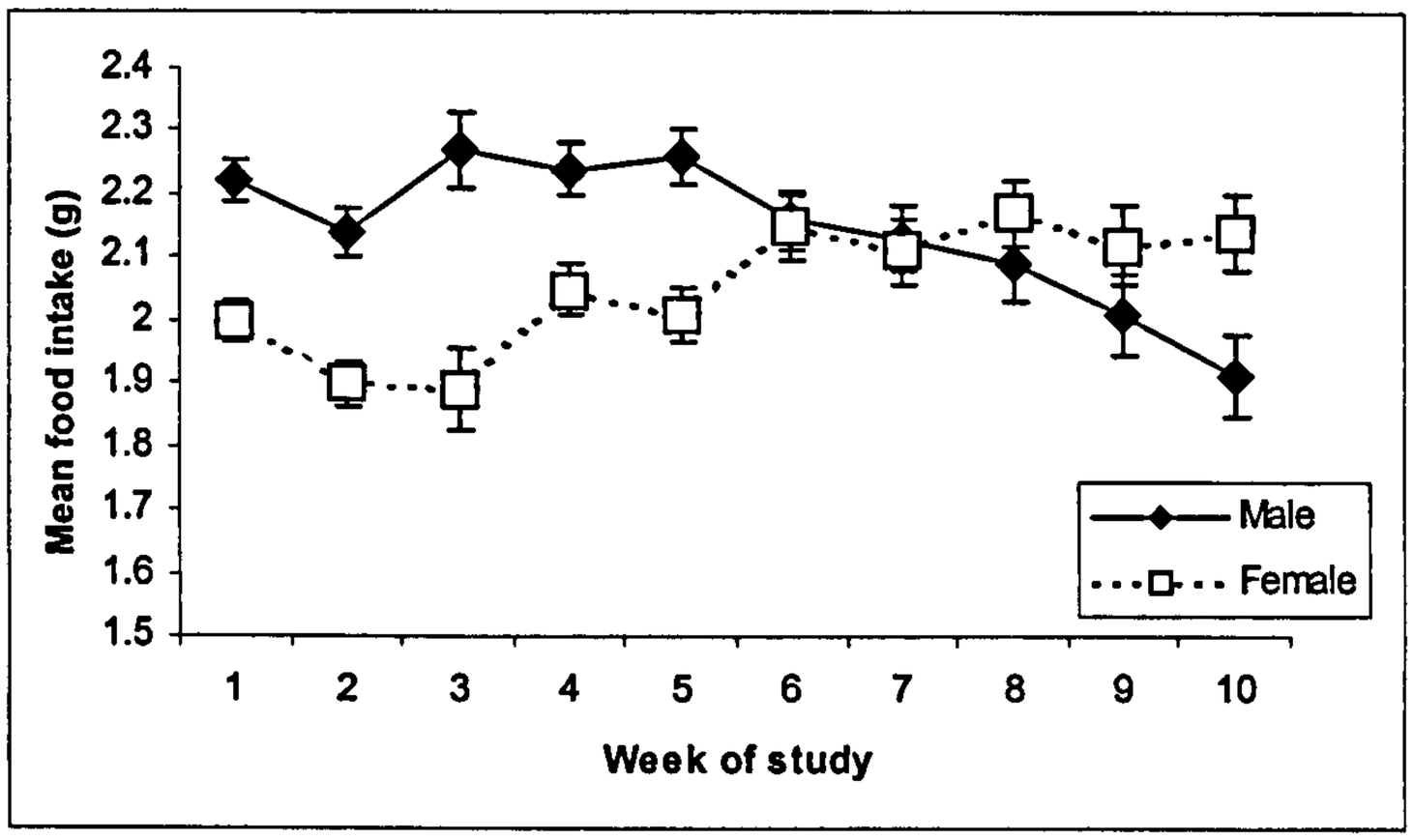


Figure 3.50 Mean daily food intake (g/d) for male and female mice

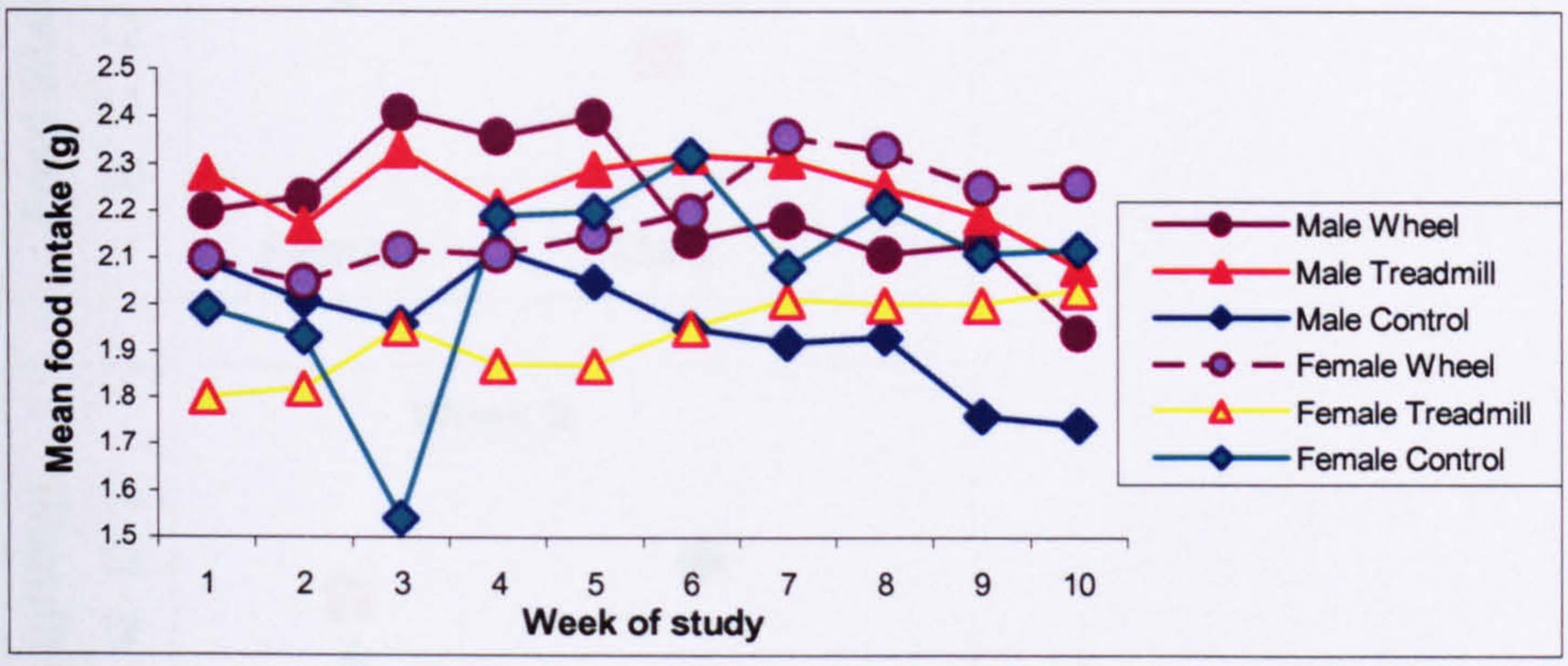
Table 3.61 Mean daily food intake (g/100g) for each week of study for male and female mice

					Probability	
Week	**Female**	**SEM**	**Male**	**SEM**	**Sex**	**Sex*Exercise**
1	13.60	0.280	12.98	0.284	0.125	0.002
2	11.60	0.417	11.40	0.439	0.771	0.014
3	11.12	0.359	10.66	0.346	0.066	0.006
4	10.77	0.418	9.80	0.412	0.102	0.178
5	10.37	0.303	9.35	0.308	0.025	0.205
6	10.19	0.395	8.45	0.366	0.003	0.078
7	10.25	0.224	8.11	0.237	<0.001	0.005
8	10.17	0.213	8.73	0.225	<0.001	0.001
9	9.72	0.245	7.44	0.248	<0.001	0.050
10	9.56	0.235	7.08	0.252	<0.001	0.118

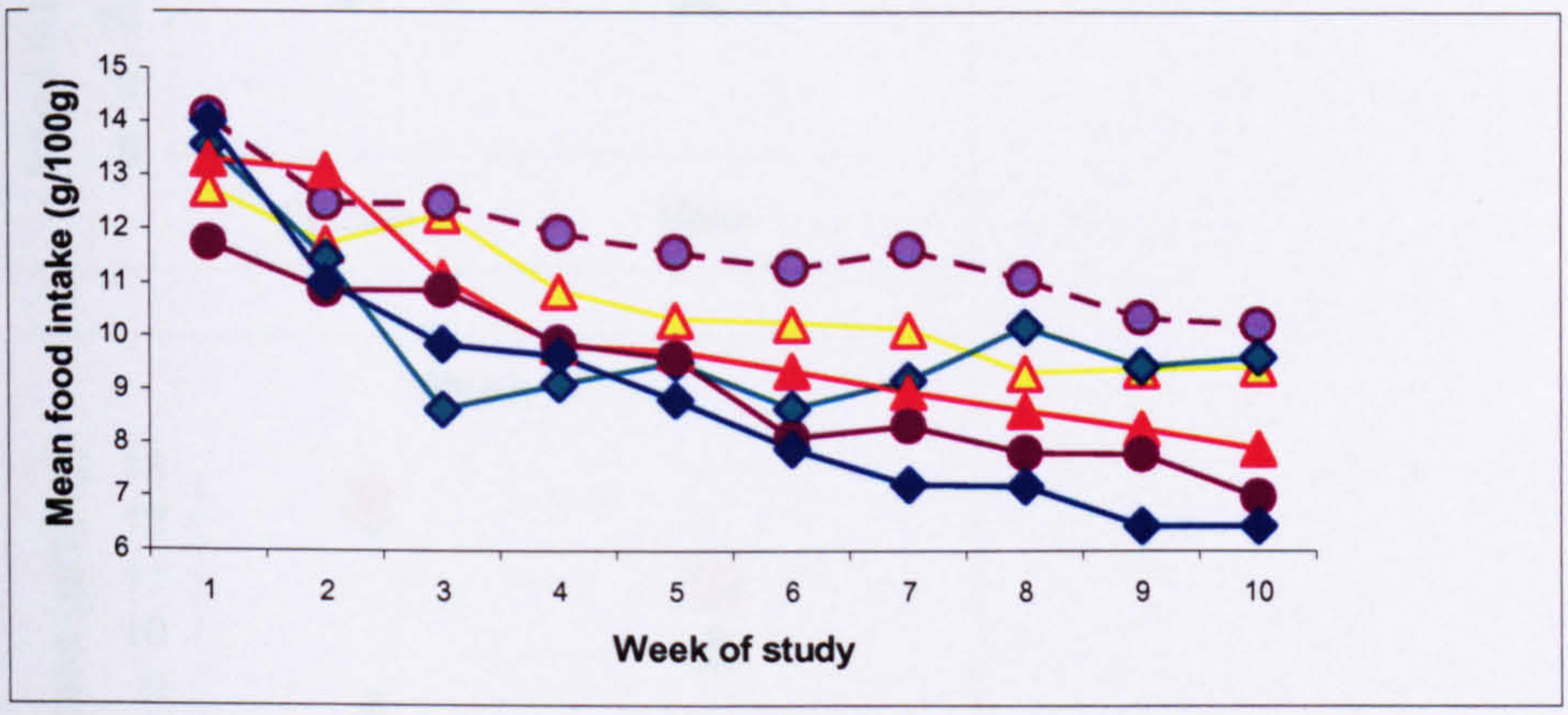
In terms of absolute intake (g food/d), male mice ate more than females during weeks 1-5 ($P<0.003$). Female mice ate significantly more during week 10 only ($P = 0.011$, Figure 3.50). Male mice consumed less food in the second half of the study compared with the first half.

When food intake was analysed on a g/100g body weight basis, female mice ate more food compared with males during weeks 5-10 (P from 0.025 to <0.001, Table 3.61).

Figure 3.51a and Figure 3.51b display food intake (g/d) and intake per 100g body mass by sex and treatment groups.



a



b

Figure 3.51 a) Comparing food intake by sex and protocol, b) food intake per 100g of body mass, by sex and treatment group

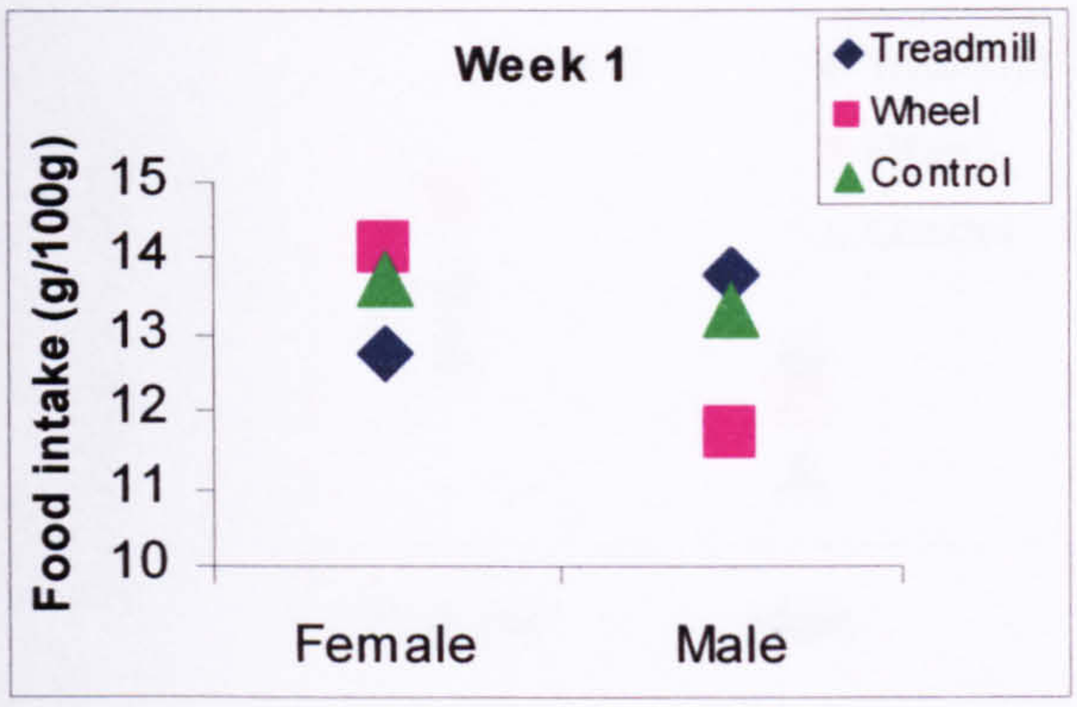


Figure 3.51 Interaction between sex and exercise on food intake in weeks 1-3

Figure 3.51 shows the interaction between sex and exercise on food intake during weeks 1-3. Female WH mice ate more food than all other groups during week 1 and 3, and more than the other female groups during week 2. During weeks 1 and 2 female WH and CON mice ate more than male WH and CON but female TR ate less than male TR mice ($P = 0.002$ and 0.014 for weeks 1 and 2, respectively). During week 3, female WH and TR ate more than male WH and TR mice, but male CON ate more food than female CON mice ($P = 0.006$).

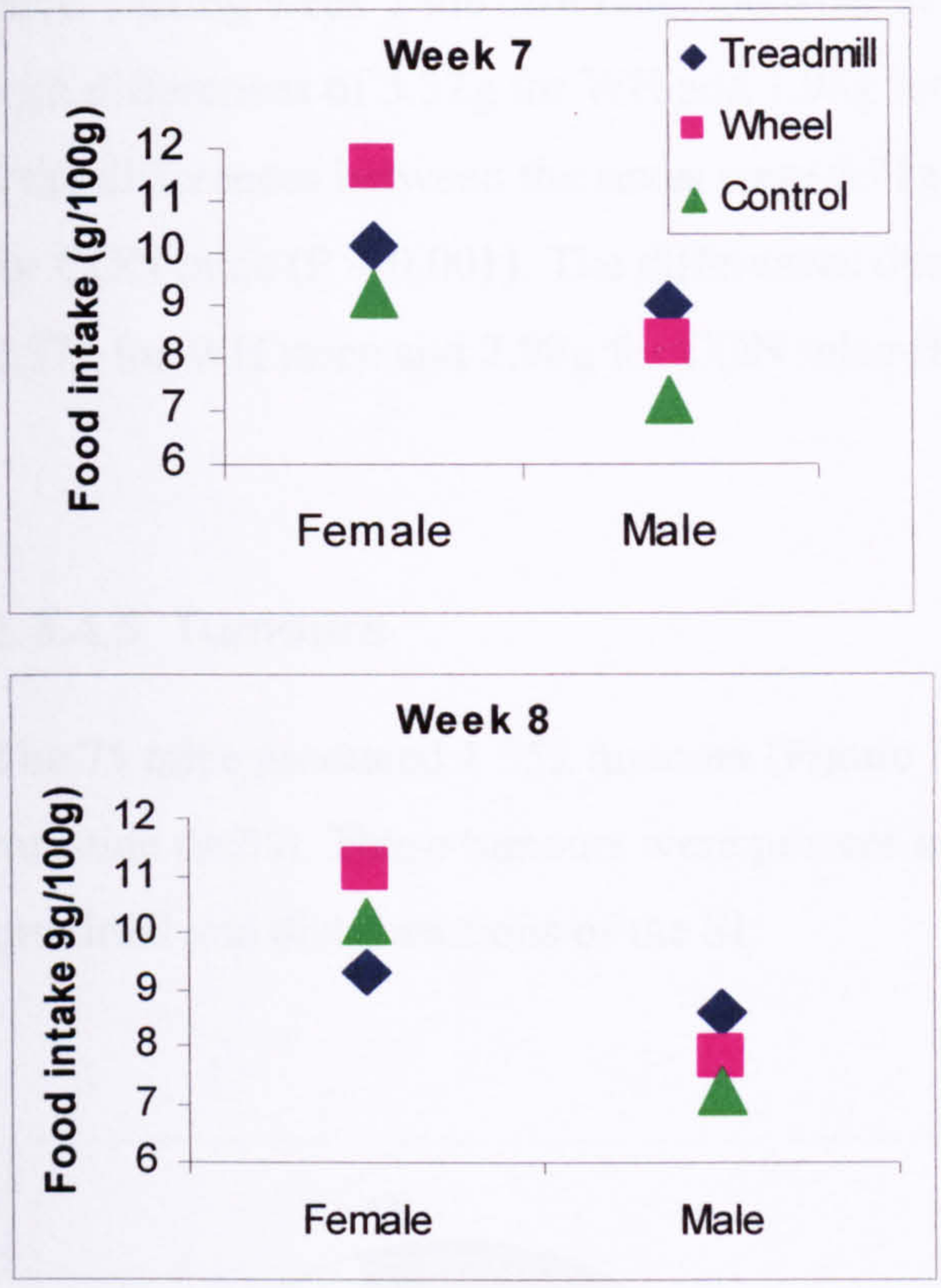


Figure 3.52 Interaction between sex and exercise on food intake in weeks 7-9

Figure 3.52 displays the interaction between sex and treatment group for food intake (g/100g body mass) in weeks 7, 8 and 9. As in weeks 1-3, female WH mice ate more food per 100g body mass than any other group. Male mice ate less food than female mice during all three weeks, in the order (greatest first): TR > WH > CON. The difference in the amount of food eaten was greater between male and female WH and CON mice than between male and female TR mice. During week 7 the difference

mice. During week 7 the difference between sexes for TR mice was 1.15g compared with differences of 3.32g for WH and 1.96g for CON mice (P = 0.005). During week 8 the differences between the sexes were 0.71g for TR mice, 3.29g for WH and 2.97g for CON mice (P = 0.001). The differences during week 9 were 1.11g for TR mice, 2.57g for WH mice and 2.98g for CON mice (P = 0.050).

3.3.4.5 Tumours

The 75 mice produced 1 352 tumours (Figure 3.54), with the majority in the small intestine (96%). These tumours were present in approximately equal numbers in the proximal and distal sections of the SI.

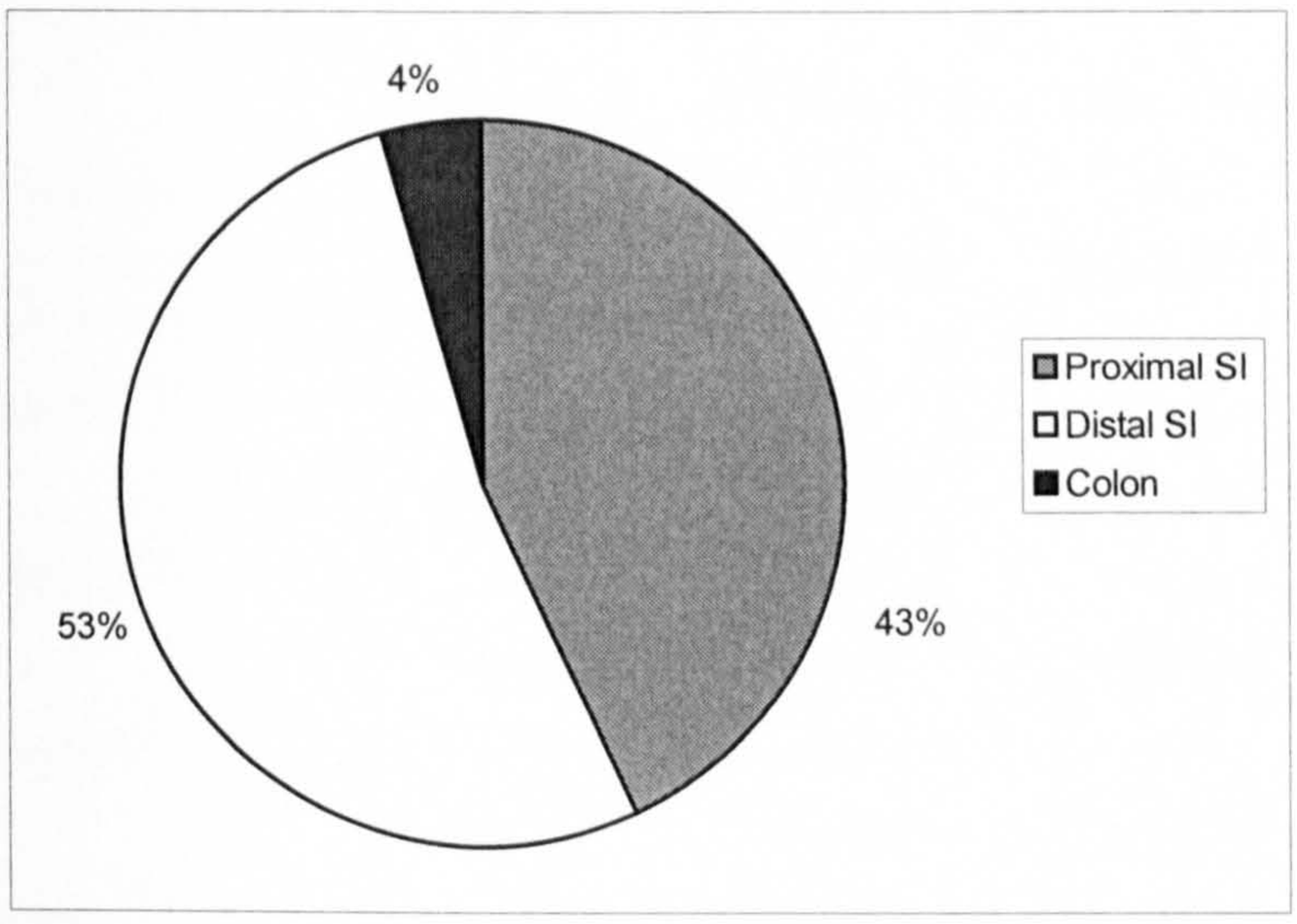


Figure 3.54 Anatomical distribution of tumours in ES3

Tumour data for treatment groups are displayed in Table 3.62. In general, WH mice suffered more and bigger tumours than either TR or CON, although none of these differences were statistically significant. Numbers of small intestinal tumours were similar in all treatment groups. There were fewer colon tumours in TR mice compared with CON mice, and this difference approached significance (median values 0.0 v. 1.0, P = 0.061). In contrast, TR mice had significantly more 4mm diameter tumours than CON mice, with median values of 1.0 compared with 0.0 (P = 0.042).

Table 3.62 Mean tumour data by exercise protocol

Tumour variable	Treadmill (n = 23)	Wheel (n = 24)	Control (n = 28)	Probability of effect	
				TR v. CON	WH v. CON
Proximal SI natLog	6.8	7.9	6.2	0.763	0.190
	5.46-8.42	6.35-9.80	5.08-7.52		
Distal SI natLog	5.9	8.8	7.9	0.280	0.808
	4.38-7.95	6.62-11.70	6.02-10.30		
Total SI Log10	12.8	16.9	14.5	0.648	0.535
	10.16-16.12	13.48-21.19	11.78-17.90		
ColonKW	0.0	0.5	1.0	0.061	0.589
	0.0-3.0	0.0-5.0	0.0-4.0		
Total number Log10	13.1	17.6	15.4	0.505	0.607
	10.32-16.53	13.99-22.19	12.43-19.04		
Tumour burden (mm diameter) Log10	31.6	38.6	35.3	0.692	0.787
	25.38-39.41	31.07-47.82	28.94-43.11		
1mmKW	1.0	2.5	1.0	0.771	0.141
	0.0-32.0	0.0-31.0	0.0-17.0		
2mmKW	7.0	7.0	8.0	0.177	0.811
	1.0-16.0	3.0-30.0	1.0-22.0		
3mmKW	3.0	3.0	3.5	0.298	0.504
	0.0-9.0	0.0-9.0	0.0-20.0		
4mmKW	1.0	1.0	0.0	0.042	0.445
	0.0-13.0	0.0-4.0	0.0-4.0		

No sex differences were observed in this study (Table 3.63), where numbers of mice were more closely matched than before, and all tumour variables produced similar values for both sexes. In addition, there were no significant sex*exercise interactions.

Table 3.63 Mean tumour data for male and female mice

Tumour variable	Female (n = 40)	Male (n = 35)	Probability of effect
Proximal SI ^{natLog}	7.2	6.5	0.440
	6.07-8.44	5.45-7.80	
Distal SI ^{natLog}	7.0	8.0	0.471
	5.59-8.84	6.32-10.19	
Total SI^{Log10}	14.5	14.6	0.908
	12.15-17.28	12.31-17.94	
Colon^{KW}	0.0	1.0	0.246
	0.0-5.0	0.0-4.0	
Total number ^{Log10}	15.0	15.6	0.851
	12.53-17.94	12.87-18.90	
Tumour burden (mm diameter) ^{Log10}	33.7	36.8	0.516
	28.49-39.74	30.82-43.98	
1mm^{KW}	2.0	1.0	0.623
	0.0-32.0	0.0-17.0	
2mm^{KW}	7.0	8.0	0.315
	1.0-30.0	1.0-22.0	
3mm^{KW}	3.0	4.0	0.577
	0.0-9.0	0.0-20.0	
4mm^{KW}	1.0	1.0	0.739
	0.0-13.0	0.0-4.0	

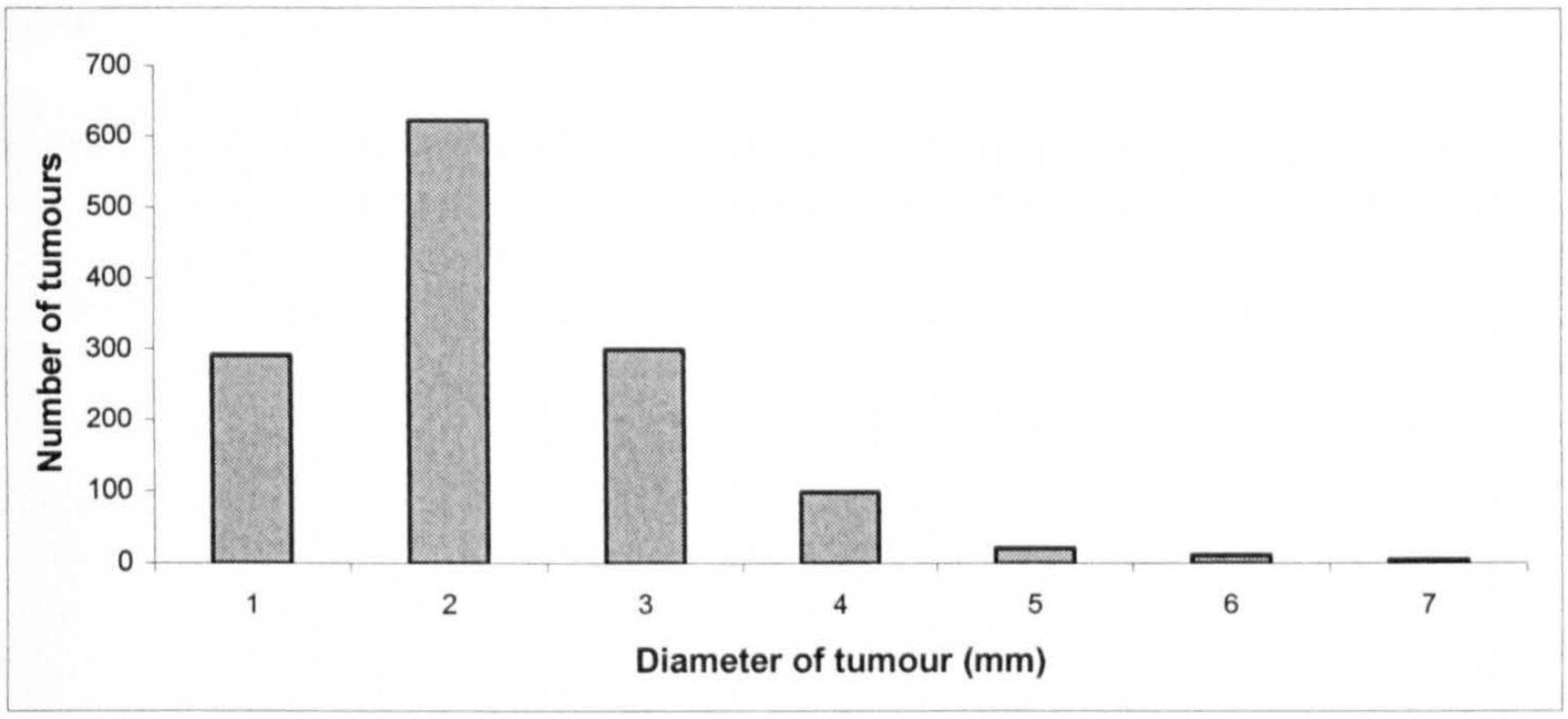


Figure 3.55 Distribution of tumour sizes in ES3

Figure 3.55 shows the distribution of tumour sizes in this study, which was positively skewed, with most tumours having a diameter of between 1 and 3mm. The largest tumours were 7mm diameter.

3.3.4.6 Relationship between fathers and number of tumours in their offspring

The great variation in tumour number between mice prompted an assessment of the relationship fathers played in the number of tumours produced by their offspring. Table 3.64 shows some of the tumour data from Table 3.62 categorised according to father. Some mice sired more offspring than others, some produced more Mins than wild types, and some were used only once, if they were old or the number of mice required for the study had been reached. As a consequence, the number of mice in the study sired by each father ranged from 1 to19. Table 3.64 shows that the fathers had a significant effect on both the tumour number and tumour burden of their offspring (P = 0.046 and 0.012 respectively), with the worst affected mice having 3.5 times the tumour burden of the least affected mice.

A highly significant positive correlation was observed between tumour burden and tumour multiplicity ($r = 0.95$, $P < 0.001$, Figure 3.56).

Table 3.64 Tumour data (mean and 95% CI)according to father

Father code	Number of offspring in study	Tumour burden (mm diameter) of offspring^{Log10}	Total number of tumours of offspring ^{Log10}
1	3	70.0	27.7
		40.07-122.22	15.15-50.59
2	11	28.8	12.7
		21.51-38.67	9.22-17.39
3	9	49.5	21.2
		35.92-68.33	14.95-29.96
4	19	42.9	18.7
		34.12-53.83	14.62-23.94
5	1	39.8	15.6
		15.09-105.04	5.47-44.53
6	12	26.9	11.9
		20.37-35.56	8.82-16.11
7	7	31.5	14.5
		21.86-45.33	9.75-21.44
8	2	20.8	7.91
		10.51-41.23	3.79-16.54
9	12	30.4	13.9
		23.02-40.18	10.27-18.75
Probability	-	**0.012**	**0.046**

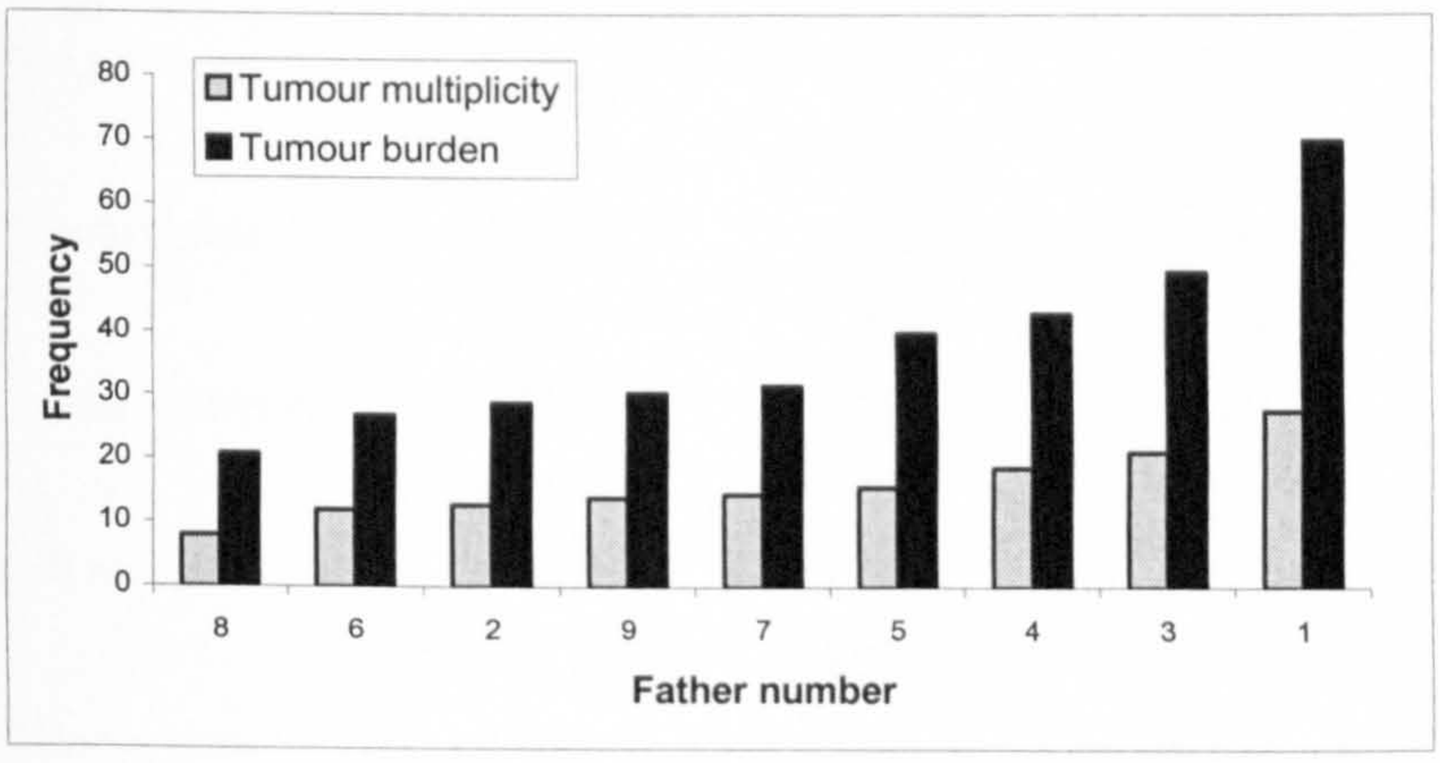


Figure 3.56 Relationship between total number of tumours and tumour burden in study mice, by father

3.3.4.7 Organs

Table 3.65 shows data for the intestinal organs of mice by exercise treatment group.. TR mice had colons that were significantly shorter than those of CON mice (4 v. 5cm, P = 0.010). No other weights were significantly different between the treatment groups.

