



## **The Effects of Age and Aerobic Training on T Helper Lymphocyte Proliferation**

### **Author**

Broadbent, Suzanne

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# **The Effects of Age and Aerobic Training on T Helper Lymphocyte Proliferation**

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Suzanne Broadbent

Bachelor of Education,  
Bachelor of Exercise Science (Hons)

School of Physiotherapy and Exercise Science

Griffith University – Gold Coast

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## Abstract

Deficiencies in immune responses can lead to increases in the rate of infections and chronic diseases, such as cancer. Critical to the adaptive immune response is the activation of the T helper (Th)/CD4<sup>+</sup> cell, the subsequent production of interleukin 2 (IL-2), expression of IL-2 and transferrin receptors (IL-2R, TfR) and transcription of genes resulting in DNA synthesis and T cell clonal expansion. The CD4<sup>+</sup> lymphocyte response is impaired with ageing. Recent evidence suggests that moderate, regular aerobic training may increase the responsiveness of CD4<sup>+</sup> lymphocytes to antigenic and mitogenic challenge, and thereby improve immune function in the older individual. Large volumes of chronic endurance training, and also high intensity training, may adversely affect the immune response, leading to immunosuppression and increased risk of infections. Impaired immune function and increased rates of URTI are found in athletes who undergo large volumes of training, often at high intensity. **Purpose:** To investigate if long-term aerobic training improved the immune response in men and women aged 65 to 75 years and, and to investigate if long-term endurance training depressed the immune response in male athletes aged 23 to 36 years. **Methods:** T helper lymphocyte proliferation was assessed monthly, by inducing the expression of CD25 (IL-2R $\alpha$ ) and CD71 (transferrin) receptors with phytohemagglutinin (PHA). Percentage of CD4<sup>+</sup> cells positive for the receptors, and the receptor density, were measured using two colour flow cytometry. Concentrations of intracellular calcium (Ca<sup>2+</sup>) and iron (Fe<sup>3+</sup>) were also measured monthly to determine the effect of endurance training on intracellular Ca<sup>2+</sup> ([Ca<sup>2+</sup>]<sub>i</sub>) and Fe<sup>3+</sup> ([Fe<sup>3+</sup>]<sub>i</sub>) within the CD4<sup>+</sup> lymphocyte signal transduction pathway. **Results:** After twelve months of moderate aerobic training the percentage of CD4<sup>+</sup> lymphocytes positive for CD25 increased in males aged 65 to 75 years, but not in females. There was no training effect on the density of CD25 in either gender, nor was there a training-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>, total intracellular [Ca<sup>2+</sup>] from endoplasmic reticulum stores ([Ca<sup>2+</sup>]<sub>t</sub>) or [Fe<sup>3+</sup>] in this age group. Significant month to month variations in leucocyte, erythrocyte and haemoglobin concentration, mean corpuscular haemoglobin concentration, haematocrit, platelets, CD25 expression, CD71 expression, [Ca<sup>2+</sup>] and [Fe<sup>3+</sup>] were documented for both trained and untrained male and female groups. Aerobic capacity increased significantly with training for both men and women, with increases in  $\dot{V}O_{2\text{ peak}}$ , peak power and peak ventilation ( $p < 0.05$ ). Twelve months of chronic endurance training produced significantly lower haemoglobin, mean corpuscular haemoglobin and platelet concentration for six ([Hb]) and nine months ([MCHC], platelets) of the

year in Ironman-distance triathletes, compared to sedentary males aged 23 to 36 years. There was no evidence of immunosuppression in the trained group, with no significant differences between groups in the percentage of CD4<sup>+</sup> cells positive for CD25. The trained group showed a significantly higher density of CD25 receptors in October, January and June, suggesting a better immune response during these months. Endurance training did not effect [Ca<sup>2+</sup>] or [Fe<sup>3+</sup>]. The trained group did not show a reduced leucocyte concentration, and reported significantly fewer cases of URTI in twelve months than their sedentary counterparts. The 23 to 36 years age group showed seasonal changes in haematological and immunological indices similar to older individuals, indicating that autumn, late winter and late spring are periods of reduced immunocompetency. **Conclusion:** Twelve months of moderate intensity training significantly increased functional capacity in older men and women, and the percentage of CD4<sup>+</sup> lymphocytes expressing CD25 in older men, thereby improving the lymphoid immune response. Twelve months of endurance training significantly increased CD25 density in CD4<sup>+</sup> lymphocytes in Ironman triathletes compared to sedentary young males. The monthly changes in immune variables in young and older subjects suggested that autumn, late winter and late spring might be periods where individuals were more at risk of succumbing to infections due to decreased lymphocyte responsiveness. Summer months appeared to be a period of increased lymphocyte responsiveness and proliferation.

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## Statement of Originality

This work has not previously been submitted for any degree or doctoral candidature at any university. To the best of my knowledge and belief, the dissertation contains no material previously written or published by another person except where due reference is made in the thesis itself.

Signature: \_\_\_\_\_

Suzanne Broadbent



## Supervisor's Certificate

This is to certify that the thesis entitled "The Effects of Age and Aerobic Training on T Helper Lymphocyte Proliferation" submitted by Suzanne Broadbent in the fulfilment for the degree Doctor of Philosophy is ready for examination.

Professor G C Gass: \_\_\_\_\_

Dr N R Morris: \_\_\_\_\_

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## List of Abbreviations

ABS: Australian Bureau of Statistics  
 ACSM: American College of Sports Medicine  
 ACTH: Adrenocorticotrophic hormone  
 ADP: Adenosine diphosphate  
 AIHW: Australian Institute of Health and Welfare  
 Ala: Alanine  
 ALP: Altered ligand peptide  
 AM: Acetylmethylester  
 ANOVA: Analysis of variance  
 AP-1: Activating Protein-1, transcription factor  
 APC: Antigen presenting cell  
 Arg: Arginine  
 ATP: Adenosine triphosphate  
 ATPS: Atmospheric temperature pressure saturation  
 $\beta_2$ AD: Beta adrenergic receptor  
 BAPTA: 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid –  $\text{Ca}^{2+}$  chelator  
 Bp: Base pairs  
 BTPS: Body temperature pressure saturated  
 BV: Blood volume  
 $\text{Ca}^{2+}$ : Calcium ion  
 $[\text{Ca}^{2+}]_i$ : Calcium concentration, intracellular (cytoplasmic pools)  
 $[\text{Ca}^{2+}]_t$ : Calcium concentration, total intracellular (cytoplasmic plus ER stores)  
 cAMP: Cyclic adenosine monophosphate  
 CD: Cluster of differentiation (or cluster designation)  
 CD2: T lymphocyte adhesion molecule (also NK and thymocyte adhesion molecule)  
 CD3: T cell receptor  
 CD4: T Helper lymphocyte marker  
 CD8: T cytotoxic/suppressor lymphocyte marker  
 CD11a: LFA-1 subunit, cell adhesion glycoprotein  
 CD18: Adhesion molecule, combines with all CD11 subunits in all leucocytes  
 CD22: B lymphocyte marker, adhesion molecule  
 CD25: Interleukin- $2\alpha$  receptor  
 CD28: T cell co-stimulator molecule  
 CD44: Adhesion molecule, most cell types  
 CD45RA<sup>+</sup>: Leucocyte common antigen, isoform found in “naïve” T cells  
 CD45RO<sup>+</sup>: Leucocyte common antigen, isoform found in “memory” T cells  
 CD71: Transferrin receptor  
 CD80: T lymphocyte activator, co-stimulator with CD86  
 CD86: Major T lymphocyte co-stimulatory molecule, interacting with CD28 and CD80  
 CD95: With *Fas* antigen, mediates apoptosis-inducing signals in activated T and B cells

CD122:  $\beta$  unit of CD25 receptor  
CDP: Cytidine diphosphate  
CHO: Carbohydrate  
CIF:  $\text{Ca}^{2+}$ -influx factor  
*C-Kit*: isoform (see *Kit*)  
CNS: Central nervous system  
CO: Cardiac output  
CON A: Concanavalin A  
CRAC:  $\text{Ca}^{2+}$ -release-activated  $\text{Ca}^{2+}$  channel  
CSF: Colony stimulating factor  
CV: Cardiovascular  
Cys: Cysteine  
DAG: Diacylglycerol  
dATP: 2'-deoxyribo-adenosine triphosphate  
dGTP: 2'-deoxyribo-guanosine triphosphate  
DMSO: Dimethyl-sulphoxide  
DNA: Deoxyribonucleic acid  
DTH: Delayed-type hypersensitivity response  
DTTP: 2'-deoxyribo-thymidine triphosphate  
ECG: Electrocardiograph  
EGTA: Ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid ( $\text{Ca}^{2+}$  chelator)  
EPO: Erythropoietin  
ER: Endoplasmic reticulum  
ERK: Extracellular signal-regulated kinase  
*Fas*: Gene – initiates apoptosis  
FCM: Flow cytometry  
FCS: Foetal calf serum  
 $\text{Fe}^{3+}$ : Ferric iron  
 $\text{Fe}^{2+}$ : Ferrous iron  
[ $\text{Fe}^{3+}$ ]: Ferric iron concentration  
[ $\text{Fe}^{3+}$ ]<sub>i</sub>: Ferric iron concentration, intracellular  
FEV<sub>1</sub>: Forced expiratory volume  
FITC: Fluorescein isothiocyanate  
fMLP: N-formyl-methionyl-leucylphenylalanine  
*Fos*: FBJ osteosarcoma virus gene - transcription oncogene  
FSH: Follicle stimulating hormone  
FVC: Forced vital capacity  
*Fyn*: Formerly src/yes-related novel gene - protein tyrosine kinase p59 (*src* family)  
GH: Growth hormone  
GHR: Growth hormone receptor  
Glu: Glutamine  
Grb2: Growth factor receptor-bound protein 2

GTP: Guanosine triphosphate  
Hb: Haemoglobin  
HbA<sub>1c</sub>: Glycosylated haemoglobin  
Hct: Haematocrit  
hGH: Human growth hormone  
His: Histidine  
HIV: Human immunodeficiency virus  
HLA: Human leucocyte antigen  
HLA/DR: Human leucocyte antigen, MHC class II  
HPA: Hypothalamic-pituitary-adrenal axis  
HPT: Hypothalamic-pituitary-thyroid axis  
HSA: Heat stable antigens  
HSP: Heat shock protein  
Hz: Hertz  
ICAM: Intracellular adhesion molecule  
Ig: Immunoglobulin  
IGF-IR: Intracellular growth factor I receptor  
IL: Interleukin  
IL-2: Interleukin-2  
IL-2R: Interleukin-2 receptor CD25  
INF $\alpha$ : Interferon alpha  
INF $\beta$ : Interferon beta  
INF $\gamma$ : Interferon gamma  
ITAM: Immunoreceptor tyrosine-based activation motif  
IP3: Inositol 1,4,5 – triphosphate  
IRE: Iron responsive element  
IRP: Iron regulatory protein  
ITAM: Immunoreceptor tyrosine-based activation molecule  
JAK: Janus kinase  
JNK: *jun*-NH terminal kinase  
*Jun*: “Ju-nama” - transcription oncogene, avian sarcoma virus 17  
KCl: Potassium chloride  
kDA: Kilodalton  
*Kit*: KL tyrosine kinase – receptor for stem cell factor  
Km: Kilometre  
LAK: Lymphokine-activated killer  
*Lck*: Lymphoid cellular protein tyrosine kinase p56 (*src* family)  
LFA-1: Leucocyte-function-associated antigen 1 (CD11a/CD18), adhesion molecule  
LFA-3: Leucocyte-function-associated antigen 3 (CD58), adhesion molecule  
LSD: Least significant difference  
Lys: Lysine  
M: Molar

mAB: Monoclonal antibody  
MAPK: Mitogen-activated protein kinase  
MCHC: Mean corpuscular haemoglobin concentration  
MCV: Mean cell volume  
MEK: MAP/ERK kinase - mitogen-activated protein kinase  
MHC: Major histocompatibility complex  
 $\mu$ M: Micromolar  
mM: Millimolar  
MOPS: 3-[N-Morpholino]propanesulphonic acid  
mRNA: Messenger ribonucleic acid  
MW: Molecular weight  
NADH: Nicotinamide adenine dinucleotide (reduced form)  
NADPH: Nicotinamide adenine dinucleotide phosphate  
NFAT: Nuclear factor of activated T cells  
NF- $\kappa$ B: Nuclear transcription factor  
NH<sub>2</sub>: Amino terminus of a polypeptide chain  
NK: Natural killer cells  
nM: Nanomolar  
*Oct.*: Octamer motif  
PAF: Platelet activating factor  
PBS: Phosphate buffered saline  
PC: Pearson correlation  
PE: Phycoerythrin  
PGA: Prostaglandin  
PGE<sub>2</sub>: Prostaglandin E<sub>2</sub>  
PHA: Phytohemagglutinin  
Phe: Phenylalanine  
PIP<sub>2</sub>: Phosphatidylinositol 4,5-bisphosphate  
PI3-K: Phosphoinositide 3 kinase  
PKC: Protein kinase C  
PLC: Phospholipase C  
PMA: Phorbol 12-myristate 13-acetate  
PMCA: Plasma membrane-activated channels  
PTK: Protein tyrosine kinase  
PV: Plasma volume  
*Raf.*: "Murine transforming retrovirus" oncogene – protein serine kinase (MAPK path)  
RAG 1 & 2: Recombination-activating genes  
*Ras.*: "Rat sarcoma" oncogene superfamily- GTP-ase  
RBC: Red blood cells  
RCV: Red cell volume  
*Ref.*: "Reticuloendotheliosis virus" oncogene – NF $\kappa$ B family  
RER: Respiratory exchange ratio

RNA: Ribonucleic acid  
 RPMI: Roswell Park Memorial Institute  
 SAPK: Stress-activated protein kinase  
 SERCA: Sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPases  
 SH: *Src* homology  
 SLP-76: SH2-domain-containing leukocyte protein 76  
 SOS: "Son-of-sevenless" gene – activates *ras* by stimulating GTP-GDP exchange  
 SPSS: Statistical Package for Social Sciences®  
 SR: Sarcoplasmic reticulum  
*Src*: Rous sarcoma virus – transforming gene for sarcoma-producing kinase superfamily  
 SRE: Serum response element  
 SRF: Serum response factor  
 STAT: Signal transducer and activator of transcription  
 sTfR: Soluble transferrin receptor  
 STPD: Standard temperature pressure dry  
 SV: Stroke volume  
 T<sub>3</sub>: Triiodothyronine  
 T<sub>4</sub>: Thyroxine  
 TCGF: T cell growth factor  
 TCR: T cell receptor CD3<sup>+</sup>  
 Tc/s: T cytotoxic/suppressor cell CD8<sup>+</sup>  
 TfR: Transferrin receptor CD71  
 TG: Thapsigargin  
 TGF-β: Transforming growth factor β  
 Th1: T helper cell 1 CD4<sup>+</sup> - produce IL-2, INF-α, IL-12  
 Th2: T helper cell 2 CD4<sup>+</sup> - B cell activation, produce IL-4,5,6,10,13  
 TNF: Tumour necrosis factor  
 TPA: 12-O-tetradecanoylphorbol-13-acetate  
 TR: Trained  
 TRE: TPA response element  
 Tyr: Tyrosine  
 UDP: Uridine diphosphate  
 URTI: Upper respiratory tract infection  
 UT: Untrained  
 $\dot{V}_E$ : Ventilation  
 $\dot{V}_{\text{CO}_2}$ : Volume of carbon dioxide  
 $\dot{V}_{\text{O}_2 \text{ max}}$ : Maximum oxygen consumption  
 $\dot{V}_{\text{O}_2 \text{ peak}}$ : Peak oxygen consumption  
 WBC: White blood cells  
 WHO: World Health Organization  
 ZAP-70: Zeta-associated protein (Syk-family PTK)



## General Navigation of the Thesis

There are two experiments, Experiment 1 and Experiment 2. Given the complexity of the experimental design, particular attention has been given to the use of headings and sub-headings to assist the reader to clearly and easily identify the physiological responses with a given experiment. To assist the reader's navigation of the thesis, the Methods, Results and Discussion are divided into separate sub-sections for Experiment 1 and Experiment 2. For each experiment, the text is laid out in the same order:

- Subject characteristics
- Haematology
- Immunology
- Intracellular Calcium
- Intracellular Iron

The Results and Discussion sections are more detailed, with sub-sections laid out in the following manner:

- Subject Characteristics (general health; blood pressure; peak oxygen uptake; peak heart rate; peak power and ventilation)
- Haematology (leucocytes; erythrocytes; haemoglobin concentration; mean corpuscular haemoglobin concentration; haematocrit; platelets)
- Immunology (IL-2 receptor/CD25 expression; Transferrin receptor/CD71 expression; "double positive" CD4<sup>+</sup>/CD25/CD71 expression)
- Intracellular Calcium (cytosolic calcium; total intracellular calcium)
- Intracellular Iron

Within the Results section, the Figures with an asterisk denote significant differences ( $p < 0.05$ ) between male Untrained and Trained groups (Experiment 1 and 2) and female Untrained and Trained groups (Experiment 2). To avoid crowding the Figures, significant differences between consecutive monthly sampling points, within each group (male Untrained, male Trained, female Untrained, female Trained) are not shown in each Figure but are mentioned in the text preceding the Figure, for each variable.

Within the Results section, each dependent variable is analysed in the following manner:

- Significant differences pre- and post-study (subject characteristics, haematology)
- Between-group significant differences (ie between male Untrained and Trained, and female Untrained and Trained groups in Experiment 1; male Untrained and Trained groups in Experiment 2) and at which months these differences occurred
- Significant differences occurring between consecutive months (month to month) for each group, in each experiment

Due to the sheer volume of material, tables from the SPSS statistics programme, showing significant differences between groups at each sampling point, and between months for each group, have been omitted from the Results section. Copies of the tables can be provided to the examiners upon request.

Within the Discussion section, each dependent variable is discussed in the following manner:

- Significant differences pre- and post-study
- Significant differences between untrained and trained groups
- Significant differences between genders
- Significant differences between months for each separate group



## **1.0 Introduction**

### **1.1 Background**

The human immune system is both a specific and non-specific defence network that responds to, and attempts to eliminate, infectious agents. Deficiencies in immune responses can lead to increases in the rate of infection, and chronic diseases such as autoimmunity, cancer and cardiovascular disease. The incidence of chronic diseases in Australians over the age of 45 years is increasing, and particularly so in those aged over 65 years (A.I.H.W. 2000). With the percentage of the population aged over 60 years (22%) increasing, and the human lifespan lengthening, chronic diseases such as cancers (Schindowski et al., 2001; Slattery and Potter 2002), cardiovascular disease (Wannamethee et al., 2000; Wang et al., 2002) and osteoporosis (Buchner 1997) are becoming more prevalent. Chronic diseases associated with ageing now consume a disproportionately large proportion of health care costs. It is estimated that 35% of the Australian health care budget is consumed by people over the age of 60 years (A.I.H.W. 2000). The Australian Bureau of Statistics (McLennan 1998) reported that 68% of males and 78% of females over 60 years had one or more chronic health conditions, and accounted for 51% of all hospital bed days.