Table 3.65 Intestinal organ measurements of each treatment group (mean and SEM)

Organ dimension	Treadmill (n = 23)	Wheel (n = 24)	Control (n = 28)	Probability of effect	
				TR v. CON	WH v. CON
Slaughter weight (g)	23.0	23.5	24.0		
	0.71	0.70	0.65	0.488	0.810
Length (cm)					
SI	29.0	27.6	27.5	0.101	0.983
	0.550	0.538	0.498		
Colon[KW]	4.0	4.5	5.0	0.010	0.106
	3.5-6.0	3.5-5.0	3.5-6.0		
Weight (g)					
Full SI	1.024	1.005	0.974	0.239	0.540
	0.025	0.024	0.022		
Proximal SI	0.626	0.627	0.607	0.600	0.574
	0.017	0.016	0.015		
Distal SI	0.348	0.364	0.376	0.280	0.764
	0.015	0.014	0.013		
Full Caecum	0.250	0.247	0.233	0.453	0.581
	0.012	0.012	0.011		
Caecal tissue	0.098	0.095	0.104	0.559	0.245
	0.004	0.004	0.004		
Caecal contents	0.152	0.152	0.129	0.146	0.146
	0.010	0.009	0.009		
Full colon	0.233	0.229	0.227	0.899	0.982
	0.011	0.011	0.011		
Colonic tissue	0.160	0.169	0.159	0.985	0.358
	0.006	0.006	0.006		
Colon contents	0.072	0.060	0.068	0.888	0.715
	0.009	0.008	0.008		

When the weights of these organs are expressed as % final body mass, there were no statistically significant differences (Table 3.66).

Table 3.66 Intestinal organs of exercise groups as % final body mass (mean and SEM)

Organ (% final body mass)	Treadmill (n = 23)	Wheel (n = 24)	Control (n = 28)	Probability of effect TR v. CON	Probability of effect WH v. CON
Proximal SI	2.77	2.76	2.56	0.237	0.263
	0.105	0.100	0.093		
Distal SI	1.52	1.59	1.59	0.655	0.996
	0.067	0.064	0.059		
Caecum^{Log10}	0.42	0.40	0.43	0.936	0.453
	0.388-0.456	0.372-0.436	0.398-0.461		
Colon^{KW}	0.70	0.69	0.65	0.325	0.180
	0.461-1.190	0.501-1.320	0.500-0.961		

The weights of other organs taken at necropsy are displayed in Table 3.67. WH mice had hearts 10% heavier than those of CON mice (0.229 v. 0.204g, P = 0.021). The weights of thymus and adrenal glands were very similar between groups, although left adrenals were consistently heavier than right (P = 0.027).

When the weights of these non-intestinal organs were expressed as % final body mass (Table 3.68), the weight of hearts in WH mice remained significantly heavier than those of CON mice (1.0% v. 0.9%, P = 0.033), and the difference in the weight of the liver between these two treatment groups also became significant, at 4.5% for WH mice and 4.2% for CON mice (P = 0.004). Weights of hearts and livers of TR mice were between those of WH and CON groups. There were no significant differences between exercise groups for any of the other organs.

Table 3.67 Non-intestinal organ measurements of each treatment group (mean and SEM)

Organ weight (g)	Treadmill (n = 23)	Wheel (n = 24)	Control (n = 28)	Probability of effect	
				TR v. CON	WH v. CON
Liver	0.987	1.049	1.004	0.861	0.388
	0.028	0.027	0.025		
Heart	0.219	0.229	0.204	0.219	0.021
	0.007	0.007	0.006		
Brain[KW]	0.478	0.473	0.472	0.072	0.594
	0.439-0.520	0.446-0.591	0.447-0.513		
Thymus	0.061	0.059	0.061	1.000	0.922
	0.003	0.003	0.003		
Left adrenal gland	0.0074	0.0072	0.0069	0.789	0.955
	0.00058	0.00057	0.00053		
Right adrenal gland [Log10]	0.0060	0.0052	0.0058	0.944	0.578
	0.0050-0.0072	0.0043-0.0062	0.0049-0.0068		
Haematocrit (%)	26.2	26.8	24.1	0.663	0.498
	2.00	1.92	1.77		

Table 3.68 Non-intestinal organs as % final body mass of exercise groups (mean and SEM)

Organ	Treadmill (n = 23)	Wheel (n = 24)	Control (n = 28)	Probability of effect	
				TR v. CON	WH v. CON
Liver	4.31	4.49	4.20	0.367	0.004
	0.066	0.06	0.06		
Heart	0.96	1.008	0.863	0.190	0.033
	0.045	0.044	0.040		
Brain	2.13	2.10	2.01	0.223	0.419
	0.059	0.057	0.053		
Thymus	0.26	0.25	0.26	0.994	0.777
	0.014	0.013	0.012		
Left adrenal	0.032	0.031	0.028	0.314	0.642
	0.002	0.002	0.002		
Right adrenal [Log10]	0.026	0.022	0.024	0.669	0.612
	0.023-0.030	0.019-0.026	0.021-0.028		

Male mice tended to have longer and heavier intestinal organs than females (Table 3.69). The weight of the full caecum and caecal contents were heavier in males (P = 0.019 and 0.001 respectively) and also the full colon, colon contents and colon tissue (P = 0.004, 0.023 and 0.030 respectively).

Table 3.69 Intestinal organ measurements of males and female mice (mean and SEM)

Organ dimension	Female (n = 40)	Male (n = 35)	Probability
Slaughter weight (g)	21.7 0.54	25.8 0.58	<0.001
Length (cm)			
SI	27.6 0.424	28.5 0.453	0.130
Colon[KW]	4.5 3.5-6.0	4.5 4.0-6.0	0.148
Weight (g)			
Full SI	0.989 0.019	1.011 0.020	0.407
Proximal SI	0.621 0.013	0.616 0.013	0.768
Distal SI	0.367 0.011	0.360 0.012	0.618
Full Caecum	0.228 0.009	0.260 0.010	0.019
Caecal tissue	0.102 0.003	0.096 0.003	0.278
Caecal contents	0.126 0.007	0.164 0.008	0.001
Full colon	0.212 0.009	0.249 0.009	0.004
Colonic tissue	0.155 0.005	0.171 0.005	0.030
Colon contents	0.056 0.007	0.078 0.007	0.023

When the intestinal organs were expressed as % final body mass, the direction of significance was reversed (Table 3.70). Female mice had heavier proximal and distal SI, heavier caecal tissue and heavier colonic tissue compared with males. These differences were highly significant for proximal SI, distal SI and caecum (all P<0.001). The difference in weight of colon tissue approached significance (P = 0.069).

Table 3.70 Male and female intestinal organs as % final body mass

Organ	Female (n = 40)	Male (n = 35)	Probability
Proximal SI	2.91	2.44	<0.001
	0.079	0.084	
Distal SI[KW]	1.70	1.42	<0.001
	0.050	0.053	
Caecum [Log10]	0.46	0.372	<0.001
	0.435-0.492	0.348-0.397	
Colon	0.70	0.63	0.069
	0.502-1.320	0.461-1.224	

The weights of liver and heart were significantly heavier in males compared with females (Table 3.71), although females had a slightly heavier brain and a significantly heavier thymus gland.

When these data were expressed as % final body weight, female mice had significantly heavier liver, brain, thymus and left adrenal gland (Table 3.72).

Table 3.71 Non-intestinal organ weights of male and female mice

Organ weight (g)	Female (n = 40)	Male (n = 35)	Probability
Liver	0.949	1.087	<0.001
	0.006	0.022	
Heart	0.196	0.240	<0.001
	0.006	0.006	
Brain[KW]	0.477	0.472	0.301
	0.439-0.520	0.447-0.591	
Thymus	0.067	0.053	<0.001
	0.002	0.003	
Left adrenal	0.0073	0.0069	0.520
	0.00044	0.00047	
Right adrenal[Log10]	0.0055	0.0057	0.716
	0.0048-0.0064	0.0049-0.0066	
Haematocrit (%)	28.3	22.5	0.009
	1.49	1.58	

Table 3.72 Non-intestinal organs as % final body mass

Organ	Female (n = 40)	Male (n = 35)	Probability
Liver	4.40	4.25	0.034
	0.053	0.056	
Heart	0.92	0.96	0.497
	0.035	0.037	
Brain	2.24	1.89	<0.001
	0.045	0.047	
Thymus	0.30	0.21	<0.001
	0.010	0.011	
Left adrenal	0.034	0.026	0.001
	0.001	0.002	
Right adrenal[Log10]	0.03	0.02	0.122
	0.023-0.029	0.020-0.025	

3.3.4.8 SCFA

The concentrations of SCFA present in the colon contents of mice of each exercise group are shown in Table 3.73. The concentration of acetate was significantly greater in CON samples compared with both TR and WH mice samples(P = 0.021 and 0.009, respectively), but concentrations of other SCFA were similar between the groups and not statistically (P>0.05) different. However, the total concentration of colonic SCFA was greater in CON samples compared with both TR (P = 0.044) and WH (P = 0.034) mice samples.

Table 3.73 SCFA concentrations (mean and 95% CI) (µmol·g colon contents) by exercise protocol

Individual SCFA (µmol·g colon contents)*	TR (n = 17)	WH (n = 19)	CON (n = 22)	Probability of effects	
				TR v CON	WH v CON
Acetate	17.70	17.18	25.06	0.021	0.009
	14.59-21.48	14.30-20.63	21.15-29.70		
Propionate	2.66	2.90	2.83	0.853	0.970
	2.21-3.21	2.43-3.47	2.40-3.34		
Isobutyrate	0.33	0.34	0.36	0.747	0.852
	0.26-0.41	0.27-0.42	0.30-0.44		
Butyrate	0.84	0.87	0.77	0.918	0.851
	0.57-1.24	0.60-1.25	0.54-1.08		
Isovalerate	0.60	0.57	0.53	0.496	0.714
	0.50-0.71	0.49-0.67	0.46-0.61		
Valerate	0.35	0.42	0.42	0.658	0.999
	0.26-0.48	0.31-0.56	0.32-0.55		
Total concentration	22.91	22.80	30.27	0.044	0.034
	20.93-27.34	20.94-26.96	27.97-35.35		

***all Log10 transformed, data shown are mean and 95% CI**

When expressed as molar proportions, there were several differences in SCFA between the exercise groups (Table 3.74). Acetate proportion was again greater in CON compared with TR and WH mice (829 v. 777 and 759 mmol/mol respectively). For all other SCFA proportion, the inverse association was observed, with CON mice

having a smaller proportion of these SCFA than both TR and WH mice, significantly ($P<0.05$) so for propionate, isobutyrate and isovalerate (Table 3.74).

Table 3.74 Molar proportions (mmol/mol total SCFA) of colonic SCFA by exercise group, mean and SEM/95% CI

Molar proportion	TR	WH	CON	Probability of effects	
				TR v CON	WH v CON
Acetate	777	759	829	0.034	0.002
	15.8	15.0	13.9		
Propionate[Log10]	116	127	93	0.059	0.004
	100.5-134.8	110.8-146.3	82.1-106.1		
Isobutyrate [KW]	15	15	9	0.004	<0.001
	6.5-36.2	5.9-33.3	0.0-17.8		
Butyrate[KW]	43	46	37	0213	0.158
	14.1-87.6	11.6-83.7	0.0-84.7		
Isovalerate [Log10]	26	26	18	0.0006	0.001
	22.7-30.8	22.1-29.5	15.5-20.2		
Valerate	16	16	12	0.334	0.231
	2.3	2.2	2.0		

Data shown are: acetate and valerate – mean and SEM, propionate and isovalerate – mean and 95% CI, isobutyrate and butyrate – median and range

Table 3.75 shows the concentrations of colonic SCFA in samples from male and female mice, which were significantly different for acetate ($P = 0.018$) and butyrate ($P = 0.049$). Male samples had a greater concentration of acetate (23.01 v. 17.54 μmol·g colon contents) but a lower concentration of butyrate (0.62 compared with 1.06 μmol·g colon contents). When expressed as molar proportions, sample from male mice again had more acetate and females a greater proportion of butyrate (Table 3.76). In addition, samples from female mice had a significantly greater proportion of isovalerate than did males (25 v. 20, $P = 0.041$).

Table 3.75 SCFA concentrations (mean and 95% CI) (µmol·g colon contents) by sex

Individual SCFA (µmol·g colon contents)*	Female (n = 30)	Male (n = 28)	Probability
Acetate	17.54	23.01	0.018
	15.00-20.51	19.57-27.06	
Propionate	2.73	2.88	0.573
	2.38-3.14	2.50-3.33	
Isobutyrate	0.35	0.33	0.642
	0.30-0.41	0.28-0.40	
Butyrate	1.06	0.62	0.049
	0.79-1.41	0.46-0.84	
Isovalerate	0.56	0.57	0.775
	0.49-0.63	0.50-0.65	
Valerate	0.46	0.34	0.126
	0.36-0.57	0.27-0.44	
Total concentration	23.01	28.31	0.046
	21.43-26.46	26.30-32.72	

***all Log10 transformed, data shown are mean and 95% CI**

Table 3.76 Molar proportions (mmol/mol total SCFA) of colonic SCFA by sex

Molar proportion	Female	Male	Probability
Acetate	767	816	0.011
	13.0	13.5	
Propionate^{Log10}	119	102	0.088
	105.5-133.8	90.1-115.2	
Isobutyrate ^{KW}	14	12	0.367
	0.0-36.2	5.9-21.1	
Butyrate^{KW}	59	27	0.012
	0.0-87.6	2.8-83.7	
Isovalerate ^{Log10}	25	20	0.041
	21.5-28.0	17.8-23.3	
Valerate	17	12	0.118
	1.75	1.81	

Data shown are: acetate and valerate – mean and SEM, propionate and isovalerate – mean and 95% CI, isobutyrate and butyrate – median and range

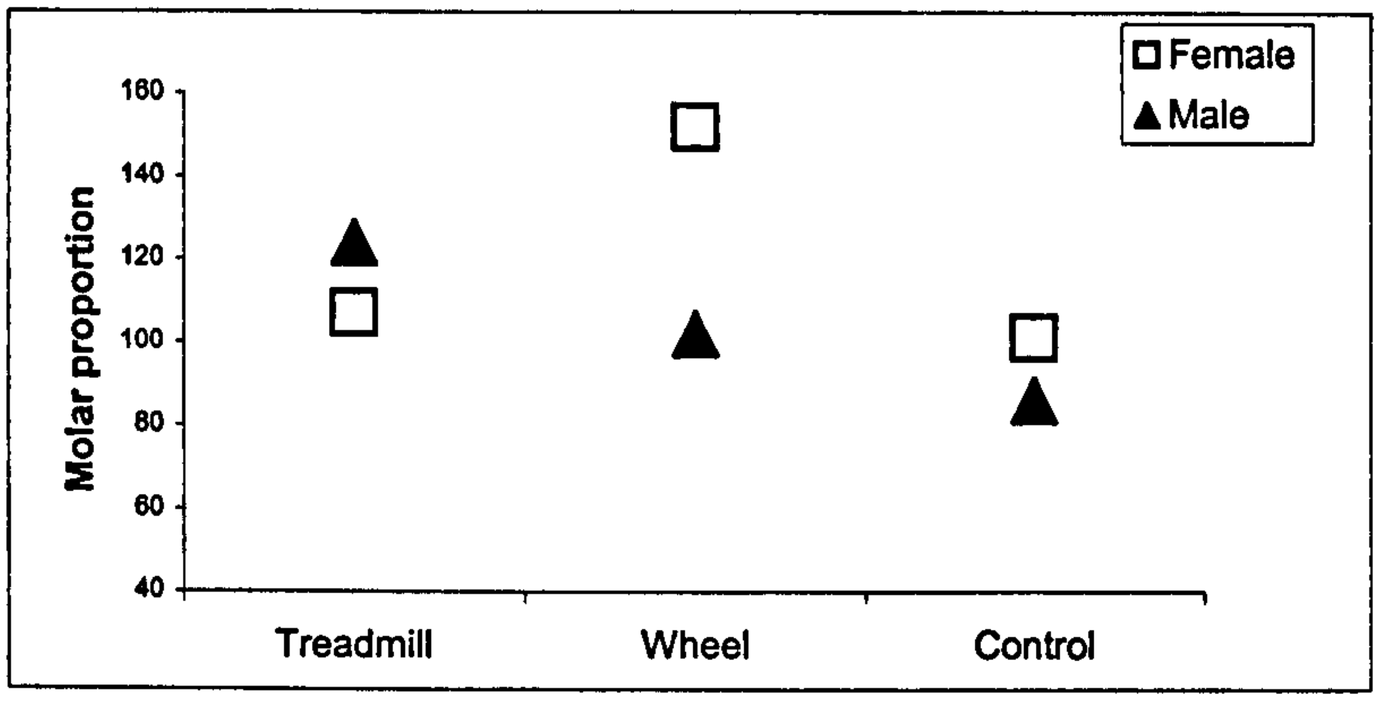


Figure 3.57 Interaction between sex and exercise on propionate molar proportion (mmol/mol total SCFA)

There was an interaction between sex and exercise protocol for the proportion of propionate only (P = 0.028), with samples from female WH and CON mice having a greater proportion of propionate than their male counterparts, whilst female TR mice had a smaller proportion than male TR (Figure 3.57).

3.3.4.9 Transit time (TT)

There was no significant difference in caecal transit time between exercise groups, with the median values at around 3.5 hours (Table 3.77). =

Table 3.77 Caecal transit time of treatment groups (h)

Group (n)	Median transit time^{KW}	Range	P (v. CON)
Treadmill (22)	3.17	0.79-10.84	0.533
Wheel (24)	3.89	0.50-23.40	0.584
Control (27)	3.48	0.39-13.67	-

Despite considerable inter-animal variation in caecal transit time for both sexes, on average, males had TT which was significantly slower than that of female mice (P = 0.004, Table 3.78).

Table 3.78 Caecal transit time of male and female mice (h)

Group (n)	Median transit time[KW]	Range	Probability
Female (39)	3.18	0.39-23.40	0.004
Male (34)	4.07	1.25-20.14	

3.3.4.10 Non-exercise physical activity (NEPA)

Activity of singly-housed mice was monitored continuously using the InfraMot device for 23h periods. For CON and TR mice, the other hour in the 24h period was spent in the imposed exercise regime and associated activities. The numbers of animals studied and the total number of recordings for each exercise group are summarised in Table 3.79, and varied between one and 23 recordings per animal. Inter- and intra-animal variation over the recording period was great, as shown by Figure 3.58.

Table 3.79 Summary of NEPA recordings for each exercise group

Exercise group	Number of animals studied (female, male)	Total number of recordings
CON	6 (3, 3)	77
TR	9 (2, 7)	85
WH	12 (8, 4)	54

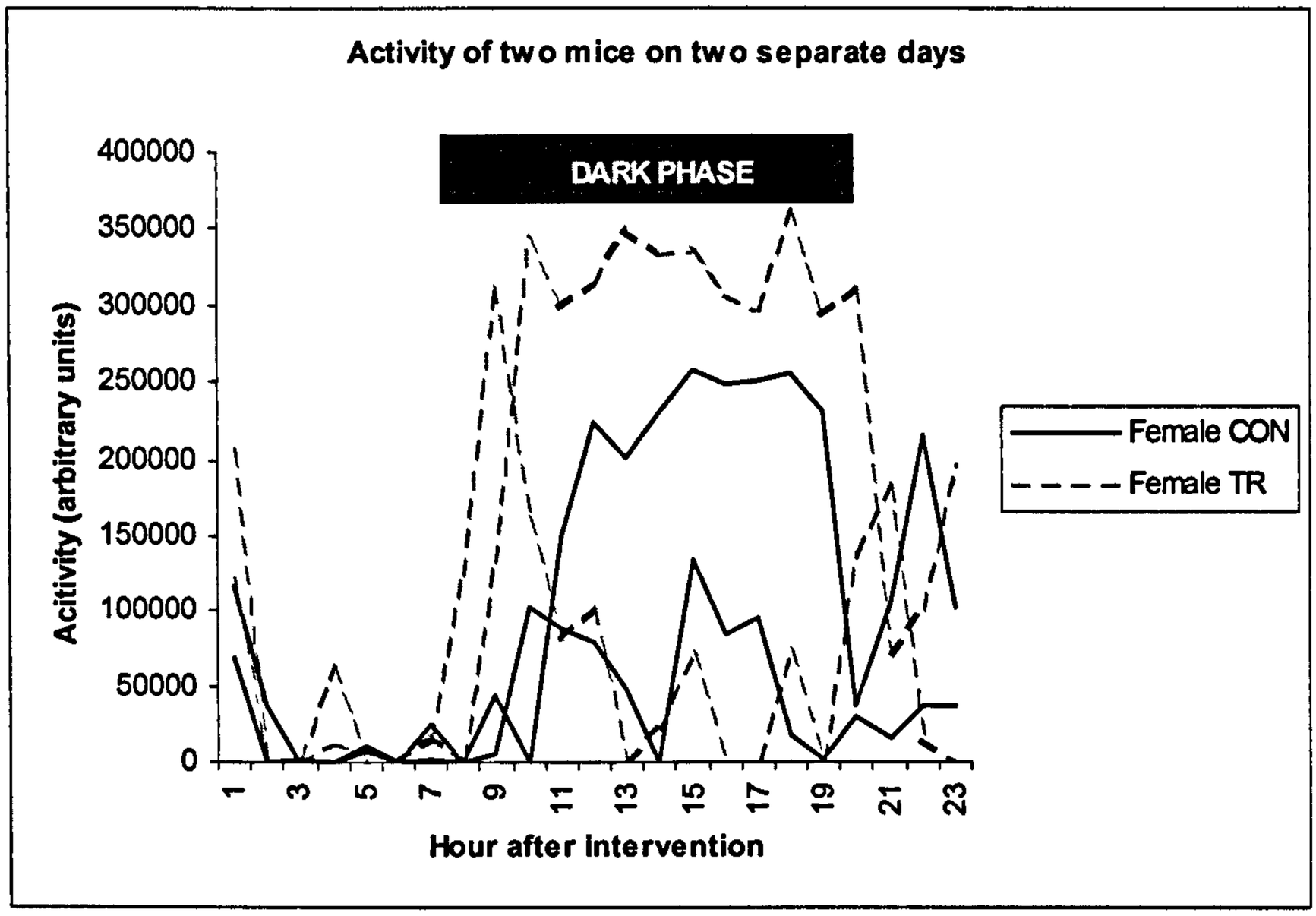


Figure 3.58 Inter- and intra-animal variation in cage activity

The NEPA variables analysed were total activity in 23 hours ("total 23h activity"); time spent sleeping (classed as zero activity); activity in the dark period (12h) and activity in the light (11h).

Analysis of activity – Treadmill v. Control mice

The difference in total activity in 23h was highly significant ($P = 0.001$) between the treatment groups, with TR mice recording 32% more activity during the recording period than CON mice (Table 3.80). The bulk of this activity appears to have been during the dark phase, where TR mice were 40% more active ($P < 0.001$) than CON mice. There was no difference in activity during the light phase ($P = 0.687$) or in time spent sleeping ($P = 0.327$, Table 3.80).

Table 3.80 Activity measurements (arbitrary units x10^3) by treatment group (mean and 95% CI)

Activity variable	Treadmill	Control	Probability
Total 23h activity^{Log10}	984	671	0.001
	849 - 1 141	575 - 785	
Light phase ^{Log10}	140	151	0.687
	110 - 180	117 - 195	
Dark phase ^{Log10}	752	454	<0.001
	646 - 874	388 - 532	
Sleep (min)	649.0	672.3	0.327
	616.9 - 681.1	639.7 - 704.9	

For all aspects of the activity recording, female mice were more active than males (Table 3.81). Male mice recorded just 38% of the activity in 23h as females (P<0.001) and the female mice were significantly more active in both light and dark periods (P = 0.006 and <0.001, respectively). This increased activity by female mice was associated with a significantly shorter time spent sleeping than for male mice (579 v. 722 min, P<0.001 Table 3.81).

Table 3.81 Activity measurements by sex (arbitrary units x10^3) (mean and 95% CI)

Activity variable	Female	Male	Probability
Total 23h activity ^{Log10}	1 442	552	<0.001
	1 223 – 1 700	477 - 640	
Dark phase ^{Log10}	1 148	366	<0.001
	963 - 1 368	316 - 425	
Light phase ^{Log10}	199	116	0.006
	152 - 260	92 - 146	
Sleep (min)	578.5	722.4	<0.001
	544.1-612.9	692.6-752.3	

There were interactions between sex and exercise protocol for total activity (Figure 3.59a), dark activity and time spent sleeping (P = 0.016, 0.020 and 0.044 respectively). On all occasions female mice were more active than males, and

treadmill mice were more active than controls, but the difference in 23h activity was greater between male CON and TR mice than their female counterparts. Male CON mice slept more than male TR mice (762 v. 698min), whereas female CON slept less than female TR mice (567 v. 599min, Figure 3.59b).

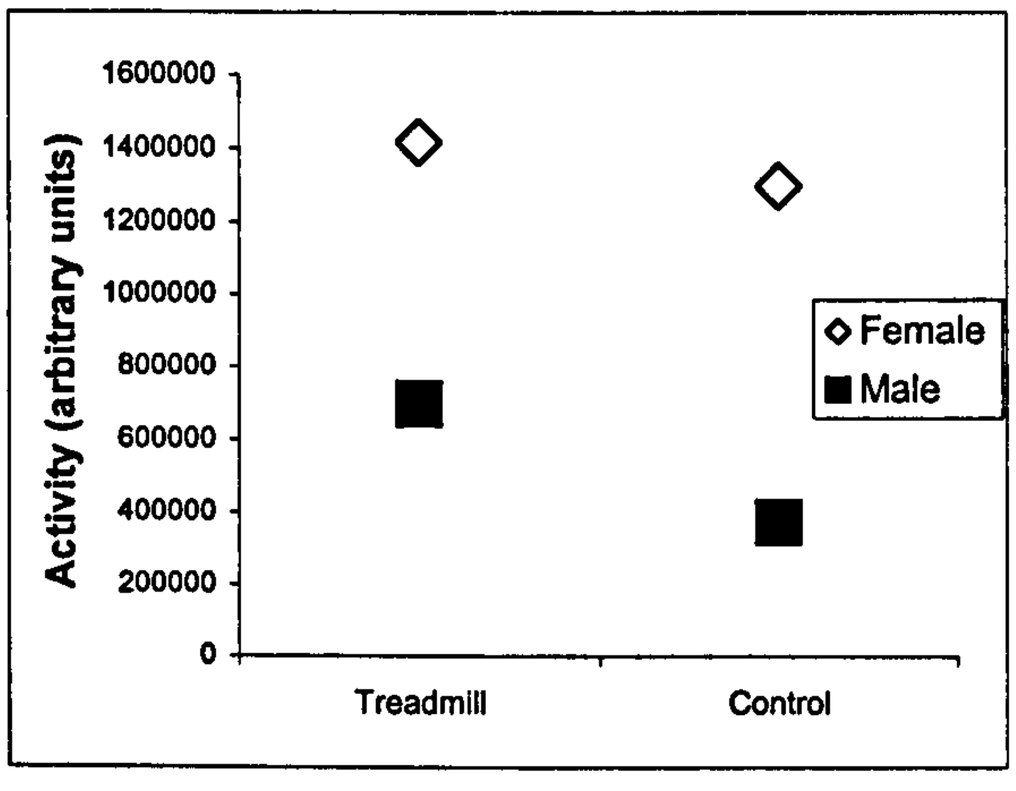
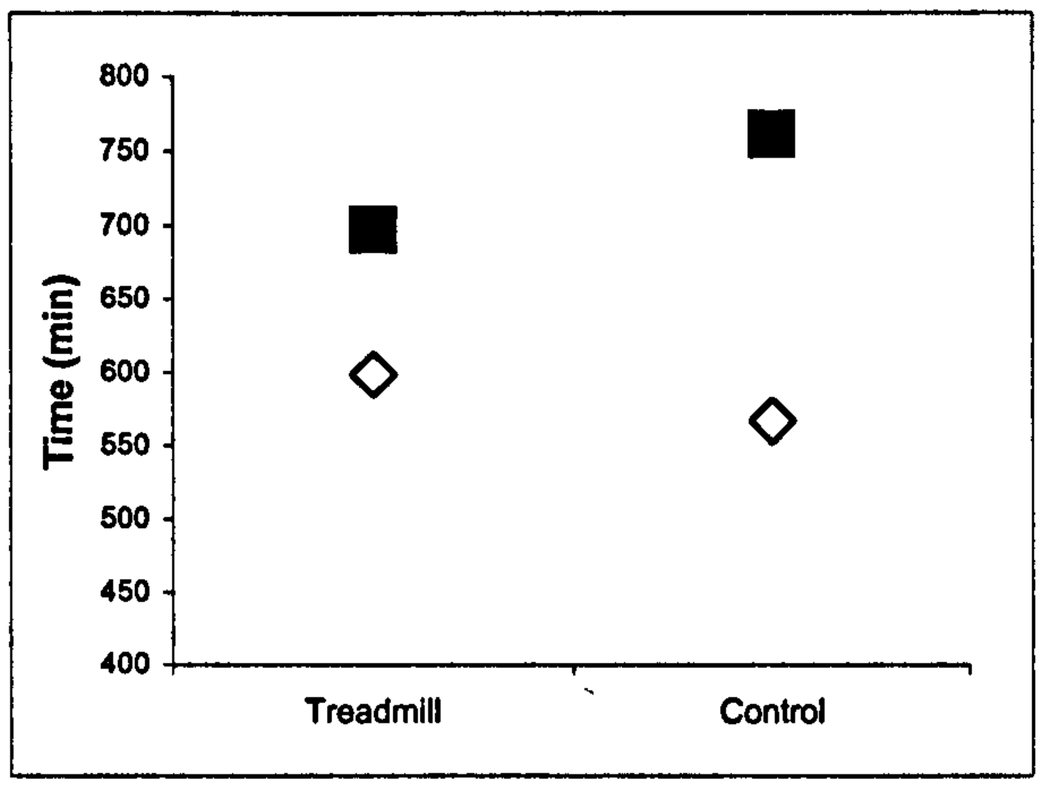


Figure 3.59a) Interaction between sex and exercise on total activity in 23h (geometric means)



b) Interaction between sex and exercise on sleep times

Analysis of activity – Wheel v. Control mice

Total activity was significantly greater in CON compared with WH mice (803 $x10^3$ v. 256 $x10^3$counts, P <0.001), as was activity in both the light and the dark phases (P<0.001 for both, Table 3.82). WH mice slept for less time than CON and the difference approached statistical significance (609 v. 655 min, P = 0.065).

Table 3.82 Activity measurements of WH v. CON (arbitrary units $x10^{3)}$ (medians and range)

Activity variable	Wheel	Control	Probability
Total 23h activity[KW]	257	803	<0.001
	10 - 884	35 - 4 091	
Light phase[KW]	33	178	<0.001
	20 - 661	1 - 227	
Dark phase[KW]	215	605	<0.001
	15 - 3 719	8 - 795	
Sleep (min)	609	655	0.065

The direction of significance for activity when measured according to sex did not alter from the TR v CON measurements, with females more active (P<0.001) at all periods except the light (P = 0.238) and sleeping for a shorter amount of time (P<0.001). There was a slight interaction between sex and exercise on sleep time (P = 0.081), where sleep times were similar in females of both treatment groups, but male CON mice spent more time asleep than male WH.

3.3.4.11 Stress marker (ADH mRNA expression)

Only 9 intact brains, all from male mice, were available for analysis. There was no difference between the exercise groups (Table 3.83), although TR mice had the lowest optical density, and therefore the lowest amount of expression.

Table 3.83 ADH mRNA expression in male mouse hypothalamus (mean and SEM)

Variable (nett optical density)	Treadmill (n = 2)	Wheel (n = 3)	Control (n = 4)	Probability	
				TR v CON	WH v CON
ADH	209.5	213.7	214.0	0.576	0.996
	3.8	3.1	2.7		

3.3.5 Summary

Table 3.84 Summary of results from the third experiment (P<0.05)

	Effect of exercise regime (compared with Control)	
Variable	**Treadmill**	**Wheel**
Body weight gain	No difference	No difference
Food intake	Increased	Increased
Tumours		
-colon	Decreased (0.061)	No difference
-other sites	No difference	No difference
Total number of tumours	No difference	No difference
Tumour burden	No difference	No difference
Intestinal organ weights (%)	No difference	No difference
Non-intestinal organ weights (%)		
-liver	No difference	No difference
-heart	No difference	Increased
-other organs	No difference	Increased
SCFA total concentration	Decreased	Decreased
-Acetate	Decreased	Decreased
-Propionate/Isobutyrate/butyrate/Isovalerate/valerate	No difference	No difference
SCFA molar proportion		
-Acetate	Decreased	Decreased
-Propionate	Increased (0.059)	Increased
-Isobutyrate/isovalerate	Increased	Increased
-Butyrate/valerate	No difference	No difference
Transit time	No difference	No difference
NEPA		
-total	Increased	Decreased
-dark phase	Increased	Decreased
-light phase	No difference	Decreased
-sleep time	No difference	Decreased
Stress markers - ADH	No difference	No difference

3.3.6 ES3-specific discussion

3.3.6.1 Treadmill performance

The altered treadmill protocol saw "just" 7 of 31 mice dropping out, with most then running every day. This is a vast improvement on the previous studies and could be due to either the speed being increased more quickly in this study (though previous performances suggest that is not always a factor) or the reduced duration of daily running sessions. The performance of the male mice was particularly surprising, given their previous form. There is evidence to suggest that strain may play a part in this, as Lerman et al. (2002) reported that male C57BL/6 mice, out of six strains of mice, ran slowest and stopped most often during a treadmill performance test. Conversely, the C57BL/6 mice ran further and faster in running wheels than the other strains (Lerman et al., 2002). The next challenge will be to have mice running quickly **and** for a longer time. The time may continue to be a problem, as observations of the wheel-running mice in this study showed that they tended to run in short bursts, a behaviour also reported by De Bono et al., (2006) and found to last ~150s at an average speed of 3km/h, considerably faster than most treadmill protocols, which rarely go beyond 1.2km/h. These combined observations offer an explanation for the poor treadmill performance of some mice.

3.3.6.2 Wheel-running

Unlike the previous study (ES2), female mice did not perform significantly better than male mice on the wheels, although females consistently ran further. The daily average distance was less than in ES2 (2.63km v. 3.46km), with most of the decrease due to less running by the females mice (down from 4.53km in ES2 to 3.32km in ES3, whereas male mice ran 2.1km in ES2 and 1.95km in ES3). The increases in running distance observed in ES2 when the large wheels were present also occurred in this study. Large variation between animals was also present, which results in heterogeneous exercise exposure. This could possibly be avoided by selecting male mice that run well and successively breeding from them, as running performance can be selected (Swallow et al., 1998a). Mice selected for high running ability were found

to have greater VO_{2max} than sedentary controls (Swallow et al., 1998b), indicating improved cardiorespiratory responses. The genes responsible for high running ability in a comparable rat model were investigated using a microarray approach, with samples from the left ventricle (Lee et al., 2005). The authors found several potential candidates to be upregulated, including those in energy pathways and glucose uptake, which may produce more efficient glucose utilisation and muscle contractions, increasing cardiac performance (Lee et al., 2005). Animals with this phenotype might produce a less varied response to the running wheel, and might permit a dose-response analysis of tumourigenesis.

3.3.6.3 Organs

Adrenal and thymus glands were measured as a marker of activity of the hypothalamic-pituitary-adrenocortical (HPA) axis. The left adrenal gland is known to be larger than the right, a finding borne out by this study. Other researchers have found that exercise abolishes this difference due to an increase in size of the right adrenal gland (Droste et al., 2003). This indicates an increase in HPA axis activity (though should not be confused with the stress-like changes caused by forced or endurance exercise), and is due to the increase in glucocorticoid levels at the start of the dark phase, when most wheel running activity took place (Droste et al., 2003). A decrease in thymus weight was also observed by this group, again due to the increase in glucocorticoid secretion.

However, no such changes in right adrenal size or reduction in thymus weight were observed in this study and it might be useful to measure glucocorticoid levels in future studies, to see if they are comparable with studies where an alteration in adrenal size was observed.

3.3.6.4 Parental influence on offspring tumours

The observation of 3 siblings each with an unusually large number of tumours prompted the investigation of the variation in tumourigenesis between offspring of different sires. The finding that the father had a huge influence on the tumour number

and burden of its offspring is perhaps not too surprising given the fully penetrant autosomal dominant nature of Min inheritance. The finding may have an effect on studies where the mean number of tumours produced is quite low, as results could be skewed by a few mice bearing many more tumours than the rest of the study population. Knowing how many tumours the fathers had could be vital information for controlling the variability inherent in studies such as this. The offspring of sires with similar numbers of tumours could be used to maintain the colony, and perhaps generate a colony where the numbers of tumours produced by each mouse were more similar. The effect of mothers was not examined, but they may pass on modifier genes (such as *Mom1* or *Mom2*, discussed in Chapter 1.7.8) to their offspring, a hypothesis that could be tested by genotyping.

3.3.6.5 Transit time

Transit time has not been measured in animal exercise studies before, though it has been mooted as a possible reason for decreased incidence of colon cancer in the physically active, as potential carcinogens are in contact with the mucosa for shorter periods of time (Quadrilatero & Hoffman-Goetz, 2003). The human studies are not all directly comparable as some measured colon transit (Liu et al., 1993), others measure mouth-to caecum transit (Scott & Scott, 1994) and others measure total transit (Robertson et al., 1993).

In this study, median caecal transit time was unaffected by the exercise protocol, though the range was smaller for TR compared with WH and CON. It may be more valuable to measure total transit in these mice, as most tumours were generated in the small intestine, and none in the caecum. Another potential problem comes from the small amounts of caecal matter available and another method may be more suitable, such as the use of indigestible markers.

3.3.6.6 NEPA

As NEPA has not been measured before on exercising animals, the results of this experiment were particularly exciting, and could be interpreted in several ways.

Treadmill mice were significantly more active than Control mice, and this was in some ways surprising, as it was thought that after being made to run for 30 minutes the mice would perhaps decrease the amount of activity in their home cage. Such a compensatory change in NEPA could have accounted for the lack of difference in tumour number in response to treadmill running. If the Control mice had been more active, it could have been due to having been confined to a control lane for 30 minutes.

The use of a computerised tracking system allowed de Visser et al., (2005) to separate time spent running in a wheel from time spent on the cage floor or sheltering. Mice with access to a running wheel spent significantly less time on the cage floor than non-running counterparts, but had a greater total cage activity (including wheel running). The InfraMot system used in this study was not able to distinguish between wheel-running and other activity, and may even have been unable to monitor wheel activity if the infrared sensor was blocked by the wheel. The observation of increased activity by CON compared with WH compares favourably with those of de Visser et al (2005). Unfortunately, a literature search has not yielded similar evidence regarding treadmill-running and cage activity.

People who undertake physical activity in their leisure time are known to have a healthier lifestyle in general (Schroder et al., 2003), and those who take exercise may be more active generally, though studies do not distinguish between these types of "leisure" activity. A year-long exercise intervention in post-menopausal women found that adherence to the protocol was increased in women who were already intending to start exercising, or who had undertaken physical activity in the 3 months prior to randomisation (Irwin et al., 2004).

Female mice were more active than males in all categories, and the shorter sleep time may reflect this. Whether this was due to increased time spent nest building or on general cage activity cannot be determined by this experiment, and others measuring voluntary activity (de Visser et al., 2005) have used only female mice, although De Bono et al., (2006) reported that the increased wheel running of female mice compared with male mice happened in the second half of the dark period, and may suggest that males were sleeping at this time.

3.3.6.7 Stress markers

The number of mice in this analysis was smaller than intended, however the technique worked well and can be used in future studies. Despite the small number of samples, the lack of difference in ADH expression between exercising and control mice suggests that neither the treadmill nor the wheel running produced a stress response in the mice. Stress activates ADH neurons in the paraventricular nucleus of the hypothalamus, increasing circulating concentrations of ADH (Genuth, 1998). This ultimately causes an increase in plasma cortisol concentrations, resulting in increased blood pressure, cardiac output and increased glucose utilisation by the central nervous system (Genuth, 1998). The lack of stress response suggests that treadmill-running should not produce any adverse effects in this group of mice.

3.4 Combined results

Data from the three studies involving the treadmill were pooled to give greater statistical power and re-analysed. To assess the effect of the particular study on the outcome, "study" was included as a fixed factor in the analysis, as each study varied slightly from the others. Where a variable category contains transformed data, mean and 95%CI are given; where data from a category were all normally distributed, mean and SEM. Data measured by non-parametric methods are presented as the median and the range.

The main outcome criteria of tumour load, food intake, body weight, organ weight, body composition and SCFA are discussed in detail.

3.4.1 Comparison of studies

3.4.1.1 Tumour data

The tumour data for all three studies are shown in Table 3.85. The number of tumours generated by each study was relatively stable, with the exception of the increase in proximal SI tumours observed in study 3 (P=0.007). The was a slight increase in the total number of tumours generated (P = 0.063), with mice in the first study having

fewer tumours than mice in the second study, and mice in the third study having more tumours still (10.9, 13.5 and 15.2 tumours, respectively).

Table 3.85 Combined tumour data, by study

Variable	Study group			Probability of difference between studies
	Study 1	Study 2	Study 3	
Tumours	**n = 18**	**n = 62**	**n = 75**	
Proximal^{Sqrt}	5.5	5.5	7.4	
	4.1-7.2	4.7-6.4	6.5-8.3	0.007
Distal SI^{Log10}	5.1	7.1	7.5	
	3.6-7.3	5.9-8.6	6.3-8.9	0.160
Total^{Log10}	10.6	12.9	14.7	
	8.1-13.8	11.2-15.0	12.9-16.6	0.073
Colon^{KW}	0.0	0.0	0.0	
	0-1	0-3	0-5	0.258
Total number^{Log10}	10.9	13.5	15.2	
	8.4-14.2	11.7-15.5	13.4-17.3	0.063
Burden (mm diameter)^{Log10}	32.1	35.5	34.8	
	25.1-41.2	31.0-40.6	31.0-39.2	0.791

Data shown are mean and 95% CI except for colon, which are the medians and ranges

3.4.1.2 Weight gain, food intake and body composition

The amount of weight gained by the mice in each study remained constant, with the slight increase shown by mice in study 1 reflecting the longer study (12 weeks as opposed to 10). However, the average food intake decreased with each study, even though the mice were fed the same diet (Table 3.86).

With respect to body composition, there was a significant effect of study on water, mineral and non-fat organic matter proportions, with body water higher in study 1 mice and mineral and non-fat organic matter greater in study 2 mice (Table 3.87, body composition was not analysed in study 3 mice). However, the proportion of fat in the mice was of most interest, and this variable was unaltered between studies.

Table 3.86 Combined weight and food intake data, by study

Variable	Study group			Probability of difference between studies
	Study 1	Study 2	Study 3	
	n = 18	n = 62	n = 75	
Total weight gain^{Sqrt} (g)*	8.4	7.9	7.2	
	6.73-10.29	6.98-8.85	6.42-8.00	0.203
Food intake over study (g/d)	2.39	2.18	2.09	
	0.05	0.02	0.02	<0.001

*** initial weight included as a covariate**

Table 3.87 Body composition of mice by study

Variable	Study group		Probability of difference between studies
	Study 1	Study 2	
Body composition (%)	n = 18	n = 56	
Water	55.9	52.3	
	1.49	0.85	0.045
Fat	29.6	25.7	
	2.26	1.29	0.155
Mineral	2.8	3.8	
	0.26	0.15	0.001
Non-fat organic matter ^{KW}	11.6	17.8	
	3.05-19.12	6.04-33.98	<0.001

3.4.1.3 Organ weights

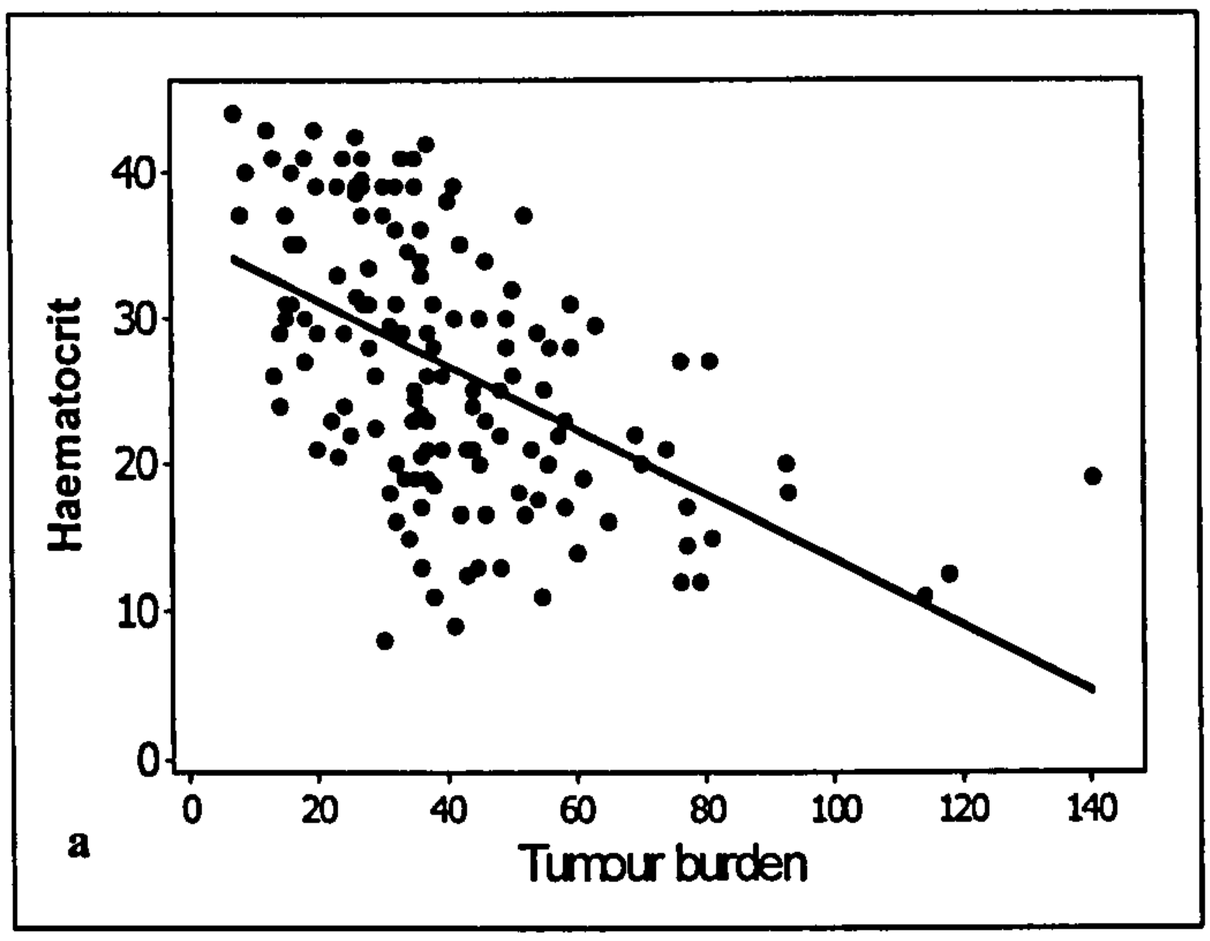
When expressed as % final body mass, liver weights for all studies were consistent, but mice in the third study had heavier hearts, which may reflect the increased number of mice running (Table 3.88). The spleens of mice from study 1 were significantly heavier than those of mice from study 2 (1.13 % v. 0.67%, P = 0.001). When spleen data was analysed with that of tumour burden and haematocrit, a heavier spleen was

associated with a reduced haematocrit (Figure 3.60c), and both spleen weight and haematocrit were predictive of tumour burden, with decreased haematocrits and increased spleen weights associated with a greater tumour burden (Figure 3.60a and b, $P<0.001$ and $P = 0.001$ respectively).

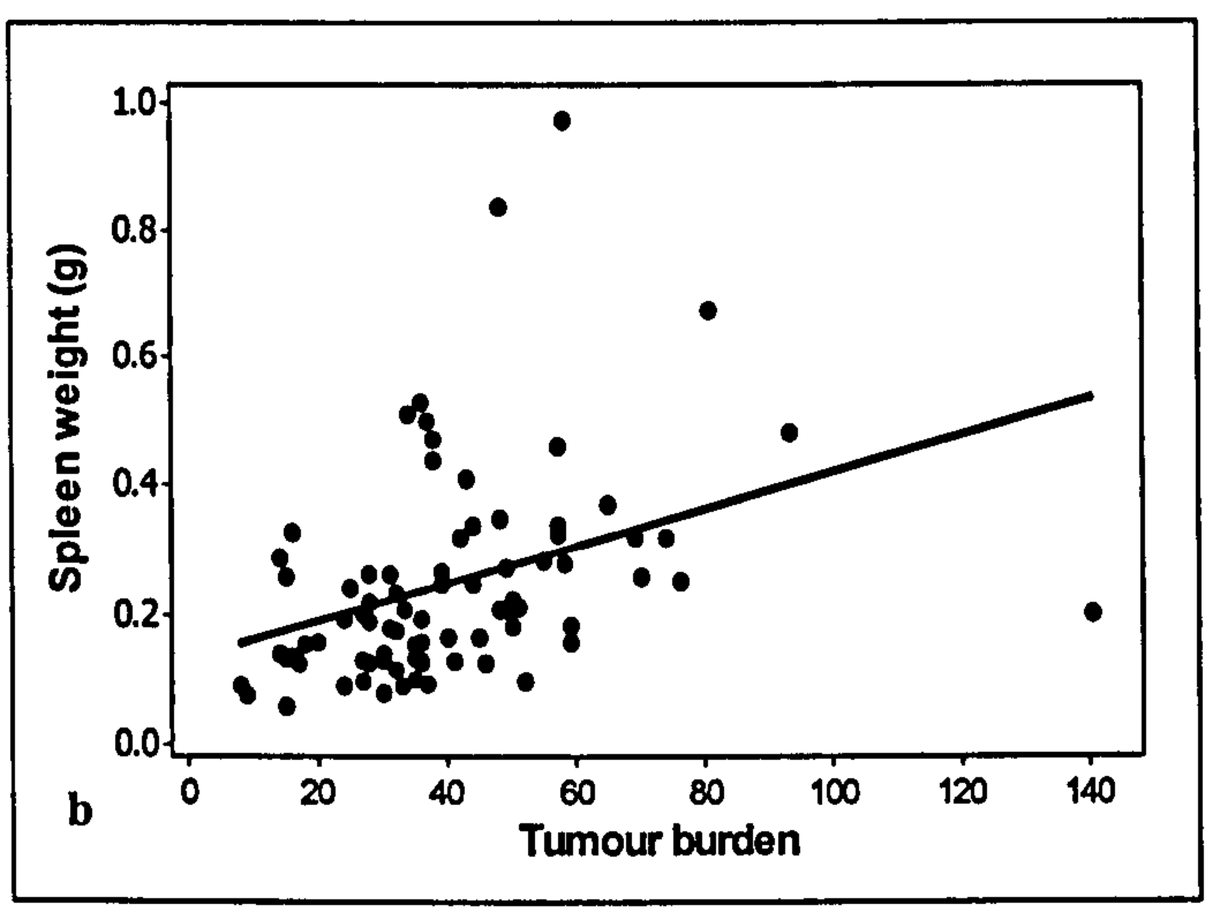
The regression equation is tumour burden = 73.6 - 1.25 *haematocrit. ($R^2 = 0.27$, $P <0.001$).

Table 3.88 Extra-intestinal organ weight data, by study (mean and 95% CI)

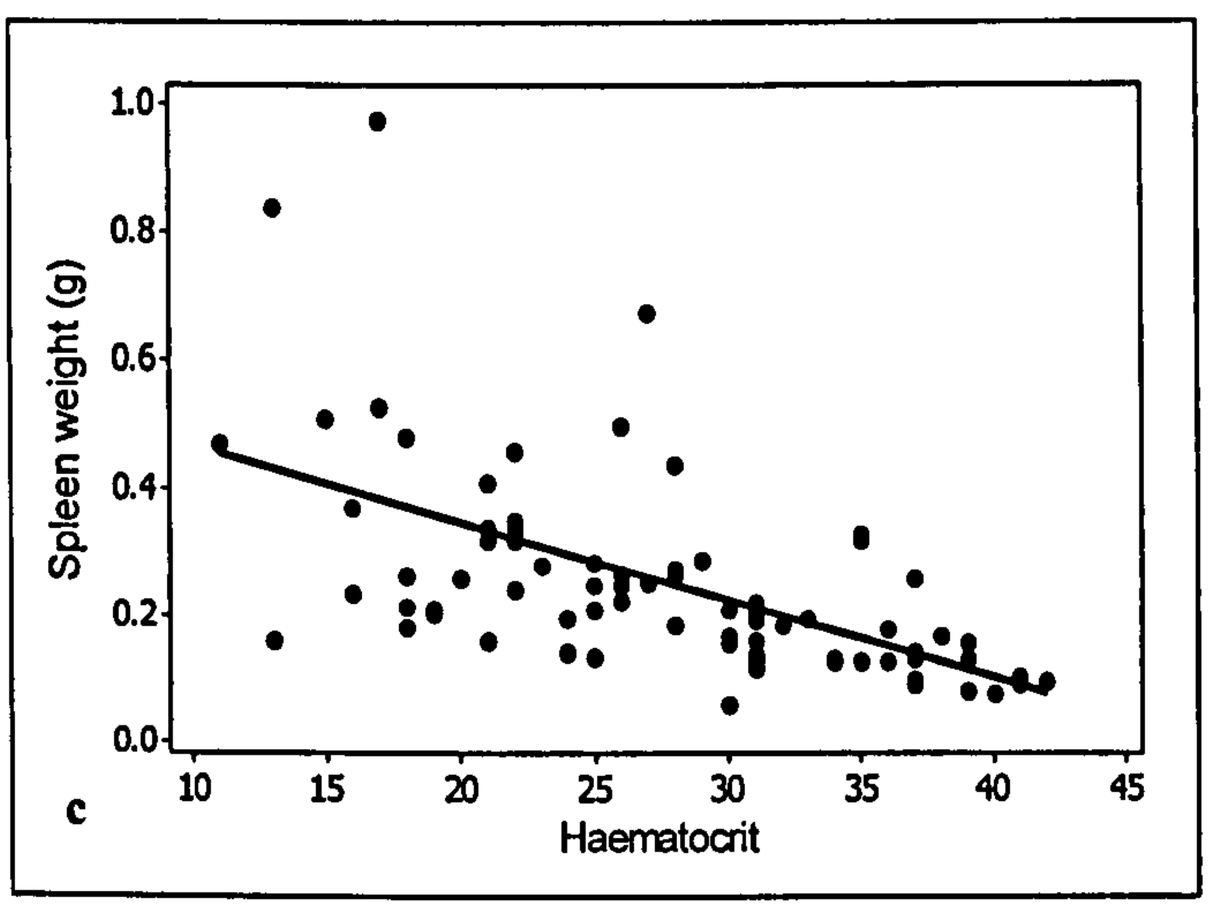
	Study group			
Variable	**Study 1**	**Study 2**	**Study 3**	**Probability of difference between studies**
Organ (% final body mass)	**n = 18**	**n = 62**	**n = 75**	
Liver	4.42	4.36	4.34	
	4.25-4.59	4.27-4.45	4.26-4.42	0.704
Heart	0.69	0.78	0.94	
	0.60-0.79	0.73-0.83	0.90-0.99	<0.001
Spleen ^{Log10}	1.13	0.67		
	0.87-1.46	0.58-0.77	-	0.001
Haematocrit (%)	26.3	29.1	26.0	
	22.4-30.2	27.0-31.2	24.1-27.9	0.086



r = -0.53
P<0.001
n = 154



r = 0.36
P = 0.001
n = 80



r = -0.60
P<0.001
n = 80

Figure 3.60 Relationships between (a) haematocrit and (b) spleen weight and tumour burden, and (c) between spleen weight and haematocrit, with fitted regression lines. N is less for (b) and (c) as spleen weights were not measured in ES3

3.4.2 Comparison of sexes

Sex differences occurred for several variables, some of which are presented here.

3.4.2.1 Tumour data

In general, tumour data for the sexes was similar (Table 3.89), and the only statistically significant sex effect was on colon tumours, where female mice had fewer than males (median 0.0 v. 1.0, P = 0.004), but the range was greater in females. The higher number of proximal SI tumours in females compared with males approached significance (6.8 v. 5.9, P = 0.072). All other variables were not different.

Table 3.89 Effect of sex on tumour variables

Variable	Sex		
	Female n = 87	Male n = 68	Probability of sex effect
Tumours			
Proximal[Sqrt]	6.8	5.9	
	6.1-7.6	5.1-6.7	0.072
Distal[Log10]	7.1	7.3	
	6.1-8.4	6.1-8.7	0.772
Total SI[Log10]	13.7	13.6	
	12.2-15.5	11.8-15.5	0.832
Colon[KW]	0.0	1.0	
	0-5	0-4	0.004
Total number[Log10]	14.2	14.3	
	12.6-16.0	12.5-16.4	0.945
Burden (mm diameter)[Log10]	34.9	36.0	
	31.3-39.0	31.8-40.7	0.718

Data shown are mean and 95% CI except for colon, which are the medians and ranges

3.4.2.2 Weight gain, food intake and body composition

Male mice were heavier than females during each study and also gained more weight (Table 3.90), although the food intake of the sexes was not significantly different, and neither was the composition of the carcasses.

Table 3.90 Total weight gain and average food intake, by sex

Variable	Sex		
	Female n = 87	Male n = 68	Probability of sex effect
Total weight gain^{Sqrt} (g)*	6.8 6.1-7.5	8.9 8.0-9.8	0.001
Food intake (g/d)	n variable but all 3 studies used		
Average intake over study	2.13 0.02	2.18 0.03	0.151
Body composition (%)	n = 43	n = 31	
Water	54.1 0.95	52.8 1.12	0.151
Fat	25.3 1.43	26.6 1.69	0.484
Mineral	3.9 0.18	3.4 0.21	0.065
Non-fat organic matter ^{KW}	17.5 3.05-19.12	17.8 6.04-34.00	0.814

*initial weight included as a covariate

3.4.2.3 Organ weights

In general, male mice had heavier organs than females, but when expressed as % final body mass, female mice had heavier organs than males (both intestinal and extra-intestinal). Liver weight was heavier in female mice compared with males, but there

were no differences in heart or spleen weight (Table 3.91), and haematocrit was significantly lower in male mice compared with females (P <0.001).

Table 3.91 Extra-intestinal organ weights of male and female mice (mean and 95% CI)

Variable	Sex		
	Female	Male	
Organ (% final body weight)*	n = 87	n = 68	Probability of sex effect
Liver	4.48	4.18	
	4.40-4.56	4.09-4.27	<0.001
Heart	0.85	0.84	
	0.81-0.90	0.79-0.89	0.553
Spleen	0.80	0.81	
	0.68-0.95	0.66-1.00	0.791
Haematocrit (%)	29.3	23.7	
	27.5-31.1	21.6-25.7	<0.001

*** all Log10 transformed**

3.4.2.4 SCFA

Colonic SCFA concentrations were mostly not different between the sexes, with acetate concentration the only statistically significant difference between the two (P = 0.001), with a greater concentration observed in male mice samples compared with females (23.01 v. 17.54 μmol·g colon contents). Male samples also had a greater concentration of total SCFA (28.31 v. 23.01 μmol·g colon contents, P = 0.007). Several differences in molar proportion of colonic SCFA existed, with male mice having a greater proportion of acetate than female mice (P = 0.031), and a corresponding decrease in the other SCFA, significantly so for butyrate, isovalerate and valerate (Table 3.92).

Table 3.92 Colonic SCFA (mmol/mol total SCFA) of male and female mice

Variable	Sex		
	Female	Male	
SCFA* (molar proportion)	n= 72	n = 62	Probability of sex effect
Acetate	773	802	
	490.5-891.5	658.6-918.0	0.031
Propionate	120	111	
	40.4-372.4	40.6-213.2	0.223
Isobutyrate	14	14	
	0.0-36.2	5.9-28.7	0.288
Butyrate	54	34	
	0.0-453.9	0.0-172.1	0.038
Isovalerate	23	20	
	21.5-25.2	18.2-21.6	0.008
Valerate	15	11	
	1.0	1.1	0.023

*** all Kruskal Wallis test except isovalerate – Log10 transformed, and valerate – normally distributed, therefore median and range displayed for acetate, propionate, isobutyrate nad butyrate, mean and 95% CI for isovalerate, and mean and SEM for valerate**

3.4.3 Effects of treadmill- and wheel-running exercise

3.4.3.1 Tumour data

The treadmill protocol affected many of the tumour variables (Table 3.93), with decreases in distal SI, total SI, colon and total number of tumours and also tumour burden (P values of 0.03 – 0.062) compared with control mice. There was no significant (P>0.05) effect of wheel running on any of the measured variables.

Table 3.93 Effect of exercise protocol on tumourigenesis

Variable	Treatment group			Probability of effect	
	Treadmill	Wheel	Control		
Tumours	n = 41	n = 49	n = 65	TR v CON	WH v CON
Proximal^{Sqrt}	5.6	6.7	6.0		
	4.6-6.7	5.6-8.0	5.2-6.9	0.814	0.469
Distal^{Log10}	5.6	6.3	7.7		
	4.4-7.1	4.9-8.0	6.4-9.4	0.062	0.587
Total SI^{Log10}	10.8	13.2	14.0		
	9.1-12.9	9.6-18.2	12.1-16.2	0.052	0.837
Colon^{KW}	0.0	0.0	1.0		
	0.0-3.0	0.0-5.0	0.0-4.0	0.049	0.213
Total number^{Log10}	11.1	13.7	14.6		
	9.3-13.2	11.4-16.5	12.6-16.9	0.030	0.798
Burden (mm diameter)^{Log10}	29.9	34.8	38.1		
	25.3-35.2	29.4-41.3	33.2-43.7	0.041	0.587

Data shown are mean and 95% CI except for colon, which are the medians and ranges

3.4.3.2 Weight gain, food intake and body composition

Wheel mice were on average 0.8g heavier than control mice at the start of the project ($P = 0.036$), but CON mice gained more weight overall than either TR or WH mice although there was no significant ($P>0.05$) effect of exercise regime on total weight gain when using initial weight as a covariate (Table 3.94).

Food intake when averaged over the whole study was not significantly different between TR and CON, but WH mice ate significantly more than CON mice ($P<0.0001$, Table 3.94).

Body composition was similar in all treatment groups, although the proportion of non-fat organic matter was less in TR mice compared with CON ($P<0.001$, Table 3.94).

Table 3.94 Weight gain, food intake and body composition of mice by exercise group

Variable	Treatment group			Probability of effect	
	Treadmill	Wheel	Control		
	n = 41	n = 49	n = 65	TR v CON	WH v CON
Total weight gain^{Sqrt} (g)*	7.57	7.65	8.27		
	6.49-8.73	6.51-8.87	7.32-9.26	0.618	0.597
Average food intake over study (g/d)	2.15	2.37	2.14		
	0.03	0.03	0.03	0.869	<0.0001
Body composition (%)	n = 16	n = 32	n = 26		
Water	52.4	56.2	53.7		
	1.48	1.09	1.46	0.748	0.235
Fat	31.7	24.3	27.1		
	2.25	1.65	2.21	0.234	0.447
Mineral	2.9	2.5	3.4		
	0.26	0.19	0.26	0.277	0.916
Non-fat organic matter^{KW}	15.3	17.6	17.8		
	3.05-20.03	3.65-28.25	14.68-33.98	<0.001	0.274

*** initial weight was included as a covariate**

3.4.3.3 Organ weights

There was no effect of the treadmill protocol on any of the organs measured, with the exception of the spleen, which was lighter than in CON mice (P = 0.0003, Table 3.95). Wheel mice had heavier proximal SI portions, heavier livers and lighter spleens than CON mice (P values from <0.001 to 0.035).

Both WH and TR mice had a greater haematocrit than CON mice (P = 0.013 and 0.004, respectively, Table 3.95).

Table 3.95 Extra-intestinal organ weights by exercise protocol (mean and 95% CI)

Variable	Treatment group			Probability of effect	
Organ (% final body mass)	Treadmill n = 41	Wheel n = 49	Control n = 65	TR v CON	WH v CON
Liver	4.32	4.57	4.23		
	4.21-4.43	4.46-4.68	4.14-4.32	0.361	<0.001
Heart	0.80	0.84	0.79		
	7.32-0.86	0.77-0.90	0.73-0.84	0.951	0.300
Spleen ^{Log10}	0.64	0.85	1.20		
	0.50-0.81	0.66-1.10	0.99-1.44	0.0003	0.035
Haematocrit (%)	29.2	28.2	23.9		
	26.6-31.8	25.5-30.9	21.8-26.0	0.004	0.013

3.4.3.4 SCFA

Significant differences in individual SCFA concentration between exercise groups occurred only for acetate, with samples from CON mice having a greater concentration than those from WH mice (23.6 v. 19.2 μmol·g colon contents, P = 0.016). CON mice also had the greatest total concentration of SCFA in their samples, significantly greater than WH mice only (P = 0.046).

There were differences between exercise groups for the molar proportions of all the colonic SCFA measured except valerate Table 3.96). Acetate proportion was greater in CON samples compared with both TR and WH mice (P = 0.020 and 0.006 respectively). For propionate, isobutyrate and isovalerate, CON samples had significantly smaller proportions than WH samples, whilst TR samples had a greater proportion of butyrate than CON mice (P = 0.002) and WH mice samples (Table 3.96).

Table 3.96 Colonic SCFA (mmol/mol total SCFA) of mice by exercise group

Variable	Treatment group			Probability of effect	
SCFA* (molar proportion)	Treadmill n = 34	Wheel n = 52	Control n = 48	TR v CON	WH v CON
Acetate	782	773	809		
	490.5-848.6	506.4-918.0	658.6-908.3	0.020	0.006
Propionate	111	125	106		
	40.3-211.9	40.6-372.4	56.6-168.7	0.424	0.001
Isobutyrate	14	15	12		
	6.5-36.2	5.9-33.3	0.0-28.7	0.194	0.002
Butyrate	61	47	36		
	14.1-453.9	0.0-186.3	0.0-110.8	0.002	0.192
Isovalerate	19	22	18		
	17.3-21.5	19.9-23.7	16.5-20.3	0.668	0.018
Valerate	12	12	10		
	1.4	1.1	1.4	0.286	0.153

*** all Kruskal Wallis test except isovalerate – Log10 transformed, and valerate – normally distributed, therefore median and range displayed for acetate, propionate, isobutyrate nad butyrate, mean and 95% CI for isovalerate, and mean and SEM for valerate**

3.4.3.5 Wheel running

When the second and third studies were combined, female mice ran, on average, more than males, as shown in Table 3.97.

Table 3.97 Combined wheel-running data

Distance variable (km)	Females n = 26	SEM	Males n = 23	SEM	Probability
Daily distance	3.94	0.37	2.03	0.39	0.001
Total (10weeks)	274.3	25.23	139.2	26.79	0.001
Small wheel	99.04	9.24	47.76	9.81	<0.001
Large wheel	175.26	20.20	91.42	21.45	0.007

Mice from the third study ran further than those in the second study, though the difference was statistically significant only for the distance run in the small wheel, with mice in the second study running 98km and those in the third study running 49km (P = 0.001).

There was no correlation between total running distance and tumour number (r = -0.070) or with distance and tumour burden (r = -0.088).

3.5 Discussion - Investigations into treadmill- and wheel-running exercise

3.5.1 Comparison of studies

Although differences between studies existed for some variables, in general the main variables of interest were constant (tumours, body weight gain, body fatness, SCFA).

3.5.1.1 Growth, body composition and food intake

There were small differences between individual studies in the amount of weight gained, as displayed in Table 3.98. At first glance it is perhaps surprising that there were no significant differences in body weight between the groups, given the extra energy expenditure of the TR and WH mice.. In both ES2 and ES3, WH mice gained slightly less weight in week 6 than in the weeks before or after. This corresponds with the week that the wheels were changed, and the distances covered increased, indicating that perhaps the mice had not altered their energy intake accordingly.

Table 3.98 Total weight gained (g) by each treatment group in each study

Study	Treadmill	Wheel	Control
ES1	9.2	-	11.1
ES2	9.2	8.3	8.1
ES3	7.2	7.1	8.1

N.B. ES1 was a 12 week study, ES2 & ES3 were 10 weeks

The weight of 37/155 mice decreased in the final 1-3 weeks of the study and was associated with an increased total number of tumours (mean 18.8 compared with 13.1 for mice that had not lost weight, P = 0.001), a decreased haematocrit (19.7 v. 29.0, P <0.001), and an increased spleen weight (0.325 v. 0.231g, P = 0.044, ES1 and ES2 only). Taken together, these observations suggest that the weight loss is indicative of tumour formation. As with many human cancer patients, Min mice develop cachexia, where both fat and muscle tissue is lost (Mehl et al., 2005a). The presence of tumours is thought to cause cachexia by a variety of mechanisms, including physical obstruction, metabolic disturbances of various organs, an increase in resting energy expenditure and decreased appetite (Van Cutsem & Arends, 2005), the last of which was often observed concurrently in the mice that lost weight.

For reasons unknown, mice in ES1 ate more than those in ES2 or ES3. The diet was the same so should not have been more "palatable" and the environmental conditions were identical.

3.5.1.2 Treadmill-running

Treadmill performances increased dramatically from ES1 to ES3, with many more mice adhering to the protocol for the length of the study and running at greatly increased speeds, probably helped by the shorter duration of running in ES3. The number of male mice completing the treadmill protocol in ES3 was especially pleasing, given their previous poor performances. Initially (ES1 and ES2), mice were run quite slowly in the training periods, as due to their small size it was thought that to force strenuous exercise on them might cause injury, and indeed, several damaged toenails were suffered. However, reports from others of fatalities on the treadmill (Colbert et al., 2003), were thankfully not experienced. Given the improved performance in ES3, it would seem acceptable to run the mice at faster speeds when they are young, provided the time is increased steadily and the mice observed for injuries.

The mice were all run during the light, although they are nocturnal. This may have accounted for some of the poor performances, but as they were run at the time when normal cleaning and feeding routines were being carried out, they would have been disturbed at this time anyway. In the studies by Mehl et al., (2005b) and Colbert et al. (2003), mice were run under red lights in the dark cycle, but both studies produced

mice that could not finish the protocol, suggesting that running with the lights on is not a significant factor in poor running performance.

As mentioned in the Discussion to ES3, mouse strain may affect treadmill-running performance, with C57BL/6 mice performing badly in a treadmill test but performing well in running wheels, compared with six other strains of mice (Lerman et al., 2002). The differences in treadmill performance between male and female C57BL/6 mice have also been reported by Konhilas et al., (2004), with females reaching greater maximum speeds and stopping less frequently than male mice. This may influence future studies with respect to selection of sex/strain, or expectation of homogeneous exercise exposure.

3.5.1.3 Wheel running

Wheel-running performances, though varied, exceeded expectations and are comparable with those of other studies of C57BL/6JMin mice (Table 3.99).

Most mice responded well to the presence of the wheel, though some ran very little. However, as part of the purpose of the wheel was to offer a voluntary, non-stressful alternative to the treadmill, this inter-animal variation has to be accepted.

Table 3.99 Comparison of wheel-running distances (km/d) by male and female mice

Study	Female	Male
ES2	4.5	2.1
ES3	3.3	1.9
Colbert et al. (2003)*	3.1	2.6
Mehl at al. (2005b)	3.5	4.7

***2-3wk quarantine period only**

The larger wheels seem to have suited the male mice better, and the female mice did not respond to the change to the larger wheels as well as the males, a consideration for future studies. Although the distance run by female mice decreased in ES3, combined distances were still greater for females compared with males. The reason for this is unknown, and while it is the converse of that observed by Mehl et al. (2005b), is in

accord with the findings of Colbert et al (2003) and Koteja, Swallow et al. (1999). In tests of voluntary wheel performance, female C57BL/6J mice run further and faster than males of the same strain (Konhilas *et al.*, 2004; De Bono *et al.*, 2006). Evidence for wheel-running performance in C57BL/6J mice is mixed, with some reporting almost 8km run per night by male mice (Lerman et al., 2002), significantly more than six other strains of mice, whilst others (Lightfoot et al., 2004) report approximately half that distance, placing C57BL/6J mice tenth out of 13 strains.

3.5.2 Comparison of sexes

3.5.2.1 Growth and food intake

As expected, male mice gained more weight than females, both overall and in each study, although as a % increase, both sexes gained the same amount of weight in ES3 (Table 3.100).

Table 3.100 Total weight gained (g) by male and female mice in each study

Study	Female	Male
ES1	7.1 (47%)	11.4 (62%)
ES2	7.5 (43%)	11.3 (51%)
ES3	6.9 (68%)	8.2 (68%)

N.B. ES1 was a 12 week study, ES2 & ES3 were 10 weeks

With respect to food intake, although male mice ate more than females in absolute terms, as a percentage of their body weight, females consumed 20% more food than males (10.3 v. 8.4 g/100g, $P<0.001$). This may reflect the increased NEPA in the home cage compared with males (with males recording just 38% of the activity of females).