### **1.2 Ageing**

Frequently ageing involves a progressive decline in health and an increase in morbidity (Shepherd 1990; Wayne et al., 1990; Pedersen 1997; Wang et al., 2002). Ageing is a function of both genetic and environmental factors (Bruunsgaard and Pedersen 2000; Schindowski et al., 2001). Positive genetic endowment may be seen with the longevity of successive generations, but interaction with the environment also contributes to the ageing process. Environmental factors also contribute to successful ageing and include a diet low in saturated fats, no smoking, low pollution and maintaining high levels of physical activity (Green et al., 1991; Thune and Furberg 2001). A healthy, responsive immune system is one of the genetic factors that contribute to a longer lifespan. Both reduced levels of physical activity, and immune cell senescence, are associated with the increase in chronic diseases and increased morbidity (Shepherd and Shek 1995; Schindowski et al., 2001; Wang et al., 2002). Immune cell senescence is associated with ageing and is reflected by a decline in T cell responsiveness, and an increased risk of infection. T helper

(CD4<sup>+</sup>) cells are vital in the initiation of specific immune responses, producing interleukin 2 (IL-2), a cytokine (or immune cell messenger) essential for the lymphocyte proliferative sequence.

### 1.3 Role of Exercise Training

It is becoming increasingly well established that regular and moderate intensity exercise can attenuate the decline in cardiovascular and metabolic function that occurs with ageing (for review see Section 2.9). For example, many symptoms of cardiovascular disease such as high blood pressure, atherosclerosis and reduced coronary blood flow can be attenuated by regular, moderate intensity exercise (Kohl 2001; McGuire et al., 2001; Yamamoto et al., 2001; Wang et al., 2002). Regular exercise is also prescribed for the sufferers of Type II diabetes and osteoporosis (Wilmore et al., 2001a,b; Wood et al., 2001). What is less well established is the effect of regular, moderate endurance-type exercise on the immune function in older individuals. Recent evidence suggests that moderate aerobic training may improve the immune response in older individuals by increasing the number or mitogenic responsiveness of CD4<sup>+</sup> cells (Gabriel et al., 1993; Shinkai et al., 1995; Woods et al., 1999). It has been hypothesized that moderate intensity exercise rather than no exercise, or high volume and/or high intensity exercise can decrease the rate of infection among all age groups (Nieman et al., 1994).

The relationship between exercise intensity and rate of infection has been modelled as a “J” curve, with the lowest rates of infection (i.e. upper respiratory tract infection or URTI) found in individuals who exercised at a moderate intensity and volume (Heath et al., 1991; Nieman et al., 1994). Evidence is emerging that moderate aerobic training may improve immune function and maintain cardiovascular and metabolic function in older individuals, thus reducing the increased incidence of chronic diseases (Woods et al., 1999; Spirduso and Cronin 2001; Adamopoulos et al., 2003; LeMaitre et al., 2004). While successful ageing involves the interaction of both environmental and genetic factors, it would seem reasonable to suggest that regular, moderate intensity exercise during the lifespan of an individual not only reduces the likelihood of cardiovascular disease and other chronic diseases associated with the later years, but also improves immune cell function (Bemben 1998; Pedersen et al., 1999; Adamopoulos et al., 2003).

## 1.4 Intensity or Volume?

As we age, the level of recreational sport and moderate intensity exercise common in younger age groups often diminishes with the increasing responsibilities of family and work (A.I.H.W. 2000). Organisations such as The Heart Foundation encourage people of all ages to continue with regular exercise as primary and secondary intervention strategies. The growth of Masters sporting events has provided motivation and an acceptable social framework for older individuals to maintain regular physical activity past middle age.

An increasing proportion of younger people participate in endurance sports that involve large volumes of aerobic training, often combined with sessions of higher intensity training. Many endurance athletes and coaches believe that large training volumes of high intensity exercise coupled with strenuous competition result in a higher incidence of infection. Some studies have shown that marathon runners and competitive swimmers undertaking large volume and high intensity training have a higher rate of URTI (Heath et al., 1991). However, there are some who do not support the theory that high volume/high intensity training results in an increase in URTI (Shephard and Shek 1999; Fricker et al., 2000; Pyne et al., 2000). While the “J” curve model suggests that both high volume and high intensity training may depress the immune response, few, if any, studies have quantified the effects of high volumes of endurance training on the CD4<sup>+</sup> cell proliferative sequence of endurance athletes and older individuals. If CD4<sup>+</sup> lymphocytes in endurance athletes and older individuals are less responsive to antigenic or mitogenic challenge, the ability of lymphocytes to replicate and mount an immune response is compromised, increasing the risk of infections, and possibly cancers in later years (Pyne 1994; Bembem 1998).

## 1.5 Experiments 1 and 2

Previous research has not been able to conclusively demonstrate that moderate intensity training improves the lymphoid immune response, or that long-term endurance training has an immunosuppressive effect on leucocytes. Most coaches and athletes believe that a positive correlation exists between high volume/high intensity training and an increase in URTI. Conclusive evidence to clarify the relationship between exercise dose and immune response is therefore important for both older individuals and young endurance athletes.

This thesis examined the effects of long-term training on the immune responses of two age groups. Experiment 1 investigated the effects of twelve months of moderate intensity aerobic training on males and females aged 65 to 75 years. Experiment 2 investigated the effects of high volume endurance training in males aged 23 to 36 years. Highly specific steps within the CD4<sup>+</sup> lymphocyte proliferation sequence were assessed in both experiments, as indicators of the adaptive immune response to long-term aerobic training.

## **1.6 Aims and Hypotheses**

### **1.6.1 Justification of the study**

Outcomes from the proposed research will have significant implications for older individuals and for those athletes involved in endurance events such as marathons, triathlon and cycling. Participation in endurance sports has increased during the last two decades, yet much of our current knowledge on the effects of such exercise upon the immune function has been limited to incremental exercise and short-term training studies (Mackinnon 1992; Pedersen 1997). Some have proposed that immuno-suppression may result from over-training (Mackinnon 2000), possibly from the immuno-suppressive action of high concentrations of cortisol, and decreased mucosal IgA concentrations (Gleeson et al., 1999; Pyne et al., 2000). Others have suggested that high volume/high intensity “excessive” training might impair lymphocyte activation (Mooren et al., 2001b). There are few studies that have investigated the effects of high volume endurance training on the lymphocyte proliferation sequence as a means of assessing the immune responses in athletes. Similarly, there are little data available that demonstrate the long-term outcomes of moderate intensity endurance training and immune function in males and females aged 65 to 75 years.

### **1.6.2 Aims**

The aims of this thesis were to:

- Quantify and determine the effects of long-term aerobic training on the CD4<sup>+</sup> lymphocyte response in two age groups – males and females aged 65 to 75 years, and males aged 23 to 36 years
- Quantify and determine the effects of long-term aerobic training on CD25 and CD71 receptor expression on CD4<sup>+</sup> lymphocytes

- Quantify and determine the effects of long-term aerobic training effects on  $[Ca^{2+}]$  and  $[Fe^{3+}]$  in  $CD4^+$  lymphocytes

### 1.6.3. Hypotheses

#### 1.6.3.1. Experiment 1

Experiment 1 investigated the effects of twelve months of moderate aerobic training on the proliferative response of  $CD4^+$  lymphocytes in men and women aged 65 to 75 years.

The overall experimental hypothesis was:

**Moderate aerobic training for twelve months will increase  $CD4^+$  lymphocyte proliferation in males and females aged 65 to 75 years**

The following specific experimental hypotheses ( $H_1$ ,  $H_2$ ,  $H_3$ ,  $H_4$ ) were proposed for the various dependent measures.

$H_1$ : Moderate aerobic training for twelve months will significantly increase peak oxygen consumption, peak power, and peak ventilation in males and females aged 65 to 75 years.

$H_2$ : Moderate aerobic training for twelve months will significantly increase the proliferative response of  $CD4^+$  lymphocytes to Phytohemagglutinin in males and females aged 65 to 75 years, as assessed by the expression of CD25 and CD71 receptors.

$H_3$ : Moderate aerobic training for twelve months will significantly increase the intracellular concentration of  $Ca^{2+}$  in  $CD4^+$  lymphocytes in males and females aged 65 to 75 years.

$H_4$ : Moderate aerobic training for twelve months will significantly increase the intracellular concentration of  $Fe^{3+}$  in  $CD4^+$  lymphocytes in males and females aged 65 to 75 years.

#### 1.6.3.2. Experiment 2

Experiment 2 investigated the effects of twelve months of endurance training on the proliferative response of  $CD4^+$  lymphocytes in males aged 23 to 36 years.

The overall experimental hypothesis was:

**Endurance training for twelve months will decrease  $CD4^+$  lymphocyte proliferation in males aged 23 to 36 years**

The following specific experimental hypotheses (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>) were proposed for the various dependent measures.

H<sub>1</sub>: Endurance training for twelve months will significantly decrease the concentration of leucocytes in males aged 23 to 36 years.

H<sub>2</sub>: Endurance training for twelve months will significantly decrease the proliferative response of CD4<sup>+</sup> lymphocytes to Phytohemagglutinin in males aged 23 to 36 years, as assessed by the expression of CD25 and CD71 receptors.

H<sub>3</sub>: Endurance training for twelve months will significantly decrease the intracellular concentration of Ca<sup>2+</sup> in CD4<sup>+</sup> lymphocytes in males aged 23 to 36 years.

H<sub>4</sub>: Endurance training for twelve months will significantly decrease the intracellular concentration of Fe<sup>3+</sup> in CD4<sup>+</sup> lymphocytes in males aged 23 to 36 years.

## 2.0 Literature Review

### 2.1 Structure and Function of the Immune System

Human beings are constantly exposed to a stream of microorganisms capable of disrupting normal bodily processes. Fortunately, we are provided with a complex array of protective mechanisms that can prevent diseases caused by microorganisms, and prevent or minimise the harmful effects of toxins produced by bacteria and plants (Alcami and Koszinowski 2000; Goldsby et al., 2000). The human immune system is remarkably complex and adaptive, and is able to generate an enormous variety of cells and molecules capable of recognising and eliminating a diverse range of foreign molecules and abnormal human cells.

The immune system is comprised of primary and secondary lymphoid organs, and a diverse network of both specific and non-specific immune cells which occupy lymphoid tissues and circulate in body fluids (Roitt et al., 1996). Functionally, the immune system involves both recognition and response. Immune cells are able to discriminate between foreign molecules and the body's own cells and proteins (Eales 1997; Goldsby et al., 2000). Once a foreign molecule or pathogen has been recognised, a variety of immune cells and molecules combine in an *effector response* to eliminate or neutralise the invading pathogen. Later exposure to that same pathogen will induce a *memory response*, characterised by a more rapid, specific and heightened immune response to eliminate the pathogen and prevent disease.

Immunity has both innate (non-specific) and adaptive (specific) components that operate with other physiological systems (Roitt et al., 1996). Innate immunity is provided by the skin and linings of the respiratory, gastrointestinal and urinary tracts. Chemical barriers (lysozymes, acute phase proteins, complement) and physiological barriers (pH and temperature) are also non-specific host defences. Immune cells such as monocytes, macrophages, dendritic cells, granulocytes and natural killer (NK) cells recognise foreign molecules but their response does not accelerate with repeated exposure (Boron and Boulpaep 2003).

In contrast, adaptive immunity displays specificity in response, as well as memory (Goldsby et al., 2000). There is an adaptive immune response against a foreign molecule (antigen), with antibodies produced within five to six days after exposure to that antigen. Repeated exposure to

that antigen results in a memory response that is usually faster and stronger than the first exposure, and therefore more effective than the initial response (Boron and Boulpaep 2003). The cellular participants in the adaptive immune response are T and B lymphocytes, antibodies and other molecules such as cytokines and lymphokines. Most pathogens encountered by humans are eliminated by innate mechanisms within several days (Goldsby et al., 2000). The lymphoid adaptive response is triggered if the pathogen eludes destruction, thus both innate and adaptive responses function as a highly interactive and co-operative system.

The T and B lymphocytes of the adaptive immune system can be further sub-divided into cell subsets, each with a specific role in the immune response (Boutin et al., 1997; Constant and Bottomly 1997). T helper ( $CD4^+$ ) lymphocytes express the membrane glycoprotein  $CD4^+$ . When activated,  $CD4^+$  cells secrete numerous low molecular weight proteins termed cytokines, which promote proliferation and differentiation in other leucocytes (Goldsby et al., 2000). T cytotoxic cells express the  $CD8^+$  glycoprotein and when activated, are able to differentiate into effector cells that destroy virally-infected cells and tumour cells (Roitt et al., 1996; Boron and Boulpaep 2003). B lymphocytes are activated by cytokines produced by  $CD4^+$  cells. B cells interact with antigen through their membrane-bound immunoglobulin (antibody), and will differentiate into antibody-secreting plasma cells or long-living memory B cells. The cells of the immune system are represented in Figure 1.

Antigen recognition by B cells and T cells differs according to Major Histocompatibility Complex (MHC) recognition. The MHC is a tightly linked cluster of genes arrayed within a long section of DNA on chromosome 6 (in humans), whose products are involved in discrimination between self and non-self (Goldsby et al., 2000). For example, the multiple loci of the MHC help determine whether transplanted tissue is accepted as self (histocompatible) or rejected as foreign (histoincompatible). In humans the MHC is referred to as the Human Leucocyte Antigen (HLA) complex. The MHC plays a central role in the development of both humoral and cell-mediated immune responses. Most T cells recognise antigen only when presented in conjunction with a MHC molecule (Eales 1997; Goldsby et al., 2000). MHC molecules act as antigen-presenting structures, so the MHC molecules expressed by an individual influence the range of antigens to which that individual's  $CD4^+$  and  $CD8^+$  lymphocytes can respond.

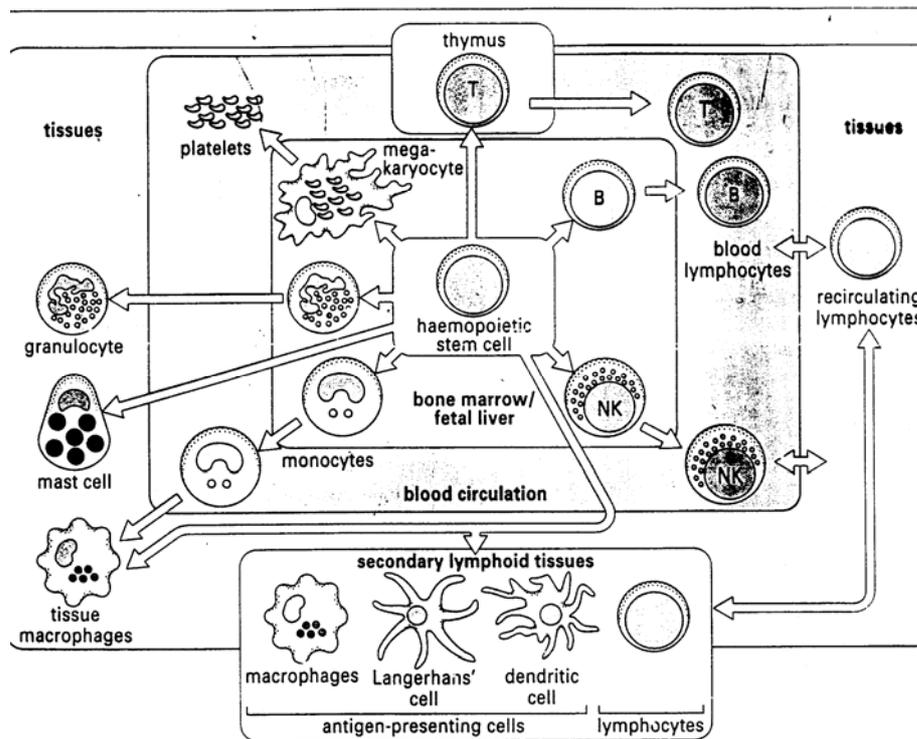


Figure 1: The Human Immune System (Roitt et al., 1996). Lymphocytes, Neutrophils, Eosinophils, Basophils and Monocytes originate from haemopoietic stem cells in the bone marrow. T Lymphocytes differentiate in the thymus and migrate to peripheral lymphoid organs. B lymphocytes differentiate in bone marrow. Plasma cells differentiate from B cells during immune responses. Macrophages differentiate from monocytes and migrate into tissues. Mast cells differentiate from stem cells and bone marrow cells.

MHC genes are organised into regions encoding three classes of molecules – Class I MHC genes encode glycoproteins expressed on the surface of nearly all nucleated cells, and their major role is presentation of peptide antigens to  $CD8^+$  lymphocytes; Class II MHC genes encode glycoproteins expressed primarily on antigen-presenting cells or APC's (macrophages, dendritic cells, B cells), where they present processed antigenic peptides to  $CD4^+$  lymphocytes; Class III genes generally encode a variety of secreted proteins with immune functions, such as components of the complement system and pro-inflammatory molecules (Goldsby et al., 2000; Boron and Boulpaep 2003).

Class I and II molecules have some similar structural features, and both have roles in antigen presentation. Both Class I and II types of membrane glycoproteins are highly specialised antigen-

presenting molecules and form extremely stable complexes with antigenic peptides, displaying them on the cell surface for recognition by T cells (Eales 1997). Class II molecules contain two different polypeptide chains (33-kDa and 28-kDa) which associate by noncovalent interactions. The membrane-bound glycoprotein contains external domains, a transmembrane segment, and a cytoplasmic anchor or tail (Boron and Boulpaep 2003). The external domains are categorized as  $\alpha 1$  and  $\alpha 2$  on one chain, and  $\beta 1$  and  $\beta 2$  on the other chain. The  $\alpha 1$  and  $\beta 1$  domains form the cleft for antigen-binding (Goldsby et al., 2000). A central core of 13 amino acids determines the ability of a peptide antigen to bind to the cleft, and its binding characteristics (Goldsby et al., 2000). The great diversity and sequence variation of MHC molecules may influence the cell's ability to recognise a particular peptide.