3.5.2.2 Tumours

The only difference between the sexes in the combined analysis was in the number of colon tumours, as measured by non-parametric methods, with female mice suffering fewer colon tumours. However, colon tumours made up less than 4% of the total

tumours in this analysis, suggesting that the use of both sexes in the experiment should not have distorted the data.

3.5.3 Comparison of treadmill- and wheel-running groups

3.5.3.1 Growth, body composition and food intake

This investigation found no effect of either treadmill or wheel running exercise on body weight, both weekly and at death. This is in accord with Colbert et al. (2000) and Mehl et al. (2005b). In contrast, Colbert et al. (2003) found that treadmill-running mice were significantly lighter than non-running controls on weeks 5-8 of their 8 week study, and also at death.

Given the lack of difference in body weight between exercise groups, it is perhaps not surprising that there were no significant differences in body composition, although analysis in the study by Colbert et al. (2003) found that treadmill exercise decreased body fat and increased bone mineral density. Other studies in mice have reported decreased body fat with voluntary wheel-running (Swallow *et al.*, 2001; Droste *et al.*, 2003), decreased body fat mass with treadmill running (Bhattacharya et al., 2005), but others have reported no difference in carcass energy content after 6 weeks of wheel access (Bell & McGill, 1991). A complication in drawing conclusions from body composition analysis in Min mice was the weight loss experienced by many mice towards the end of the study. This cachexia (as mentioned previously) will decrease both fat stores and muscle tissue and may explain the difference between this study and the shorter study of Colbert et al (2003).

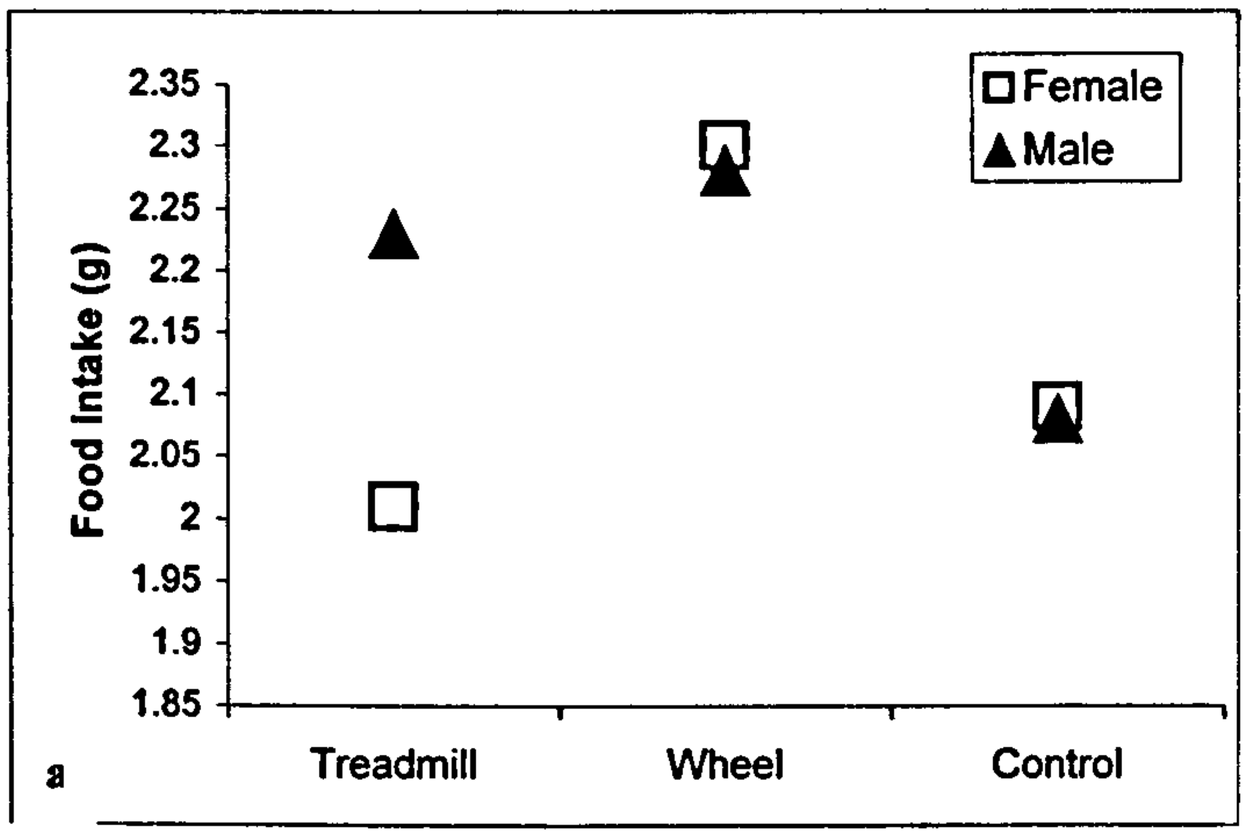
The exercise protocol influenced food consumption. In accord with the findings of other groups (Cohen *et al.*, 1988; Reddy *et al.*, 1988; Koteja *et al.*, 1999) wheel-runners consumed more food than either TR or CON mice. When considering the distances run each day by the WH mice, this is perhaps not surprising. In the week that the wheels were changed (week 6), the consumption of male WH mice dropped, which in addition to the increased distance run that week would account for the decrease in weight that week. The reason for the decreased intake could be due to the novel object in the cage causing stress, or the preference for running over eating that

week, as food intake increased again the following week, though this conjecture was not tested.

Given the increased energy expenditure of the TR mice, one might assume they would therefore consume more food. As they did not consistently eat more than the CON mice in ES1 or ES2, this suggests that either the energy content of the high fat diet was providing sufficient energy for their needs or that the mice did not expend more energy than the controls may have used on voluntary activity in the home cage. TR mice in ES3 consumed more food than CON, and this may be a reflection of the increased speeds, although the time spent running was much less than in ES1 and ES2. However, overall, TR mice did not consume more food than CON mice and this suggests that the treadmill protocol could be increased.

Neither study by Colbert and colleagues (2000 and 2003) reported food intake of the animals. However, the second study (Colbert et al., 2003) measured intake of control mice and pair-matched exercising animals in the following week to that amount "to prevent over-consumption" by the exercising mice. However, Reddy (1988) and Thorling (1993; 1994) found that exercising rats (both forced and voluntary) ate more food than their sedentary counterparts. Increases of 13, 22 and 25% more, respectively, were reported. Mehl and colleagues (2005b) reproduced the findings of this study with wheel running mice eating more than controls and treadmill runners eating a similar amount as controls (exact amounts were not reported), supporting the theory that wheel-running mice and rats undertake more activity compared with their treadmill-running counterparts.

An interaction between sex and exercise protocol existed for both food intake (g/d, $P = 0.009$)) and intake per 100g body mass ($P = 0.016$), with slightly different outcomes for the two measurements (Figure 3.61). Mean daily food intake was greater for TR males than females, and similar for CON and WH mice of both sexes, with WH mice eating more than the other groups. However, when expressed as g/100g mouse, female mice ate more than their male counterparts, with female WH eating more than the other groups (Figure 3.61), reflecting the differences in body mass between the sexes.



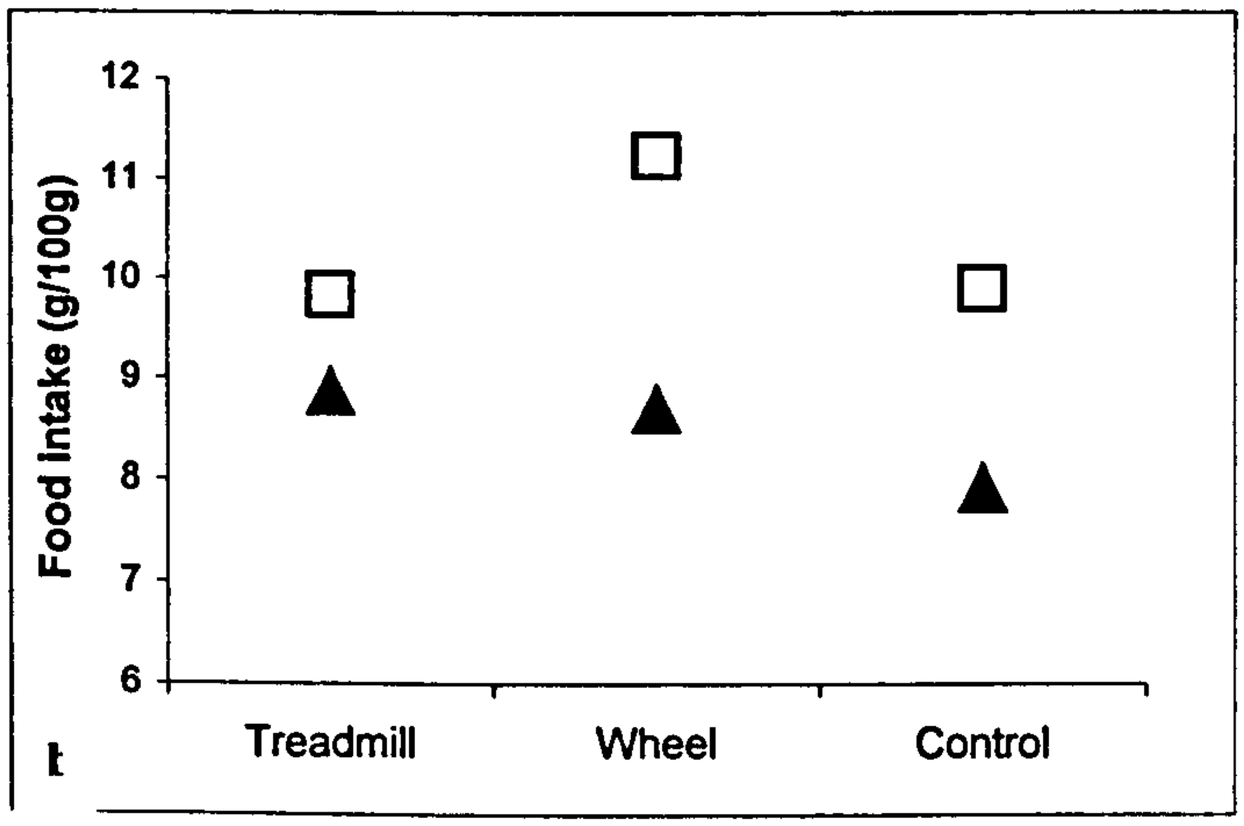


Figure 3.60 Interaction between sex and exercise protocol on mean daily food intake (a) g/d and (b) g/100g mouse

3.5.3.2 Tumours

The combined data for these studies found a slight reduction in tumour numbers for treadmill mice compared with controls, with TR mice having on average 11.1 tumours, compared with 14.6 for controls and 13.7 for wheel-runners. This was a surprising finding, as it might have been expected that given the variability in responses to the treadmill a decrease in tumour would not have been observed. More surprising was the lack of effect of the wheel-running protocol, as the increased distances run in wheels compared with those run on the treadmill might have been expected to be beneficial.

Table 3.101 compares the total number of tumours in the published studies of Min mice and exercise. Mehl et al. (2005b) found an effect of treadmill exercise on tumour

number in male mice, but not wheel-running. Treadmill runners suffered 29% fewer polyps than controls and had 38% fewer large polyps (classed as ≥1mm). The tumour numbers differed considerably both from those in our studies and those of the Colbert et al. studies (2000; 2003). Mehl et al., (2005b) reported that male treadmill runners had an average of 66 ± 9 polyps compared with 13 seen in this study, and male controls had 93 ± 7 tumours compared with 15 in this study. This contrasts with the numbers of tumours in the first study by Colbert et al. (2000), where the mean number of tumours for different treatment groups was between 25 and 41. The 2003 study generated 6-9 tumours/mouse. Mice from the 2000 study were bred in-house after purchase of sires from the Jackson Laboratories, the mice from the 2003 study were purchased from the Jackson laboratory. This disparity in tumour number reflects the slight genetic diversity that occurs with separation of breeding colonies, differing diets and exposure to various environmental challenges (Dove *et al.*, 1997; Newman *et al.*, 2001; Corpet & Pierre, 2003) and creates a problem when attempting to compare studies. The presence or absence of modifier genes (which can affect the severity of the Min phenotype (Dietrich et al., 1993) was not examined in this thesis. The large inter-animal variation may also disguise any true effects of the treatment.

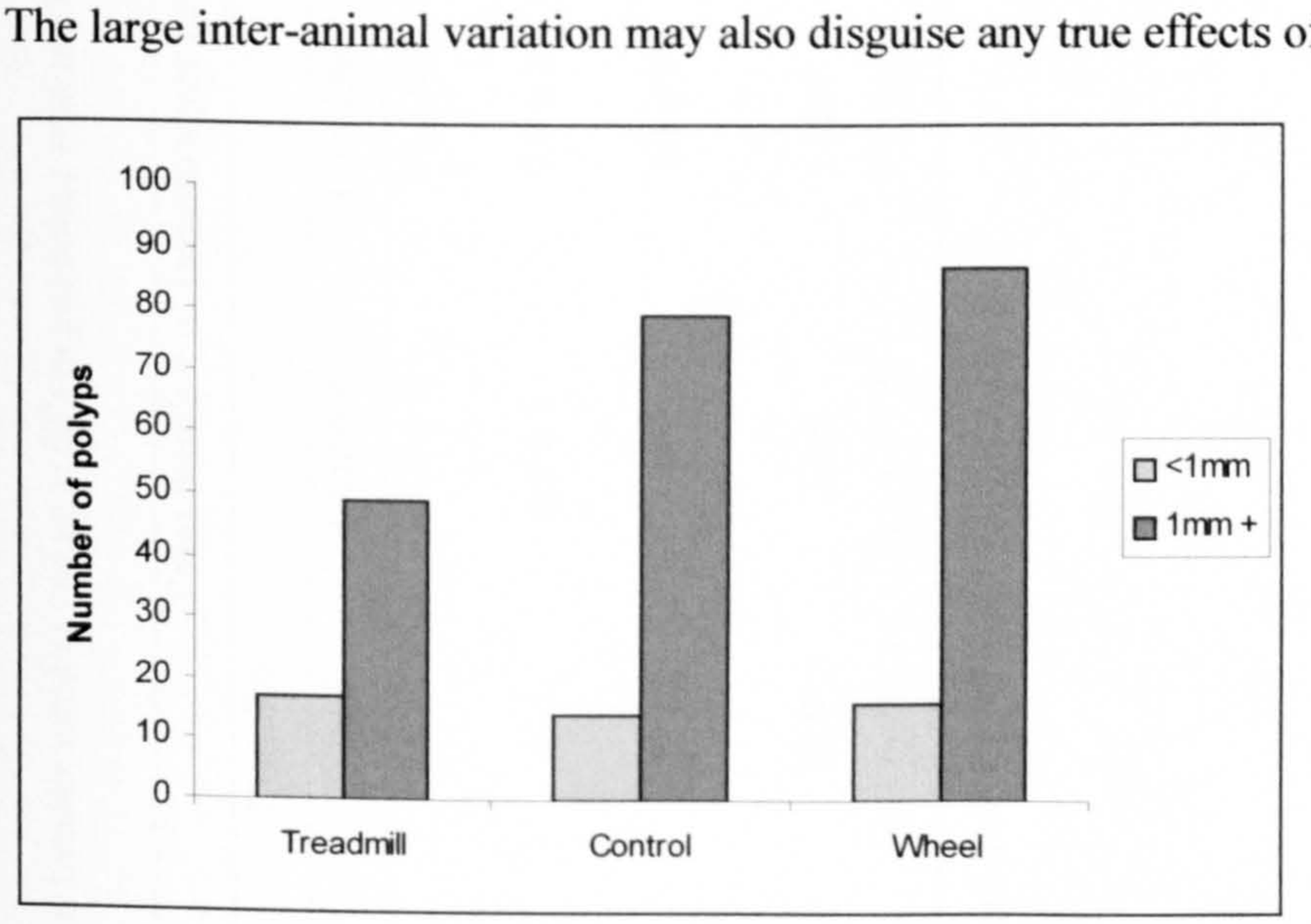


Figure 3.61 Distribution of tumour sizes reported by Mehl et al., (2005b)

Table 3.101 Comparison of total tumour number (mean ± SE) in published studies of Min mice and exercise

	Total number of tumours per mouse						Probability of effects	
	Females			Males				
Reference	Treadmill	Wheel	Control	Treadmill	Wheel	Control	TR v CON	WH v CON
Colbert et al. (2000)	40.9 ± 4	-	38.0 ± 6	24.8 ± 4	-	36.8 ± 5	0.44	-
Colbert et al. (2003)	6.4 ± 1	-	6.5 ± 1	7.0 ± 1	-	8.8 ± 1	>0.05	
Mehl et al., (2005b)*	-	-	-	66 ± 9	103 ± 8	93 ± 7	0.04 (males only)	>0.05
This study**	11.2 (9.0-14.0)	16.9 (13.6-21.0)	14.5 (12.1-17.4)	12.4 (9.5-16.2)	13.2 (10.5-16.6)	16.7 (13.6-21.0)	0.03	0.80

*** female polyp data not published (P>0.05), ** mean and 95% CI**

This disparity in tumour number may be due to genetic variation, as mentioned earlier, but may also be a consequence of staining the tissue (Mehl et al., 2005b) and examining it with a microscope to identify tumours. Using this method, approximately 10-20 tumours/group were identified as <1mm, which greatly increases the number of tumours that can be identified (Figure 3.62). This does, however, still leave the majority of tumours sized ≥1mm (49 ± 6 in treadmill-runners and 79 ± 6 in controls). The lower limit for sizing of tumours in our studies is 1mm, but staining may reveal smaller tumours. The treatments may be having an effect at the microscopic level, and staining would allow us to identify a greater number of tumours and perhaps show a treatment effect at an earlier stage of tumourigenesis.

There was a difference in anatomical site of tumours between the published studies and our results. Mehl et al., (2005b) reported that 95% of tumours were in the small intestine, of which 25% were in the proximal and 75% in the distal portion. Anatomical distribution was unaffected by exercise. 96% of the tumours generated per animal in this thesis were in the small intestine. 46% of these were in the proximal section, 54% in the distal. This approximately 50-50 split was maintained by TR and WH mice, but CON mice showed a tendency towards increased distal tumours (P = 0.062, Table 3.93).

The only significant interaction between sex and exercise group was on proximal SI tumours. Female TR and WH mice suffered from more proximal tumours than their male counterparts, but female CON mice had fewer proximal SI tumours than male CON (P = 0.037).

3.5.3.3 Organ dimensions

WH mice had the heaviest livers, perhaps indicative of the greater food intake leading to an increased storage of glycogen or fat.

The weight of the spleen was heavier in CON mice compared with TR and WH mice (P = 0.0003 and 0.035 respectively for organ as % body mass). This splenomegaly may reflect either the greater number of tumours in CON mice or the anaemia that all the mice suffered from, shown by the significantly lower haematocrit of the CON

mice (Moser et al., 1990). It is also possible that the TR and WH mice had reduced levels of systemic inflammation, a condition that causes spleen enlargement as lymphocytes are generated (Mehl et al., 2005b). However, a significant positive correlation was found between spleen weight and tumour burden in this study (r = 0.36, Figure 3.60), suggesting that the heavier spleen was in fact caused by tumour load. This is reinforced by data from the study by Mehl et al. (2005b), which measured heart and spleen weight and found that spleen weight of male treadmill-running Min mice was decreased by 35% and the spleen of male wheel-running mice decreased by 47% compared with non-running controls. This would correspond with the difference in tumours between exercising and control male animals. No differences in spleen weight were detected in female mice, and no differences in tumour number were found. Spleen weights from that study were much lighter than those in this study (TR 91mg, WH 75mg and CON 141mg, compared with TR 165mg, WH 218mg and CON 304mg), which is surprising given the increased tumour burden of the animals in the Mehl (2005b) study. The correlation between haematocrit and tumour burden suggests a use for the haematocrit as a useful surrogate for tumour burden in mice before they are killed, as only a small amount of blood is required. It could provide an estimate of tumour burden half-way through a study, or to confirm the necessary killing of a mouse before the end of a study due to morbidity.

An unexpected result was that TR and WH mice did not have a heavier heart mass than CON mice, although WH mice had the heaviest hearts. Heart weight of treadmill-running mice was not significantly different from control mice in the study of Mehl et al. (2005b), which supports the findings of this study, though female wheel-runners had increased heart weight (Mehl et al., 2005b). This is in accord of the findings of Konhilas et al, (2004), who reported that female wheel-runners significantly increased cardiac mass compared with males, though wheel-running produced a significant increase in mass-adjusted heart weight in males also.

3.5.3.4 SCFA

Resistant polysaccharides are fermented in the large intestine by the resident anaerobic bacterial flora. The products of this fermentation include short chain fatty acids, which are monocarboxylic hydrocarbons. SCFA are absorbed by the intestinal

lumen both passively and actively, along with an increase in Na^+ and Cl^- absorption (Kles & Chang, 2006). Absorbed SCFA are then oxidised by the epithelial cells and used as fuel. Acetate, propionate and butyrate occur in the greatest concentrations, and butyrate has been shown to display dual properties. In normal cells it is the preferred energy source and induces proliferation, although both low and high concentrations inhibit proliferation (Csordas, 1995), but in colon cancer cell lines butyrate increases apoptosis and induces differentiation (Csordas, 1995).

The colon contents of TR mice in this study contained a greater proportion of butyrate than those of CON mice (P = 0.002, Figure 3.63). TR mice also suffered fewer distal tumours (P = 0.062) and colon tumours (P= 0.049).

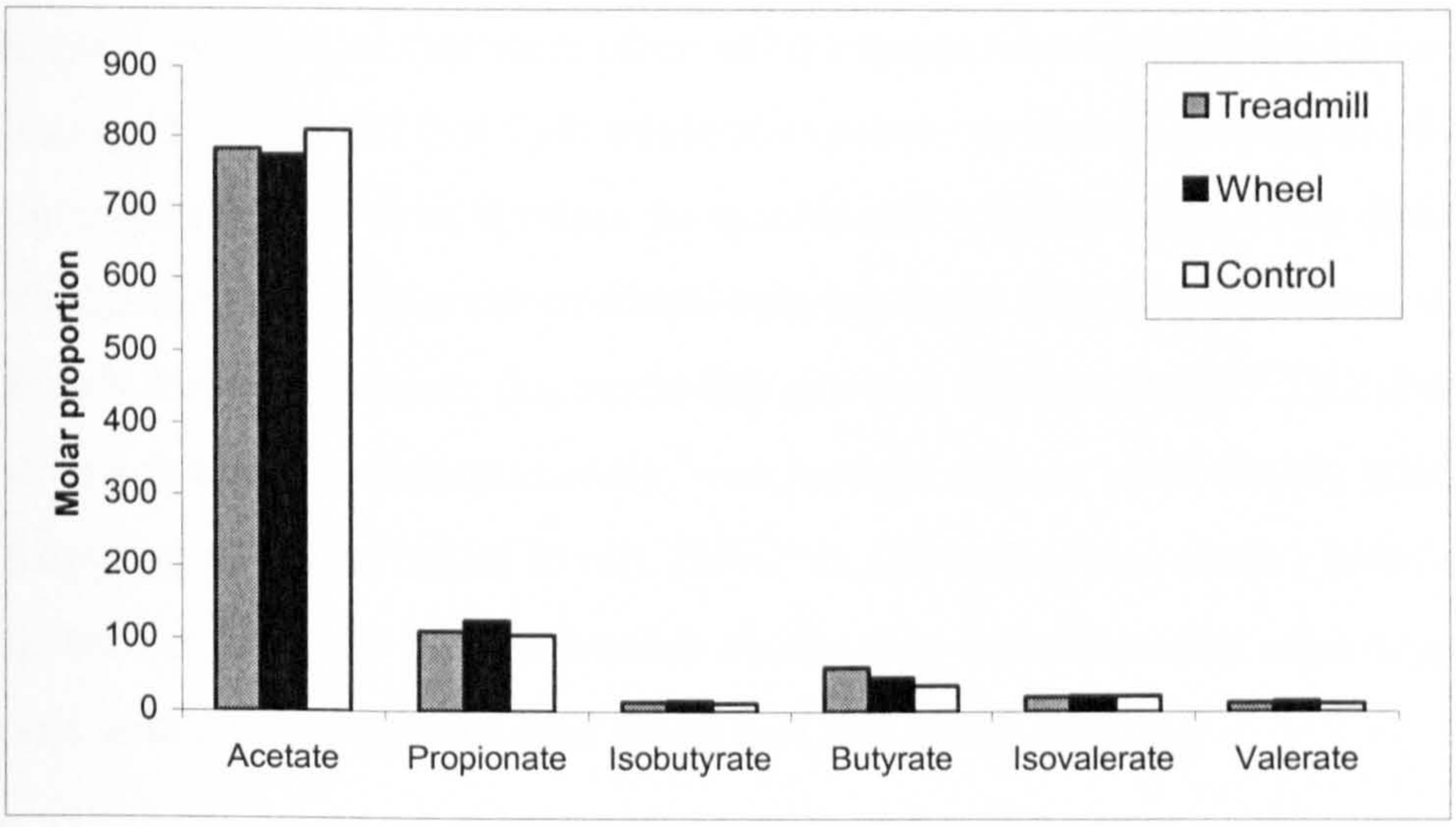


Figure 3.63 Molar proportions of SCFA (mmol/mol total SCFA) for each exercise treatment group

Diet is known to alter SCFA concentrations, particularly polysaccharides, but no information on the effect of exercise on SCFA has been reported. Exercise may have altered the proportions of gut bacteria, promoting growth of the anaerobes that produce butyrate, though this proposal has not been tested. The greater proportion of butyrate in TR mice might help to explain the reduction in tumour number seen in this group of mice. As fermentation occurs in the caecum there may be some back flow of SCFA into the small intestine, accounting for the decrease in distal SI tumours observed. It is perhaps more likely that butyrate acts indirectly, by increasing

immunosurveillance (Pierre *et al.*, 1997; Armstrong & Mathers, 2000) or targeting cells from the basal surface and decreasing inflammation or angiogenesis (Williams et al., 2003). Butyrate is known to increase with decreased transit time(Lewis & Heaton, 1997), so a decreased transit time due to exercise (although not observed in this study) could have led to an increase in colonic butyrate concentrations.

3.6 Critique of study and suggestions for further research

As detailed in the Materials and Methods, some mice in this study changed treatment group in the first 2 weeks (the training period). This was to prevent wasting the animals, as the mice that were taken off the treadmill would otherwise have been destroyed. It was felt that their minimal exposure to exercise would allow them to move groups. However, it raises the question of why these particular mice would not run e.g. did they dislike the treadmill-running more than the other mice, or were they already forming tumours that made this exertion uncomfortable? This question cannot be answered without further study, which might require immediately killing and dissecting mice that refuse to run. However, the lack of correlation between running distance and tumour number/burden observed in wheel-running mice suggests that even with a heavy tumour load some exercise is still possible.

Data were reanalysed with mice placed in one of the following five groups: 1) treadmill only (TR), 2) wheel only (WH), 3) control only (CON), 4) was treadmill but moved to control (TR-to-CON), 5) was treadmill but moved to wheel (TR-to-WH). Using the General Linear Model of ANOVA and Dunnett's test, groups were compared with CON.

There was no difference in tumour number ($p = 0.698$) or tumour burden ($p = 0.149$) between mice that had changed groups and mice that had not, so broadly speaking, mice that had changed groups were not affected differently to the other mice in the study. When the tumour variables were re-analysed compared with the control group the significant effect of the treadmill exercise was removed, but the tend towards decreased total number of tumours was still present (11.7 v. 15.4, $p = 0.077$). The decrease in significance is presumably because the TR-to-CON mice, whilst not being significantly different from CON, were more affected (15.5 tumours on average), so

removing them from the CON group decreases the overall number of tumours in the CON group. However, this does not help to explain why mice that would not run on the treadmill but were given a wheel which they used (Figure 3.64), had in general *fewer* tumours than WH or CON (13.7 on average, compared with 15.2 for WH and 15.4 for CON).

It should be noted though, that numbers of mice in the "changed" groups were considerably less than those in the "unchanged" categories, and this complicated issue deserves further exploration, perhaps by increasing the numbers of mice that have a short treadmill exercise exposure followed by access to a wheel.

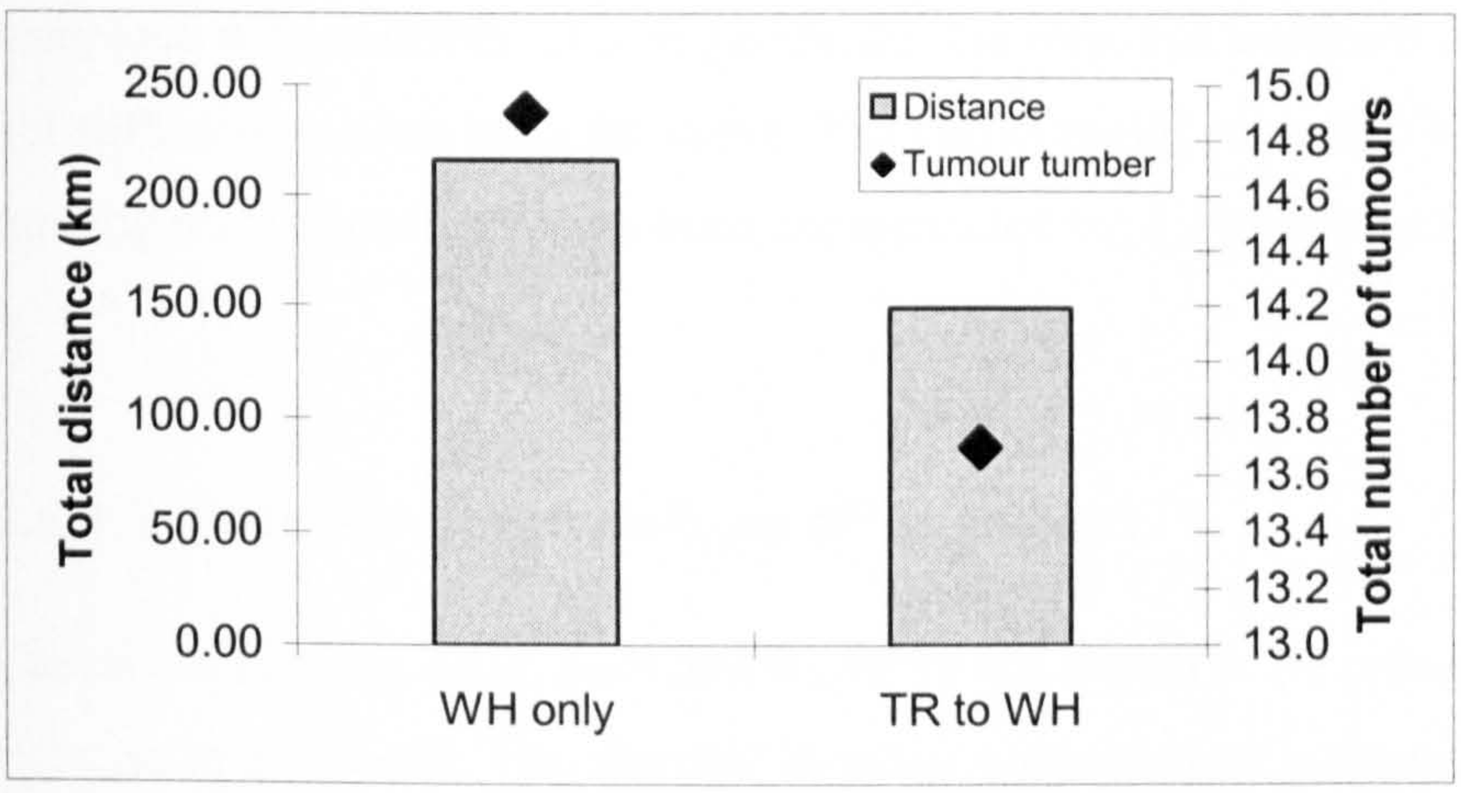


Figure 3.64 Mean total distances run (km) by WH mice (n = 43) and TR-to-WH mice (n = 6), with corresponding total tumour number

3.6.1 Ability of the study to address the hypotheses

3.6.1.1 Hypothesis 1 – Treadmill- or wheel-running exercise will reduce tumour number compared with controls

There was no consistent effect of exercise across the studies, with no effect of treadmill running in the pilot study, a trend towards decreased total number of tumours in ES2, a trend towards decreased colon tumours in ES3 but overall decreased tumour number and burden. With respect to wheel running, ES2 showed a

trend towards decreased distal tumours, no effects were seen in ES3 (though running distances were less), and overall no difference was observed compared with control. The reasons for this apparent paradoxical finding that forced exercise should reduce tumour number, and conversely, why the exercise involved in voluntary wheel running should have no effect on tumourigenesis, have yet to be elucidated.

3.6.1.2 Hypothesis 2 – Exercised mice will have lower body fat than controls

No difference in body fat was discovered in treadmill or wheel running mice compared with controls. This suggests that the imposed treadmill exercise regime was not sufficient to alter body fat stores. The extra energy expended by the wheel-running mice appears to have been compensated for by increased food intake.

3.6.1.3 Proposed mechanisms of protection

Caecal transit time, CCP, IL-6 and IL-10 do not appear to be consistently altered with exercise in this thesis. The increase in molar proportion of butyrate observed in TR mice may present one explanation for the decreased tumour number observed in this group.

3.6.2 Conclusions and suggestions for further research

This series of experiments was performed to assess the impact of exercise on intestinal tumourigenesis in the Min mouse. Despite a modest reduction in tumour number in treadmill-running mice, there was no consistent evidence to suggest that exercise affected tumourigenesis to any significant extent, in agreement with previous studies (Colbert *et al.*, 2000; Colbert *et al.*, 2003). The lack of effect of wheel-running suggests that there are other factors at work, such as hormonal or neuroendocrine, that deserve further investigation.

The disparity between rat and Min mouse studies needs to be addressed, and the main difference between the two appears to be the lower body weight (and loss of fat mass,

where measured) by exercising rats, whereas exercising mice, in general, maintained a body weight similar to that of controls. This suggests that imposed exercise regimes need to be higher for mice than rats. This difference in energy economy of the mice can be further explored using caloric restriction, as reducing the available food energy reduced total tumour number in Min mice (Mai et al., 2003) and of tumours >2mm (Kakuni et al., 2002). More accurate measurement of food intake is possible through the use of metabolic cages, and indirect calorimetry will provide more accurate estimates of energy expenditure. Different strains of mice have different resting energy expenditures (Funkat et al., 2004), so it is likely that a species difference exists, which may explain, in part, the differences between rat and mouse studies. Serial body fat measurements can be made using MRI (Tang et al., 2000), to counteract any loss of body fat that occurs with increasing illness in Min mice.

One new technique that may help assessing the impact of exercise regimes in the future is the mouse endoscope (Becker et al., 2005). This miniature endoscope, with an external diameter of 1.9mm allows the researcher to monitor the progress of ACF and tumours in the colon. This endoscope was designed for use with mice developing colitis; if it were possible to use this equipment to examine the small intestine (where most Min tumours occur), it would be extremely valuable. Another alternative could be to use AOM-treated Min mice, which generate more colon tumours than normal Min mice (Suzui et al., 2002). Exercise effects which have an impact on humans may become more obvious with a greater number of colon tumours, allowing us to find a more accurate level of beneficial activity.

Chapter 4

4 Effects of diet and exercise on body fatness in mice

4.1 Introduction

Dietary fat and obesity are closely implicated in the development of colon cancer, as discussed in Chapter 1. Obesity is associated with chronic inflammation, which generates several measurable biomarkers, such as tumour necrosis factor-alpha (TNF-α) and various interleukins, as mentioned in Chapter 1 (Opal & DePalo, 2000).

Bell et al., (1997), showed that access to an exercise wheel for 8 weeks caused a significant reduction in body fat in female Swiss Albino mice even when fed a high fat diet ($P<0.001$). All exercising mice ran well, averaging 14.5km/day (n =13/group), with no significant difference between low and high fat fed mice. Providing access to an exercise wheel for animals fed a high fat diet decreased body fat to values similar to those of non-exercised mice fed a low-fat diet (12.6% v. 13.9%). Exercised mice fed high fat diets were not significantly heavier than their low fat-fed exercising counterparts, but high fat non-running mice were significantly heavier than low fat-fed non-running mice (33g v. 28g).

The following study was undertaken to investigate in more detail the effect of a high or low-fat diet on wheel running and on body composition. In particular, attempts were made to quantify non-exercise physical activity (NEPA), which has not been measured previously in studies of this kind. This is potentially important since animals undertaking exercise through wheel running (or treadmill running) may have different amounts or patterns of NEPA from mice without access to such exercise devices. This may have implications for energy balance and tumourigenesis. Also, a previous study (ES2) suggested that (possibly due to the oestrus cycle) female mice have a different pattern of running compared with male mice, and this observation was examined more closely in the following study.

4.2 Aims

- To quantify the effects of high and low fat diets on wheel running, body composition and NEPA in mice
- To investigate the effect of such diets on the inflammatory marker, TNF- α
- To test for relationships between running distance and stage of oestrus in female mice

4.3 Hypotheses

- Mice fed high fat diets will have more body fat than those fed low fat diets
- Wheel-running mice will have less body fat and heavier hearts than non-running counterparts
- High fat-fed mice will have higher plasma concentrations of TNF- α

4.4 Specific methods

4.4.1 Sample size

The software package nQuery Advisor (Statistical Solutions Ltd, Cork, Ireland) was used to determine the required sample size to detect a difference at the P = 0.05 level. The power calculation was based on the results of Bell *et al*, (1997) whose paper was the basis for this study. Four groups of size 8 were calculated to be required.

4.4.2 Animals

32 six-week-old C57BL6/J female mice were purchased from Harlan (Oxon, UK). They were housed in groups of 8 mice and fed chow (RM3 pellets, SDS, Essex, UK) *ad libitum* for two weeks to acclimatise them to their new accommodation. Other housing conditions were as stated in General Materials and Methods (Chapter 2). Mice were weighed twice each week on Monday and Thursday.

4.4.3 Diet and exercise groups

At 8 weeks old mice were randomised to either high fat (HF) or low fat diets (LF) (n= 16 for each group). Mice were then split into wheel (WH) or control (CON) groups (n = 8 for each) and individually housed in cages. Wheels were 5 ½ " Comfort Wheels as used previously (Chapter 3).

4.4.4 Diet composition

The formulation of the test diets was based on Bell et al. (1997) as summarised in Table 4.1.

Table 4.1 Composition (g/kg) of high and low fat test diets

Ingredient	High fat (HF)		Low fat (LF)		Supplier
	g/kg	g/MJ	g/kg	g/MJ	
Cornstarch	253	27.0	500	32.5	Sigma
Casein	241	13.0	200	13.0	Sigma
Sugar	181	9.8	150	9.8	Co-op
Beef fat	145	7.8	0	-	Co-op
Corn oil	60	3.2	50	3.2	Co-op
Cellulose (Alphacel)	60	3.3	50	3.3	MP Biomedicals
Mineral mix AIN-93-G-MX	42	2.3	35	2.3	MP Biomedicals
Vitamins AIN-93-VX	12	0.6	10	0.6	MP Biomedicals
Methionine	4	0.2	3	0.2	MP Biomedicals
Choline	2	0.1	2	0.1	MP Biomedicals
Butylated hydroxytoluene	0.04	0.0022	0.01	0.0006	Sigma

Mice were offered 6g of diet daily. Food refusals were measured over two weeks of the study. Water was freely available.

4.4.5 Oestrus washes

A previous study (ES2) had highlighted the apparently cyclical nature of distances run by female mice and it was thought that this might reflect different stages in their oestrus cycle (approximately 3-5 days). Stage of oestrus cycle may be determined by the type and quantity of cells present in the vagina. To measure this, a fine tip pastette (Alpha Laboratories Ltd, Hants, UK) containing sterile saline was placed into the vagina. One drop of saline was squirted in and out of the vagina to collect the cells. The drop was placed onto a microscope slide and left to air dry before being stained with 0.1% methylene blue. Slides were examined under a microscope and stage of oestrus determined (Figure 4.1) (Short & Woodnott, 1969).

Slides were categorised as stage 1-4 of oestrus as follows: 1- (not in oestrus) mainly leucocytes present, possibly a few epithelial cells; 2- (approaching oestrus) mainly epithelial cells, few leucocytes; 3- (oestrus) no leucocytes, just cornified cells); 4- (post-oestrus) cornified cells and leucocytes.

Depending on when the sample is taken, stages may not always be clear-cut.

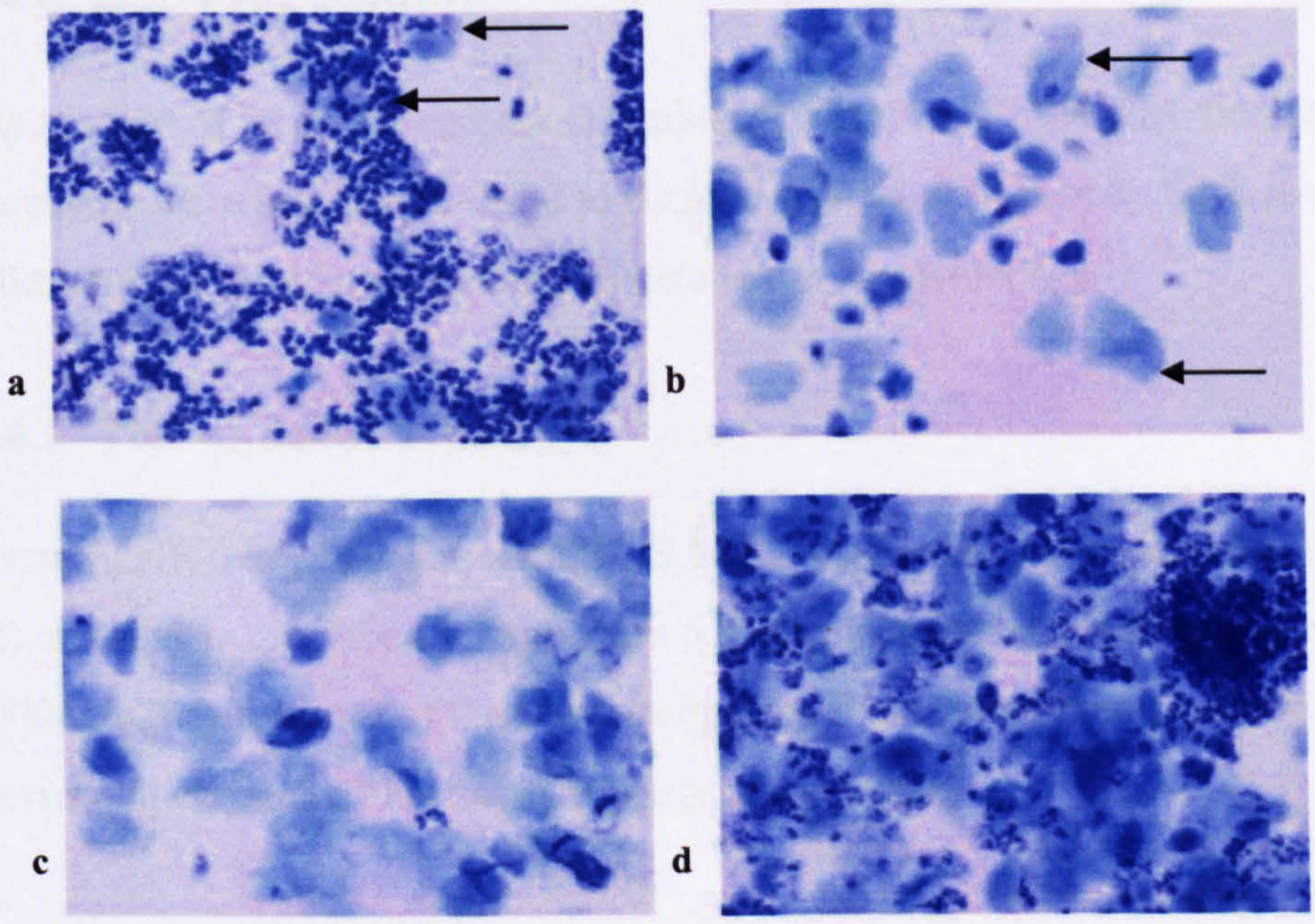


Figure 4.1 Oestrus stages: a) stage 1. A few epithelial cells (upper arrow) are present amongst the majority leucocytes (lower arrow); b) stage 2 into 3. The leucocytes have disappeared, some epithelial cells are still present (upper arrow) but most cells are cornified cells (lower arrow); c) stage 3 (oestrus). Only cornified cells are present; d) Stage 4 (post-oestrus). Leucocytes are now present again amongst the cornified cells. All x200.

Vaginal washes for characterisation of oestrus stage were collected from each mouse on 17 consecutive days at the same time each day. Wheel revolutions were recorded immediately before the samples were taken.

4.4.6 NEPA

Non-exercise physical activity (NEPA) by each mouse was monitored by InfraMot for 24 hours on two separate occasions.

4.4.7 Necropsy and sampling

Mice were killed after 8 weeks of study, using the methods described in General Methods and Materials (Chapter 2). Blood was collected into EDTA tubes and stored on ice until separation. Whole blood was centrifuged at x5000 for 10 minutes and the plasma pippetted into a fresh eppendorf tube and frozen at –20°C until use. Final body weight, liver and heart weights were recorded. Livers and hearts were snap frozen in liquid nitrogen and stored at –80°C. Carcasses were stored at –20°C.

4.4.8 Body fat content

The amount of fat in the carcass excluding the heart and liver was measured. Carcasses were first freeze-dried to remove water and then body fat was extracted following the Soxhlet procedure, as detailed in Appendix 6.3.

4.4.9 TNF-α determination

Plasma TNF-α was measured by ELISA (Ready-Set-Go!, eBioscience, cat # 88-7324-22), method as described in Appendix 6.9. The standard curve ranged from 8pg/ml to 1000pg/ml. Sensitivity of the test was 8pg/ml. Plates were read on a Molecular Devices Spectramax Plus using Spectramax software.

4.5 ES4 Results

4.5.1 Wheel running data

All the mice used their running wheels, and most mice ran a similar amount each week, with no decrease towards the end of the study. The range of distances varied between mice, as shown in Figure 4.2.

Although HF mice as a group ran further on weeks 1, 2, 7 and 8 whilst LF mice ran further on weeks 3, 4,5, and 6 (Table 4.2 and Figure 4.3), none of the between-diet differences was statistically significant (P>0.05). The similarity in running distance between groups was demonstrated at the end of the study, when after 8 weeks, LF mice had run further than HF mice by just 7km (374km v. 367km, P = 0.905, Figure 4.4).

Table 4.2 Mean daily distance (km) run each week by HF and LF mice

Week	High fat	Low fat	SEM	Probability of effect of diet
1	4.64	2.46	1.11	0.187
2	9.28	6.75	1.60	0.282
3	8.24	10.41	1.49	0.320
4	7.06	9.10	1.46	0.341
5	6.69	7.61	1.16	0.583
6	5.67	6.62	0.81	0.424
7	5.66	4.91	1.04	0.621
8	6.08	5.30	0.84	0.525
Total in 8 weeks	**367.3**	**374.0**	**38.99**	**0.905**

n = 8 for each group

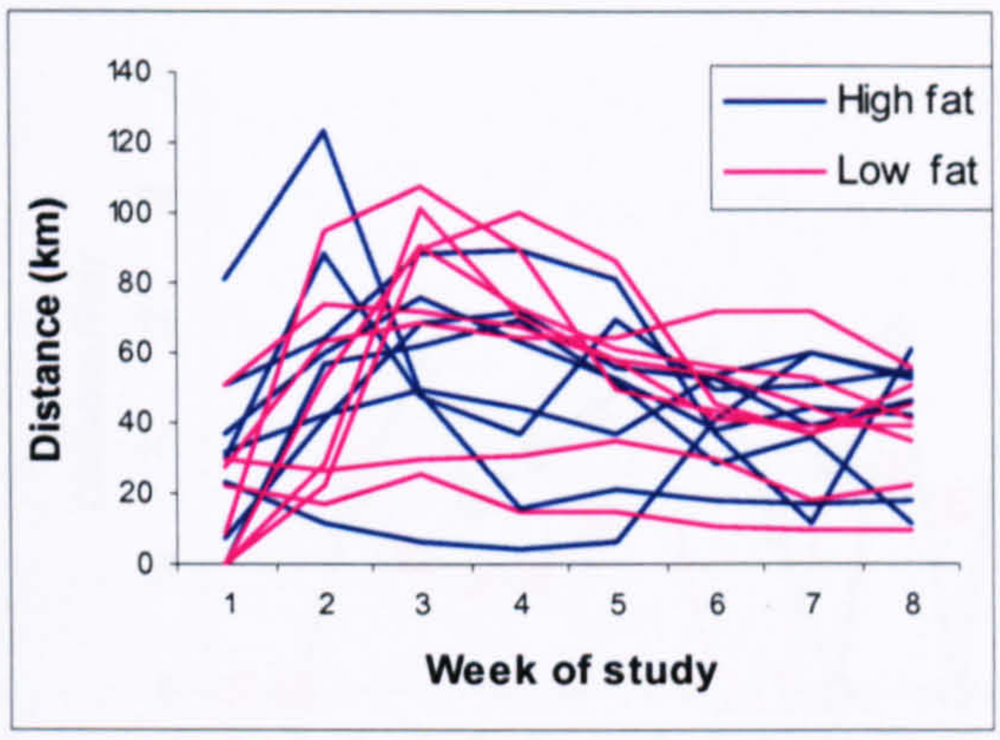


Figure 4.2 Total distances run in each week by individual mice

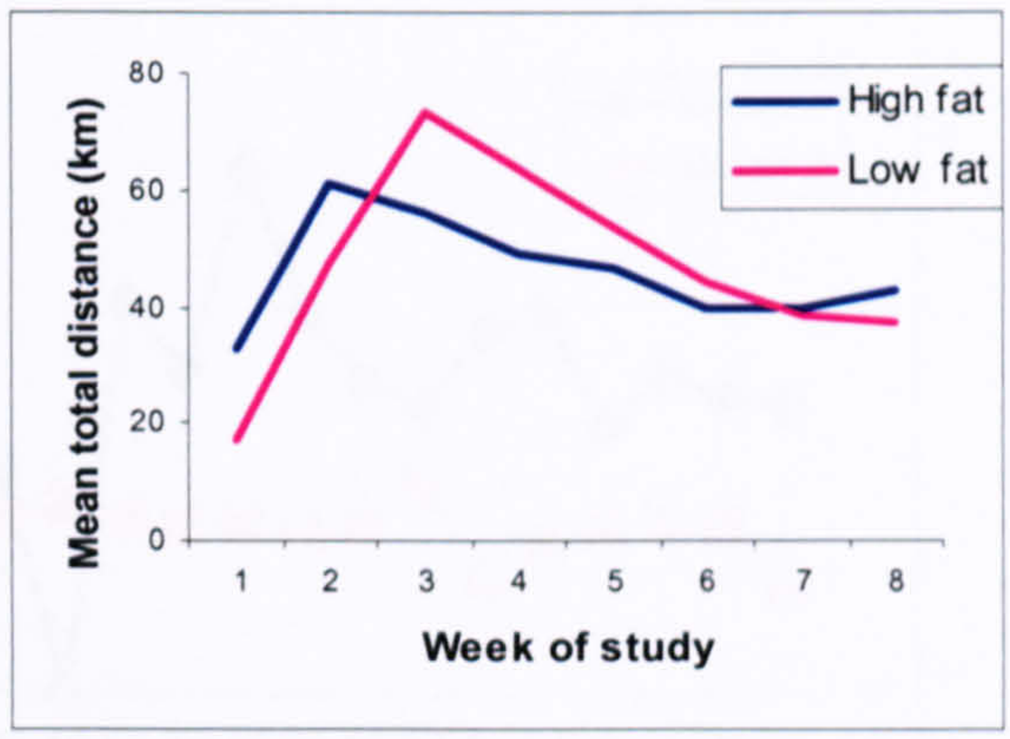


Figure 4.3 Mean total distance run each week, by diet group

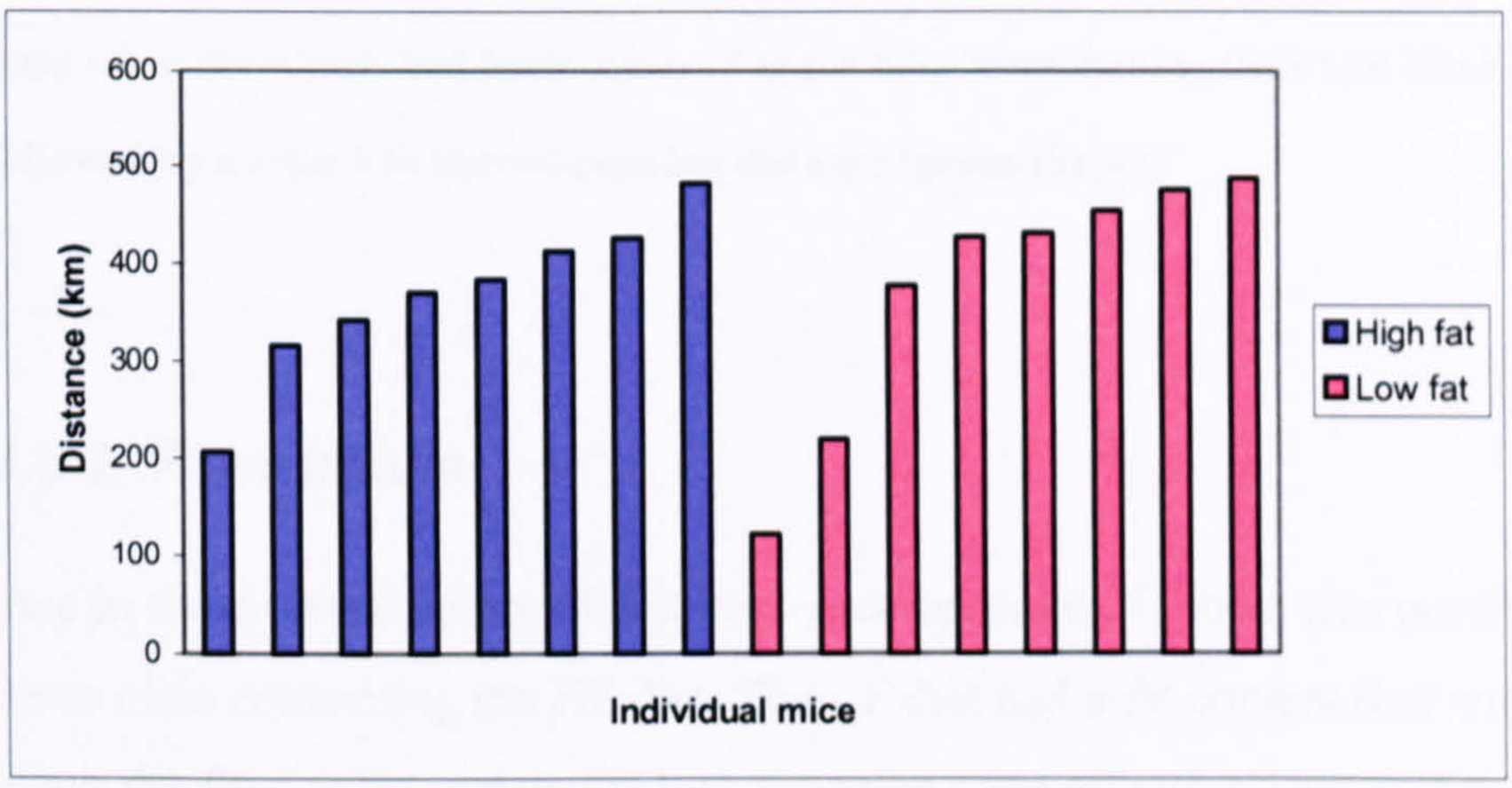


Figure 4.4 Distances run in 8 weeks by individual mice in each diet group

Two mice ran so much that their feet started to bleed, so wheels were removed on 3 and 2 separate days, respectively (Figure 4.5) to give the animal's feet time to recover. When the wheels were returned, the mice continued to run as normal and no further ill effects were observed.

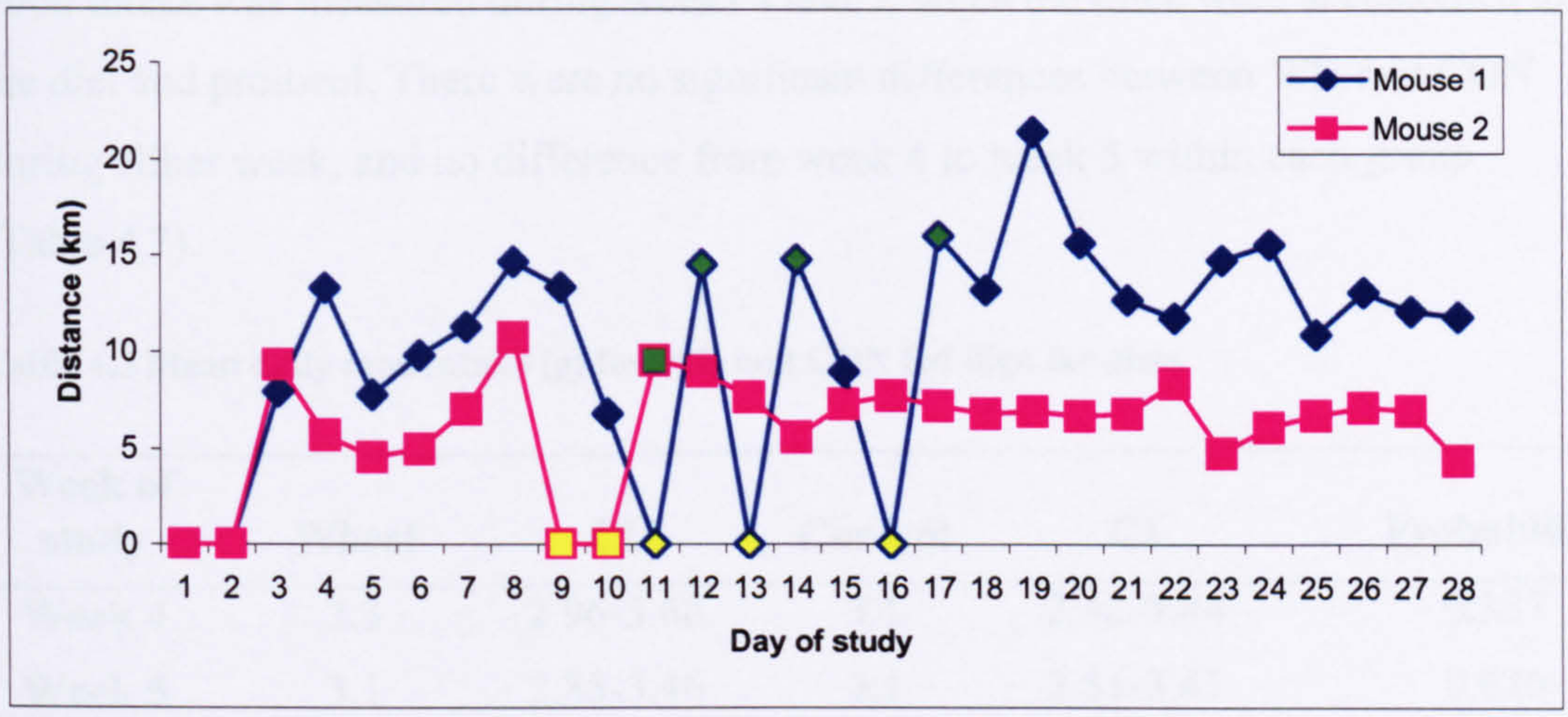


Figure 4.5 Daily distance run by 2 individual mice. The yellow shapes at distance zero are the days when the wheels had been removed as the mice were making their feet bleed. This was followed by a return to normal running distance (green shapes)

4.5.2 Food intake

Due to the physical nature of the food, measurement of intake was possible only for those mice consuming the HF diet. The LF diet had a fat content that was too low to allow the food to be compressed, so the mice were effectively given a powder diet. This resulted in the food being spread around the cage, including throughout the sawdust, on the cage bars, in the bedding and in the mouse's fur. In preliminary studies, attempts were made to prevent this spillage by mixing the LF diet with water but it was found that the diet solidified when liquid was added and the diet became too hard for the mice to eat. If a mouse urinated into the pot it was not possible to remove all of the diet to weigh it.

Although the HF diet was not solid, it had a more crumbly texture that allowed the mice to pick up small lumps to eat. There was a problem with scattering from some HF mice, but in general any uneaten diet remained in the food pot.

To determine the extent of scattering, the indigestible marker chromic oxide was added to trial samples of food. The chemical is bright green and stands out well against the other cage contents. This showed the LF diet being well scattered.

Food intake was measured during weeks 4 and 5, when the mice were accustomed to the diet and protocol. There were no significant differences between WH and CON during either week, and no difference from week 4 to week 5 within each group (Table 4.3).

Table 4.3 Mean daily food intake (g) for WH and CON fed high fat diets

Week of study	Wheel	CI	Control	CI	Probability
Week 4	3.3	2.96-3.68	3.1	2.42-3.84	0.337
Week 5	3.1	2.85-3.46	3.1	2.81-3.41	0.826
P	0.152	-	0.640	-	-

n = 8 for each group

4.5.3 Body weights

Initial weights ranged from 15.6g to 20.4g and final weights ranged from 17.8g to 33.2g. Weekly weights and weekly weight gain of all mice are displayed in Figure 4.6. Due to the large difference in initial weights, these values were used as a covariate in the analysis of weekly weights.

One HF CON mouse gained substantially more weight than any other mouse (see Figure 4.6). Analyses were also run with data for this mouse removed in case of bias, but values did not alter greatly. For weekly WH v. CON weights, the only difference was at week 1, with WH starting heavier than CON (18.27g v. 17.24g, P = 0.015).

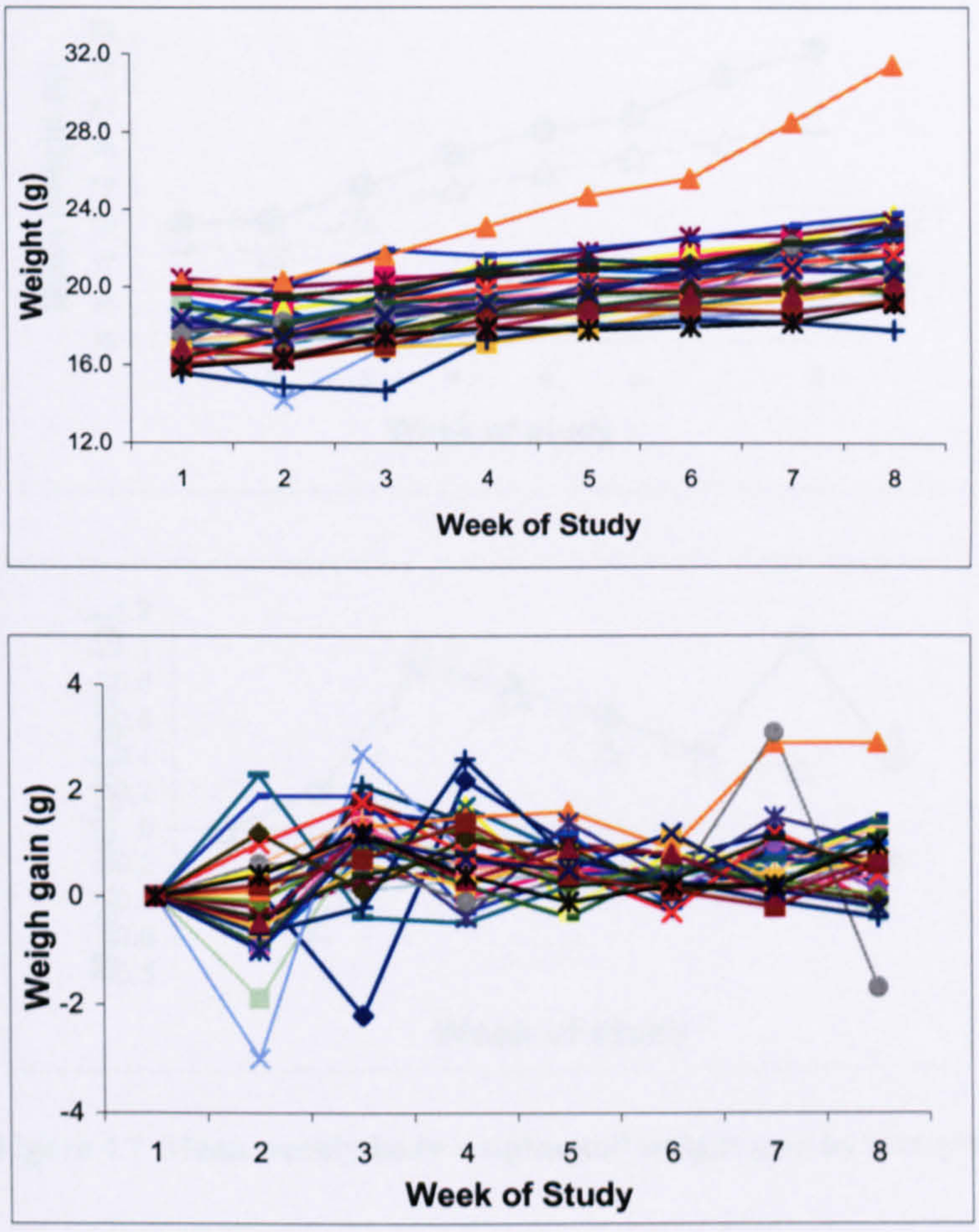
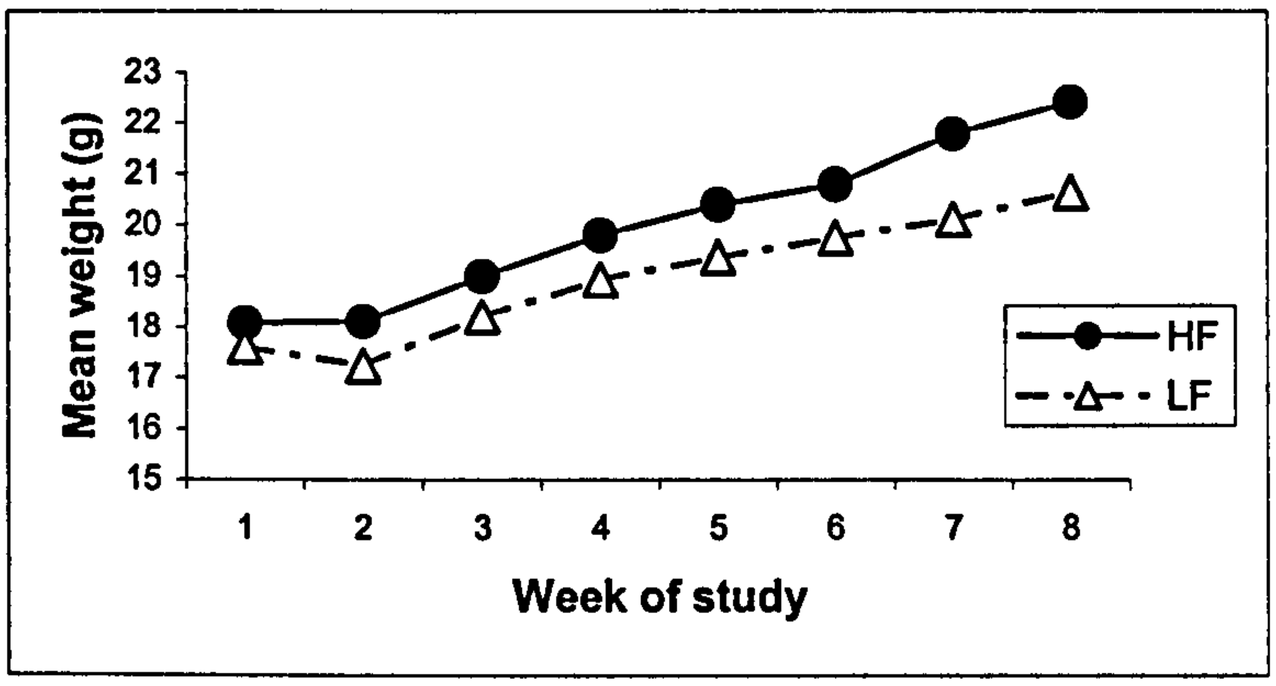


Figure 4.6 Weekly weights and weight gain of all mice (n = 32)

Weight differences between diet groups were significantly different on every week except the first, with HF mice heavier than LF at every week (Figure 4.7 and Table 4.4). This did not change even with the heavy mouse removed, although some P-values altered slightly, with week 3 showing the only alteration in statistical significance (P = 0.043 changed to P = 0.057).

Weight gain was significantly different between the dietary groups on weeks 2 and 7 (P = 0.036 and 0.005 respectively) only. On both occasions HF mice gained more weight (Figure 4.7).



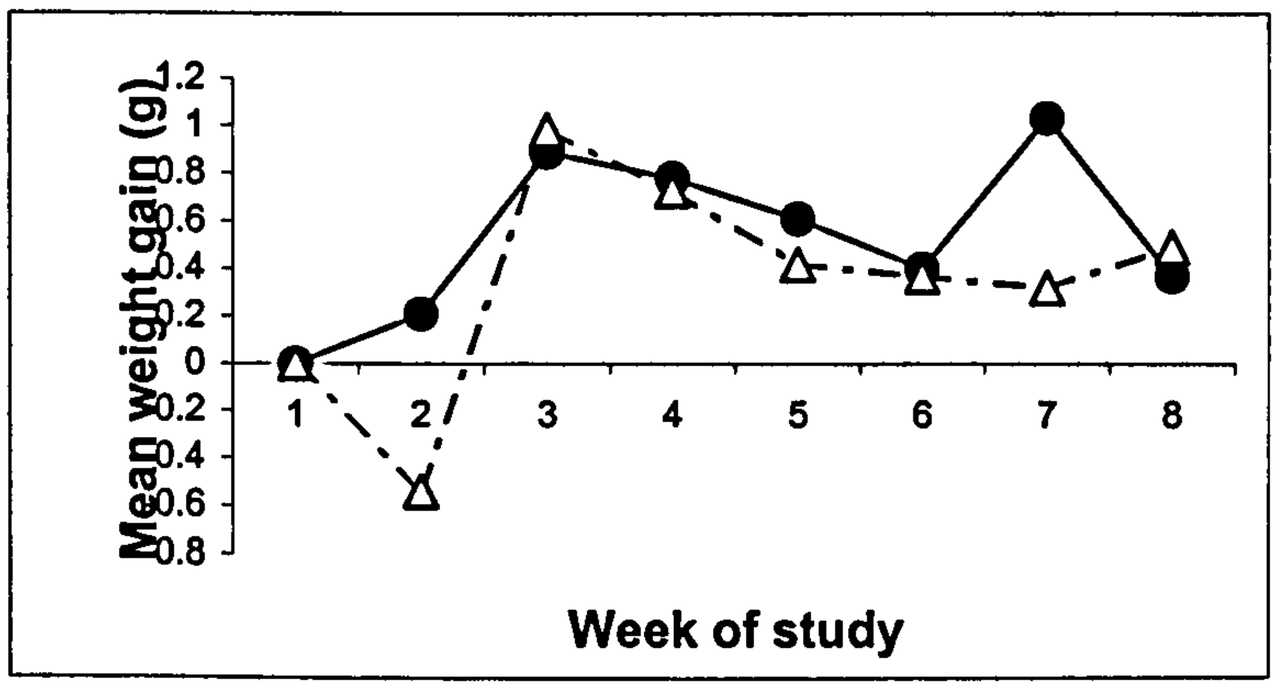
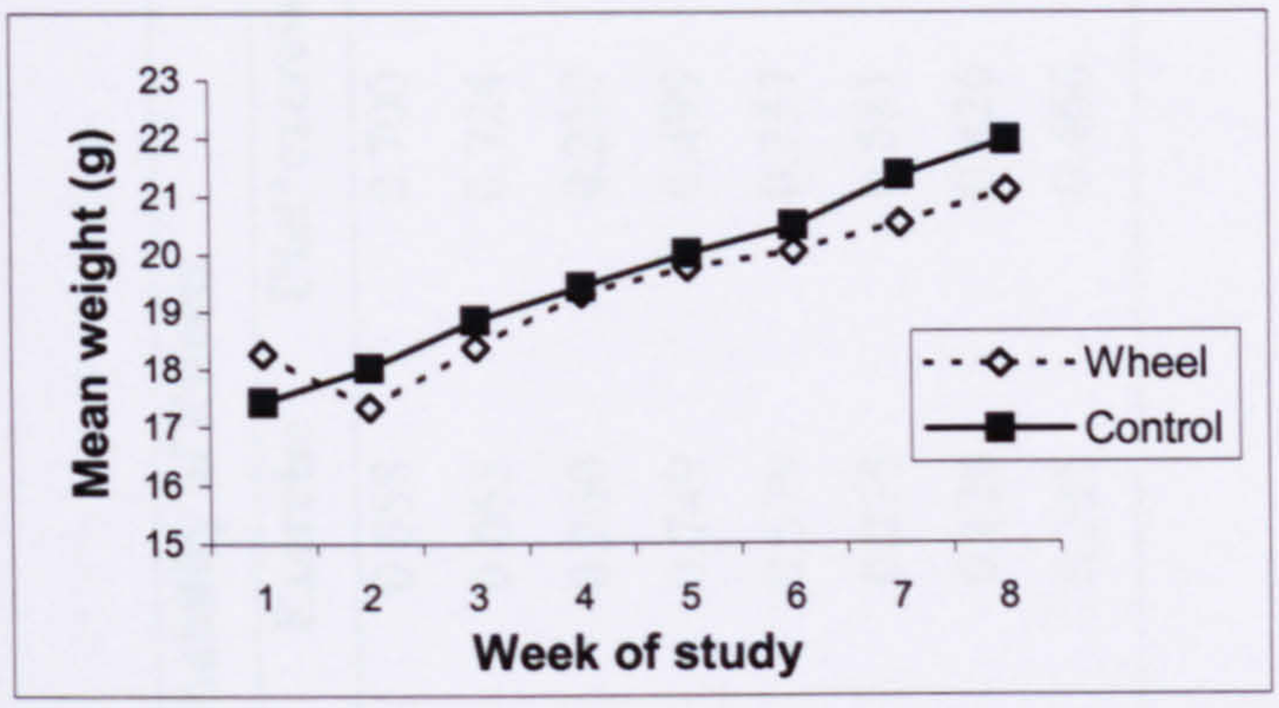


Figure 4.7 Mean weekly body weights and weight gain by diet group

Weights were not significantly different between WH and CON on any week (Figure 4.8) and weight gain was significantly different between exercise groups for the first week ($P = 0.024$) only, during which WH mice lost on average 0.56g compared with a gain of 0.23g by the CON mice.