The expression of MHC is regulated by various transcription factors and promoter motifs, as well as some cytokines. The interferons (IFN)  $-\alpha$ ,  $-\beta$  and  $-\gamma$  and tumour necrosis factor (TNF) increase the expression of Class I MHC, while IFN- $\gamma$  increases Class II on some non-APC's (Goldsby et al., 2000). IL-4 increases Class II molecules on B cells. Both MHC II transactivators and transcription factors bind to the promoter region of Class II genes (Goldsby et al., 2000; Boron and Boulpaep 2003). Both  $CD4^+$  and  $CD8^+$  T cells can recognise antigen only when it's presented with a self-MHC molecule on the membrane of another cell, a feature termed self-MHC restriction (Goldsby et al., 2000). A  $CD4^+$  Th cell will only be activated in the presence of an antigen-APC complex that shares the same Class II MHC alleles as the  $CD4^+$  cell. Therefore, antigen recognition by the  $CD4^+$  cell is class II MHC restricted.

Th lymphocytes can be further subdivided into T helper 1 (Th1) and T helper 2 (Th2) subgroups on the basis of cytokine production (Boutin et al., 1997; Uyemura et al., 2002). Th1 cells produce IL-2, IL-12, IFN- $\gamma$  and TNF, resulting in T cell proliferation and macrophage activation (cell-mediated immunity). Th2 cells produce IL-4, IL-5, IL-6, IL-10 and IL-13, cytokines which augment antibody responses and aid humoral immunity (Goldsby et al., 2000; Uyemura et al., 2002), as illustrated in Figure 2 by the author. These two Th cell populations are cross regulatory (i.e. IL-2, IL-12 and IFN- $\gamma$  inhibit Th2 responses, and IL-4 and IL-10 are antagonistic to Th1 cells) (Mosmann and Sad 1996; Uyemura et al., 2002).

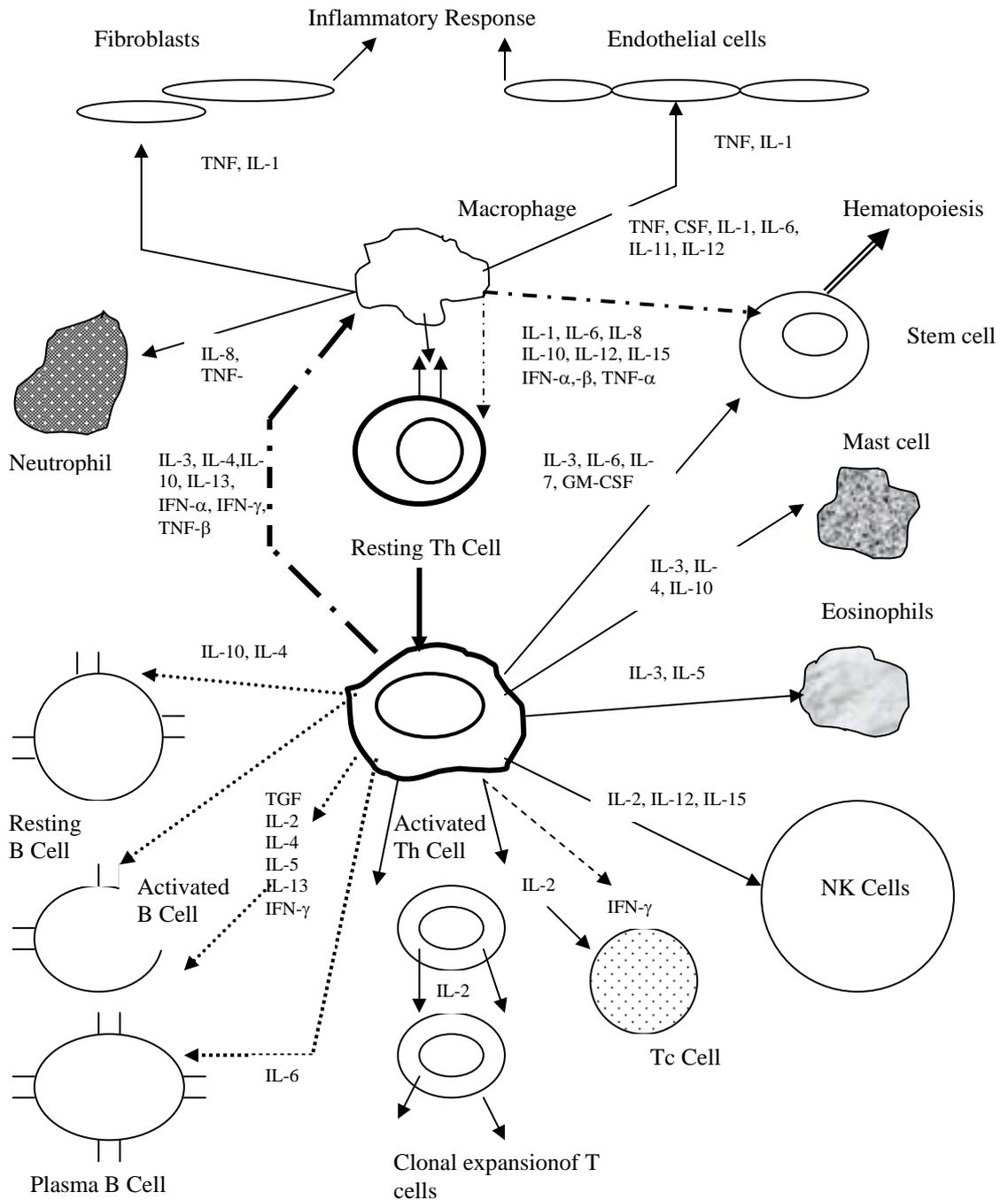


Figure 2: Cytokine Regulation of T Cell Activation.

Normally lymphocytes exist as resting cells in the “Go” phase of the cell cycle (Figure 3), until stimulated to divide by the presentation of an antigen (Pedersen 1997; Goldsby et al., 2000). During a primary immune response, an antigen localised in lymphoid tissue is degraded by an APC, usually a macrophage or dendritic cell. “Naïve” CD4<sup>+</sup> lymphocytes recognise the antigen, progress through the cell cycle after stimulation by the cytokine IL-1 and co-stimulation of the CD28 receptor, and rapidly proliferate (Eales 1997; Pedersen 1997).

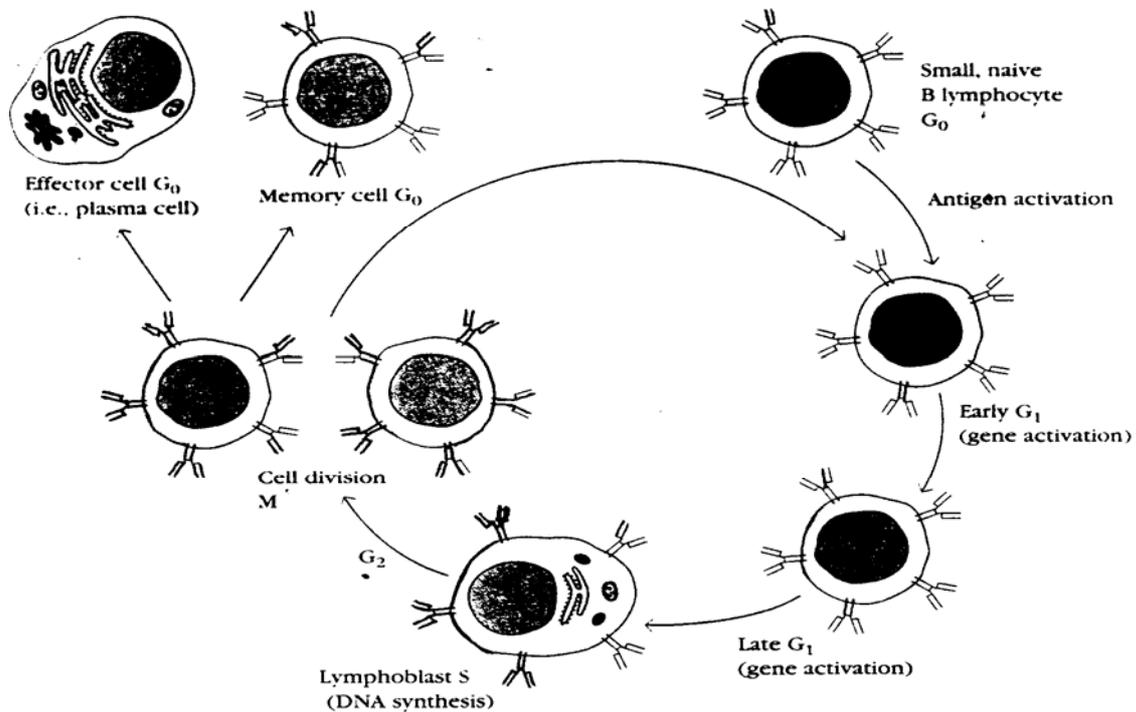


Figure 3: Lymphocyte Growth Cycle (Goldsby et al., 2000). A small resting (un-primed or naive) lymphocyte resides in the G<sub>0</sub> phase of the cell cycle. At this stage, B and T lymphocytes cannot be distinguished morphologically. After antigen activation, a B or T cell enters the cell cycle and enlarges into a lymphoblast, which undergoes several rounds of cell division, and eventually generates effector cells and memory cells.

The CD28 antigen is a homodimer of disulphide-linked chains, with a molecular weight of 44 kDa each (Tortorella et al., 2002). CD28 is expressed on approximately 95% of CD4<sup>+</sup> and 50% of CD8<sup>+</sup> circulating T lymphocytes, and provides a major co-stimulatory signal for T cell activation through the interaction with CD80 or CD86, members of the B7 molecule family that are expressed on the surface of APC (Tortorella et al., 2002). There is growing evidence that T lymphocytes from older individuals are characterised by a marked decline in CD28 expression and CD28-dependent co-stimulatory activity, which is correlated with changes in T cell phenotypes (Eales 1997; Vidan et al., 1999; Tortorella et al., 2002).

“Naïve” CD4<sup>+</sup> cells differentiate into effector Th cells which proliferate and produce various cytokines (eg IL-2) which activate other lymphocytes (T cytotoxic/suppressor cells/CD8<sup>+</sup>, NK cells, B cells) (Farber 1998). IL-2 has an autocrine effect by activating more CD4<sup>+</sup> cells to produce IL-2, and inducing the expression of IL-2 receptors (IL-2R) on CD4<sup>+</sup> lymphocytes, shown in Figure 4 by the author. Most activated effector CD4<sup>+</sup> cells die an activation-induced death, but a subset of antigen-specific T cells will persist as memory cells (Farber 1998). The CD4<sup>+</sup> lymphocyte is vital to the adaptive immune response, since it is the only lymphocyte which produces IL-2, critical to the CD4<sup>+</sup> proliferative sequence and immune cascade by way of its activation of antigen-specific Th and Tc/s cells, and B lymphocytes (Goldsby et al., 2000). Factors which adversely affect CD4<sup>+</sup> cell development or activation will compromise immune function.

B lymphocytes bearing the marker CD22<sup>+</sup> become long-living memory B cells, possibly living for years, which may allow a more rapid response when exposed to the same antigen (Eales 1997; Pedersen 1997). After exposure to the same antigen, the activated B lymphocyte multiplies and differentiates to form a clone of daughter cells which secrete antibodies of the same class and specificity as expressed on the surface of the parent cell (Eales 1997; Goldsby et al., 2000). The immune system is altered, so that the next time the same antigen is encountered, a faster and more aggressive secondary immune response occurs. In this case, the class of antibody may alter (Boron and Boulpaep 2003). Initially IgM antibody is produced but with differentiation, the class of antibody will change to IgG, IgA or IgE depending on the site of presentation. The specific receptors on the B cell are immunoglobulins, anchored in the cell membrane (Eales 1997). The T cell receptor (TCR CD3<sup>+</sup>) is also bound in the membrane of T cells and consists of a complex collection of polypeptides, of which two disulphide-linked chains give antigen specificity (Lefranc 1994; Boron and Boupaep 2003).

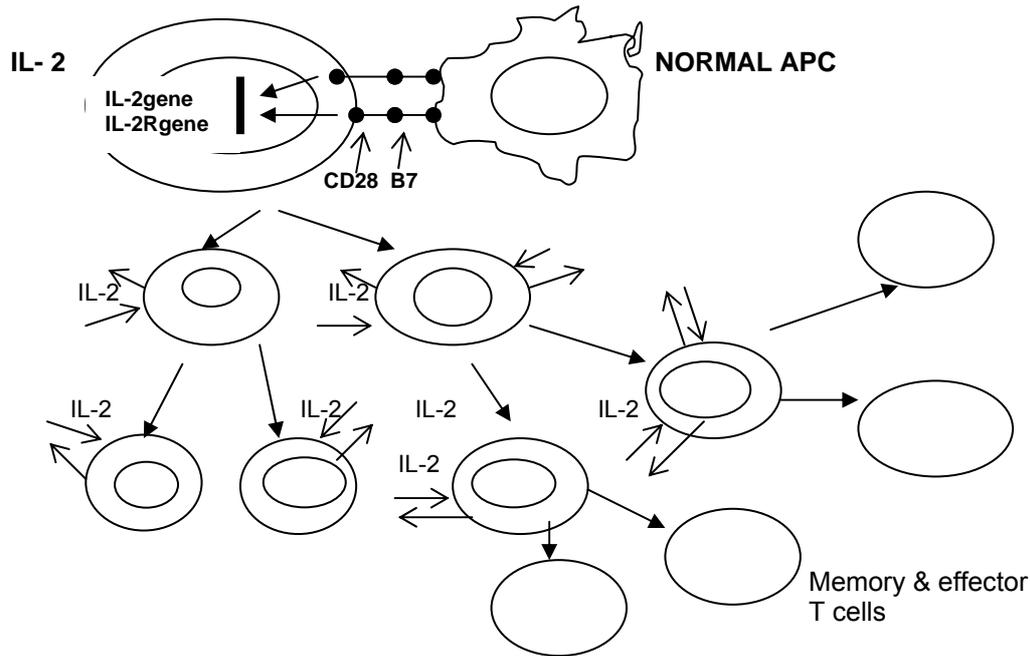


Figure 4: IL-2 Production and Autocrine Response of T helper Lymphocytes. Upon the binding of antigen to the TCR/CD3<sup>+</sup>, activation of co-stimulator molecules CD28 and B7 occurs; transcription of IL-2 gene and IL2R/CD25<sup>+</sup>; IL-2 produced, with an autocrine effect on other T lymphocytes. More IL-2 is produced, and further transcription of the CD25 receptor occurs.

### 2.1.1 T Cell Maturation, Differentiation and Activation

The lymphocytes are found throughout the body in blood and lymph fluid, and are organised in lymphoid tissue known as the lymphoid system (Figure 1) (Eales 1997; Roitt et al., 1996). During foetal development, cells migrate from the liver to the bone marrow to become common precursors, or stem cells (Roitt et al., 1996). Stem cell division is controlled by cytokines (e.g. colony stimulating factor). Cytokines are molecular mediators that modify the function of other cells, including the cells that produced them (Mackinnon 1992), to form pre-T and -B cells. Thymocytes, (or immature pre-T cells displaying no surface molecules characteristic of mature T cells), enter the thymus, attracted by a factor secreted by the thymic endothelium (IL-3). Epithelial cells in the thymus produce several hormones including thymulin, thymosin, thymosin  $\beta$ 4 and thymopoietin, which are needed for the differentiation of thymic precursors into mature cells (Eales 1997; Goldsby et al., 2000).

The thymocytes proliferate and differentiate into mature T cells in the outer cortex of the thymus (Goldsby et al., 2000). Differentiation involves a series of stages in which the thymocytes develop cell-surface phenotypic features (adhesion molecule CD44, IL-2 $\alpha$  -chain CD25, stem cell growth factor c-Kit) (Demaison et al., 1998). However, TCR genes remain unarranged until expression of these phenotypic markers decreases, at which stage, thymocytes re-arrange their genes (Goldsby et al., 2000). Thymocytes that productively re-arrange both the  $\gamma$  and  $\delta$  chain genes develop into double negative CD3<sup>+</sup>  $\gamma\delta$  cells. Overall, double negative  $\gamma\delta$ -cells account for less than 5% of thymocytes. However, the remainder of the double negative thymocytes follow a different developmental pathway by re-arranging their TCR  $\beta$ -chains (Chattopadhyay et al., 1998; Boron and Boulpaep 2003). These thymocytes rearrange their TCR  $\beta$ -chain genes to combine with the pre-T $\alpha$  chain, and then these chains associate with the CD3<sup>+</sup> molecules to form a pre-T cell receptor (Goldsby et al., 2000).

The pre-TCR is thought to recognise some intra-thymic ligand, which causes a signal transduction through the CD3<sup>+</sup> complex that activates the protein tyrosine kinase *Lck* (Garcia and Miller 1996; Lewis et al., 1997; Goldsby et al., 2000). This process suppresses further  $\beta$ -chain gene re-arrangement, enhances re-arrangement of the  $\alpha$ -chain gene, and selects thymocytes displaying the  $\beta$ -chain for further maturation. The process also induces the progression of the CD4-CD8 double positive T cell state (Lewis et al., 1997; Boron and Boulpaep 2003). Double positive T cells begin to proliferate, and the recombination-activating genes RAG-1 and RAG-2 are transcriptionally active (Goldsby et al., 2000). When RAG-1 and RAG-2 activity decreases,  $\alpha$ -chain genes are expressed. This whole proliferative phase contributes to T cell diversity by generating a clone of cells with a single TCR  $\beta$ -chain arrangement (Boron and Boulpaep 2003). Each cell within this clone can then re-arrange different  $\alpha$ -chains, allowing greater diversity. This T cell differentiation process takes about 17 days (Goldsby et al., 2000). Double positive cells expressing both CD3 and the  $\alpha\beta$  TCR appear at day 17 and achieve maximum concentration at the time of birth. An estimated 98% of all thymocytes don't mature and die by apoptosis, which has been attributed to non-productive TCR gene re-arrangement or a failure to survive thymic selection (Goldsby et al., 2000; Boron and Boulpaep 2003). Double positive thymocytes expressing the  $\alpha\beta$  TCR-CD3 that survive, develop into either single positive CD4 or CD8 thymocytes (Goldsby et al., 2000).

During positive selection the RAG-1 and RAG-2 proteins, required for gene re-arrangement and modification, continue to be expressed (Goldsby et al., 2000). The immature thymocytes in a clone expressing a given  $\beta$ -chain continue to re-arrange TCR  $\alpha$ -chain genes, and the resulting TCR's are then selected for self-MHC recognition (Boron and Boulpaep 2003). Only those cells whose  $\alpha\beta$  TCR hetero-dimer recognises a self-MHC molecule are selected for survival (Goldsby et al., 2000). Thymocytes not positively selected are unable to interact with epithelial cells in the thymic cortex and do not receive the protective signal that prevents cell death by apoptosis (Zhang and Herman 2002).