Weights by exercise and diet group are displayed in Figure 4.9 and Table 4.4. There was no interaction between diet and exercise on any week. However, there was a significant interaction between diet and weight gain at week 4 ($P = 0.008$) only, which is displayed in Figure 4.10. In week 4, HF WH mice gained more weight than LF WH, but LF CON gained more weight than HF CON.



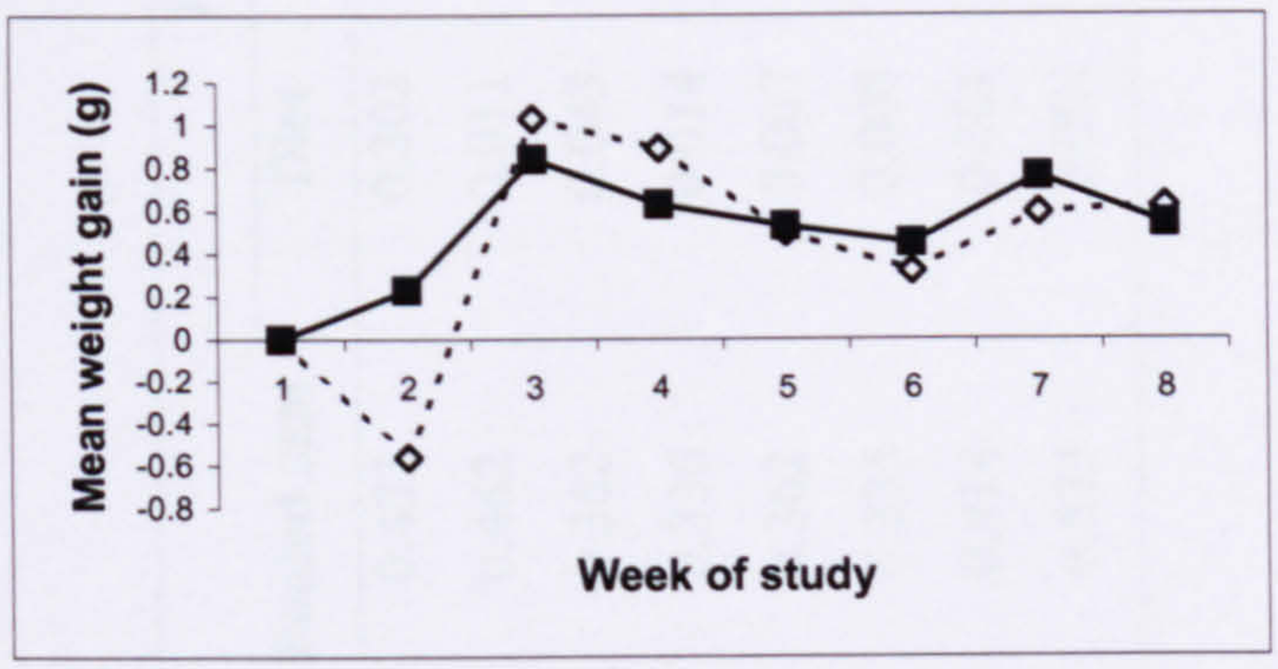
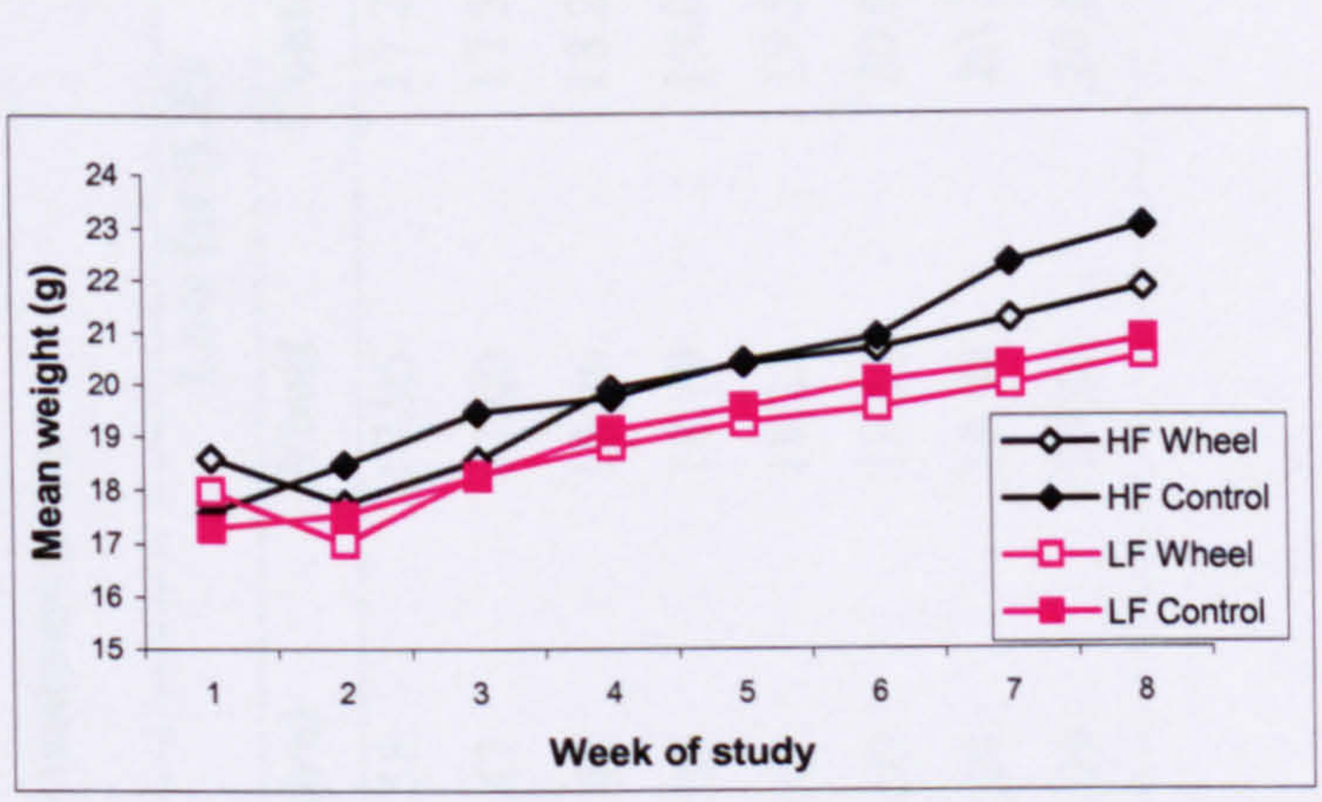


Figure 4.8 Mean weekly body weights and weight gain of mice by exercise group (n = 16 for each group)



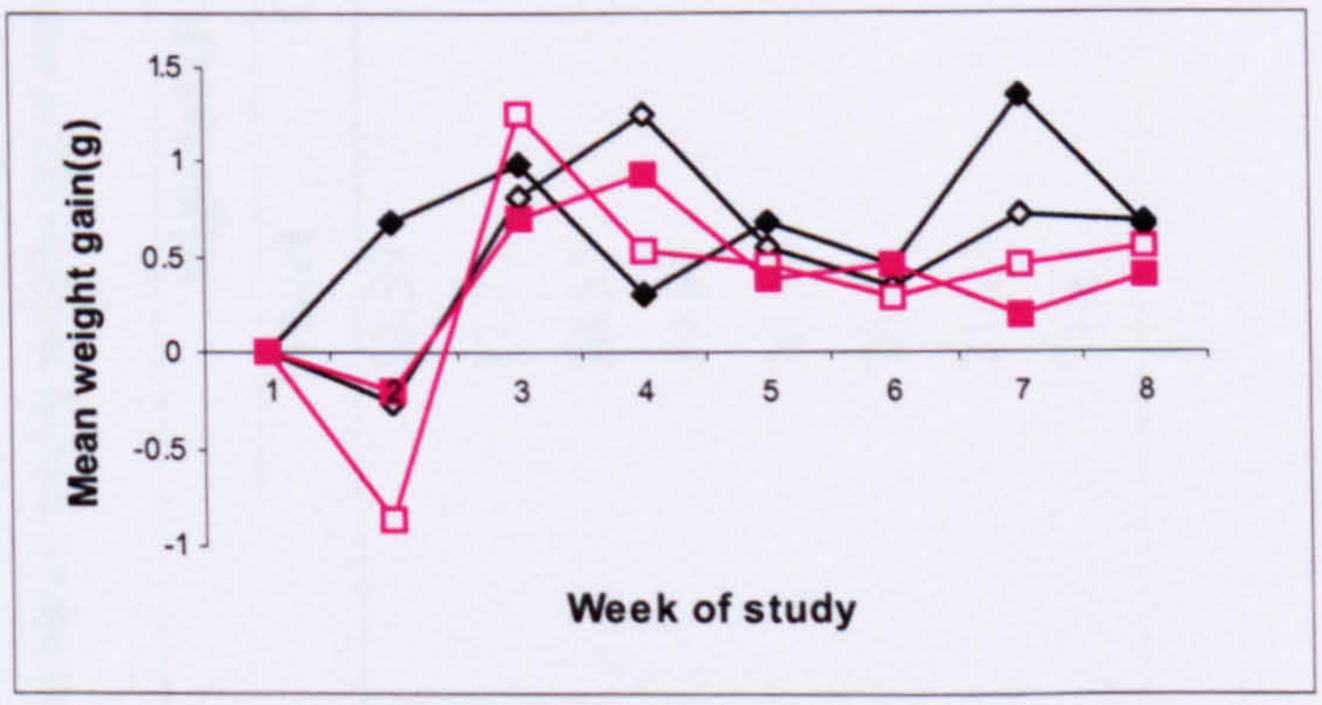


Figure 4.9 Mean weekly body weight and weight gain for all groups (n = 8 for each group)

Table 4.4 Mean weekly weights (g) of mice by treatment group

	High fat (HF)		Low fat (LF)			Probability of Effects		
Week	Wheel	Control	Wheel	Control	Pooled SEM	Diet	Exercise	Diet*exercise
1	18.59	17.58	17.95	17.28	0.433	0.302	0.058	0.700
2	17.73	18.47	17.00	17.52	0.462	0.011	0.065	0.724
3	18.55	19.44	18.26	18.22	0.362	0.043	0.260	0.210
4	19.89	19.72	18.79	19.08	0.336	0.014	0.749	0.499
5	20.38	20.42	19.23	19.52	0.362	0.007	0.579	0.737
6	20.67	20.89	19.51	20.02	0.335	0.005	0.275	0.681
7	21.23	22.29	19.94	20.33	0.414	0.001	0.128	0.426
8	21.81	22.99	20.49	20.82	0.573	0.004	0.215	0.466

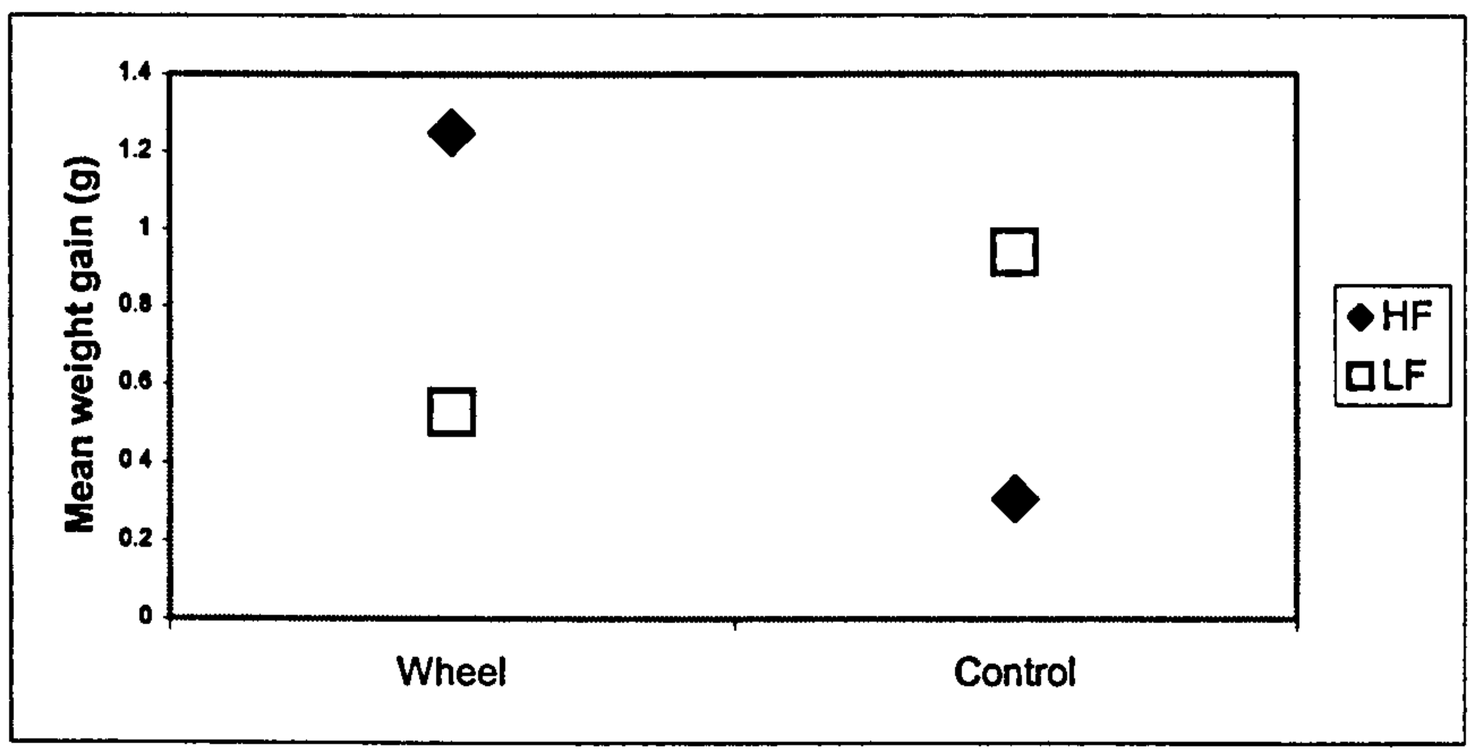


Figure 4.10 Interaction between diet and exercise on weight gain in week 4

4.5.4 Organ weights

Hearts and livers were removed for weighing and these weights were also calculated as percentage of final body mass. There were significant ($P<0.05$) effects of diet on both heart and liver weights (Table 4.5). HF-fed mice had heavier hearts compared with LF-fed mice (0.170 v. 0.140g, $P = 0.040$), and also heavier livers (1.039 v. 0.925g, $P = 0.025$) but when these weights were expressed as % final body mass, differences were non-significant ($P>0.05$, Table 4.6). Wheel-running mice had heavier hearts than control mice (0.169 v. 0.138g, $P = 0.023$), and this effect persisted when expressed as % final body mass ($P = 0.009$,Table 4.6). There was no statistically significant effect of exercise on liver weight.

Table 4.5 Organ weights of mice by exercise and diet group (mean and 95% CI)

	High fat		Low fat		Probability of Effects		
Variable (g)	Wheel	Control	Wheel	Control	Diet	Exercise	Diet*exercise
Heart ^{Log10}	0.174	0.160	0.163	0.120	0.040	0.023	0.151
	0.151-0.202	0.138-0.185	0.141-0.189	0.103-0.139			
Liver	1.052	1.026	0.909	0.941	0.025	0.955	0.564
	0.954-1.149	0.928-1.123	0.811-1.006	0.843-1.038			

Table 4.6 Organ weights as % final body mass (median and range)

	Diet		Exercise		Probability of effect	
Organ as % body mass	High fat	Low fat	Wheel	Control	Diet	Exercise
Heart ^{KW}	0.727	0.635	0.724	0.603	0.163	0.009
	0.450-1.271	0.541-1.361	0.614-1.361	0.450-1.271		
Liver ^{KW}	4.676	4.701	4.709	4.650	0.763	0.970
	3.657-5.811	3.656-5.037	3.656-5.811	3.657-5.037		

4.5.5 Body composition

Body composition measurements were made on the carcass after the removal of the heart and liver.

Individual body fat values ranged from 7.7% in a LF WH to 37.8% in a HF CON mouse but the differences between either diet or exercise groups) were not statistically significant (P>0.05 Figure 4.11, Table 4.7). When exploring diet and exercise groups further, mice had body fat in the following order (greatest first): HF CON, HF WH, LF CON, LF WH with percentages of 14.6, 13.7, 12.9 and 10.7 respectively.

There was no interaction between diet and exercise on body fat.

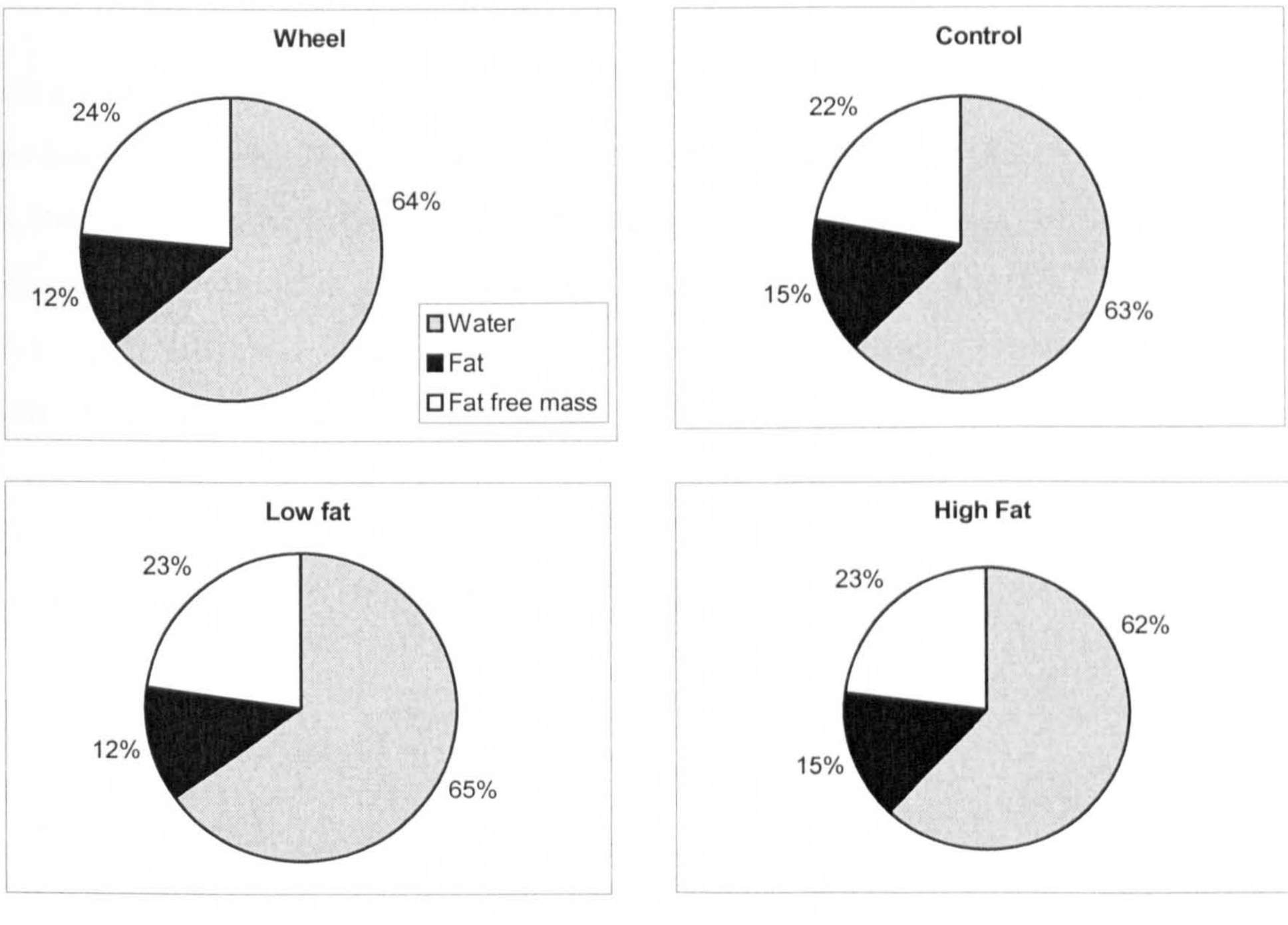


Figure 4.11 Body composition data by diet and exercise group (arithmetic means)

LF mice had significantly more body water than HF mice (65.4 v. 62.3%, P = 0.019) but there was no significant difference between CON and WH mice (64.4 v. 64.5%, P = 0.880, Figure 4.11 and Table 4.7).

Fat-free dry matter (FFM) contributed a similar proportion to total body mass in all groups, with no statistically significant differences (Table 4.7 and Figure 4.11).

The data included the mouse that grew much more than any other (finishing the study 9g heavier than the next heaviest mouse). This mouse had a carcass constituting 38% body fat, 45% water and 17% FFM. As these values were markedly different from the other mice (the next greatest percentage of body fat was 21% in a LF CON mouse), analyses were performed after excluding this mouse to observe whether this one mouse had skewed the results. Table 4.8 shows the data obtained. P-values were increased, but the difference in body water between HF and LF remained significant (63.1 v. 64.9%, P = 0.039).

On a g/mouse basis, the only statistically significant difference was of dietary group on body water, with HF mice containing 0.8g more than LF mice (13.1 v. 12.3g P = 0.004), the converse of the observation when data were expressed as % of carcass. The amount of fat in HF mice approached significance compared with LF, 2.8v. 2.3g, P = 0.081. There was no effect of exercise group on any of the variables and no interactions were observed (Table 4.9).

Table 4.7 Body composition data for all mice (n = 16/group)

	Diet		Exercise		Probability of Effects		
Variable	**HF**	**LF**	**WH**	**CON**	**Diet**	**Exercise**	**Diet*exercise**
% body fat [Log10]	14.1	11.7	12.1	13.7	0.107	0.292	0.598
	12.1-16.5	10.1-13.7	10.3-14.2	11.7-16.1			
% water[KW]	62.3	65.4	64.5	64.4	0.019	0.880	-
	44.9-67.3	64.3-67.3	59.6-67.3	44.9-67.3			
% FFM[KW]	23.2	23.5	23.4	23.1	0.498	0.243	-
	17.4-27.7	12.2-27.8	22.4-25.2	12.2-27.8			

Body fat data are means and 95% CI, water and FFM are medians and ranges

Table 4.8 Body composition data excluding the heaviest mouse (n)

	Diet		Exercise		Probability of Effects		
Variable	**HF (15)**	**LF (16)**	**WH (16)**	**CON (15)**	**Diet**	**Exercise**	**Diet*exercise**
% body fat [Log10]	13.2	11.7	12.1	12.8	0.209	0.569	0.178
	11.6-15.1	10.3-13.4	10.6-13.8	11.2-14.6			
% water	63.1	64.9	64.1	64.0	0.039	0.972	0.686
	61.9-64.3	63.7-66.1	62.8-65.4	62.7-65.3			
% FFM[KW]	23.3	23.5	23.4	23.2	0.664	0.363	-
	21.8-27.7	12.2-27.8	22.4-25.2	12.2-27.8			

Body fat and water data are means and 95% CI, FFM are medians and ranges

Table 4.9 Body composition (g/mouse) excluding the heaviest mouse (n) (mean and SEM)

Variable (g)	HF		LF		Probability of Effects		
	WH (8)	CON (7)	WH (8)	CON (8)	Diet	Exercise	Diet*exercise
Water	13.3	12.9	12.4	12.3	0.004	0.344	0.575
	0.25	0.26	0.25	0.25			
Fat	2.9	2.7	2.1	2.5	0.081	0.758	0.248
	0.27	0.29	0.27	0.27			
FFM	1.3	1.1	1.1	1.1	0.160	0.390	0.361
	0.09	0.09	0.09	0.09			

4.5.6 TNF-alpha

Concentrations of TNF-α were low in all plasma samples. Concentrations of TNF-α were lower in WH than CON mice (median values 44.8 v. 49.7pg/ml respectively). LF and HF mice had identical median TNF- α concentrations of 48.2pg/ml (Table 4.10 and Figure 4.12). Neither the difference between WH and CON nor between HF and LF mice approached significance ($P > 0.05$).

The ELISA required 100μl of plasma per sample. Blood samples from four mice did not produce enough plasma for duplicate tests and for three mice there was less than 100μl in the duplicate sample. When analyses were re-run including only samples for which there was enough plasma for two complete measurements, there were only marginal alterations in the median values of TNF-α concentration (Table 4.11). The difference between WH and CON mice became even less significant, with the CON concentration becoming closer to that of WH mice. The difference between HF and LF mice widened, though not statistically ($P<0.05$) so, with the median LF concentration decreasing and the HF concentration remaining the same (44.8 v. 48.2 pg/ml, Table 4.11 and Figure 4.13).

Table 4.10 TNF- α concentrations of mice by diet and exercise group (pg/ml) (median and range)

Variable	Exercise (n)		Diet (n)		Probability of effects	
	Wheel (16)	Control (16)	HF (16)	LF (16)	Exercise	Diet
TNF-α^{KW}	44.8	49.7	48.2	48.2	0.141	0.752
	40.6-57.3	42.2-84.9	43.3-65.4	40.6-84.9		

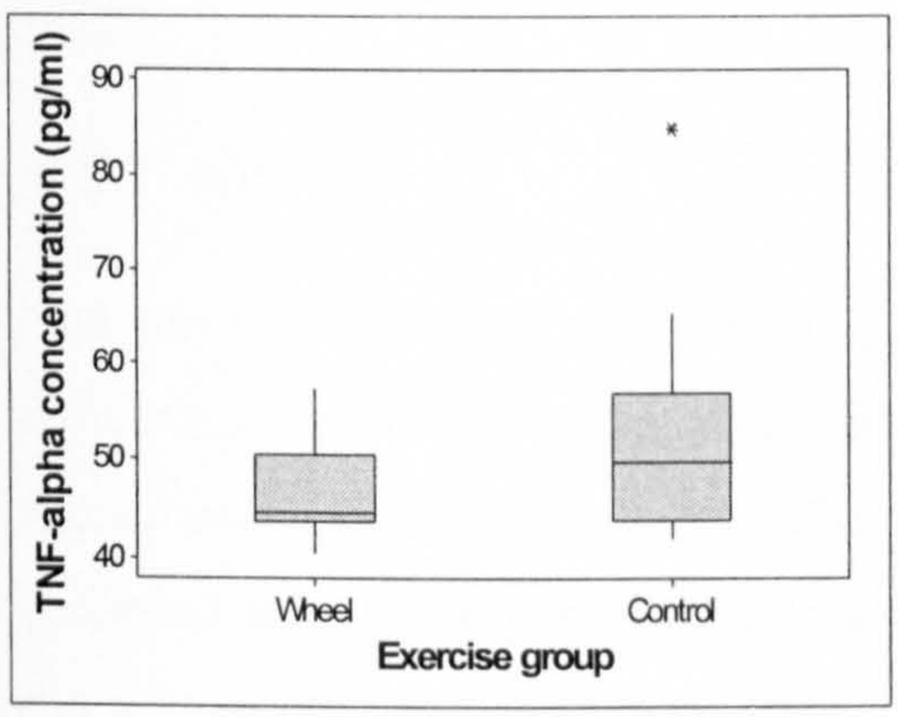


Figure 4.12 Concentrations of TNF-α by exercise and diet group

Table 4.11 TNF-α concentrations (pg/ml) for samples with complete duplicates only (median and range)

Variable	Exercise (n)		Diet (n)		Probability of effects	
	Wheel (15)	Control (9)	HF (14)	LF (10)	Exercise	Diet
TNF-α^{KW}	44.7	48.9	48.2	44.8	0.355	0.178
	40.6-57.3	42.2-65.4	43.3-65.4	40.6-51.4		

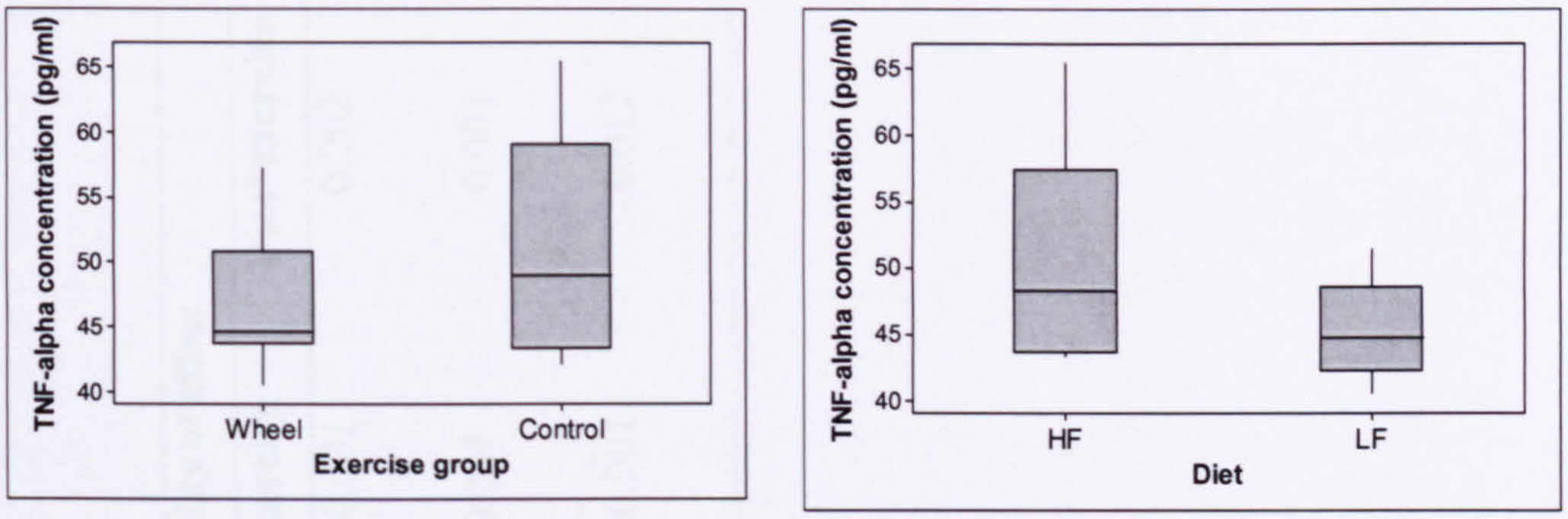


Figure 4.13 Concentrations of TNF-α for samples with complete duplicates only

4.5.7 NEPA

The non-exercise physical activity (NEPA) of each mouse was recorded twice over the duration of the study using the InfraMot, for 24 hours on each occasion. One mouse from each treatment group (HF CON, HF WH, LF CON, LF WH) was recorded each day. Figure 4.14 shows the differences in activity by individual mice in the first and second recordings. There were very large between-mouse differences but only small differences in activity levels between the two days of recordings, and overall the between-day differences were not significant (P = 0.368).

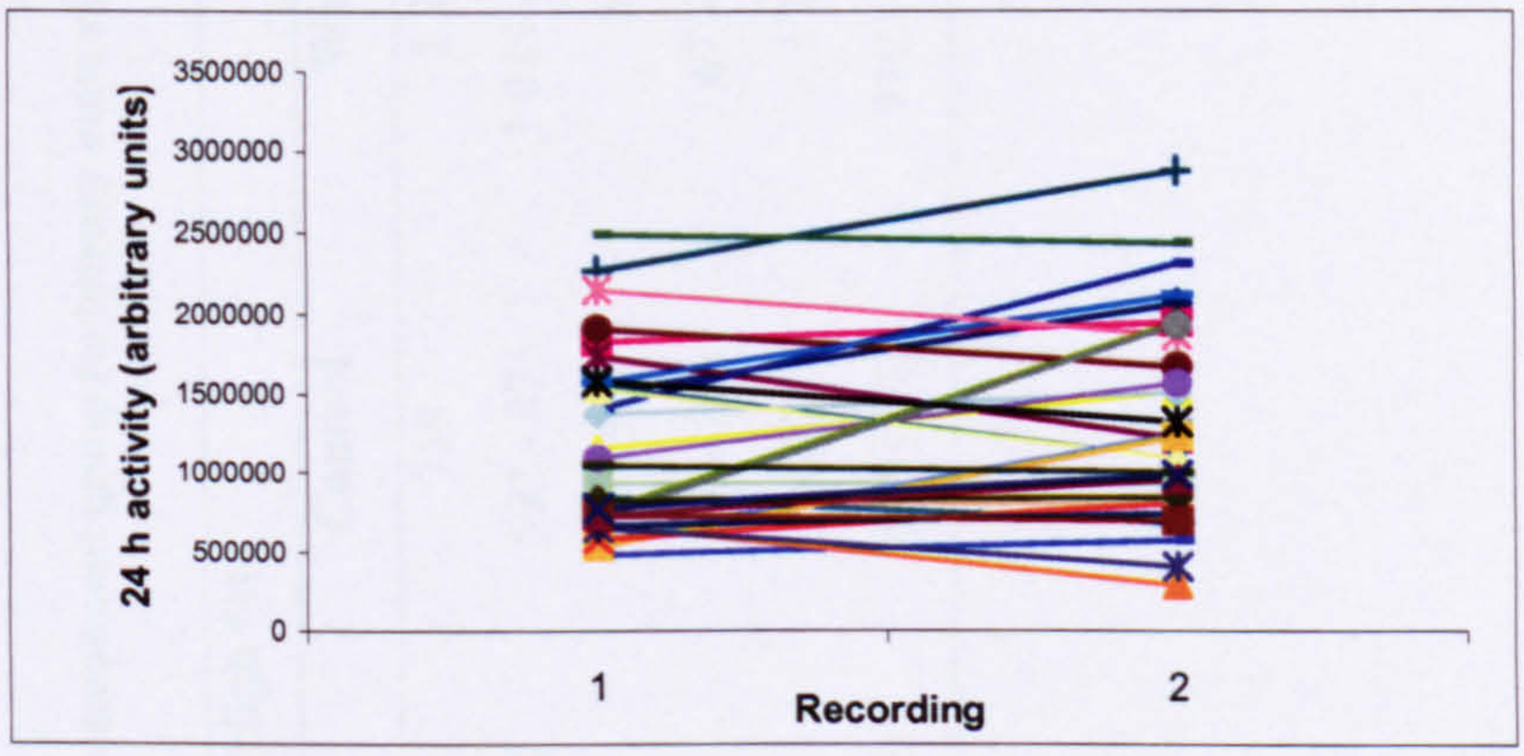


Figure 4.14 Differences in total activity by individual mice in each 24h recording

Table 4.12 NEPA of mice by each treatment group (arbitrary units x10^3) (mean and 95% CI)

	High fat		Low fat		Probability of effect		
Variable	**Wheel**	**Control**	**Wheel**	**Control**	**Diet**	**Exercise**	**Diet*exercise**
Total 24h activity ^{Log10}	1 694 1 393 - 2 061	719 592 - 875	1 239 1 019 - 1 507	1 014 834 - 1 233	0.908	<0.001	0.002
Light-phase $_{Log10}$	80 65 - 99	85 69 - 106	82 67 - 102	193 157 - 240	0.002	0.001	0.001
Dark-phase $_{Log10}$	1 607 1 292 - 1 999	622 500 - 774	1 143 919 - 1 422	791 636 - 984	0.728	<0.001	0.012

n = 8 for each group

Table 4.12 shows the differences in activity of the mice by treatment group. Whilst there was no effect of diet on total 24h activity, HF WH recorded the most activity, followed by LF WH and LF CON. HF CON were the least active over the 24h recording period. As expected, there was a significant ($P<0.001$) effect of exercise treatment on 24h activity, with both LF and HF WH recording more activity than their sedentary counterparts. CON mice recorded 59% of the activity of WH mice (855 $x10^3$ v. 1 449 $x10^3$ counts, Figure 4.15).