Positive and negative thymocyte selection presents a paradox. Positive selection allows thymocytes reactive with self-MHC molecules to survive, and those thymocytes not self-reactive to die by apoptosis (Goldsby et al., 2000; Zhang and Herman 2002). However, negative selection eliminates thymocytes bearing high-affinity receptors for self-MHC molecules alone, or self-antigen presented by self-MHC, which results in self-tolerance. If both positive and negative selection use the same criteria and respond to identical signals, both processes would not allow T cells to mature (Goldsby et al., 2000). Obviously there are other factors that prevent the two processes from eliminating all T cells. To resolve the paradox of positive and negative selection, two hypotheses or models are proposed.

The first hypothesis, the *avidity model*, proposes that the differences in the strength of the signal received by the thymocyte during interactions between its TCR's and the MHC-complex of APC's determine the outcome of positive and negative selection (Goldsby et al., 2000). Signal strength is determined by the avidity of the MHC-peptide and TCR interactions, and avidity depends upon both the affinity of the interaction between TCR and MHC-peptide complexes, and the density of the relevant peptide-MHC complexes on the APC (Alberola-Ila et al., 1997; Goldsby et al., 2000). High avidity interactions result in strong signals, and the avidity model suggests that all thymocytes with TCR's that bind moderately to a wide variety of peptide-MHC complexes available in the thymus are selected for survival. Thymocytes whose receptors react strongly with peptide-MHC complexes are induced to die by apoptosis (Boron and Boulpaep 2003). Thus the surviving thymocytes are those whose TCR's bind weakly self-MHC peptide complexes on APC's and thereby avoid negative selection. The positively selected cells can then leave the thymus as

mature T cells with self-MHC-restricted receptors. Experiments using foetal thymic organ cultures in mice have shown that TCR-MHC peptide interactions can be varied according to the concentration of peptide added to the cultures (Goldsby et al., 2000). An increase in the concentration of TCR-MHC peptide results in a more avid response, and greater numbers of TCR-MHC complexes being displayed. Fewer CD8<sup>+</sup> cells appear as the dose of stimulating peptide increased. Indeed, more CD8<sup>+</sup> cells appeared with a lower dose of peptide, despite fewer TCR-MHC complexes being displayed (Goldsby et al., 2000).

The second hypothesis, the *differential signalling model*, presents a qualitative rather than quantitative approach, emphasising the nature of the signal given by the TCR rather than its strength (Goldsby et al., 2000). The *differential signalling model* suggests that some MHC-peptide complexes deliver a weak or partial-activation signal, while other complexes deliver a complete signal (Constant and Bottomly 1997; Goldsby et al., 2000). Positive selection occurs when a weak or partial signal is delivered and negative selection occurs when a full signal is delivered. For example, foetal thymic organ culture experiments show that if co-receptor CD8 expression is increased (i.e. a full signal), the concentration of mature CD8<sup>+</sup> cells declines, suggesting negative selection has increased (Goldsby et al., 2000; Boron and Boulpaep 2003). Dendritic cells and macrophages bearing Class I and Class II MHC molecules interact with thymocytes bearing high-affinity receptors for self-MHC alone, or self-antigen plus self-MHC molecules (Eales 1997; Goldsby et al., 2000). The interaction involves the TCR of the thymocyte undergoing selection and MHC molecules on the mediating cells. The exact process of negative selection is still unknown but unsuitable thymocytes appear to undergo apoptosis, and self-reactive T cells are eliminated (Zhang and Herman 2002).

Positively selected thymocytes pass from the cortex to the medulla of the thymus (Boron and Boulpaep 2003). They are released and re-enter the bloodstream to settle in the lymph nodes and peripheral lymphoid tissue. Activation of T cells is initiated by interaction of the TCR-CD3<sup>+</sup> complex with a processed antigenic peptide bound to either a Class II MHC molecule (for CD4<sup>+</sup> cell activation) or a Class I MHC molecule (for CD8<sup>+</sup> cell activation) (Hayball and Lake 2000; Patrick et al., 2000). The signal transduction processes initiated by CD4<sup>+</sup> lymphocyte activation are discussed in section 2.4.

Any interference in thymic activity may adversely affect T cell development (Boron and Boulpaep 2003). Thymic involution occurs with age, resulting in a decrease in the size of the thymus, and the gland's ability to produce new CD4<sup>+</sup> T cells (Shinkai et al., 1996; Ginaldi et al., 2000). The result is an increase in "memory" or clonally expanded T cells (CD45RO<sup>+</sup>) relative to "naïve" or new T cells (CD45RA<sup>+</sup>), which have yet to encounter antigen (George and Ritter, 1996; De Boer and Noest 1998; Ginaldi et al., 2000). The age-related reduction in "naïve" T cells suggests that the primary immune response to new antigens may be compromised in older individuals (Song et al., 1993; de Boer and Noest 1998; Suh 2002).

## 2.2 T Lymphocyte Memory and Cell Senescence

The primary response of CD4<sup>+</sup> lymphocytes to an antigen may be influenced by several factors, including the type of antigen, route of entry and type of APC (Eales 1997; Goldsby et al., 2000). The primary response, mediated by cytokines, will depend partly on the type of CD4<sup>+</sup> cell (Th1 or Th2) activated and whether antigen-specific "memory" cells are present (Boutin et al., 1997; Tao et al., 1997). Peripheral Th cells are also classified as either "naïve", "effector" or "memory" cells (Eales, 1997; Farber 1998). The majority of "memory" cells within the peripheral group are usually short-lived and antigen-specific memory may depend on re-stimulation of long-living "memory" cells by antigen (Tao et al., 1997; Farber 1998). "Naïve" cells only secrete IL-2 on initial antigenic stimulation, whilst "memory" cells may display defined cytokine profiles that correspond to either Th1 or Th2 profiles (Pawelec and Solana 1997). These Th1 and Th2 profiles stimulate different types of immune response. Th1 lymphocytes promote T, B and NK cell differentiation due to IL-2, IL-12, IFN- $\gamma$  and TNF $\alpha$  production while Th2 lymphocytes promote the production of immunoglobulins by B cells (Farber 1998; Cousins et al., 2002). The Th2 response tends to occur after an initial Th1 response, if the initial response is not successful.

The ability of the long-living "memory" CD4<sup>+</sup> cells to respond more quickly and aggressively to antigen stimulation does not appear to be due to the expression of TCR's with increased affinity for antigen, but to the altered expression of adhesion molecules (LFA-1, LFA-3, CD44, CD2) (Tortorella et al., 2002). The altered expression results in enhanced interaction with APC's, and increased expression of IL-2R which allow a more rapid response to secreted IL-2 after

lymphocyte activation. The rapidity of secondary responses is due to the proportion of T “memory” cells that are long-lived (Farber 1998; Tortorella et al., 2002).

Replicative senescence is characteristic of all normal somatic cells, whereby cells undergo a finite and predictable number of cell divisions before reaching an irreversible state of growth arrest (Effros and Pawelec, 1997; Hwang 2002). Replicative senescence is a consequence of cell division rather than chronological time, and depends upon the cell origin, age and genetic background of each individual (Effros and Pawelec, 1997; Tarazona et al., 2000). The limited replicative lifespan is termed the Hayflick Limit (Hayflick and Moorhead 1961). Research has shown that the majority of “memory” T cells divide more rapidly than “naïve” T cells, reaching cell senescence sooner (Effros and Pawelec 1997). “Naïve” Th cells undergo 41 population doublings before reaching cell senescence, whereas “memory” cells undergo only 17 population doublings (Effros and Pawelec, 1997). The mechanism for counting cell divisions may be attributed to the telomeres, repetitive DNA sequences at the end of chromosomes which are responsible for genomic stability and the protection of chromosome ends from exonucleolytic degradation (Effros and Pawelec, 1997; De Boer and Noest, 1998). The ends of linear chromosomes can’t be fully replicated with each cell division, so that lymphocytes lose telomeric DNA at the rate of 40-100 base pairs per population doubling (De Boer and Noest, 1998; Effros and Pawelec, 1997). A critical telomere length may signal cell-cycle arrest. Chronic immune stimulation resulting in clonal exhaustion, marked by telomere shortening may contribute to immunodeficiency (Effros and Pawelec, 1997).

“Naïve” Th cells have been estimated to divide only once every 3.5 years (a figure provided by rates at which patients treated with radiotherapy lose lymphocytes with chromosome damage) (De Boer and Noest, 1998). However, “memory” Th cells divide once every 22 weeks, suggesting that telomeric shortening occurs at a greater rate with “memory” cells, thereby contributing to immunodeficiency (De Boer and Noest, 1998). Indeed, telomere shortening, defective IL-2 production and the loss of co-stimulation molecules from the persistence of antigen are factors increasingly used to define replicative senescence in T cells (Tarazona et al., 2000).

Both human and animal studies have shown that T cell senescence is associated with an increased susceptibility to tumours (Mazzeo 1993; Pawelec et al., 1995). These results suggest

that older individuals, who have a higher percentage of memory lymphocytes and greater rate of cell senescence (De Boer and Noest, 1998; Tarazona et al., 2000) may also be at greater risk of developing tumours. Indeed, Frisch et al (1985) found an increased incidence of breast and reproductive system cancers in older female non-athletes compared to older former-collegiate athletes. (Figure 5A, 5B). Older male non-athletes showed an increased incidence of colon cancer compared to older ex-athletes (Frisch et al., 1985).

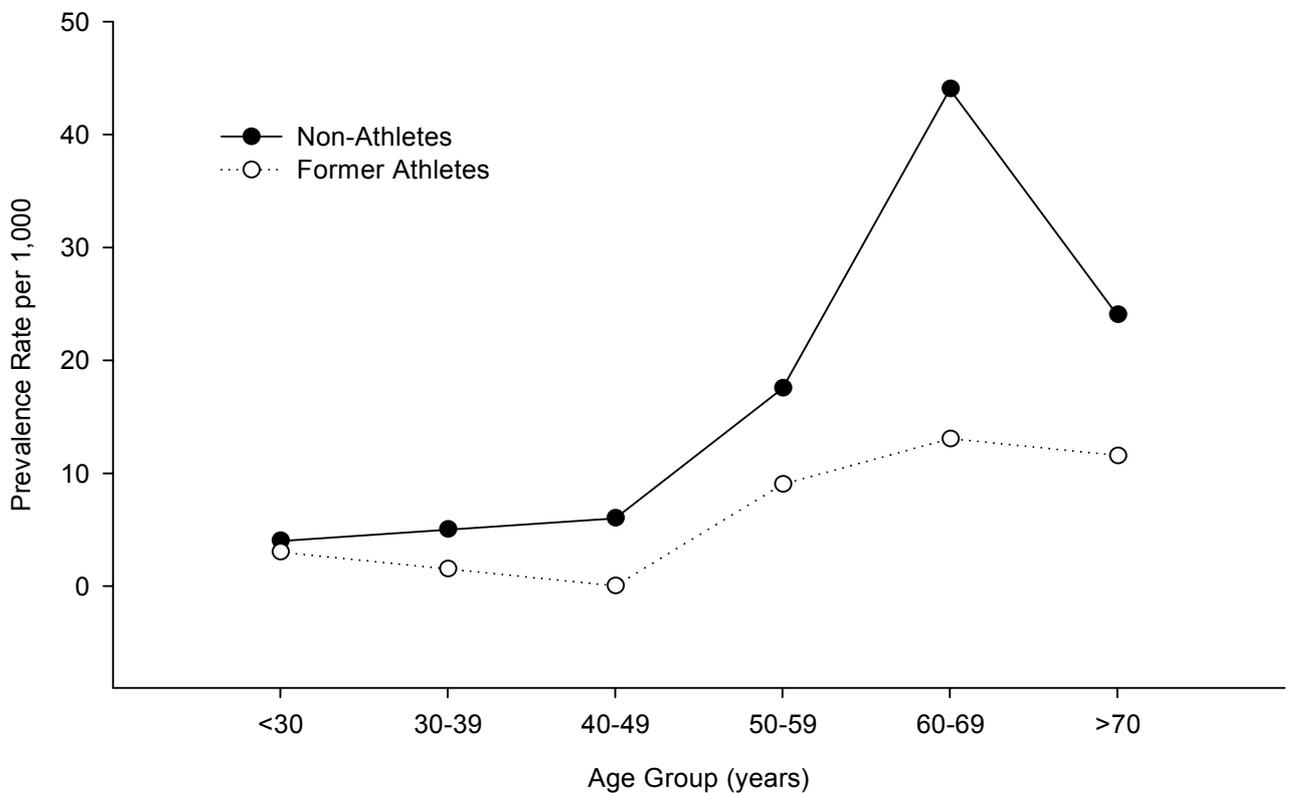


Figure 5A: Prevalence of Cancer of the Reproductive System in Female Former Collegiate Athletes and Non-Athletes (Frisch et al., 1985)

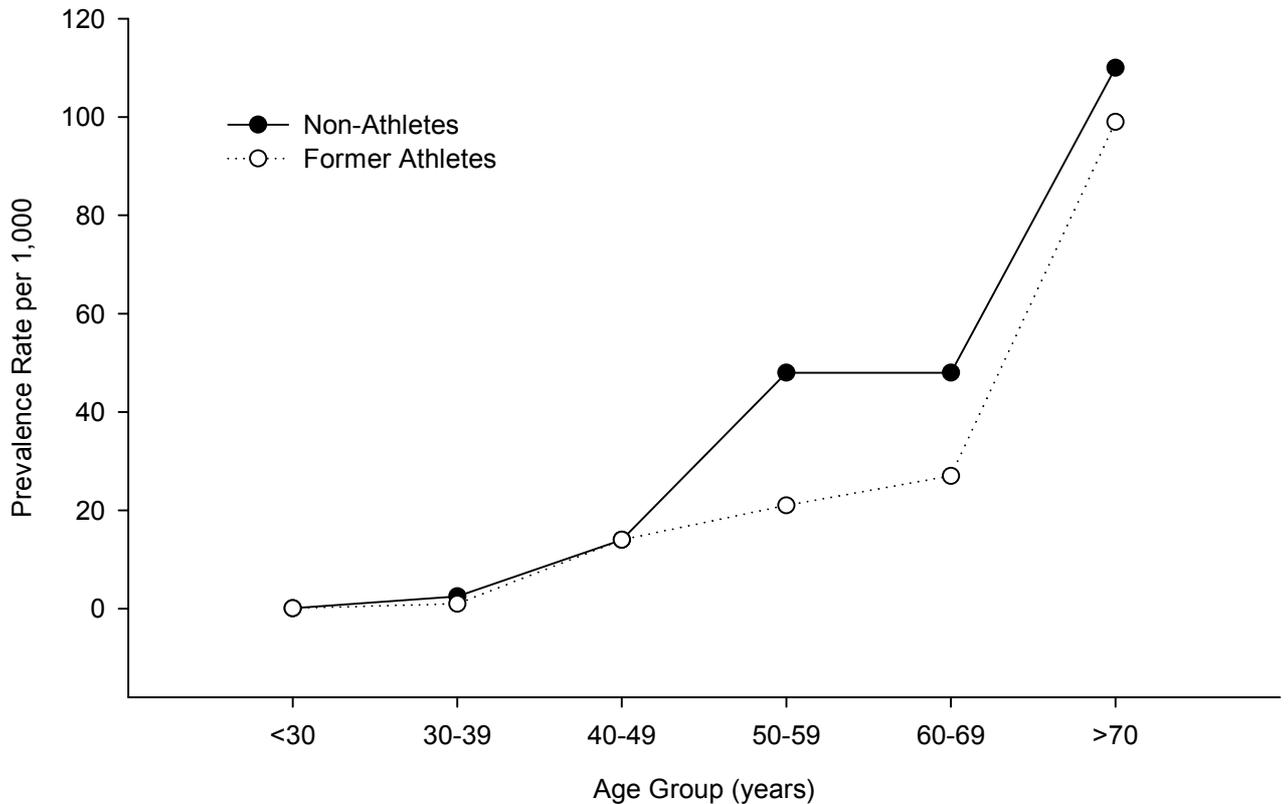


Figure 5B: Prevalence of Breast Cancer in Female Former Collegiate Athletes and Non- Athletes (Frisch et al., 1985).

Cell division of “memory” Th cells is the dominant maintenance mechanism for the T cell pool in human adults (De Boer and Noest, 1998). With ageing, clonal T cell populations contain steadily decreasing frequencies of “memory” cells responsive to antigenic/mitogenic stimulation, thereby associated reduced proliferative ability. Decreased proliferation and IL-2 production in response to mitogens have been associated with down-regulation and decreased receptor expression of the Th co-stimulator CD28, and with telomere shortening in T lymphocytes from older age groups (Boulougouris et al., 1999; Tarazona et al., 2000; Tortorella et al., 2002). However, surface markers for cell lineage, adhesion pathways, activation antigens, (such as CD25<sup>+</sup>) and other receptor structures appear to remain relatively normal with ageing (Effros and Pawelec, 1997; Uyemura et al., 2002).

While “naïve”, “effector” and “memory” T cells display surface antigens and activation markers CD45R, CD44 and L-selectin, effector and “memory” cells have an increased expression of CD44 (Eales 1997; Effros and Pawelec 1997; Farber 1998). CD28 co-stimulation is needed for effector T cell generation from “naïve” cells, but either CD28 or heat-stable antigens (HSA) can generate “memory” cells (Farber 1998). The co-stimulatory signal from HSA is much weaker than CD28, suggesting that “memory” cells can be generated with a lower stimulus (Farber 1998). Therefore, “memory” T cells may be engaged in low-grade responses to persisting antigens and remain semi-activated. Depending on the priming conditions, most “memory” cells decay in a matter of weeks. It is probable that the maintenance of memory depends upon the persistence of antigen. For example, follicular dendritic cells are known to retain antigen on their surface for months (Mosmann and Sad 1996; Tarazona et al., 2000). Viral antigens may also remain in tissues in low concentrations after the primary response. Constant semi-activation and cell division with the resultant decrease in telomeric length and loss of genomic stability may explain why the immune response is not as effective in older individuals as in younger individuals (Tarazona et al., 2000).

### **2.3 T Cell Activation and the Role of Mitogens**

The activation of the immune response is dependent upon vital biochemical events that initiate intracellular signalling and the production of cytokines. If there are age or training-related deviations in IL-2R and T<sub>H</sub>R expression and intracellular calcium flux, DNA replication in CD4<sup>+</sup> cells and T cell clonal expansion may be compromised, as might subsequent immune responses (Koretzky 1997; Farber 1998; Schindowski et al., 2001).

The primary immune response has been outlined in Section 2.2. Th cells are activated by a processed antigen binding to the TCR-CD3<sup>+</sup>, in conjunction with CD28 co-stimulation and IL-1 release by the APC (Goldsby et al., 2000). The binding ligand may be an antigen, or it may be a mitogen, which does not have to be combined with an APC (Pereira 1994). Mitogens, derived from plants, are used because their binding properties are almost identical to those of antigens (Pereira 1994). The binding sites of the mitogen interact with complementary TCR on the CD4<sup>+</sup> cell surface, binding with carbohydrate moieties of glycoproteins or glycolipids exposed on the outer cell membrane (Goldsby et al., 2000).

In lymphocytes, mitogens will trigger blast formation and cell division, activation of CD8<sup>+</sup> cells, alterations of movement of receptors on the cell membrane, and the release of soluble substances such as cytokines, interferon, immunoglobulins and prostaglandin (Pereira 1994; Goldsmith and Green 1994). The mitogens commonly used in immunology are Concanavalin A (Con A), pokeweed and Phytohemagglutinin (PHA). Con A can stimulate all T cells, but PHA specifically stimulates CD4<sup>+</sup> cells to divide and produce IL-2, hence its use in Th cell proliferation assays (Tharp and Preuss 1991; Pereira, 1994). The binding of PHA to a TCR initiates the tyrosine kinase phosphorylation and Phosphatidylinositol biphosphate (PIP2) hydrolysis signal transduction pathway, resulting in transcription of IL-2 genes and expression of IL-2R (Lewis et al., 1997).

## 2.4 T Cell Receptors and Signal Transduction Mechanisms

The TCR is a complex collection of polypeptides with two disulphide-linked chains (either  $\alpha$ - $\beta$  or  $\gamma$ - $\delta$ ) that are non-covalently associated with CD3<sup>+</sup> proteins at the cell surface (Lefranc 1994; Koretzky 1997; Goldsby et al., 2000). The CD3<sup>+</sup> molecule participates in signal transduction after the interaction of the T cell with antigen, but does not influence interaction with the antigen. T cells express either  $\alpha$ - $\beta$  chains or  $\gamma$ - $\delta$  chains, the former type making up 90% of mature T cells (Lefranc 1994). The TCR has a molecular weight of approximately 96 kDa, with the  $\alpha$  chain weighing 43-49 kDa, and the  $\beta$  chain 38-44 kDa ( $\gamma$  chain weighs 40-55 kDa, the  $\delta$  chain 40-45 kDa) (Goldsmith and Green 1994; Goldsby et al., 2000).

The associated CD3<sup>+</sup> complex is comprised of five invariant polypeptide chains that associate to form three dimers: a  $\gamma$  chain (25 kDa),  $\delta$  chain (20 kDa), and  $\epsilon$ - (20 kDa),  $\xi$ - (16 kDa) and  $\eta$ - (21 kDa) chains (Lefranc 1994). The chains form a heterodimer of gamma and epsilon chains ( $\gamma\epsilon$ ); a heterodimer of delta and epsilon chains ( $\delta\epsilon$ ); and a homodimer of two zeta chains ( $\xi\xi$ ) or a heterodimer of zeta and eta chains ( $\xi\eta$ ) – 90% of of the CD3<sup>+</sup> complexes incorporate the  $\xi\xi$  dimer, and the remainder have the  $\xi\eta$  dimer (Goldsby et al., 2000). The TCR can be thought of as four dimers with the  $\alpha\beta$  or  $\gamma\delta$  TCR heterodimer determining the ligand-binding specificity, and the CD3<sup>+</sup> dimer required for expression of TCR and signal transduction (Goldsby et al., 2000). The  $\gamma$ ,  $\delta$  and  $\epsilon$  chains of CD3<sup>+</sup> are members of the immunoglobulin superfamily, each containing an Ig-like extracellular domain followed by a transmembrane region and a cytoplasmic domain of more than

forty amino acids (Goldsmith and Green 1994). The  $\xi$  and  $\eta$  chains have a different structure – a short external region of nine amino acids, a transmembrane region and a long cytoplasmic tail containing 113 amino acids in the  $\xi$  chain and 155 amino acids in the  $\eta$  chain (Goldsmith and Green 1994; Goldsby et al., 2000). The transmembrane region of all the CD3<sup>+</sup> chains contains a negatively charged aspartic acid residue. These charged groups enable the CD3<sup>+</sup> complex to interact with one or two positively charged amino acids in the transmembrane region of each TCR chain (Goldsby et al., 2000), (Figure 6).

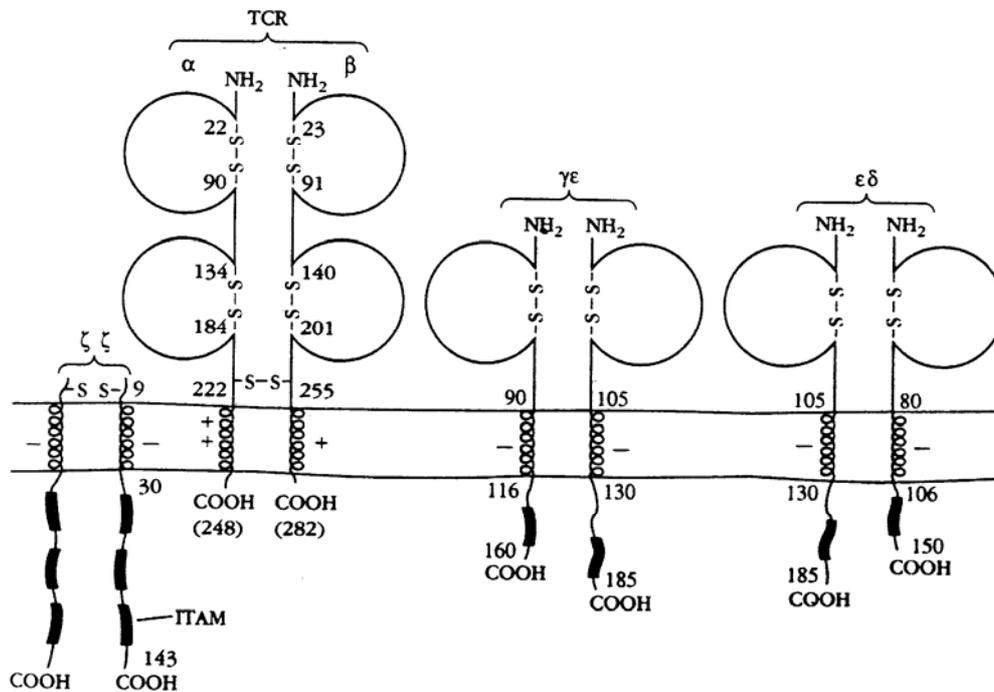


Fig 6: TCR-CD3<sup>+</sup> complex (Goldsby et al., 2000). The CD3<sup>+</sup> complex consists of the  $\zeta\xi$  homodimer (alternatively the  $\gamma\eta$  heterodimer) plus  $\gamma\epsilon$  and  $\delta\epsilon$  heterodimers. The external domains of the  $\gamma$ ,  $\delta$ , and  $\epsilon$  chains of CD3 are similar to the immunoglobulin fold, which facilitates their interaction with the T cell receptor and each other. Ionic interactions also may occur between oppositely charged transmembrane regions in the TCR and CD3 chains. The long cytoplasmic tails of the CD3 chains contain a common sequence, the immuno-receptor tyrosine-based activation motif (ITAM), which functions in signal transduction.

The cytoplasmic tails of the CD3<sup>+</sup> chains contain a motif called the immunoreceptor tyrosine-based activation motif (ITAM). This motif is also found in some other receptors, and has been shown to interact with tyrosine kinases and play a vital role in signal transduction. The  $\gamma$ ,  $\delta$  and  $\epsilon$  chains contain a single copy of the ITAM, whilst the other chains contain three copies of the motif. Closely associated with the CD3<sup>+</sup> complex are the *zeta associated protein* (ZAP-70) and phosphotyrosines *fyn* and *lck* (1-3) of the *src* family, involved in phosphorylation of the TCR and subsequent

activation of further intracellular signalling proteins responsible for eventual CD4<sup>+</sup> lymphocyte DNA transcription (Lewis et al., 1997). The *lck* protein tyrosine kinase is bound to the intracellular domain of CD3<sup>+</sup>, and is translocated into close association with the cytoplasmic tails of the TCR upon the binding of an antigen to the TCR (Alberola-Ila et al., 1997; Goldsby 2000). (Fig 7).

Recognition of the antigen-MHC complex is mediated by the TCR-CD3<sup>+</sup> complex but various accessory molecules also play an important role in T cell activation (Lewis et al., 1997). CD4<sup>+</sup> T cells recognize antigen combined with Class II MHC molecules. CD4<sup>+</sup> is a 55-kda monomeric membrane glycoprotein containing four extracellular Ig-like domains, a hydrophobic transmembrane region, and a long cytoplasmic tail containing three serine residues, capable of phosphorylation (Boulougouris et al., 1999). The extracellular domains bind to the membrane-proximal  $\beta$ 2 domains of MHC molecules on APC's. Once bound, the CD4<sup>+</sup> molecule undergoes a conformational change, allowing its membrane-proximal domains to interact with the domains of adjacent CD4<sup>+</sup> molecules, eventually forming tetramic structures consisting of four Class II dimers with attached CD4<sup>+</sup> (Boulougouris et al., 1999; Goldsby et al., 2000). This cross-linkage of the TCR's on the CD4<sup>+</sup> cell may be necessary for trans-membrane signalling events. The interaction between MHC Class II molecules and CD4<sup>+</sup> appear to increase the avidity of the binding of the TCR to a MHC complex (Boron and Boulpaep 2003).

Other co-receptor molecules also play an important role in TCR affinity for MHC complexes. CD2, LFA-1, CD28 and CD45R do not interact with the peptide-MHC complex, but bind independently to other ligands on APC's or target cells (Zuckerman et al., 1998; Tarazona et al., 2000; Tortorella et al., 2002). During T cell activation there is a transient increase in the expression of accessory molecules, cementing the contact between interacting cells (Koretzky 1997; Tao et al., 1997). This allows cytokines to be transferred more effectively. CD28 is especially important as a Th cell co-stimulator. It is thought that older people are less responsive to immunological challenge because they possess fewer active CD28 molecules rather than fewer TCR or IL-2R (Chiricolo et al., 1995; Vidan et al., 1999; Kronin et al., 2000; Tortorella et al., 2002). The binding of the antigen to the TCR, and the MHC II B7 molecule to the CD28 receptor, initiates phosphorylation of tyrosine and serine residues (ZAP-70 and CD3- $\eta$  respectively) and the consequent activation of the IP3-K and Mitogen Activated Protein Kinase (MAPK)-Ras signal transduction pathways (Lefranc 1994; Goldsmith and Greene, 1994; Alberola-Ila et al., 1997; Farber 1998). The culmination of these

pathways is the activation of the Nuclear Factor of Activated T Cells (NFAT) and the production of IL-2, the expression of IL-2 receptors (CD25) and transferrin receptors (CD71) on the T cell surface, and subsequent mitosis (Goldsmith and Green 1994; Boutin et al., 1997; Alberola-Ila et al., 1997).

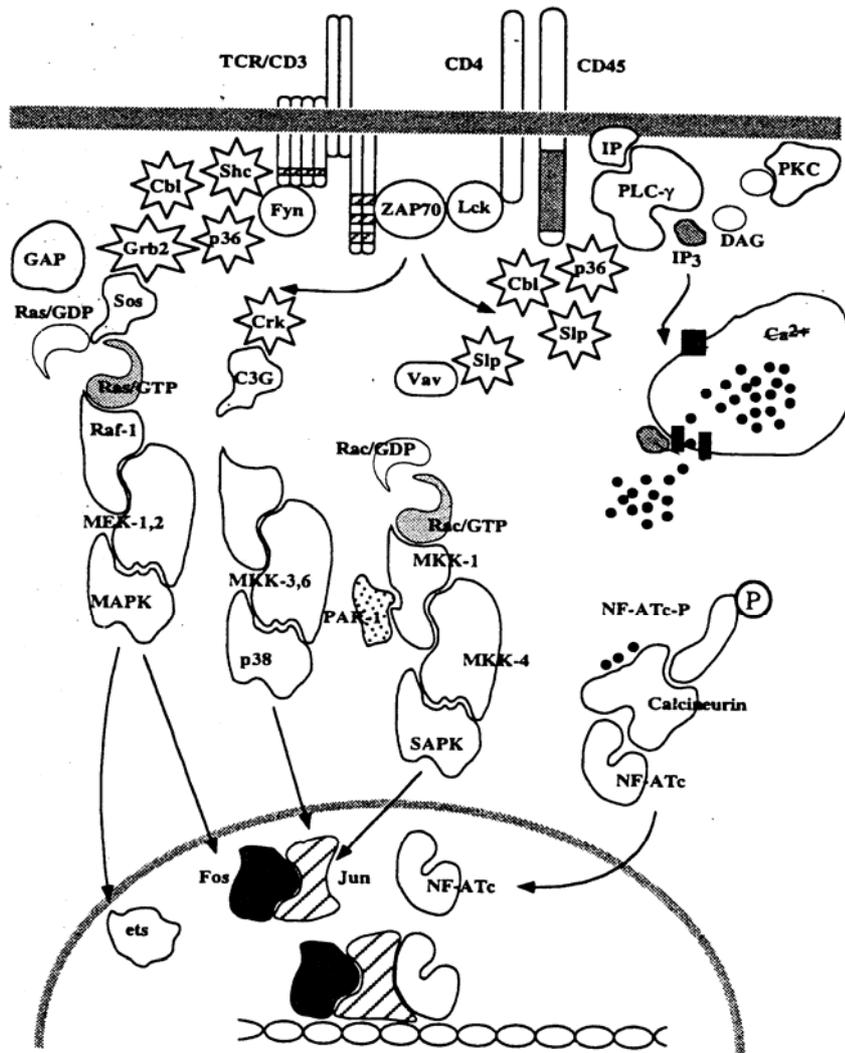


Fig 7: T Cell Activation and Signal Transduction Pathways (Alberola-Ila et al., 1997). TCR-ligand binding induces activation of tyrosine kinases of the *src* and *syk* families. Phosphorylated adaptor proteins and ITAM motifs in the cytoplasmic tails of the CD3 molecules recruit different effector pathways e.g. GTP binding proteins and PLC- $\gamma$ . PLC- $\gamma$  induces  $\text{Ca}^{2+}$  mobilisation. GTP-binding proteins control a series of Ser/Thr kinase cascades (MAPK). The effectors regulate the function of transcription factors (e.g. NFAT, *jun*, *fos*, etc).

Once IL-2 has bound to its receptor, it initiates a signal cascade that triggers TfR expression and DNA replication, involving the action of the holoenzyme ribonucleotide reductase (Stryer 1995; Jordan and Reichard 1998). Clonal expansion of T cells, with the same specificity, results. This expansion also produces a series of cytokines that control the immune response, (eg IL-2, IL-4, IL-

7) (Roitt et al., 1996). The result is the production of specific antibodies by plasma B cells (Lane et al., 1991).

There are two important signal transduction events involved in T cell activation and eventual DNA replication and lymphocyte proliferation. The first event is the activation of the MAPK-Ras and PIP2 pathways following the binding of an antigen/mitogen to the TCR (Alberola Ila et al., 1997; Ma et al., 2002). The second event is the activation of the PIP2 pathway following the binding of IL-2 to the IL-2R (Alberola-Ila et al., 1997; Lewis 2001). If there are any defects in the distinct molecular events of the signalling pathways, as suggested by research on ageing (Whisler et al., 1996a,b; Morford et al., 1997), then the activation of IL-2 transcription elements in the nucleus will be affected and therefore lymphocyte proliferation (Whisler et al., 1996a,b; Farber 1998).

When an antigen binds to the T cell receptor (TCR/CD3<sup>+</sup>), there is a change in receptor conformation and phosphorylation of tyrosine residues, resulting in the activation of phospholipase C (the IP3-K pathway) (Lewis 2001). There is also a transient activation of *Ras*, measured by its conversion from the GDP- to the GTP-bound state in activated cells (Ma et al., 2002). The activation of PLC causes the enzymatic hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP2) in the cell membrane, into diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), which act as second messengers (Mooren et al., 2001a,b; Lewis 2001). When IP3 is released into the cytoplasm, the IP3-gated calcium channel binds the *fyn* kinase directly and phosphorylates tyrosine residues, facilitating calcium release from the intracellular pool (Alberola-Ila et al., 1997; Lewis 2001). Maximal concentrations of IP3 stimulate Ca<sup>2+</sup> mobilisation within 100 milliseconds of addition, consistent with the rapid opening of Ca<sup>2+</sup> channels (Taylor et al., 1992).

The binding of IP3 to its receptor, and the induced Ca<sup>2+</sup> mobilisation are optimal at an alkaline pH, typically 8 (Lewis 2001). This appears physiologically important since PKC, by activating Na<sup>+</sup>-H<sup>+</sup> exchange, can increase cytoplasmic pH, and this has been implicated in the action of many growth factors (Taylor et al., 1992; Goldsby 2000). Also of interest is that submaximal concentrations of IP3 release only a fraction of Ca<sup>2+</sup> stores, whereas a maximal IP3 stimulation will release all stored Ca<sup>2+</sup> from IP3-sensitive stores (Rossier and Putney 1991; Taylor et al., 1992). If IP3 concentrations are low, Ca<sup>2+</sup> stores might not be completely emptied.

The elevation of calcium ion concentrations activate the phosphatase calcineurin, which dephosphorylates the cytosolic component of NFAT (Alberola-Ila et al., 1997; Hedin et al., 1997). NFAT migrates to the nucleus and together with other transcription factors AP-1, NF $\kappa$ B and Oct-1, induces the transcription of genes for both IL-2 and IL-2R, (Figure 7) (Boutin et al., 1997; Alberola-Ila et al., 1997; Ma et al., 2002). The secretion of IL-2, the subsequent expression of IL-2R and ultimately lymphocyte proliferation depends directly on these molecular events (Neckers and Cossman 1983; Ma et al., 2002).