The activity recorded during the light phase was affected by both diet and exercise ($P = 0.002$ and 0.001 respectively), with LF CON mice the most active group, recording twice the activity of the other 3 groups (193 $x10^3$ counts). Activity during the dark phase was unaffected by diet ($P = 0.728$) but significantly affected by exercise group ($P<0.001$), with both HF and LF WH more active than HF and LF CON.

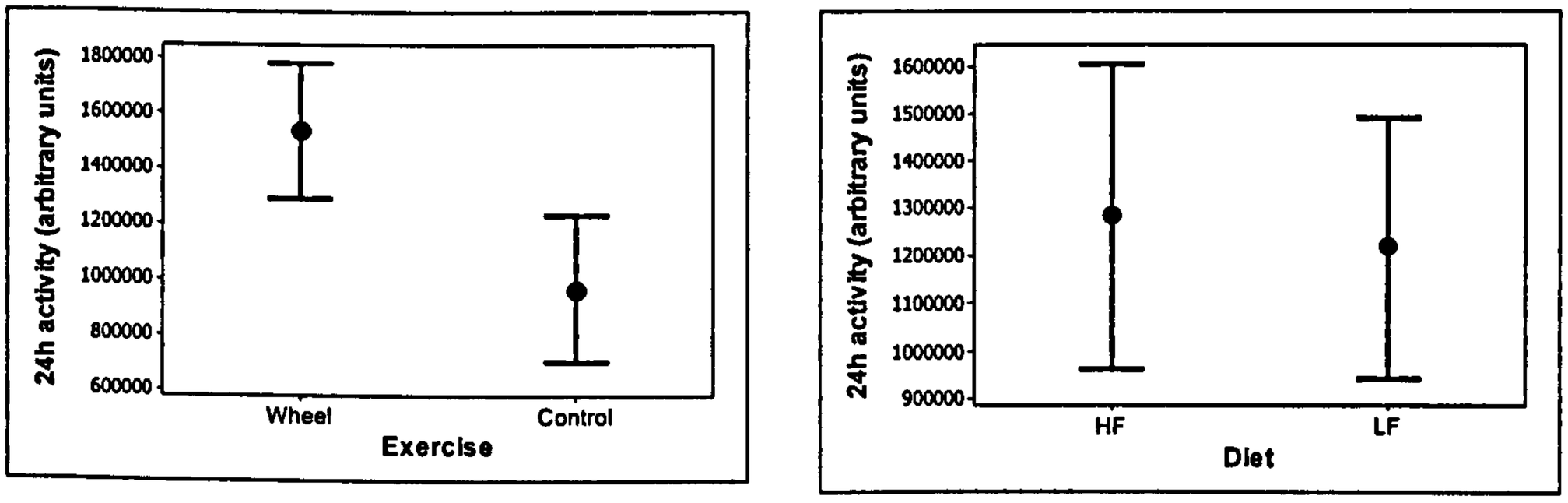


Figure 4.15 Mean and 95% CI of 24 h activity for exercise and diet groups (arithmetic means, n = 16)

Activity during the dark phase was considerably higher than activity during the light for all groups, with on average 9.5 times more activity during the dark than the light (1 133 $x10^3$ v. 119 $x10^3$ counts, arithmetic means). However, whilst LF mice were more active during the light than HF mice ($P = 0.002$), there was no significant difference in mean activity between the two diet groups during the dark phase (Figure 4.16). When averaged across both diet groups, WH mice were 1.9x more active than CON during the dark (1 355 $x10^3$ v. 701 $x10^3$, $P<0.001$).

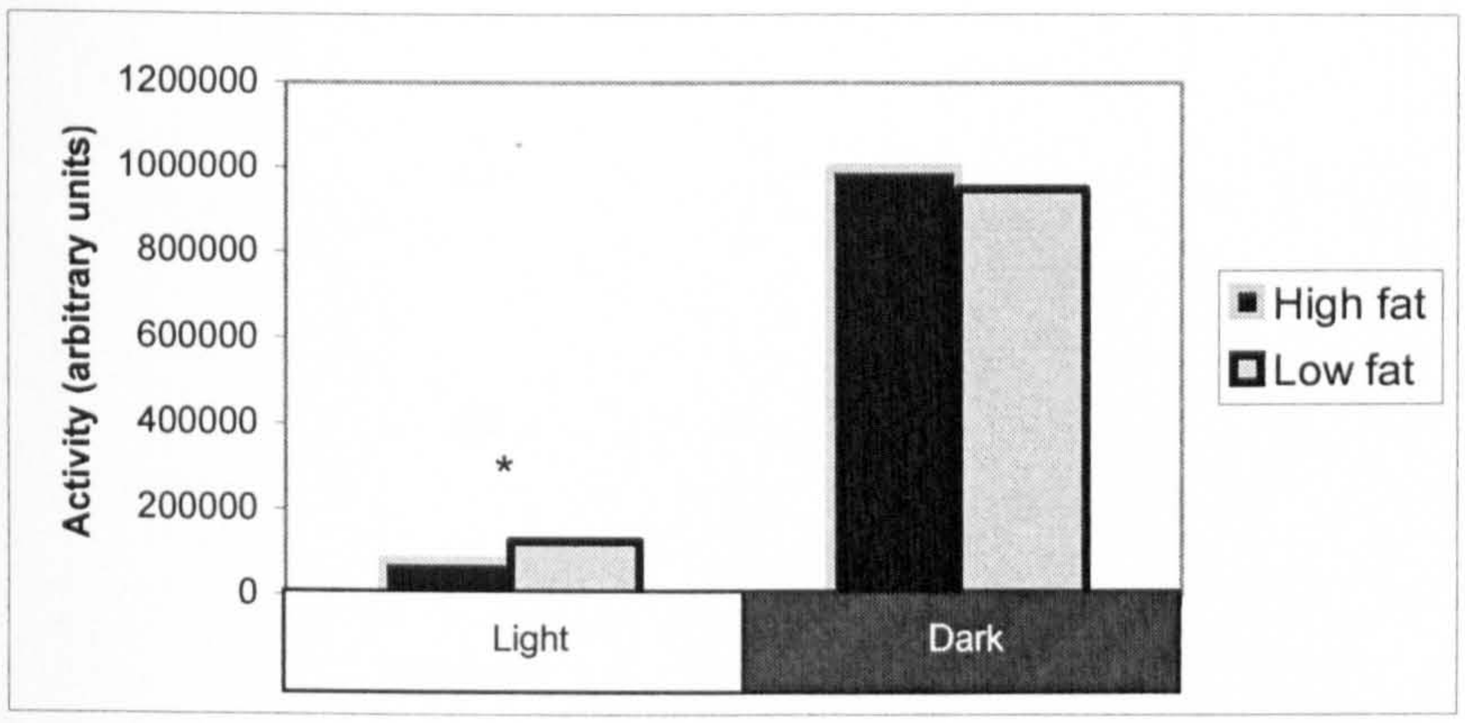


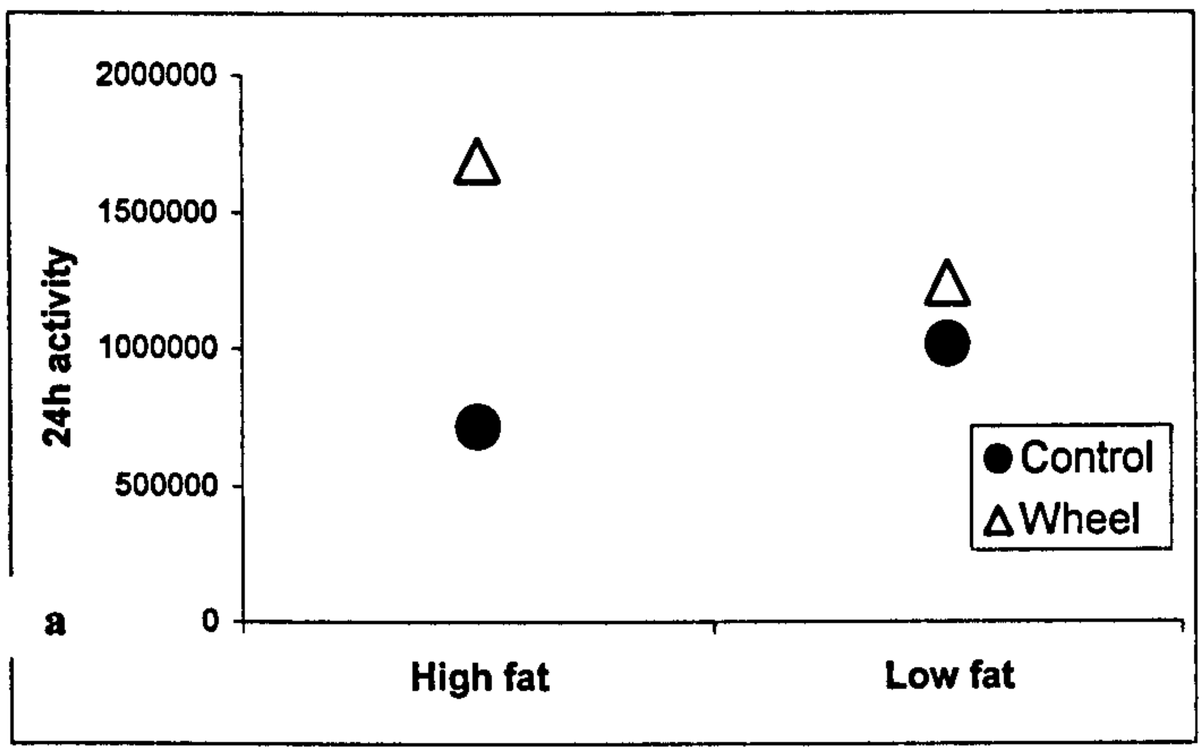
Figure 4.16 Activity during light and dark phases by HF and LF mice (* p = 0.002)

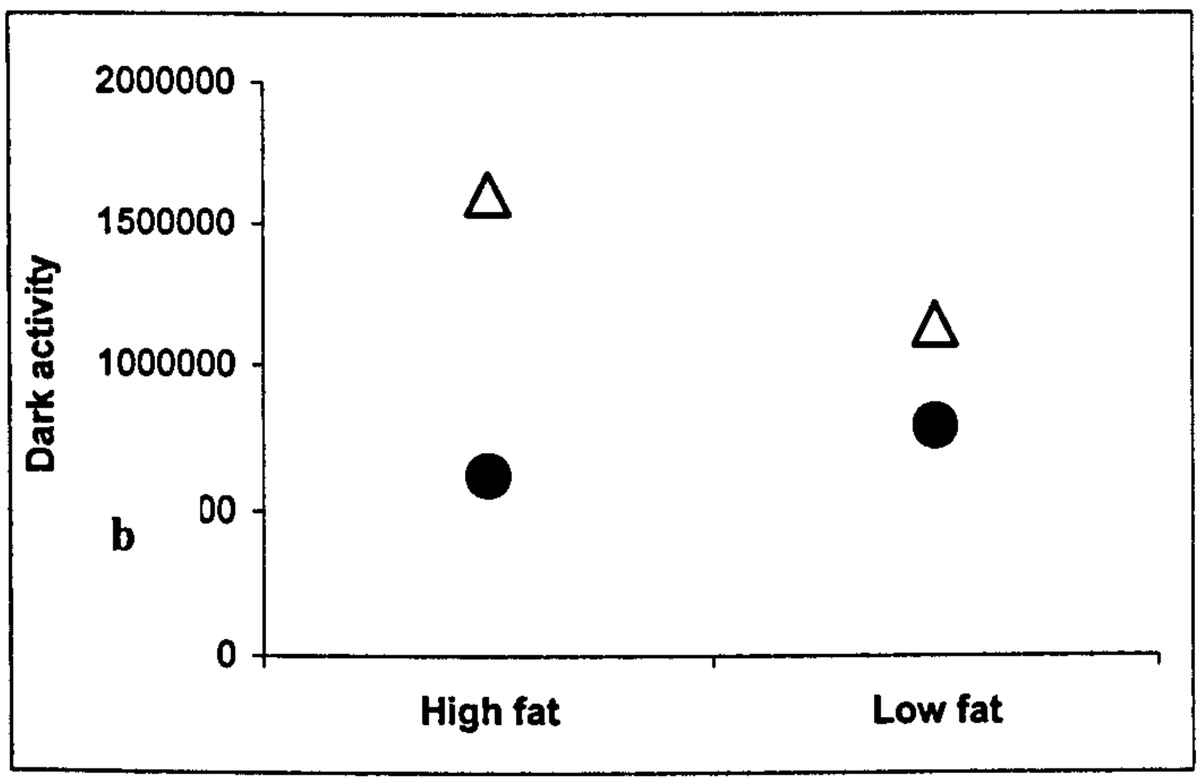
There were significant interactions between diet and exercise on all three NEPA variables; total 24h activity (P = 0.002), light-phase activity (P = 0.001), and dark-phase activity (P = 0.012, Table 4.12 and Figure 4.17).

Whilst HF WH were more than twice as active as HF CON over 24h, the activity of LF WH and LF CON was more similar, with LF WH recording 1 239 $x10^3$ counts compared with 1 014 x 10^3 of LF CON.

During the dark phase, WH mice of both diet groups were more active than the CON mice of their respective diet group, but LF CON were more active than HF CON and LF WH were less active than HF WH.

HF CON and HF WH had similar, low levels of activity during the light phase, which were matched by LF WH. However, LF CON mice recorded very high levels of activity during this phase, more than doubling the activity of the other groups (Figure 4.17).





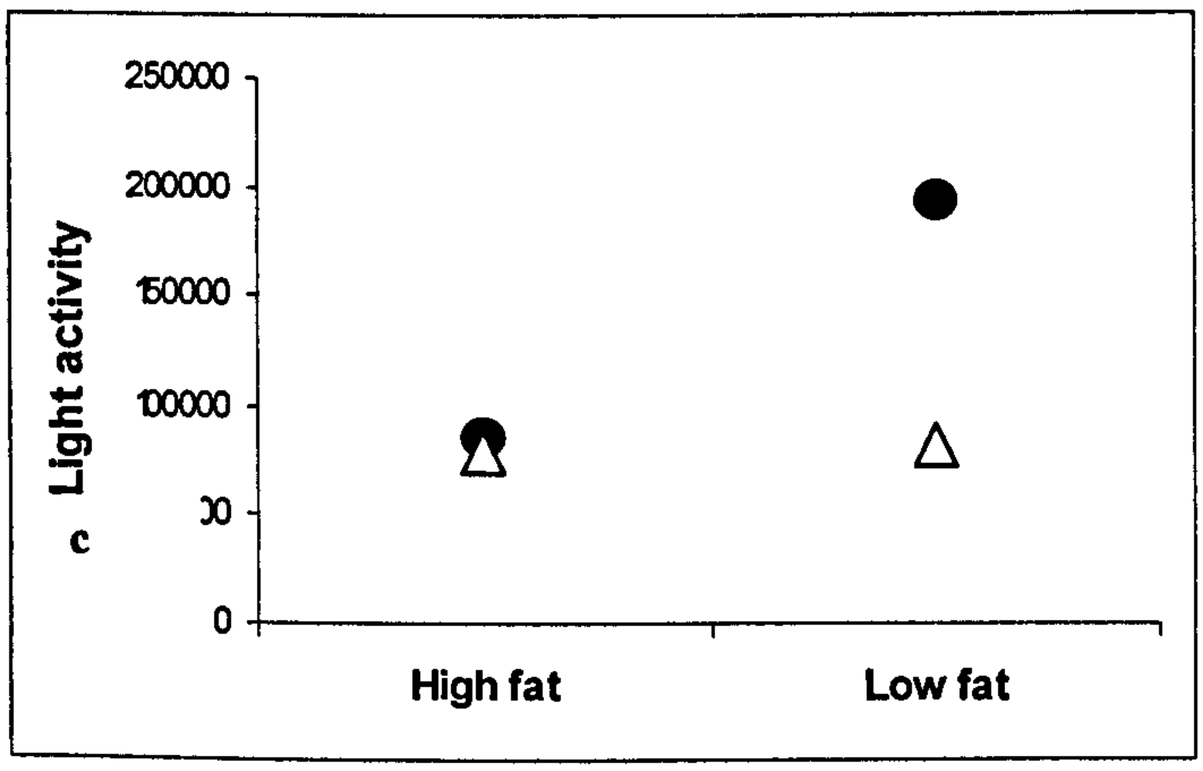
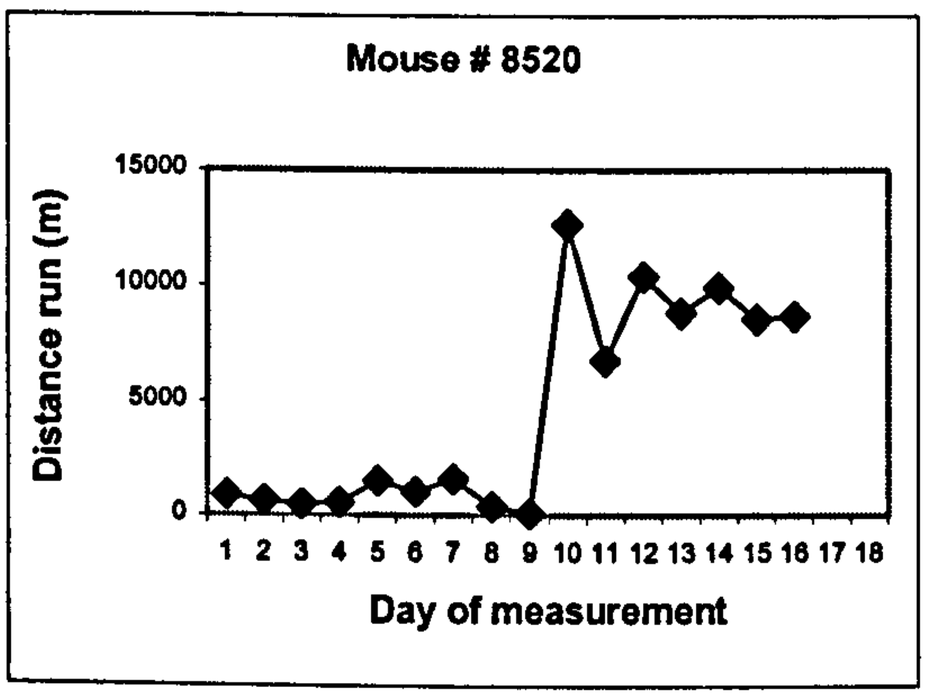


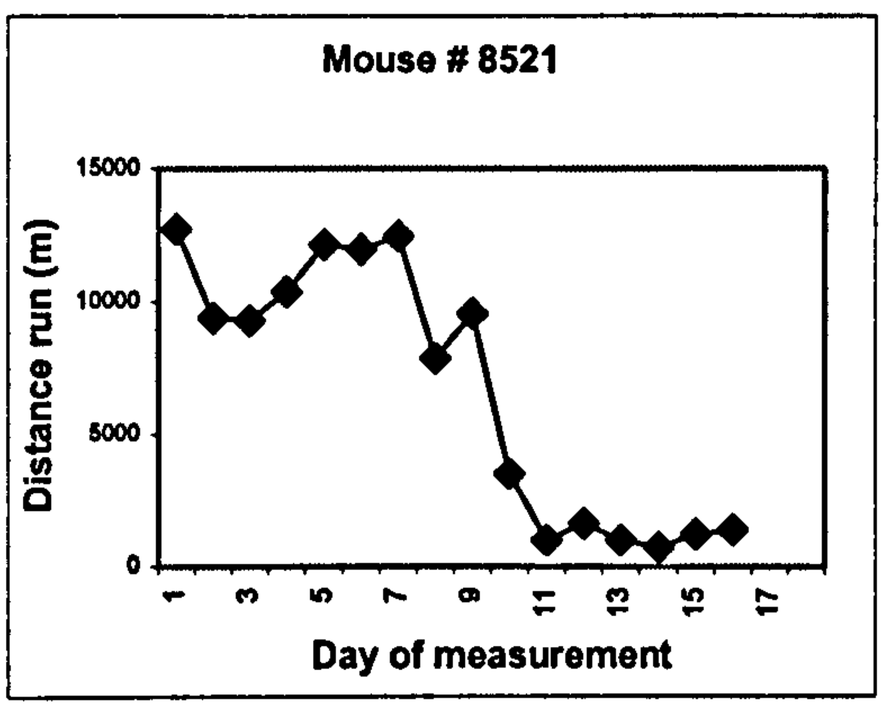
Figure 4.17 Interactions between exercise and diet for (a) 24h, (b) dark-phase and (c) light-phase activity (arbitrary units)

4.5.8 Oestrus cycle

A previous study (ES2) had discovered that some female mice demonstrated a cyclical pattern with regard to the daily distance run in wheels, when no such pattern was evident in males.

During the present investigation, the mice were assessed for stage of oestrus at the same time on each of 17 consecutive days, as described in the Specific Methods section to this chapter (4.4.5). Four main stages were identified, designated 1-4.





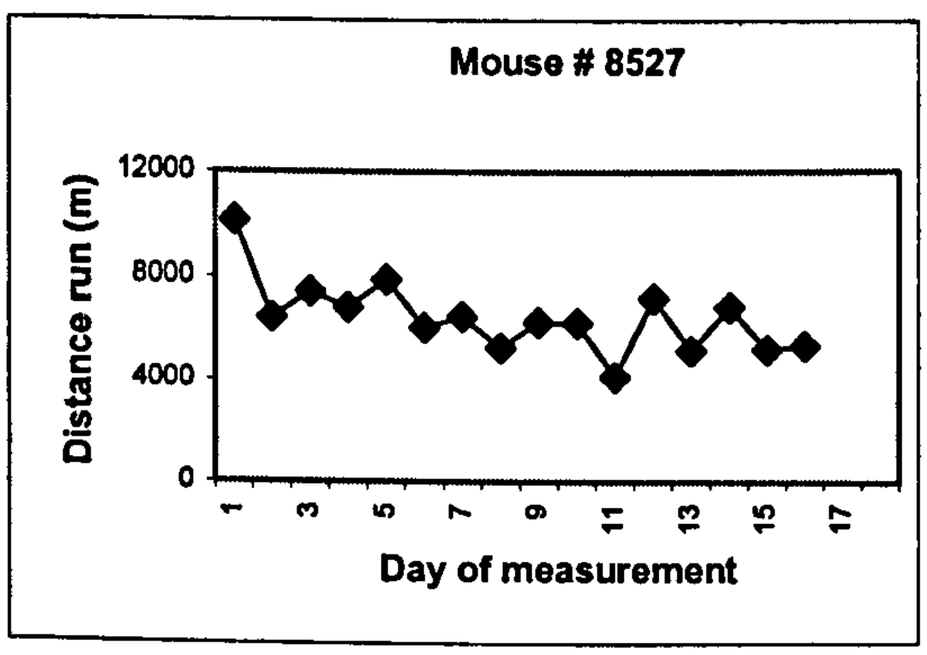


Figure 4.18 Examples of the variation in running distance and pattern in mice during the 17d period during which measurement of oestrus were made. Mice 8520 and 8521 show extreme patterns, while mouse 8527 is more typical of the patterns observed in this study

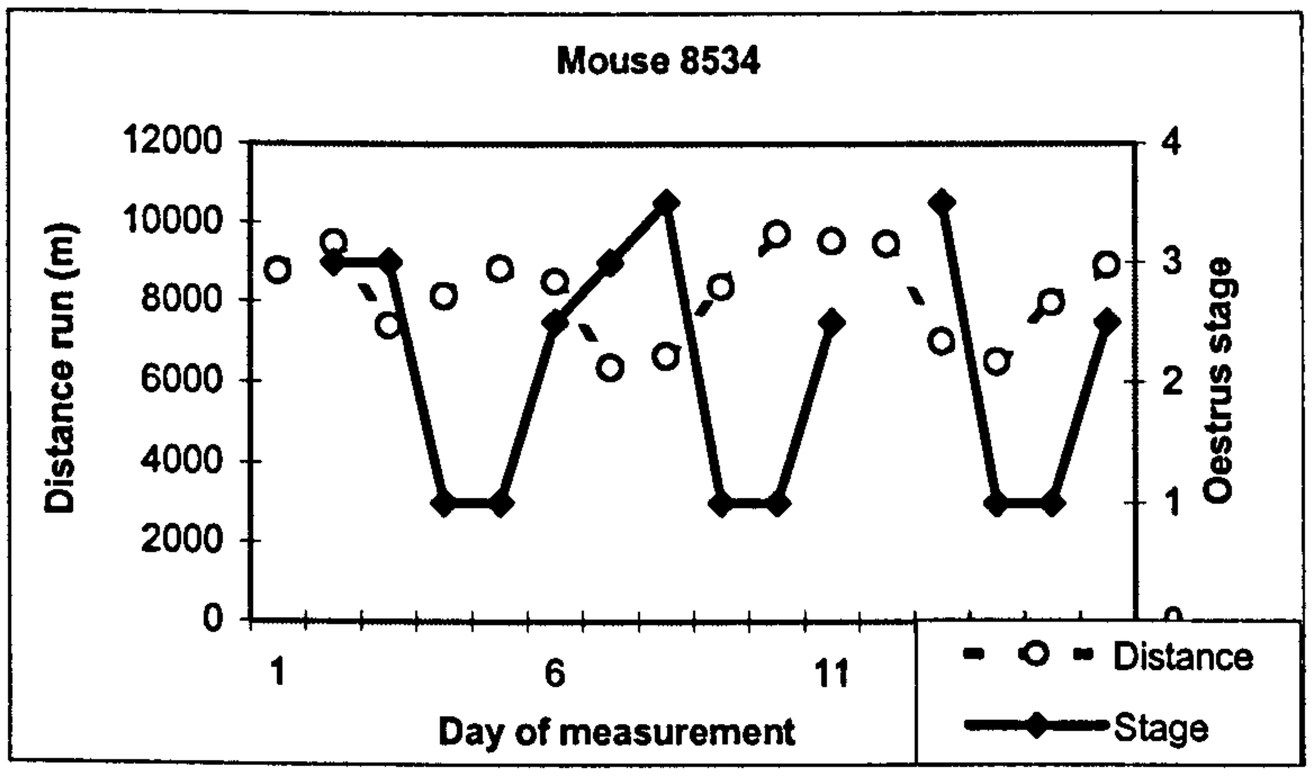


Figure 4.19 Relationship between oestrus stage and running distance

A cyclical pattern of running was not as obvious in this study as in the previous study (ES2), as shown in Figure 4.18. Mice demonstrated varying cycle lengths and time spent in each stage. Stage data were unavailable for 38 of 272 samples due to technical difficulties including the cells not taking up the stain or too few cells being present in the sample. In particular there were few slides of stage 2, either due to lack of staining or through mice passing through this stage quickly, before samples were taken.

The pattern that seemed to emerge was one of decreased running during- and post-oestrus (stage 3-4) followed by increased distance pre-oestrus (stages 1-2). Figure 4.19 shows an example of this possible link.

The difference between the distance run during each stage of the oestrus cycle was tested using one-way ANOVA with Tukey's 95% simultaneous confidence intervals. This test "provides confidence intervals for all pairwise differences between level means" (Minitab). An error rate of 5 was chosen to provide the 95% confidence intervals.

The test showed that the distances run during stages 3 and 4 were significantly difference from stage 2 ($P = 0.021$, Figure 4.20), i.e. the distance run during stage 2 (pre-oestrus) was significantly further than that run during and just after oestrus.

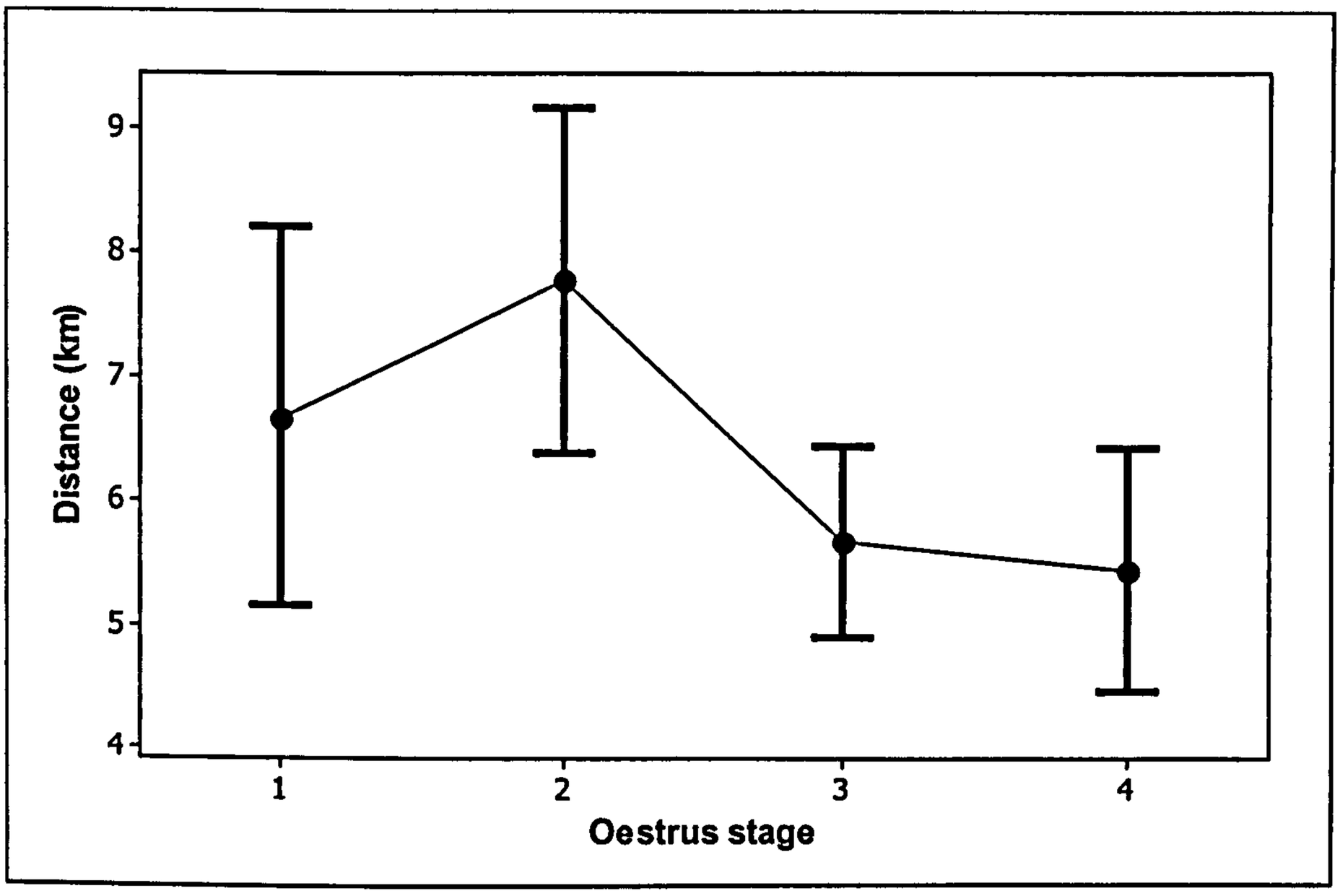


Figure 4.20 Means and 95%CI for the distances run during each stage of the oestrus cycle

4.6 Discussion

This investigation was carried out to assess the impact of diet and exercise on weight gain, body composition and inflammatory status.

4.6.1 Wheel-running and NEPA

Previous studies (ES2 and ES3) suggested that the mice would use their wheels, and indeed there were few problems with this form of exercise, although the inter-animal distances run varied considerably. The average daily distance run by HF mice was 6.6km, and by LF mice, 6.7km. Table 4.13 compares some studies of mice given access to running wheels and shows that the distances run by the mice in this study are similar to

those run by male mice in a comparison of 7 different mouse strains (Lerman et al., 2002), and almost double those run by the same strain of mice (C57BL/6) in the study of Lightfoot *et al.* (2004), although female mice of this strain ran approximately 5km/d . The studies using Min mice (which are on a C57BL/6 background) observed distances that were also much less than those of this study (Colbert *et al.*, (2003), Mehl *et al.*, (2005b) ES2 and ES3). Indeed, the distances in this study compare favourably with distances run by female mice selected and bred for their running ability, which covered 6.5km/day (Bronikowski et al., 2001). However, Bell *et al.* (1997) recorded distances of 14.9km for LF and 14.5km/d for HF-fed mice which, given the maximum average daily distance in the inter-strain study of Lightfoot *et al.* (2004) was 7.95km by C57L/J mice (10km for the females), is a high value, though the Swiss Albino mice used by Bell *et al.* were not included in the comparison.

The InfraMot system does not allow us to distinguish activity that is wheel running from any other cage activity. A literature search has not yielded any evidence of increased cage activity by mice that also have access to a wheel. For this reason, the difference in activity between WH and CON is not discussed in detail.

Unlike the small number of WH mice recorded in ES3, this study found a highly significant increased in total activity of WH mice compared with CON mice. This had been expected, but it is not possible to determine whether this increase is solely due to the running or whether the presence of a wheel in the cage caused the mice to be more inquisitive and active in exploring the wheel and the cage in general.

For each NEPA variable, LF CON mice were more active than HF CON (Table 4.12). This is in accord with Funkat et al., (2004) who reported decreased activity in C57BL/6 mice fed HF diets compared with those fed control diets, an effect also observed in rats (Bartol-Munier et al., 2006). However, Funkat et al. (2004) also reported a decrease in wheel-running activity of HF-fed mice, which is the opposite of the observations made in this study. No explanation is offered for this finding. The differences in activity may have been linked to food intake, but as LF intake was not measurable, further investigation of this phenomenon is required. To date, NEPA has not been measured in any other studies of exercise in mice.

Table 4.13 Summary of distance run (km/d) by mice given free access to exercise wheels

Mouse strain	Sex	Age (wk)	Distance (km)	Length of study (wk)	Reference
C57BL/6	Combined	9	3.8	3	Lightfoot et al., (2004)*
C57BL/6	Male	20-24	7.6	2	Lerman et al. (2002)**
C57BL6/Min	Female	5-6	3.6	2-3 (Quarantine only)	Colbert et al., (2003)
	Male	5-6	3.1	2-3 (Quarantine only)	"
C57BL6/Min	Female	4	3.5	9	Mehl et al., (2005b)
	Male	4	4.7	9	"
C57BL6/Min	Female	5	4.5	10	ES2
	Male	5	2.1	10	"
C57BL6/Min	Female	5	3.3	10	ES3
	Male	5	1.9	10	"
Swiss Albino	Female – low fat diet	6	14.9	8	Bell et al., (1997)
	Female – high fat diet	6	14.5	8	"
Outbred Hsd:ICR	Female	6-8	3.6	6 days	Bronikowski et al., (2001)
Outbred Hsd:ICR	Male	6-8	2.6	6 days	"
Outbred Hsd:ICR selected* **	Female	6-8	6.5	6 days	"
Outbred Hsd:ICR selected* **	Male	6-8	4.3	6 days	"

*** 14 strains of mice were analysed in this study, C57BL/6 shown here as this is the Min background strain, ** 7 strains of mice were analysed , *** mice have been bred for wheel-running ability**

4.6.2 Food intake

Food consumption was only measurable on the HF-fed mice, as the LF diet was powder and was scattered by the mice. There was no detectable difference in intake between WH and CON mice, which was unexpected given the anticipated extra energy expenditure of the WH mice. ES2 and ES3, Bell et al. (1997) and others (Koteja *et al.*, 1999; Bronikowski *et al.*, 2001; Mehl *et al.*, 2005b) have also reported an increase in food consumption among wheel-running mice. Bell et al. (1997) found an increased energy intake of HF mice compared with LF for both WH and CON mice, perhaps suggesting that the HF food was more palatable, as the WH mice did not need the extra energy for running, as they did not run further than the LF mice. Bell et al. (1997) did not note any problems in measuring food intake, but also did not give any detail of how it was achieved. The inherent problems in measuring food intake (as mentioned in 4.5.2) mean that the validity of our results is thrown into doubt.