Research suggests that signal transduction is impaired during the ageing process (Chiricolo et al., 1995; Garcia and Miller 1996; Whisler et al., 1996a,b; Morford et al., 1997). Garcia and Miller (1996) suggested that ageing reduces the level of phosphorylation of stimulus-response phosphoproteins (primarily ZAP-70). Experiments with aged mice showed that their CD4<sup>+</sup> cells were unresponsive to PHA, and tyrosine phosphorylation was three times lower in unstimulated cells, compared to T cells from young animals (Garcia and Miller, 1996). Furthermore, human studies by Morford et al. (1997), who examined T cells from patients with intracranial tumours, found that these patients had a marked decrease in tyrosine phosphorylation of the ZAP-70, PLC and pp100 – p56/*lck* proteins. Concentrations of these *lck* proteins were noticeably reduced. CD4<sup>+</sup> lymphocytes from patients with tumours showed decreased antigenic and mitogenic responsiveness to PHA, and less intracellular calcium mobilization than in normal CD4<sup>+</sup> cells, indicating defects in early transmembrane signalling events associated with transcription of mRNA (Morford et al., 1997).

Age-related impairments in cytosolic calcium responses and normal patterns of protein phosphorylation have been reported by Beiqing et al., (1997), Whisler et al., (1996a,b), Gadina et al., (1998), and Farber et al., (1998). Whisler et al. (1996a,b) and Beiqing et al. (1997) found that reduced production of IL-2 by PHA-stimulated T cells from older subjects was subsequent to decreased concentrations of IL-2 mRNA. This suggested that reduced IL-2 production with age may be related to underlying deviations in transcriptional regulatory proteins needed for normal cytokine production and IL-2R expression (eg reduced activation of NFAT) (Whisler et al., 1996a,b; Beiqing et al., 1997; Thoman 1997).

In approximately 30% to 50% of healthy older humans, mitogenically activated T cells displayed a substantial reduction in IL-2 production in-vitro. T cells from the remainder of the older subjects showed a similar IL-2 concentration to young subjects (Beiqing et al., 1997). The decrease in IL-2 production in the 30% to 50% of older individuals represented lower frequencies of IL-2-secreting cells rather than reductions in the overall number of T cells (Beiqing et al., 1997). These results would lend a measure of support to other research suggesting that higher serum IL-2 concentrations are associated with an increased number of “naïve” cells, while reduced IL-2 concentrations are related to an increased number of “memory” cells (Rea et al., 1996; Ginaldi et al., 2000; Chakravarti and Abraham 2002).

While ageing appears to have a negative impact on intracellular signalling in CD4<sup>+</sup> cells, research has shown that T cell proliferation can be adversely affected by elevated concentrations of cortisol, prolactin (Mann et al., 2000) and prostaglandin (Brenner et al., 1998; Ronsen et al., 2001). Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), is released from macrophages during the inflammatory response from injury, illness or heavy training (Felli et al., 1996; Kolenko et al., 1999) has been shown to down-regulate the expression of the Janus kinase 3 (JAK3), an important enzyme in the tyrosine phosphorylation and activator of transcription (STAT) 5 pathway (Kolenko et al., 1999). Suppression of JAK3 results in inhibited IL-2R expression and Th cell proliferation (Felli et al., 1996).

PGE<sub>2</sub> has also been shown to inhibit transcription of the IL-2 gene promoter by interfering with signals activating the -99 to -66 base pair octamer motif (Felli et al., 1996). The octamer motif is a composite cis-regulatory element binding the *jun* and *fos* AP-1 factors (inducible by calcium ionophores and phorbol esters), and the Oct-1 and Oct-2 transactivators (Felli et al., 1996; Goldsby et al., 2000). Hence, any interference with the octamer element antagonizes calcineurin-dependent pathways and inhibits transcription factors (*Rel/NFκB*) in the nucleus. The action of PGE<sub>2</sub> on gene transcription is also mediated by increased concentrations of cAMP (Felli et al., 1996; Kolenko et al., 1999). Since an increased concentration of PGE<sub>2</sub> is common in athletes (Pedersen 1997; Brenner et al., 1998), due to training-induced injury, local inflammation or viral/bacterial infection, it is possible that signal transduction mechanisms can be inhibited during periods of high volume and high intensity training, thereby contributing to the immunosuppression that is observed in some athletes.

## 2.5 The Interleukin-2 Receptor

When IL-2 binds to its receptor, not only does it stimulate further IL-2 production, but it also initiates another signal transduction cascade involving tyrosine kinase phosphorylation and the PIP2 hydrolysis to DAG and IP3 (Beiqing et al., 1997; Pahlavani et al., 1997). It is important to note that the IL-2R exists in low, intermediate and high affinity forms, consisting of three distinct membrane associated sub-units (Vilcek and Le 1994). These are encoded by different genes (Theze et al., 1996). The binding affinity depends on heterodimerization and heterotrimerization of subunits for intermediate and high affinity binding respectively (Goldsmith and Green, 1994), (Figure 8).

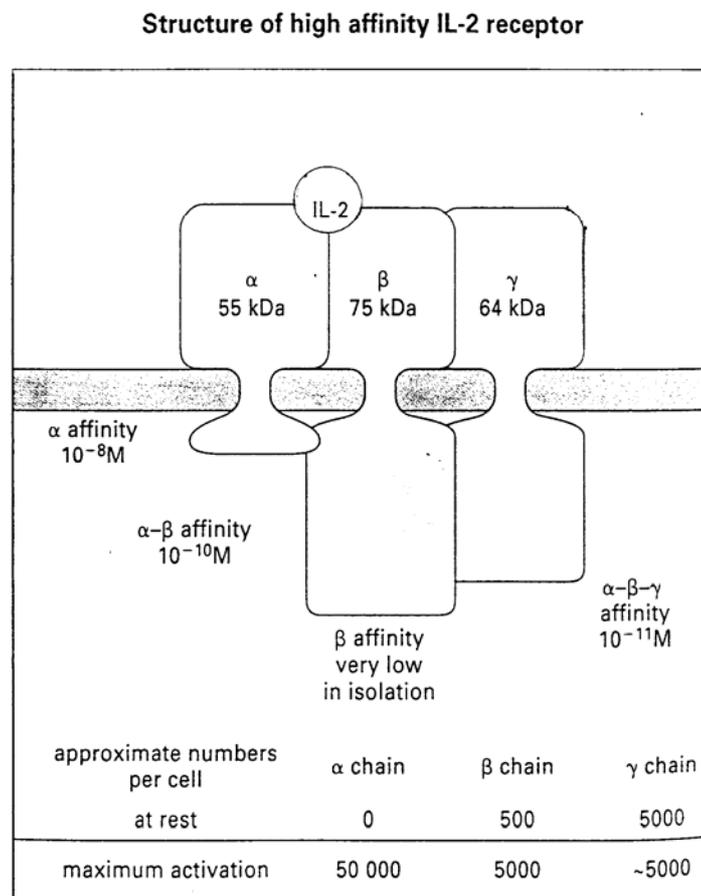


Figure 8: The IL-2 Receptor (Roitt et al., 1996). The IL-2R barely exists in un-activated cells. Upon a ligand binding to the TCR/CD3<sup>+</sup>, IL-2 and IL-2R genes are transcribed, inducing an up-regulation of α-β binding sites.

Individual subunits alone bind IL-2 with low affinity – these subunits are the α subunit (CD25 - p55kD protein), the most active of the units; βCD122<sup>+</sup> (p75kD); and γ (p64kD) unit (Theze et al., 1996). At rest, few lymphocytes express high affinity IL-2Rα, or β receptors (100-500 sites per cell and 500-1,000 sites respectively in NK cells) (Neckers and Cossman 1983; Rhind et al., 1994;

Theze et al., 1996). In a resting state, the cells are unresponsive to IL-2. Stimulation of T cells by antigen or mitogen induces a 30-100 fold transient up-regulation of IL-2R $\alpha$  chains, peaking 48 to 72 hours after stimulation (Rhind et al., 1994). The increase in  $\beta$  chains is small, resulting in more than 10,000 excess  $\alpha$  units per cell, and the formation of 2,000 to 3,000 high-affinity  $\alpha$ - $\beta$  binding sites per cell (Rhind et al., 1994). The  $\alpha$  and  $\beta$  heterodimer has the highest binding affinity, but all three subunits are required for IL-2 internalization and signal transduction. The interaction of all three subunits causes a conformational change in the  $\alpha$  -  $\beta$  subunit that alters the structure of the ligand binding site (Goldsmith and Green, 1994; Rhind et al., 1994; Theze et al., 1996). Once the ligand has bound, the IL-2R promotes CD4<sup>+</sup> cell proliferation by the phosphorylation of tyrosine residues and activation of various proteins, and eventually transcription of genes for further IL-2R and TfR (Chakrabarti and Kumar 1999; Lewis et al., 1997).

## 2.6 The Transferrin Receptor

The binding of IL-2 to its receptor precedes the expression of transferrin receptors (TfR/CD71), by approximately 24 hours (Neckers and Cossman 1983). Induction of CD71, subsequent binding of transferrin and the release of iron (Fe<sup>3+</sup>) into the lymphocyte cytoplasm leads to the initiation of DNA synthesis (Neckers and Cossman 1983; Pascale et al., 1998). Mitogen-induced lymphocyte proliferation is transferrin and iron dependent, even though lymphocytes at rest do not possess detectable TfR (Brock 1981; Pascale et al., 1998). Transferrin is an 80kD protein, synthesized in the liver, that mediates iron transport between the gut, bone marrow and tissues (Gaston et al., 1987; Rutledge et al., 1998). Each molecule of transferrin can bind two molecules of ferric (Fe<sup>3+</sup>) iron, and will bind to TfR on the lymphocyte membrane when iron is needed. The genes for both transferrin and its receptor are found on the long arm of chromosome 3 (Worwood 1995). The TfR is a disulphide-linked dimer transmembrane glycoprotein with a molecular weight of 180kD (Worwood 1995; Baynes 1996). Each receptor can bind one or two molecules of transferrin (Baynes 1996; Stryer 1995). Ferric-transferrin is bound by receptors in coated pits, which form endocytic vesicles. The vesicles fuse with endosomes, the low pH of between 5 and 6 within the endosome causing dissociation of the two Fe<sup>3+</sup> ions from the receptor into cytosolic pools (Stryer 1995; Trinder et al., 1996; Allen et al., 1998). The receptor, retaining apotransferrin (the protein free of iron), is recycled back to the plasma membrane. Here, apotransferrin is released from the

receptor when the vesicle fuses with the plasma membrane and there is a sudden shift in pH to 7.4 (Stryer 1995; Baynes 1996).

Apotransferrin has little affinity for the receptor at pH 7.4. The transferrin cycle takes approximately 12 to 16 minutes (Stryer 1995; Baynes 1996) with the amount of iron taken up by the cell determined by the number of receptors. If the individual is low in iron, more receptors are expressed (Baynes 1996; Maes et al., 1997a; Allen et al., 1998; Skikne 1998). TfR numbers are regulated at the synthetic level by the stability of their mRNA, which is sensitive to intra-cellular iron pools distributed through the cytosol (Rutledge et al., 1998). TfR numbers are also regulated at the level of degradation, by the release of the receptors from the cell by proteolytic cleavage (Baynes 1996; Rutledge et al., 1998). If TfR expression is blocked, for example, by anti-Tac antibody, the proliferative sequence is halted (Neckers and Cossman, 1983).

When lymphocytes are stimulated with an antigen/mitogen, there's a marked increase in TfR expression and transferrin-bound iron uptake, which occurs several hours prior to DNA replication (Trinder et al., 1996). Molecular events initiating the transferrin cell cycle are not fully understood, but intracellular calcium and calmodulin are involved in the internalization process, and phosphorylation and dephosphorylation of the receptor may be involved in directing receptor movement from the endosome network to the surface (Sainte-Marie et al., 1997). Treatment of the lymphocytes with the phorbol ester PMA and calcium ionophores increase the translocation of a receptor-containing compartment to the cell surface (Sainte-Marie et al., 1997; Pascale et al., 1998). Research by Sainte-Marie et al. (1997) suggested that the binding of transferrin to the TfR initiated several second-messenger pathways, which could redundantly stimulate recycling of the receptor. Artificial elevation of free internal  $\text{Ca}^{2+}$  in cells accelerated the recycling rate of TfR, and conversely, intracellular addition of transferrin produced a rise in  $\text{Ca}^{2+}$  concentration, suggesting that  $\text{Ca}^{2+}$  is a regulator of TfR recycling, and that TfR may function as a signal transduction molecule, possibly in conjunction with other membrane proteins (Sainte-Marie et al., 1997).

The expression of cell surface TfR is carefully regulated by TfR mRNA, according to the internal iron concentration and individual iron requirements of the cell (Skikne 1998; Rutledge et al., 1998). Intra-cellular iron influences the translation of ferritin mRNA and the stability of TfR mRNA (Khumalo et al., 1998). The regulation of TfR occurs by way of an interaction between iron

regulatory proteins (IRP), which sense changes in the chelatable intra-cellular iron pool, and iron-responsive elements (IRE's) located on the 5' untranslated region of the ferritin mRNA and the 3' region of TfR mRNA (Baynes 1996; Khumalo et al., 1998). The 5' of ferritin mRNA and 3' regions of TfR mRNA contains a sequence forming a stem-loop structure, the IRE (Worwood 1995). Cytoplasmic repressor proteins able to bind to this sequence are the IRP, and prevent translation when intracellular iron is abundant. In the presence of iron, the IRP display activity of the aconitase enzyme and do not bind to the IRE's. Aconitase contains iron that is not bonded to haem – its four iron atoms are complexed to four inorganic sulphides and four cysteine sulphur atoms (Stryer 1995; Baynes 1996).

Aconitase acts by dehydrating and rehydrating its bound substrate, and in this case extra cytoplasmic iron is bound in the enzyme and IRP's will not bind to IRE's (Stryer 1995; Khumalo et al., 1998). The result is increased ferritin mRNA translation and increased TfR mRNA degradation (Khumalo et al., 1998). During iron depletion, the situation is reversed. IRP loses aconitase activity and binds to IRE's, repressing ferritin mRNA translation and increasing TfR mRNA stability (Baynes 1996; Khumalo et al., 1998).

Iron is needed for DNA synthesis – it is an integral part of the holoenzyme ribonucleotide reductase (Figure 9), which catalyzes the formation of DNA precursors, deoxyribonucleotides (Stryer, 1995; Jordan and Reichard, 1998). These are formed from the reduction of ribonucleotides, the reductant being NADPH (Jordan and Reichard, 1998). The holoenzyme consists of B1 and B2 subunits plus two iron ions (Drobyski et al., 1996). The B1 subunit contains a substrate binding site, two allosteric control sites and a sulphhydryl pair that serve as immediate electron donors in the reduction of the ribose unit of the substrate (Jordan and Reichard 1998). The B2 subunit generates a free radical in each of its chains, and both units together form the catalytic site for the enzyme. If iron is deficient, the ability of the B2 subunit to produce free radicals is impaired, and the reduction of the ribonucleotide does not occur. The entire cascade of reactions, and eventual DNA synthesis will be impaired (Stryer 1995). For DNA replication to begin, both IL-2R and TfR must be expressed (Neckers and Cossman 1983; Maes et al., 1997a). Once TfR appear, the continued presence of functional IL-2R is not needed for the cell to proceed with at least one round of DNA synthesis (Brock 1981; Neckers and Cossman 1983).

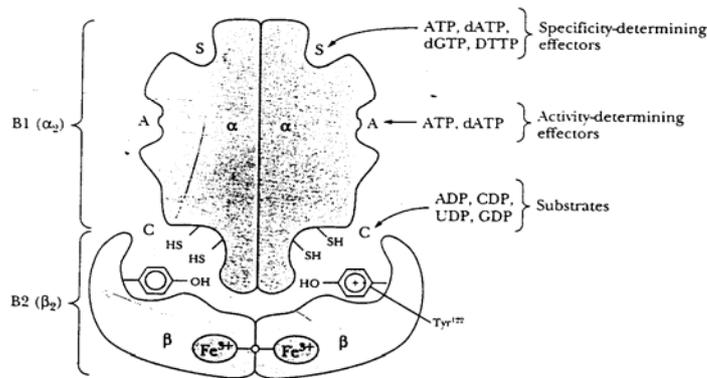


Figure 9: Structure of Ribonucleotide Reductase (Stryer 1995). Each chain of B1 contains a substrate binding site, 2 allosteric control sites and a sulfhydryl pair that act as the immediate electron donor in the reduction of the ribose unit. B2 acts in catalysis by generating a free radical in each of its chains. B1 and B2 subunits together form the catalytic sites of the enzyme.

## 2.7 The Role of Intracellular Calcium

Changes in intracellular  $\text{Ca}^{2+}$  are associated with a wide variety of cellular processes (Lewis 2001; Mooren et al., 2001a,b; Berridge et al., 2003). Cellular events such as secretion, fertilisation, cleavage, muscle contraction, nuclear envelope breakdown, activation of transcription and apoptosis are all accompanied by changes in concentrations of  $\text{Ca}^{2+}$  (Lewis 2001). Changes in intracellular calcium concentration ( $[\text{Ca}^{2+}]_i$ ), part of the IP<sub>3</sub> intracellular signaling path, represent an important link between the stimulation and activation of lymphocytes, and their immune response (i.e. proliferation, cytokine production) (Lewis 2001). When the cell is at rest, the basal  $[\text{Ca}^{2+}]_i$  is regulated at a low level in the nanomolar range, against a large electro-chemical gradient (Taylor et al., 1992; Mooren et al., 2001a,b; Berridge et al., 2003). Basal  $[\text{Ca}^{2+}]_i$  is the result of an equilibrium between calcium leakage across the plasma membrane, and membrane of intracellular calcium stores, along the electro-chemical gradient. Equilibrium is also maintained by the action of active calcium pumps in the plasma membrane (PMCA),  $\text{Ca}^{2+}$  store-activated pumps in the membrane (CRAC), and sarcoplasmic/endoplasmic reticulum pumps (SERCA) (Guse et al., 1997; Hoth et al., 2000; Lewis 2001; Bautista et al., 2002). There is a constant cycling of  $\text{Ca}^{2+}$  across both the ER and plasma membrane. Cytoplasmic  $\text{Ca}^{2+}$  is increased by either a depolarisation of

the plasma membrane, dependent upon a voltage sensor, or receptors and second messengers (eg IP3) that release  $\text{Ca}^{2+}$  from ER stores and stimulate influx across the plasma membrane.

Elevation of intracellular  $\text{Ca}^{2+}$  is one of the prime signals for T cell activation by antigen (Berridge 1989; Lewis 2001; Bautista et al., 2002; Ma et al., 2002).  $\text{Ca}^{2+}$  signals may be varied in T cells, appearing as transient spikes, oscillations or a sustained plateau. Recent research has shown that varying  $\text{Ca}^{2+}$  spikes, plateau and oscillations may activate different signalling and transcriptional pathways within cells (Torgan and Daniels 2001; Bautista et al., 2002; Lewis 2003). Different signals are derived from the interactions of multiple  $\text{Ca}^{2+}$  intracellular sources and membrane/SERCA pumps (Berridge et al., 2003). SERCA refers to five sarcoplasmic/endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase isoforms, derived from three genes (SERCA 1,2 and 3) (Lewis 2001). SERCA 1a and 1b exist in fast-twitch muscle fibres, SERCA 2a in slow-twitch and cardiac muscle, 2b in non-muscle tissue, SERCA 3 in intestine and brain tissue (Lewis 2001). The activation of the TCR is coupled to  $\text{Ca}^{2+}$  mobilisation, via the PIP2 signal path. After antigenic or mitogenic stimulation, the IP3-dependent release of  $\text{Ca}^{2+}$  from intracellular stores is followed by the release of  $\text{Ca}^{2+}$ -influx factor (CIF) from the ER (Putney et al., 2001). CIF activates CRAC channels, allowing a sustained influx of extracellular  $\text{Ca}^{2+}$  across the plasma membrane (Lewis 2001; Putney et al., 2001; Bautista et al., 2002; Mather and Rottenberg 2002).

When stimulated, intracellular  $\text{Ca}^{2+}$  concentrations increase from basal concentrations to between 800 and 1500 nM (Morris et al., 1994; Berridge et al., 2003). After the cessation of stimulation,  $[\text{Ca}^{2+}]_i$  are regulated to pre-stimulatory levels by the action of  $\text{Ca}^{2+}$ -ATPases, either PMCA or SERCA (Lewis 2001).  $\text{Ca}^{2+}$  signals affect downstream events on different timescales. Signals to stabilise contacts between T cells and APC through changes in motility and cytoskeletal re-organisation occur over a timespan of seconds (Bautista et al., 2002; Lewis 2003). Long-lasting signals acting from minutes to several hours may increase the efficiency and specificity of gene activation events, depending upon the amplitude, duration and kinetic nature of the  $\text{Ca}^{2+}$  signal (Lewis 2001; Berridge et al., 2003).

The matching of  $\text{Ca}^{2+}$  regulating and transport systems is important for optimal cellular function. Alterations in cellular  $\text{Ca}^{2+}$  signalling have been shown to be critically involved in cellular dysfunction and diseases such as Alzheimer's disease and some types of tumours (Hartmann et

al., 1994; Morford et al., 1997; Mooren et al., 2001a,b; Mooren and Volker 2001; Berridge et al., 2003).

The elevation of intracellular cytosolic  $[Ca^{2+}]_i$  is a vital trigger for T lymphocyte activation by either antigenic or mitogenic stimulation of the TCR (Lewis 2001). The binding of antigen/MHC complexes to the TCR initiates the recruitment of a series of tyrosine kinases and substrates to the TCR/CD3<sup>+</sup> complex, resulting in the phosphorylation and activation of PLC, and then the PIP2 and *ras*-dependent (MAPK) pathways (Alberola-Ila et al., 1997). IP3 generated from TCR stimulation binds to receptors in the ER membrane, opening channels that release  $Ca^{2+}$  to the cytosol.  $Ca^{2+}$  also enters the cytosol from the extracellular space (Thomas and Hanley 1994; Lewis 2001; Bautista et al., 2002).  $Ca^{2+}$  release is a highly co-operative process since multiple IP3 molecules bind to the tetrameric receptor, and  $Ca^{2+}$  released from the ER has a positive feedback effect (Lewis 2001; Putney et al., 2001). The released  $Ca^{2+}$  transient is usually in the order of about 800 to 1500 nM but returns to baseline concentrations (between 40 – 100 nM) within minutes (Marriot and Mason 1996; Hoth et al., 2000; Lewis 2001; Berridge et al., 2003). Usually,  $Ca^{2+}$  influx must be maintained for 60 to 120 minutes to efficiently activate events leading to IL-2 gene expression. Protein products of the transcription activation *c-fos* and *c-jun* oncogenes have been detected in the cell nucleus, associated with the chromatin, between five and sixty minutes after mitogenic stimulation, along with an increase in  $[Ca^{2+}]_i$  (Morgan and Curran 1989; Whisler et al., 1996a,b). Beyond sixty minutes, further antigen stimulation is needed (Lewis 2001; Mather and Rottenburg 2002).

Sustained  $Ca^{2+}$  signaling is needed to maintain NFAT in the nucleus, and in a transcriptionally activated state (Hoth et al., 2000; Lewis 2001; Berridge et al., 2003; Kotturi et al., 2003). In unstimulated cells  $[Ca^{2+}]_i$  may range from a low nM range, but after stimulation transient  $Ca^{2+}$  peaks and sustained elevations may vary from approximately 200 nM to over 1  $\mu$ M (Hoth et al., 2000; Berridge et al., 2003). The character and amplitude of the  $Ca^{2+}$  response is influenced by the type of immune cell and its state of maturation (Greimers et al., 1996), as well as by the type of antigen or mitogen (Mooren et al., 2001a,b). Lymphocytes may be activated, or guided towards anergy or apoptosis, depending upon the  $Ca^{2+}$  response (Kiang et al., 2003).

Little is known about the calcium storage compartment and how it is physiologically loaded with calcium (Lewis 2001). Evidence suggests that while calcium is available in the cytosol, larger amounts are stored in the ER (Marriot and Mason 1996; Gagne et al., 1997; Mather and Rottenberg 2002; Berridge et al., 2003). In both SR and ER, calcium is loaded from the cytosol to the store by an ion-motive ATPase, the intracellular calcium pump (Thomas and Hanley, 1994), or SERCA. The  $\text{Ca}^{2+}$  transport cycle occurs when the ATPase undergoes a conformational change, transforming high-affinity cytosol-facing ion binding sites (E1 state) to low-affinity lumen-facing binding sites (E2 state).  $\text{Ca}^{2+}$  is released from IP<sub>3</sub>-sensitive storage by the activation of the PIP<sub>2</sub> pathway and elevation of calcineurin (Alberola-Ila et al., 1997; Fomina et al., 2000). The exact mechanism of the  $\text{Ca}^{2+}$  release is still unknown, but the depletion of IP<sub>3</sub>-sensitive stores opens CRAC channels, further elevating  $[\text{Ca}^{2+}]_i$  (Fomina et al., 2000).

Whether the intracellular calcium store accessible to IP<sub>3</sub> receptor action is in a specialised domain, or just in the ER, is open to debate (Bautista et al., 2002). Several hypotheses have been suggested, including the regulation of ER stores by IP<sub>3</sub> receptors which may sense changes in  $[\text{Ca}^{2+}]$  through  $\text{Ca}^{2+}$ -binding sites on their luminal domains. This “conformational-coupling” information may then be directly conveyed by the IP<sub>3</sub> receptor physically interacting with  $\text{Ca}^{2+}$  channels in the plasma membrane (Putney et al., 2001; Bautista et al., 2002). Another hypothesis is that depletion of ER stores causes the fusion of vesicles containing CRAC channels with the plasma membrane, allowing an influx of  $\text{Ca}^{2+}$  into the cell (Putney et al., 2001).  $\text{Ca}^{2+}$  discharged from a full intracellular pool may also keep CRAC and PMCA inhibited, whereas a reduction in intracellular pools and ER stores may remove the inhibition on the  $\text{Ca}^{2+}$  channels (Fomina et al., 2000; Putney et al., 2001). While the exact mechanism for ER store  $\text{Ca}^{2+}$  entry and removal is still open to debate, the regulation of high  $[\text{Ca}^{2+}]_i$  is known to involve other organelles apart from the ER.

It is known that the mitochondria can accumulate  $\text{Ca}^{2+}$  only when cytoplasmic  $[\text{Ca}^{2+}]$  is high (e.g. over 1  $\mu\text{M}$ ) (Taylor et al., 1992; Bautista et al., 2002; Berridge et al., 2003). Mitochondria may fulfil a protective role during times of potentially damaging high  $[\text{Ca}^{2+}]$ , but are not important  $\text{Ca}^{2+}$  regulators in unstimulated cells. However, the mitochondria are able to regulate high concentrations of intracellular  $\text{Ca}^{2+}$  in stimulated cells by sequestering  $\text{Ca}^{2+}$  and then releasing the stored  $\text{Ca}^{2+}$  after  $[\text{Ca}^{2+}]_i$  falls below approximately 400 nM (Hoth et al., 1997; Bautista et al., 2002).

In non-excitabile cells, mitochondria appear to modulate  $\text{Ca}^{2+}$  flux across cell membranes by influencing signals, usually oscillations, generated by  $\text{Ca}^{2+}$  release from the ER (Hoth et al., 2000). Hoth et al. (2000) suggested that the mitochondria interacted closely with the IP3 receptor, and could modulate  $\text{Ca}^{2+}$  flux by regulating  $\text{Ca}^{2+}$ -ATPases or  $\text{K}^+/\text{Cl}^-$  channels (Hoth et al., 2000; Bautista et al., 2002). The apparent mechanism behind this regulation was the local sequestering and reduction of  $[\text{Ca}^{2+}]_i$  near sites controlling CRAC inactivation, rather than a widespread reduction of  $[\text{Ca}^{2+}]_i$  by the mitochondria (Hoth et al., 2000; Fomina et al., 2000)

The formation of IP3 after mitogenic stimulation results in only a fraction of intracellular  $\text{Ca}^{2+}$  being released from ER stores (Lewis 2001). Approximately 50% of  $\text{Ca}^{2+}$  stores are IP3-sensitive, with some variation in the percentage depending upon cell type (Taylor et al., 1992; Lewis 2001). The size of the IP3-sensitive stores depend upon concentrations of GTP and IP3. Since not all  $\text{Ca}^{2+}$  stores are sensitive to IP3 and are therefore subject to independent regulation, accurate determination of total intracellular  $\text{Ca}^{2+}$  relies on other molecules such as thapsigargin to release  $\text{Ca}^{2+}$  from all stores, (Thastrup et al., 1990; Taylor et al., 1992; Mather and Rottenberg 2002). There is growing evidence that there are specific interactions among various  $\text{Ca}^{2+}$  stores occur in response to various external stimuli, and recent research has attempted to investigate the contributions of different  $\text{Ca}^{2+}$  storage compartments to cellular responses initiated by different stimuli (Gagne et al., 1997; Mather and Rothenburg 2002; Bautista et al., 2002; Berridge et al., 2003; Lewis 2003). Bautista et al. (2002) found that SERCA, CRAC pumps and the mitochondria helped to regulate cytosolic  $\text{Ca}^{2+}$  during stimulation, and suggested that the mitochondria are able to sequester and then release  $\text{Ca}^{2+}$ , which is then taken up by the ER and eventually released through IP3 receptors. The clearance of  $\text{Ca}^{2+}$  following a rise in  $[\text{Ca}^{2+}]_i$  in T lymphocytes involves contributions from the PMCA, mitochondria and SERCA. PMCA appears to be the sole clearance mechanism with lower  $[\text{Ca}^{2+}]_i$ , whereas both PMCA and mitochondria primarily control  $\text{Ca}^{2+}$  recovery when  $[\text{Ca}^{2+}]_i$  is high, with additional clearance in a minor capacity from SERCA (Bautista et al., 2002). There is also evidence for slow leakage of  $\text{Ca}^{2+}$  from the internal stores, unrelated to the SERCA pump (Thomas and Hanley, 1994; Lewis 2001; Berridge et al., 2003).

The measurement of  $\text{Ca}^{2+}$  involves not only cytosolic calcium but also the stores in the ER. To estimate all the available calcium, a compound called thapsigargin is used. Thapsigargin is a sesquiterpene lactone isolated from the plant *Thapsia garganica* (Thomas and Hanley, 1994).



many types of body cells (Hartmann et al., 1994; Morford et al., 1997). There is evidence that endurance exercise can reverse the age-related down-regulation of SERCA in cardiac cells implicated in diastolic dysfunction (Tate et al., 1994; Cain et al., 1998). Moderate exercise undertaken by older individuals may attenuate disruptions to calcium mobilisation and the signal transduction pathway in lymphocytes and other leucocytes (Mooren and Volker 2001).

Immunosuppression attributed to repeated bouts of high intensity exercise and to long-term, high volume training undertaken by endurance athletes, could also be linked to alterations in  $\text{Ca}^{2+}$  signaling and disruptions to lymphocyte proliferation and neutrophil responses (Mooren et al., 2001a,b; Mooren and Volker 2001). There is, however, little research regarding alterations in  $[\text{Ca}^{2+}]_i$  and cellular signalling in endurance athletes, and older individuals undertaking long-term training. Further investigation is required into possible gender differences in  $[\text{Ca}^{2+}]$  regulation and training-induced alterations in  $[\text{Ca}^{2+}]$ . Such research may be useful in chronic disease prevention and the prescription of endurance training programmes.

## 2.8 The Role of Intracellular Iron

Iron is needed for lymphocyte proliferation, and it has been established that transferrin and transferrin receptors are vital in T cell DNA replication (Neckers and Cossman 1983; Brock 1981). However, TfR expression is regulated by the amount of iron available in intracellular pools, as well as the stability of mRNA. Intracellular iron appears to be found in cytosolic pools (termed “chelatable” or “labile” pools), as well as bound to cytosolic ferritin and transferrin, ligands and proteins (Thomas et al., 1999; Picard et al., 1998). Studies by Picard et al. (1998) have found that iron can be distributed from the cytosolic pools to various cellular compartments as required for transcriptional and post-transcriptional processes, cellular iron transport, expression of iron regulatory genes for ferritin and TfR, and control of the activity of iron-containing proteins (Breuer et al., 1995; Thomas et al., 1999).

Iron concentration within lymphocytes is controlled by IRE and IRP (Worwood 1995; Picard et al., 1997). Free iron, ( $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$ ), is found in intracellular pools, and is difficult to quantify due to the dynamic nature of iron metabolism within the cell (Breuer et al., 1995). Free iron is chelatable, as distinct from that bound to ligands (peptides, proteins and nucleotides) or ferritin (Breuer et al.,

1995). Breuer et al. (1995), using transferrin bound to the labelled isotope  $\text{Fe}^{55}$ , found that iron was translocated from endosomes to the chelatable iron pool, 80-85% of which consisted of  $\text{Fe}^{2+}$ , and 15-20% of  $\text{Fe}^{3+}$ . The mean transit time of ferrous iron (with a concentration of 0.3 to 0.5  $\mu\text{M}$ ) through the chelatable pool was one to two hours, and Breuer et al. (1995) hypothesized that the small percentage of  $\text{Fe}^{3+}$  suggested an intracellular mechanism of vigorous reduction to avoid cell damage. Iron rarely occurs in free form since it is a potential catalyst of oxidative tissue damage (Stryer 1995). It is usually associated with a variety of iron-binding proteins (ferritin, transferrin) or iron-containing proteins (haemoglobin, myoglobin, cytochromes, lactoferrin, haemosiderin) (Picard et al., 1998).

The significance of cytosolic iron concentrations in relation to signal transduction in lymphocytes has yet to be established, but it would appear probable that there is a link between chelatable iron dynamics and TfR expression (and therefore  $\text{CD4}^+$  cell DNA synthesis). It also seems probable that there is a relationship among TfR expression, intracellular  $\text{Ca}^{2+}$  concentration, intracellular iron concentration and signal transduction (Sainte-Marie et al., 1997). How the relationship affects overall iron status in trained and untrained subjects of different age groups has yet to be determined.

## **2.9 Physiological and Immunological Characteristics of Normal Ageing**

### **2.9.1 Ageing and the Immune System**

Normal ageing is characterised by a reduced metabolic and physiological functional capacity (A.C.S.M. 1996), an increase in coronary vascular disease and neoplasms (A.I.H.W. 2000), and an increase in chronic disease (Shinkai et al., 1996; Bacurau et al., 2000; Bruunsgaard and Pedersen 2000; Pedersen and Hoffman-Goetz 2000; Ujemura et al., 2002; Wang et al., 2002). The most frequent cause of death in men and women aged over 50 years (42% and 49% respectively) is circulatory disease, followed by neoplasms (A.I.H.W. 2000; Thune and Furberg 2001). While normal immunosurveillance provides a protective role against infectious agents and cancers, the age-associated increase in neoplasms implicates progressive immuno-senescence (Pyne 1994; Shepherd and Shek 1995; Tortorella et al., 2002).

Immunological characteristics of ageing include thymic involution, producing an increase in “memory” CD45RO<sup>+</sup> T cells as opposed to “naïve” CD45RA<sup>+</sup> T cells (Pawelec 1995), and an increase in CD8<sup>+</sup> cells and NK CD16<sup>+</sup> cells (Mazzeo 1993; Shinkai et al., 1996). Other immunological characteristics are T cell replicative senescence (Effros and Pawelec 1997; De Boer and Noest 1998), a decrease in the number of circulating CD4<sup>+</sup> T cells and B cells (Mackinnon 1992; Song et al., 1993; Shinkai et al., 1996), a decline in APC function (Uyemura et al., 2002), decreased T and B cell proliferative responses (Mackinnon 1992; Bruunsgaard and Pedersen 2000), decreased Interleukin-2 (IL-2) production following stimulation by mitogens (Whisler et al., 1996a,b; Shinkai et al., 1996; Pedersen and Hoffman-Goetz 2000) and impaired signal transduction processes (Felli et al., 1996; Whisler et al., 1996a,b; Beiqing et al., 1997; Yeo and Park 2002).

CD4<sup>+</sup> lymphocyte IL-2 production and IL-2R expression decline with age, contributing to a reduction in T cell proliferative activity, (Whisler et al., 1996a,b; Pedersen 1997). “Proliferative activity” can be defined as the ability of the CD4<sup>+</sup> cell to respond to mitogenic or microbial challenge, replicate and clonally expand (Eales 1997). T lymphocyte proliferation has been quantified *in vitro* in studies using flow cytometry (Shinkai et al., 1995; Moyna et al., 1996a,b; Pedersen 1997; Hedin et al., 1997), radioactive thymidine labelling (Tvede et al., 1993; Mitchell et al., 1996; Ceddia et al., 1999) and *in vivo* as delayed-type hypersensitivity responses (DTH) (Bruunsgaard and Pedersen 2000). The importance of the CD4<sup>+</sup> cell is due to its ability to coordinate the adaptive immune response (Eales 1997; Goldsby et al., 2000). If the function and proliferative responses of CD4<sup>+</sup> cells are impaired, either as a consequence of ageing or endurance training, the immune response may be compromised (Song et al., 1993; Hsu et al., 2002; Fu et al., 2003).