4.6.3 Body mass and composition

The lack of difference in body weights between WH and CON are in accordance with the results of ES2 and 3 but, in contrast, Bell et al. (1997) found that exercised mice were lighter than controls. The lack of difference in this study could have been due to the formation of muscle tissue by the WH mice as opposed to the fat produced by the control mice. However, this supposition was not fully supported by the body composition analysis, because although CON mice had more body fat and less non-fat dry matter (including protein from muscle), the differences between WH and CON mice were not significant.

The significant effect of diet on weekly body weights was due to the HF mice weighing more than the LF mice. The HF mice consisted of more fat and significantly more water than the LF mice (when expressed as g/mouse), and agrees with the findings of Bell et al., (1997) who used the same formulation diets as in this experiment.

Why one mouse grew more than the others, and consisted of 38% fat, is harder to explain. C57BL/6 mice have a preference for fat when offered a choice of macronutrients and a tendency to obesity (Smith et al., 2000), but the mice did not have a choice of food, and this mouse in particular did not eat more than the other mice over the recording period. The average intake for the heavy mouse on week 4 was 2.9g compared with a group average of 3.1g, and 3.4g on week 5 compared with 3.1g for the group. However, out of the 64 InfraMot recordings generated by all the mice on study, the heavy mouse recorded the least and tenth least amounts of activity for its 2 recordings. If this mouse had continued to be inactive and had increased its food consumption the weight gain could perhaps be explained. However, there was no correlation between final body weight and total 24h activity ($r = -0.07$, $P = 0.704$).

Organ weights

This study found an increase in heart weight that had not been detected in previous investigations (ES2). This increase is consistent with reports of cardiac hypertrophy in response to prolonged exercise exposure (McArdle *et al.*, 2001; Mehl *et al.*, 2005b). HF-fed mice had heavier livers than their LF-fed counterparts, a finding also seen in female Zucker rats (Jen et al., 2003), however, as % body mass there was no difference between either the diet or exercise groups in this experiment, the converse of that found in ES2 and 3, suggesting that the increased mass was simply a result of the mice being larger. Schemmel et al. (1992) found that treadmill exercise in young female rats did not change body mass or organ weights compared with controls and Jen et al., (2003) found no effect of swimming exercise on liver weight in female Zucker rats.

4.6.4 TNF-α

TNF-α was measured in this investigation as a biomarker of inflammatory status, as obesity is associated with increased levels of inflammatory proteins such as C-reactive protein and TNF-α (Panagiotakos et al., 2005b). Feeding HF diets led to a non-significant increase in TNF- α concentration compared with LF-fed mice, perhaps suggesting that the higher fat diet was causing some increase in inflammatory status, which would tie in with the increased body fat of the HF-fed mice. Other authors (Borst & Conover, 2005)

did not find an increase in serum TNF- α in young rats fed a high fat diet, even though the rats became obese. However, soluble TNF- α was increased in muscle and fat.

Physical activity is associated with decreased levels of inflammatory markers in humans (Pischon *et al.*, 2003; Colbert *et al.*, 2004; Panagiotakos *et al.*, 2005a). This study did not find a significant effect of wheel-running on plasma TNF- α, although concentrations were reduced in WH compared with CON mice. The effect of acute treadmill exercise on plasma TNF- α in C57BL/6 mice has been measured, and the authors found no difference from control animals (Colbert et al., 2001). Analysis of various tissues found no increase in muscle, liver or brain TNF-α mRNA, although fatiguing exercise produced an increase in lung mRNA (Colbert et al., 2001).

This suggests that either the exercise is not sufficient to reduce TNF- α or that the diets in this study were insufficient to induce obesity and stimulate an inflammatory state in the animals. A different marker may have been more sensitive, e.g. IL-6 is reduced in physically active people (Colbert *et al.*, 2004; Panagiotakos *et al.*, 2005a). This is thought to be due to a decrease in fat mass, as IL-6 is released from adipocytes (Pischon et al., 2003). However, IL-6 was not found to be different in earlier studies in this thesis (ES1), perhaps due to the lack of body fat.

4.6.5 Oestrus cycle

The links between oestrus and running distance have not been reported previously for laboratory mice, although similar studies exist for other species such as the golden hamster, which runs further during pre-oestrus and oestrus (Richards, 1966) and lab rats, which run most before oestrus and least immediately after (Anantharamanbarr & Decombaz, 1989). The experiment in this chapter discovered that mice ran less as they were coming into oestrus, with a low post-oestrus, then increased the distance to a high pre-oestrus, which is in accord with the findings from the rat.

The reason for this alteration in running distance is unknown and could be compared with total cage activity as measured by the InfraMot. The study in rats (Anantharamanbarr & Decombaz, 1989) also measured total energy expenditure by indirect calorimetry, but did not find a clear relationship between energy expenditure and day of oestrus cycle in

wheel-running rats; however, sedentary rats decreased their activity after oestrus. The presence of a running wheel may have marred the measurement of energy expenditure in the wheel-running rats, as they may have felt some compulsion to perform extra activity. Nevertheless, these results agree with our own and highlight the importance of long-term monitoring of wheel running behaviour in female animals to prevent making judgements on running ability that may have been influenced by the oestrus cycle.

4.7 Conclusions

This study was performed to address a number of hypotheses, and has answered them as follows:

- Mice fed high fat diets will have more body fat than those fed low fat diets.

The HF diet caused a non-significant increase in body fat.

- Wheel-running mice will have less body fat and heavier hearts than controls.

Wheel-running led to a non-significant decrease in body fat and a significantly heavier heart.

- HF mice will have higher plasma TNF-α concentrations.

TNF-α was increased non-significantly in HF mice, and non-significantly decreased in WH mice.

In the context of colon cancer, obesity and chronic inflammation are associated with increased risk (Panagiotakos et al., 2005b). Further studies of this nature should use a higher fat diet, or a mouse more predisposed to obesity, as AOM treatment in a mouse genetically altered to display both obese and diabetic phenotypes (C57BL/KsJ-*db/db*) developed more pre-malignant colon lesions compared with either heterozygotes or wild type animals (Hirose et al., 2004). Crossing *ob/ob* mice with Min mice may allow closer assessment of the effects of obesity on colon cancer development. Different inflammatory markers in adipose tissue and muscle could also be measured. There was some variability in response to the running wheel, so increased numbers of mice may help to increase the number of mice who run well.

Chapter 5

5 Summary

The three initial experiments in this thesis demonstrated that exercise does not affect tumour number in the Min mouse. This is in agreement with the findings of others (Colbert *et al.*, 2000; Colbert *et al.*, 2003) and partial agreement with Mehl *et al.* (2005). The lack of effect of exercise in Min mice compared with rats and humans may be due to the lack of progression of adenomas to carcinomas, and exercise may be beneficial in reducing the progression of adenomas. As no molecular biology was carried out on the mucosa or adenoma tissue, it is possible that exercise made changes at the tissue level which may not be observed by only assessing the number and size of adenomas. In addition, rates of apoptosis as well as proliferation would provide further important information on tissue growth, and further molecular and cytological investigation is warranted.

A constant finding was that neither a low fat diet nor exercise reduced body fat compared with high fat diets and/or lack of additional exercise. Differences in NEPA between male and female mice, and between exercising and non-exercising mice should also be investigated further to assess the impact of the exercise regime on additional cage activity. Further experimentation on energy intake and accurate assessment of energy expenditure via indirect calorimetry may provide the answer to this finding.

The lack of change of body fatness in mice in either chapter is important for understanding the lack of effect of exercise on tumourigenesis. The main difference between rat and mouse studies to date has been the decrease in body weight and fat mass in exercised rats, compared with most studies of exercised Min mice. This inability to alter energy balance suggests a different approach is required, as mentioned in earlier chapters (3.6.2). The lack of difference in body fat in mice fed diets with different fat contents may have been due to increased food intake, as low fat fed control mice also had greater NEPA.

This thesis has shown that the Min mouse model does not demonstrate plasticity in tumour number in response to exercise, and different mouse models or exercise regimes

may be required to reinforce the observations from rat and human studies of physical activity and colon cancer.

Appendices

6 Appendices

6.1 Genotyping

6.1.1 Tail Biopsy Sampling

1. Mice were placed into an induction chamber with 3% isofluorane in oxygen flowing through.

2. The drowsy mouse was placed into a face mask until an appropriate level of anaesthesia was attained, i.e. loss of pedial and tail reflexes.

3. 0.5cm of tail was removed using sharp scissors and placed in cell lysis buffer:

121.14mg 10mM TRIS, 584.4mg 100mM NaCl, 930.5mg 25mM EDTA, 100ml UPW, made to pH 8.4.

4. Bleeding was stopped by hot iron cauterisation and an "AVID" identification microtag (AVID Microchip Systems, Folsom, LA, USA) was implanted subcutaneously at hip level.

5. Analgesia (0.5ml 1:100 buprenorphine in sodium chloride) was administered subcutaneously.

6. The mouse was allowed to recover in a clean cage.

6.1.2 DNA Extraction from Tail Biopsy

1. The tissue sample was placed on Petri dish and covered with cell lysis buffer to prevent dehydration and minced using scalpel blades.

2. The tissue was transferred to an eppendorf and resuspended in 500µl Cetus PCR buffer:

5ml 5M KCl, 5ml 1M TRIS/HCl, 5ml 0.25M $MgCl_2$, 2.25ml Nonidet P40, 2.25ml Tween 20, 0.5g gelatin, 480ml UPW. [1M TRIS/HCl = 121g TRIS, 4.9mlConc HCl, 1l UPW, to pH 8.0.

3. 5µl Proteinase K (20mg/ml) was added, vortexed briefly and placed in heat block at 55°C for at least 3 h.

4. The sample was then heated to 95°C for 15 min to inactivate the Proteinase K.

5. After centrifuging for 5 min, the supernatant was removed to a new eppendorf and the pellet discarded.

6. The supernatant was used for PCR.

6.1.3 MIN PCR

1. The master mix consisted of 1.5µl Forward primer, 1.5µl reverse primer (MWG Biotech), 19µl H_2O per sample.

Min primer sequences:

Forward - TCTCGTTCTGAGAAAGACAGAAGCT,

Reverse – TGATACTTCTTCCAAAGCTTTGGCTAT

2. 3µl supernatant containing DNA was added to 22µl mastermix (using 3µl H_2O for negative control and 3µl positive MIN sample for positive control) and added to a PCR tube containing a PCR bead consisting of Taq polymerase and dNTPs (PuReTaq Ready-to-Go PCR beads, Amersham Biosciences).

3. Samples were run on a Techne Genius PCR machine as follows:

94°C 4min x1
[94°C 1min, 55°C 1min, 72°C 1min] x35
72°C 10min x1
4°C hold

6.1.4 Digestion

1. Hind III digestion mix consists of 7.0µl H_2O, 0.2µl BSA, 0.8µl HindIII, 2.0µl Buffer E (all Promega) per sample.

2. 10µl PCR product was added to 10µl digestion mix and digested in the water bath at 37°C for at least 3 h.

6.1.5 Running gel

1. Gel components for 8% acrylamide gel:

20.93ml H_2O,
2.4ml 10xTBE,
0.48ml ammonium persulphate (10%),
6ml 40% acrylamide (Sigma),
1.2µl ethidium bromide (Sigma),
192µl TEMED (Sigma).

2. Components were mixed and poured into a gel tray with a comb and left to set.

3. The gel was covered with 1xTBE.

4. 2µl dye was added to 10µl digest product, and mixed thoroughly before 10µl PCR/dye mix was added to wells.

5. 1µl dye and 5µl DNA marker (Promega) was used in the last well and the gel was run at 75V for 1.5h before being removed and read using a molecular analyst (UVI PhotoMW, UVItec Ltd, Cambridge, UK).

6.2 Western high-fat diet (Williamson et al.,(1999))

Ingredient	g/Kg	Supplier
Casein high nitrogen	279.49	ICN (901293)
Lard	250	Asda
Cornflour	250	Sigma
Sucrose	148	Co-op
Minerals AIN-93-G-MX	35	ICN (960400)
Gelatine	20	Co-op
Vitamins AIN-93-VX	10	ICN (960402)
L-cystine	3	ICN (101454)
Choline bitartrate	2.5	ICN (101384)
Cr_2O_3	2	BDH (277574Q)
t-butylhydroquinone	0.014	Sigma (B9161)

The dry ingredients were added to a mixing bowl as follows:

1. Least amount first (t-butylhydroquinone), followed by next smallest amount (Cr_2O_3) etc. and placed on setting 2 (slow speed) of mixing machine (CryptoPeerless ED12) for 90 min.

2. The lard was melted in a microwave and poured slowly into the mixer and left to mix for a further 30 min.

3. Mixed diet was scooped into a storage container and stored at -20°C until required.

6.3 Soxhlet Procedure

1. 50ml flat bottomed Soxhlet flasks were dried in an oven for 1 hour and labelled and weighed when cool.
2. Thimbles were labelled in pencil.
3. 2 thimbles and 2 cotton wool plugs were placed on balance and tared.
4. The freeze-dried carcass was cut into pieces (< 1cm) into a beaker.
5. The cut carcass was divided between thimbles and plugged with cotton wool.

   Forceps, secateurs and beaker were wiped with cotton wool before using as a plug.
6. Thimbles were weighed before placing into an extractor and fitted to soxhlet flask.
7. Petroleum ether (petroleum spirit B.Pt 40°-65°, VWR) was added until extractor had siphoned twice.
8. Extractors were attached to cooling condensers and allowed to reflux on hot plates for 6 h. [The ether evaporates and recondenses in the coils and flushes down through the thimbles, extracting fats into the bottom of the flask].
9. Solvent was then removed from the extractor until the flask contains only fat.
10. Flasks were dried for 30 min at 80°C to evaporate any remaining ether.
11. Flasks were cooled in a dessicator before reweighing.
12. Thimbles were dried overnight at room temperature before the contents were milled in a coffee grinder and extracted a second time.
13. Body fat was calculated thus:

Body fat content (g) = (dry wt of flask + fat) – (dry weight of flask)

6.4 Crypt cell proliferation (Goodlad & Wright, 1982)

1. Sample was placed initially in Carnoy's fixative and transferred to 70% ethanol after 24h and stored refrigerated until analysis. [Carnoy's = 60:30:10 ethanol:chloroform:acetic acid]
2. A small strip of mucosa was removed and transferred to 50% EtOH for 10 min r.t.., then transferred to 1M HCl for 10 min 60°C to hydrolyse.
3. Tissue was the transferred to Schiffs' reagent (Sigma) for 45-60 min r.t. to stain DNA then transferred to 45% acetic acid for up to 48h at 4°C.
4. The sample was placed on a microscope slide under stereodissecting microscope and hypodermic needles used to tease off groups of crypts from connective tissue.
5. A coverslip was placed on top (using a drop of acetic acid to prevent tissue from drying out) and pressed firmly to spread crypts.
6. Position of crypts were located under light microscope x10.
7. At x400, lengths and widths of crypts were measured. Crypts were divided into 3 equal length sections (bottom, middle and top) and the number of mitotic cells counted in each. Heavily stained nuclear bodies were usually in mitosis. Telophase appears as 2 smaller, separate bodies.
8. At least 10 crypts per sample were counted (at x400, 1 graticule mark = 2.525um).

6.5 Chromic oxide determination (Mathers & Fotso Tagny, 1994)

Reagents:

$MnSO_4.4H_2O$: 100g/L aqueous solution

Acid mixture: 30ml $MnSO_4$ solution diluted to 1L with concentrated orthophosphoric acid (H_2PO_4, specific gravity 1.75)

Potassium bromate ($KBrO_3$): 45g/L aqueous solution

$CaCl_2$: 5.47g CaCl2.6H_2O/L (gives 1000ppm Ca)

Sodium metasilicate: 7.55g $Na_2SiO_3.5H_2O$/L aqueous solution (gives 1000ppm Si)

Stock potassium dichromate: 2.4194g oven dried $K_2Cr_2O_7$ dissolved in 500ml (gives 1250ppm Cr_2O equivalent)

Preparation of chromium-containing standards (Table 6.1). These are the standard solutions for calibration of the atomic absorption spectrophotometer.

Table 6.1 Standard solutions for calibration of spectrophotometer

Standard (ppm Cr_2O_3)	Stock $K_2Cr_2O_3$ (ml)	Na_2SiO_3 (ml)	$KBrO_3$ (ml)	Acid mix (ml)
0	0	10	4	3
2	2	10	4	3
4	4	10	4	3
6	6	10	4	3
8	8	10	4	3
10	10	10	4	3

1. Components were added to 250ml volumetric flask containing 50ml distilled water, made up to 250ml and mixed well.

2. 25ml of each of the solutions prepared in (1) was taken and pipetted into 100ml volumetric flasks. 10ml $CaCl_2$ solution was added and made up to 100ml.

Procedure: The samples were dry ashed to remove the organic matter and the ash digested with an acid mixture to solubilise the chromium.

1. 10ml graduated tubes were dried and labelled with china graph pencil.
2. Sample was weighed into the tubes and dried at 105°C for at least 5 h. The tube was reweighed to calculate dry matter content (DM). (This stage was omitted in this study as samples were freeze-dried to remove water).
3. Tubes were placed in a large beaker and ashed overnight at 450°C.
4. When the furnace had cooled to 100°C, the tubes were removed and cooled in a dessicator cabinet and reweighed when cool to calculate organic matter (OM) content.
5. 2-3 anti-bumping granules, 1.2ml acid mix and 1.6ml potassium bromate solution were added to each tube. A condensing marble was placed on top of each test tube.
6. Test tubes were heated in a beaker on a hot plate until the colour changed from brown to deep clear purple and the volume reduced by about 50%.
7. Tubes were removed from heat and allowed to cool slightly in a metal rack. Before completely cool, distilled water was added and vortexed. When completely cool, volume was adjusted to 10ml and mixed thoroughly.

Dilution for samples:

0.2ml digest

0.1ml NaSi

1.0ml $CaCl_2$

3.7ml distilled water

Standard solutions were used to calibrate equipment before measuring samples in triplicate. Equipment was re-calibrated after approximately 10 samples.

Atomic absorption spectrophotometer adjustment (Pye Unicam SP9 AAS):

Wavelength	357.9nm
Lamp Current	7.5mA
Burner height	6-8
Air	30
Acetylene	25 (9psi)
Flame	rich
Band pass	1.0

6.6 SCFA

Short chain fatty acid (SCFA) determination (Mathers *et al.*, 1990; Mathers & Fotso Tagny, 1994).

Deproteinising solution (DPS):

1. 100g metaphosphoric acid (BDH) was dissolved in 350ml distilled H_2O.
2. When cold, 2.905g 3-methyl valeric acid (Sigma) was added and mixed well.
3. The solution was made up to 500ml and stirred for several hours until completely dissolved and stored in a glass bottle at 4°C.
4. The result was a 50mM 3-methyl valeric acid solution.

SCFA Standards:

1. 0.2M solutions of the following acids were prepared by dissolving the appropriate amount in 25ml distilled H_2O:

Acetic acid (BDH)	0.3002g/25ml
Propionic acid (BDH)	0.3704g/25ml
Isobutyric acid (Sigma)	0.4405g/25ml
Butyric acid (BDH)	0.4405g/25ml
Isovaleric acid (Sigma)	0.5108g/25ml
Valeric acid (Sigma)	0.5108g/25ml

2. A SCFA mixture was prepared by mixing 10ml acetic acid (final concentration 100mM), 5ml propionic acid (50mM), 1ml isobutyric acid (10mM), 2ml butyric acid (20mM), 1ml isovaleric acid (10mM) and 1ml valeric acid (10mM).

3. Standard SCFA were prepared by mixing 2ml of the above SCFA mixture with 2ml distilled H_2O and 1ml DPS to give a final concentration of:

Acetate	40mM
Propionate	20mM
Butyrate	8mM
Isobutyrate	4mM
Valerate	4mM
Isovalerate	4mM
3-MV	10mM

Procedure for SCFA determination by Gas Chromatography (ES1 only)

1. Colon contents were weighed into an eppendorf and half the weight by volume of DPS added and mixed well before storing at -20°C until required. Samples were thawed before use.
2. 200µl dH_2O was added to each sample and centrifuged at 15 000rpm for 20 min.
3. The supernatants were transferred to glass GC vials and sealed.
4. The concentrations of SCFA's in each sample were analysed by gas chromatography using a 2mm diameter column packed with a 10% SP-1200-1% phosphoric acid on 80/100 chromosorb in a PU 4550 gas liquid chromatograph.

5. 1µl of sample was drawn from the vial into a glass-barrelled needle and injected into the GC instrument.

Chromatography Conditions.

The separation was based on the following conditions:

Gas-liquid chromatograph	Phillips PU 4700
Detector	Flame ionisation
Column oven temperature	130°C
Injection port temperature	175°C
Detector temperature	175°C
Carrier gas	High purity nitrogen
Carrier gas flow rate	55-60 ml/min
Column	Glass 1.5m x 2mm i.d.
Support	Chromosorb W AW 80-100
Gas pressure at source	20 psi
Ionisation and setting	Range 100, attenuation 4

For ES2 and ES3, SCFA were analysed using a Shimadzu Gas Chromatograph GC-2014 with AutoInjector AOC-20i and Shimadzu GC Solution software. Samples were autoinjected and conditions differed as follows.

Column oven temperature	120°C
Injection port temperature	250°C
Detector temperature	275°C
Carrier gas	Nitrogen/air
Carrier gas flow rate	60.2 ml/min
Column	Alltech EC-1000, length 15m x 0.53mm i.d.
Gas pressure at source	3.9kPa
Ionisation and setting	Range 100, attenuation 4

6.7 NEPA

The InfraMot device used "passive infra-red sensors" which sense the infra-red radiation (body heat) produced by the subject and its movement over time, and in this way registered activity. Utilising the detection of infra-red radiation meant that measurements could be made in both the light and the dark (when most activity took place).

The recording interval could be altered from 1-60min, i.e. produce information every minute or in multiples of minutes up to an hour. This changes the nature of the information produced, as minutely recordings produce more accurate representations of activity patterns.

The data were stored in Excel spreadsheets which allow for easy manipulation. Figure 6.1 is an example of data recorded every minute. Although periods of activity and inactivity can be determined, and comparison between subjects is possible, the picture is not totally clear, but can easily be altered. Figure 6.2 shows the same data for the same mice but accumulated hourly, making direct comparisons between mice much easier.

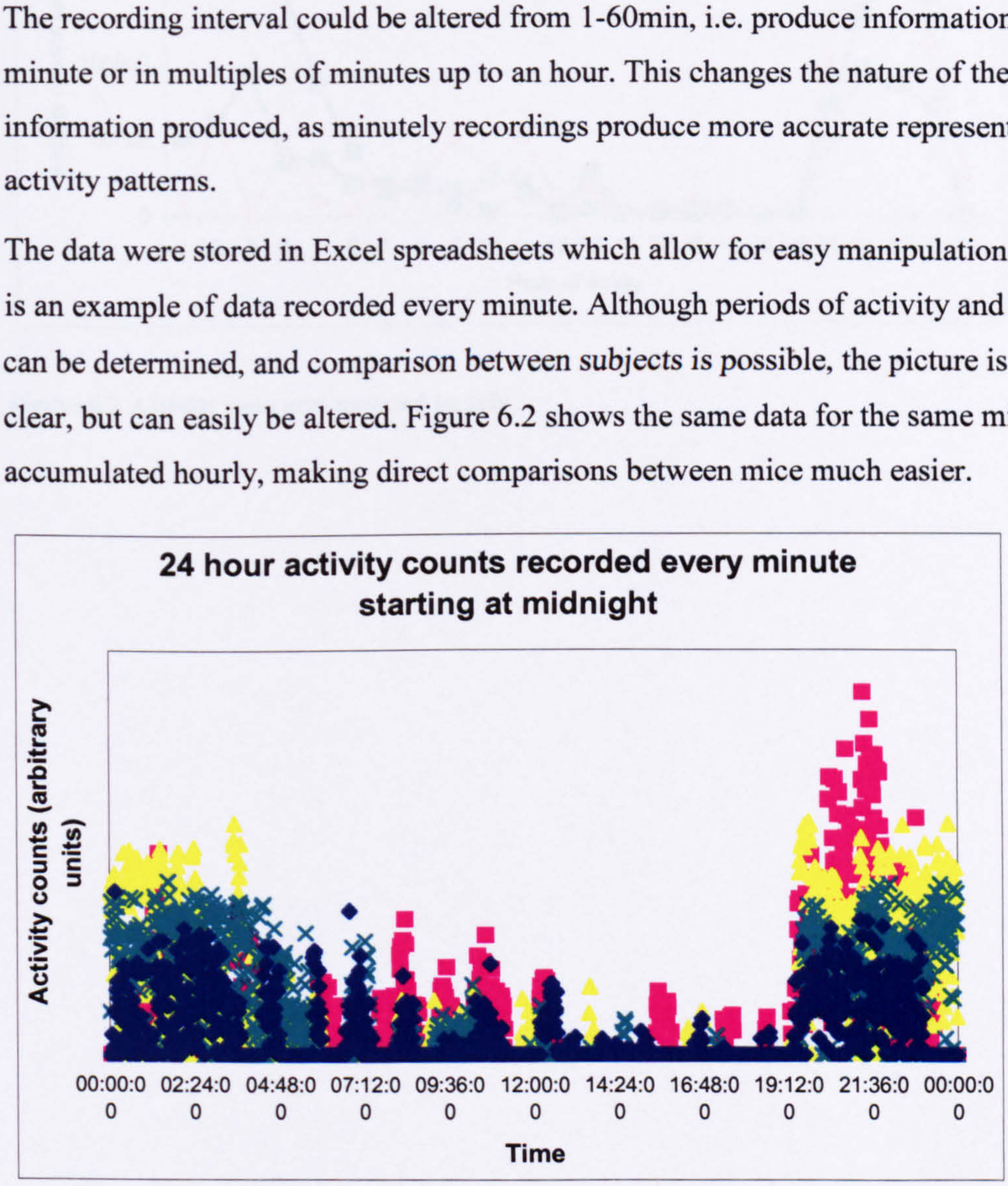


Figure 6.1 Activity data for 4 mice (different colours). Peaks of activity and troughs of inactivity are clearly visible

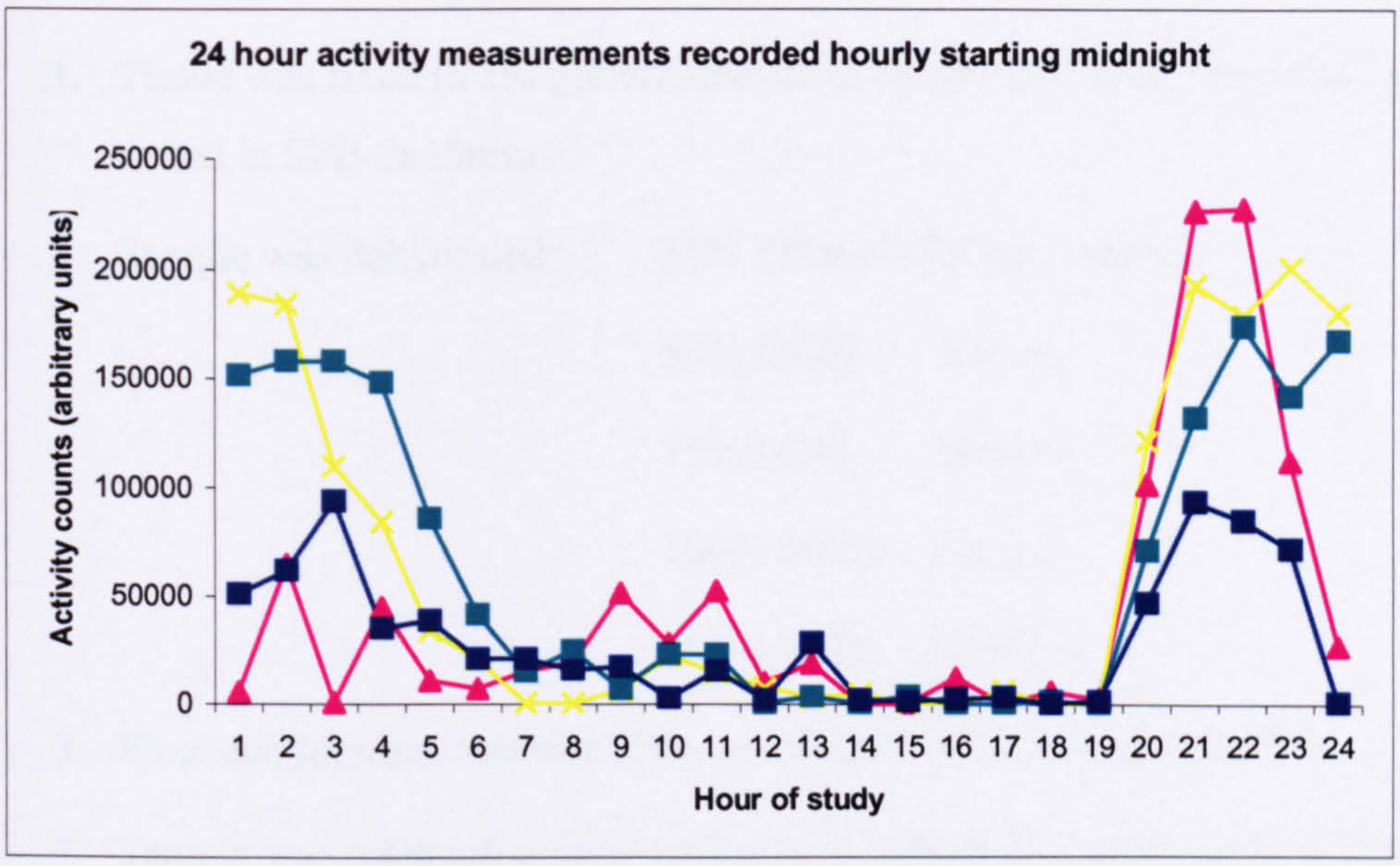


Figure 6.2 Activity data accumulated hourly

6.8 Specimen processing for scanning electron microscopy

1. Tissue was fixed in 2% gluteraldehyde in Sorensons' Phosphate Buffer (SPB) and rinsed in SPB 2x15min.
2. Sample was dehydrated:

25% Ethanol (EtOH)	30mins
50% EtOH	30mins
75%EtOH	30mins
100% EtOH	60mins
100% EtOH	60mins

3. Final dehydration was with CO_2 in a Samdri 780 Critical Point Dryer.
4. Sample was mounted on stub and coated with gold using a Polaron SEM coating unit.

Steps 3-4 performed by Biomedical E.M. Unit, Newcastle University.

6.9 ELISA protocol

Concentrations of IL-6 and IL-10 in plasma were quantified using ELISA Ready-SET-Go! Kits (Insight Biotechnology Ltd, Wembley, UK) as follows:

Contents: 10X coating buffer (C.B.);

Capture Antibody (C.A) – purified mouse IL-6/IL-10;

Standard – recombinant mouse IL-6/IL-10 (1µg/ml);

Detection Antibody – biotin-conjugate anti-mouse IL-6;

Enzyme – Avidin-HRP;

5X assay diluent;

Substrate – 1X TMB solution.

Buffers – wash buffer 1X PBS + 0.005% Tween-20, stop solution – 1M H_3PO_4, 1X PBS.

1. Coating Buffer was diluted – 2.5ml 10X C.B. to 22.5ml DI H_2O.
2. Capture Antibody was diluted – 48µl C.A. to 12ml C.B.
3. 100µl/well CA/CB mix was added to 96 well plate.
4. The plate was sealed and incubated overnight at 4°C.
5. Wells were aspirated and washed 3X with > 300µl/well Wash Buffer.
6. Plate was inverted and blotted on absorbent paper.
7. 10ml 5X Assay Diluent was added to 40ml DI H_2O.
8. 200µl/well 1X Assay Diluent was used to block the wells.
9. Plate was sealed and incubated at room temp 1h.
10. Wells were aspirated and washed 3X as step 5/6.

11. Standards were prepared: IL-6 – 5µl standard solution to 10ml 1X Assay Diluent = top standard (500pg/ml). IL-10 – 10µl standard to 5ml 1X Assay Diluent top standard (2000pg/ml).
12. 100µl/well standard was added to appropriate wells (triplicate) and 2-fold serial dilutions to top standards used to make a standard curve.
13. Plasma samples were defrosted and added 100µl/well.
14. Plate was sealed and incubated r. t. for 2 h.
15. Wells were aspirated and washed 5X as step 5/6.
16. 48µl Detection Ab was diluted in 12ml 1X Assay Diluent.
17. 100µl/well of detection Ab solution was added.
18. Plate was sealed and incubated at r. t. for 1 h.
19. Wells were aspirated and washed 5X as step 5/6.
20. 48µl AvidinHRP was diluted in 12ml 1X Assay Diluent and100µl/well added.
21. Plate was sealed and incubated at r. t. for 30 minutes.
22. Wells were aspirated and washed 7X, soaking wells in wash buffer 1-2 minutes before aspirating.
23. 100µl/well Substrate Solution was added to each well (already at working concentration).
24. Plate was sealed and incubated at r. t. 15min.
25. 50µl/well Stop Solution was added to each well.
26. Plate absorbance was read 450nm and also at 570nm to measure background levels (450-570 to give actual absorbance).

6.10 Detailed method for isolation of NK cell fraction

1. Scalpel blades and cell filters were used to reduce spleen to a single-cell suspension.

2. Cells were washed, counted and centrifuged. The supernatant was removed and the cell pellet resuspended in 90µl buffer [PBS pH 7.2 supplemented with 0.5% BSA/FCS (cold and degassed)] per 10^7 **total** cells. When the pellet contained fewer cells 90µl buffer were used.

3. 10µl of microbeads per 10^7 **total** cells were added, mixed well and incubated for 15 minutes at 10°C. For fewer cells, the same volume of microbeads was used.

4. Cells were washed by adding 10-20x labelling volume of buffer, and centrifuged at 300g for 10 minutes. Supernatant was removed completely and cell pellet resuspended in 500µl buffer per 10^8 total cells. For less than 10^8 cells 500µl buffer was used.

5. Magnetic separation:

MS separation column was attached to the MACS multistand. The collection tube was placed under column.

6. 500µl degassed buffer were applied to the top of the column and allowed to run through. Effluent was discarded and the collection tube changed.

7. Magnetically labelled cell suspension containing up to 10^7 positive cells in maximum $2x10^8$ total cells was pipetted onto the column (up to 10^8 cells per 500µl buffer). Cell suspension was allowed to run through and total effluent was collected as negative fraction.

8. The column was washed with 3x500µl buffer and the total effluent was collected as the NK cell-negative fraction.

9. The column was removed from the stand and placed over a new collection tube.

1ml buffer was applied to the reservoir of the column and cells were flushed out using the plunger provided.

10. NK cells were counted and volumes required for NK cell assay calculated.

6.10.1 Natural killer cell assay

1. Target cells (K562) were resuspended in fresh RPMI 1640 media (Gibco) and counted.

2. $2x10^6$ target cells were incubated in 200µl RPMI with 200µCi 51Cr (Amersham) for 60 minutes with agitation every 15 minutes at 37°C.

3. Cells were washed x3, and resuspended at concentration 2.5×10^4/ml.

4. 100µl labelled target cells was transferred to each well of a U-profile 96-well plate.

5. During labelling of target cells, NK cells were MACS-sorted from spleen.

6. Effector cells (NK) were added to each well at prescribed ratios.

7. Plate was incubated for 4 hours at 37°C.

8. The plate was centrifuged for 2 min at 60g. 100µl supernatant was transferred to 12x75mm polypropylene tube for γ-counting.

9. "Maximum release" was obtained by lysing target cells via freeze-thaw (2 hr -80°C + 37°C). "Spontaneous release" was obtained from supernatant of targets alone.

10. % specific lysis was calculated by subtracting spontaneous release from maximum release.

6.11 Dual COX/SDH histochemistry (Old & Johnson, 1989)

1. The COX incubation medium was prepared: 200µl cytochrome c were added to 800µl diaminobenzidine (DAB) with a small amount of catalase and vortexed.
2. The entire section was covered with 50-100µl of the medium and incubated at 37°C for 50 min.
3. Section was washed twice in PBS and any excess carefully removed with tissue.
4. SDH incubation medium was prepared: to 800µl nitroblue tetrazolium (NBT) were added 100µl sodium succinate, 100µl phenazine methosulphate (PMS) and 10µl sodium azide.
5. The entire section was covered with 50-100µl SDH medium and incubated for 45 min at 37°C.
6. Sections were then washed twice in PBS and dehydrated in ethanol:

70%, 95%, 100% and 100% again for 10min.

7. Sections were cleared twice in Histoclear and mounted in DPX.

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