Studies have documented the discrete steps leading to lymphocyte proliferation. Morford et al (1997) and Farber et al (1998) have suggested that deviations in the steps of lymphocyte proliferation, as occurs with ageing, may result in an increased risk of neoplasms. An increasing body of evidence also suggests that immuno-senescence impairs signal transduction within lymphocytes (Whisler et al., 1996a,b; Yeo and Park 2002). Ageing may reduce intracellular Ca<sup>2+</sup> concentration and flux, the activation of T cell receptors, IL-2 production and the subsequent

activation of CD4<sup>+</sup> cells, CD8<sup>+</sup> cells, NK cells and B cells (Di Pietro et al., 1993; Hartmann et al., 1994; Whisler et al., 1996a,b; Kohut et al., 2001).

There is emerging evidence that regular exercise may attenuate some aspects of immunosenescence, adding to the established benefits of moderate aerobic exercise for the older individual (Pedersen and Hoffman-Goetz 2000), reviewed in Section 2.10. What has not been established are the specific intracellular mechanisms whereby moderate aerobic training may improve the proliferative ability of lymphocytes in older individuals.

The decrease in IL-2 and IL-2R expression with ageing was central to the hypotheses for Experiments 1 and 2. IL-2R expression is a critical part of the CD4<sup>+</sup> cell proliferative sequence, while the measurement of IL-2R provides an estimate of the proliferative ability of CD4<sup>+</sup> cells when stimulated by a mitogen. The increase in “memory” cells, less responsive to mitogens and producing less IL-2, is partly responsible for the overall reduction in IL-2 (Ginaldi et al., 2000). There is some evidence that trained older subjects have higher concentrations of “naïve” CD4<sup>+</sup> cells and IL-2 production, and a greater lymphocyte proliferative response, than their sedentary counterparts (Gueldner et al., 1997; Woods et al., 1999; Pedersen and Hoffman-Goetz 2000). However, the mechanisms by which long-term aerobic exercise may affect IL-2 production and the proliferative sequence in older subjects has not yet been determined. The changes in immune function with age have been reasonably well established and are summarised by Bruunsgaard and Pedersen (2000) in Table 1.

Table 1: Changes in Immune Function with Ageing (Bruunsgaard &amp; Pedersen 2000).

**Leucocyte subsets in blood**

Leucocytes total	↔
Lymphocytes	↓
Monocytes	↑↔
Neutrophils	↑

**Lymphocyte subsets (number)**

CD4 <sup>+</sup> cells	↓
CD8 <sup>+</sup> cells	↓
B cells	↔
NK cells	↑↔

**Phenotype & T Cell Function**

Virgin T cells (CD45RA <sup>+</sup> CD62L <sup>+</sup> )	↓
Memory T cells (CD45RO <sup>+</sup> )	↑
CD28 expression	↓
Mean length of telomeres	↓
Mitogen-induced proliferation	↓
Proliferation induced by recall antigens	↓↔
Delayed-type hypersensitivity <i>in vivo</i>	↓
IL-2 production	↓↔
IFN- $\gamma$ production	↑↔
IL-10 production	↑
IL-4 production	↓↑

**Innate immunity & inflammation**

Cytotoxic activity per NK cell	↓
Pro-inflammatory & anti-inflammatory cytokines (TNF- $\alpha$ , IL-6, IL-1RA, sTNF-R; plasma concentrations)	↑

---

↑ increase; ↓ decrease; ↔ no change; IL-1RA interleukin-1 receptor antagonist; sTNF-R circulating tumour necrosis factor receptor.

**2.9.2 Ageing and Cardiovascular Function**

The metabolic, cardiovascular and functional changes that occur with ageing are well documented (Stratton et al., 1994; Brooks et al., 1996; Kohl 2001; Wood et al., 2001; Yamamoto et al., 2001). Ageing results in a decreased cardiac capillary/fibre ratio, decreased cardiac muscle and heart volume, reduced elasticity of blood vessels, decreased maximal cardiac output, increased peripheral resistance, blood pressure and cardiac afterload, decreased myocardial contractility and decreased maximum heart rate. A decline in  $\dot{V}O_{2\text{ peak}}$ , strength, power output, flexibility and bone

density are also symptomatic of normal ageing in both males and females (Brooks et al., 1996; Perini et al., 2001; Wang et al., 2002; Marcell 2003). Many of the age-associated decrements in metabolic and cardiovascular function can be positively modified by regular and moderate intensity aerobic training (Spina et al., 1993; Stratton et al., 1994; Perini et al., 2001; Spirduso and Cronin 2001; Morris et al., 2002; Sagiv et al., 2002; Wang et al., 2002; Adamopoulos et al., 2003). Moderate aerobic training results in a decrease in submaximal heart rate at a given work load, decreased resting heart rate, decreased heart rate variability, decreased exercising and resting systolic blood pressure, a faster recovering heart rate, increased stroke volume and maximum cardiac output, increased capillary and mitochondrial density, increased  $\dot{V}O_{2\text{peak}}$ , improved venous return and increased end-diastolic volume (Brooks et al., 1996; Yamamoto et al., 2001; Morris et al., 2002; Sagiv et al., 2002). Abnormal E.C.G. findings such as ST segment depression, ventricular arrhythmias and bundle branch block have also been shown to improve with aerobic training (Kasch et al., 1999; Fagard 2001; Adamopoulos et al., 2003).

Investigations of older males by Thomas et al. (1984) and Spina et al. (1993) documented respective increases in  $\dot{V}O_{2\text{peak}}$  of 12% and 22% after twelve months of moderate aerobic training. Spina et al. (1993) found a 19% increase in  $\dot{V}O_{2\text{peak}}$  in older women who also trained for twelve months. Similar increases in  $\dot{V}O_{2\text{peak}}$  were found in two groups of males aged 65 to 75 years who trained for three months on a stationary cycle at moderate intensity (50%  $\dot{V}O_{2\text{peak}}$ ) and higher intensity (70%  $\dot{V}O_{2\text{peak}}$ ) (Morris 1998). Kasch et al. (1999) documented changes in  $\dot{V}O_{2\text{peak}}$ , body fat, lean muscle mass, heart rate and blood pressure of 150 men who trained consistently for 33 years. Typically, the subjects participated in five sessions of aerobic training per week, each of 60 minutes duration, and were able to maintain  $\dot{V}O_{2\text{peak}}$  with no significant increases in percent body fat, resting heart rate and blood pressure. Kasch et al. (1999) suggested that the maintenance of aerobic capacity, lower resting and training heart rate, lower blood pressure and lean muscle mass relied on consistent, long-term moderate aerobic training through the lifespan.

Evidence suggests that regular exercise may improve cytokine production and lymphocyte proliferation, thereby reversing some of the effects of immunosenescence associated with ageing

(Guedner et al., 1997; Woods et al., 1999; Pedersen and Hoffman-Goetz 2000; Heesen et al., 2003). The question as to whether the immune system of older males and females are maintained by moderate aerobic training, as are their cardiovascular systems is not clear. Gabriel et al. (1993) found both active older males and females showed an increase in  $CD4^+/CD45RO^+$  compared to active younger subjects. It has been suggested that aerobically trained older women maintain higher concentrations of NK cells, compared to sedentary older females (Fiatarone et al., 1989; Crist et al., 1989; Nieman et al., 1993; Woods et al., 1999). Leucocytosis and the lymphocyte mitogenic responses in sedentary older men and women were compared to their sedentary younger counterparts after a treadmill test to volitional exhaustion (Ceddia et al., 1999). Ceddia et al. (1999) concluded that there was significant exercise-induced leucocytosis in both older and younger age groups, but the magnitude of leucocytosis was lower in the older group. The older male and female subjects recruited a lower percentage of  $CD4^+$  “naïve” and “memory” cells, and these cells had a lower proliferative response. However, Ceddia et al. (1999) did not account for gender differences within their experimental age groups, so no between-gender information from the older and younger individuals were reported. While the effects and benefits of long-term aerobic training on immune function in older individuals remain equivocal, the effect of gender on the immune response in older individuals undertaking long-term aerobic training is essentially unknown.

## 2.10 Modulation and Suppression of Immune Function

The immune response and T cell development can be modulated by many factors including stress, diet, disease, anaemia, medications, alcohol, smoking, age, and intensity and duration of exercise (Dhabhar et al., 1995; Dhabhar et al., 1996; Nieman 1997; Bruunsgaard et al., 2000; Schindowski et al., 2001). The decline in “naïve”  $CD4^+$  cells, an increase of “memory”  $CD4^+$  cells, and decreased production of IL-2 in response to antigenic or mitogenic challenge with age, have been documented (Mosmann and Sad, 1996; Shinkai et al., 1996; Bruunsgaard et al., 2000). Previous research suggests that regular and moderate intensity exercise may improve the response of the immune system by increasing the number or mitogenic/antigenic responsiveness of leucocytes (Gabriel et al., 1994; Shinkai et al., 1995; Pedersen and Hoffman-Goetz 2000; Fu et al., 2003). Gabriel et al. (1994) suggested that long-term aerobic training increased the concentration of “memory”  $CD4^+$  lymphocytes compared to “naïve” cells in active older subjects compared to active

young subjects, and Shinkai et al. (1995) found that the proliferative response of CD4<sup>+</sup> lymphocytes in active older subjects was greater than the response in sedentary older individuals. An earlier study by Gabriel et al. (1993) involving the mitogenic response of CD4<sup>+</sup> lymphocytes to endurance training in young athletes found an increase in the concentration of “memory” CD4<sup>+</sup> lymphocytes relative to “naïve” cells, as well as an increase in the mitogenic responsiveness of both “naïve” and “memory” CD4<sup>+</sup> lymphocytes twelve hours after a long-course triathlon.

Shinkai et al (1996) and Pedersen (1997) have proposed that moderate intensity, aerobic exercise increases immuno-responsiveness by maintaining high levels of cytokine production and NK cell activity. Kohut et al. (2001) also found that moderate intensity, aerobic training enhanced the production of Th1 cytokines (IL-2 and IFN- $\alpha$ ) in aged mice. Of most interest was the suggestion that the concentration or responsiveness of CD4<sup>+</sup> lymphocytes may be increased by regular exercise. As CD4<sup>+</sup> cells have a vital role in activating the proliferative sequence of all lymphocytes and NK leucocytes (Mackinnon 1992; Pedersen 1997) the role of repeated exercise stimulus to improve immune function was encouraging.

A J-shaped relationship between the amount and intensity of physical activity and susceptibility to upper respiratory tract infection (URTI) has been hypothesised (Douglas and Hanson 1978; Heath et al., 1991; Nieman et al., 1994; Metz 2003) and suggests that regular, moderate intensity physical activity enhances the immune response, reducing susceptibility to URTI whereas high intensity and/or high volume exercise, or a sedentary lifestyle, suppresses immune function (Shek et al., 1995; Halston et al., 2003; Whitham and Blannin 2003) (Figure 11). Support for the immuno-suppressive consequences of bouts of intense training was provided by Hack et al. (1994), Hinton et al. (1997) and Nieman (1997), who found that neutrophil function was significantly depressed after graded incremental exercise tests to volitional exhaustion and after bouts of intense interval training (>85%  $\dot{V}O_{2\text{ peak}}$ ). Others also found depression of leucocyte function after bouts of high intensity exercise (Lewicki et al., 1988; Tvede et al., 1994; Nehlsen-Canarella 1995; Nieman et al., 1994), with leucocyte concentration decreased below normal concentrations for up to 24 hours. Rohde et al. (1998) documented a decline in lymphocyte proliferative ability after three consecutive bouts of cycling at 75%  $\dot{V}O_{2\text{ peak}}$ , although the leucocytosis response (elevation in lymphocyte and neutrophil cell counts) to each exercise bout was normal. Thus a biphasic

response of lymphocytes and neutrophils may allow for a “window” of opportunity for infection (Lewicki et al., 1988; Nieman et al., 1994; Pedersen 1997; Mitchell et al., 2002). Gleeson et al. (1999, 2000) and Gleeson (2000) also suggested that a period of mucosal immune system vulnerability to URTI (decreased IgA) for trained athletes coincided with intense training undertaken immediately prior to competition. Some studies concluded that there was a greater chance of marathon runners showing symptoms of URTI after a marathon, and during periods of high volume training (running over 97 km per week). The same runners who later participated in 5 to 21 km runs reported no symptoms of URTI (Heath et al 1991; Pedersen 1997). However, much of the evidence presented was epidemiologic, based on questionnaires, and supported by infrequent clinical medical examination (Shephard and Shek 1999). A survey of masters athletes competing in distance running and endurance events found that 76% of those surveyed rated themselves less vulnerable to URTI than their sedentary colleagues, with only 1.5% rating themselves as more vulnerable to URTI (Shephard and Shek 1999). More recent promising research has seriously challenged the “J” curve model (Nieman et al., 1995a; Hinton et al., 1997; Nieman et al., 2000; Fricker et al., 2000; Dressendorfer et al., 2002; Matthews et al., 2002; Fu et al., 2003; Lakier Smith 2003; Gleeson et al., 2004a).

Nieman et al. (2000) compared of the incidence of URTI, immune cell activity and proliferative responses in elite female rowers and female non-athletes. Although proliferative responses and the concentration of NK cells was significantly higher in the rowers, there were no significant between group differences in any of the other immune cell subsets, or in the incidence of URTI. Furthermore, Pyne et al. (2000) assessed the immunoglobulin concentrations in highly trained swimmers and untrained individuals during preparation for the 1998 Commonwealth Games. Salivary IgA, IgM and IgG levels were measured as indicators of mucosal immune defences. Over the six month period of investigation, Pyne et al. (2000) found no significant differences in salivary immunoglobulins between trained and untrained individuals, nor between swimmers with and without symptoms of URTI. Furthermore, incidence of respiratory tract infection in competitive swimmers compared to the untrained group was not higher, and the respiratory infections that did exist among some swimmers did not have a significant impact on their competitive performances. Thus, the support for a clear J-shaped relationship between exercise volume and immune function remains equivocal (Shepherd and Shek 1999; Nieman et al., 2000; Pyne et al., 2000).

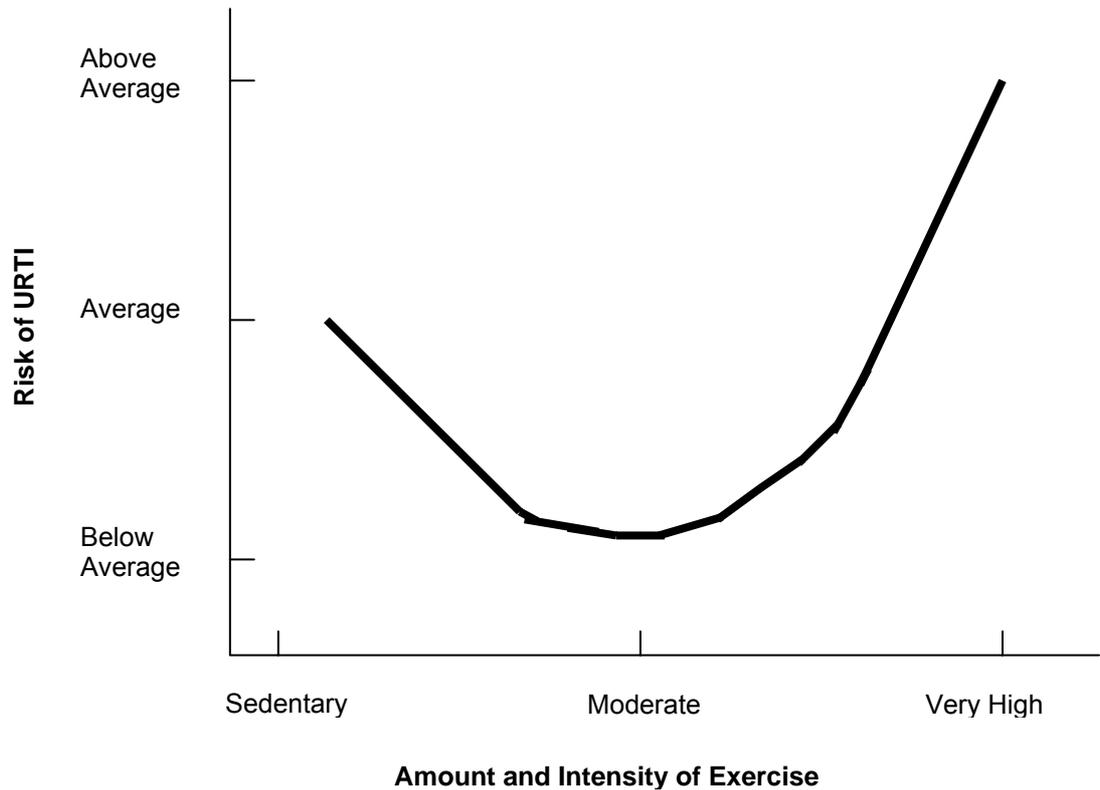


Figure 11: The “J” Curve. The risk of URTI increases as exercise volume and intensity increases (modified from Nieman et al., 1994)

Despite the increasing evidence to the contrary (Shepherd and Shek, 1999; Fricker et al., 2000; Pyne et al., 2000; Gleeson et al., 2004a), there is still a common belief by athletes and coaches that high volume/high intensity training will result in a higher incidence of URTI. What is less clear is whether the cases of URTI that do occur in athletes can be attributed to high volume/high intensity training. Furthermore, symptoms of URTI may not necessarily be higher in athletes than in the untrained population (Costill et al., 1991; Fricker et al., 2000; Pyne et al., 2000; Gleeson et al., 2004a). The longitudinal studies monitoring rowers and marathon runners, their training and the incidence of illnesses have not investigated the effects of endurance training on specific aspects of immunosuppression or immune cell activation and proliferation. The effects of differences in age, gender and athletic ability on immunosuppression and lymphocyte proliferation have also not been well documented (McCarthy and Dale, 1988; Heath et al., 1991). Immunosuppression resulting from high intensity/high volume training has been attributed to high plasma concentrations of epinephrine, norepinephrine and cortisol, often referred to in the literature as “stress hormones” (Flynn et al., 1994; Suzuki et al., 1999; Godfrey et al., 2000; Ronsen et al., 2001; Dressendorfer et al., 2002). Cortisol has been implicated in exercise-induced leucocytosis by promoting the release of polymorphonuclear leucocytes (mainly neutrophils) from

bone marrow (McCarthy and Dale, 1988; Ceddia et al., 1999; Suzuki et al., 1999; Mitchell et al., 2002). Epinephrine and norepinephrine promote the initial release of neutrophils from bone marrow, and also the increase in circulating lymphocytes and NK cells immediately after exercise (Figure 12A, 12B Pedersen 1997). The magnitude of the leucocytosis is positively correlated to the intensity of the exercise bout, and the duration of the exercise period (Pedersen 1997; Pedersen and Hoffman-Goetz 2000; Mooren and Volker 2001; Dressendorfer et al., 2002; Mitchell et al., 2002).

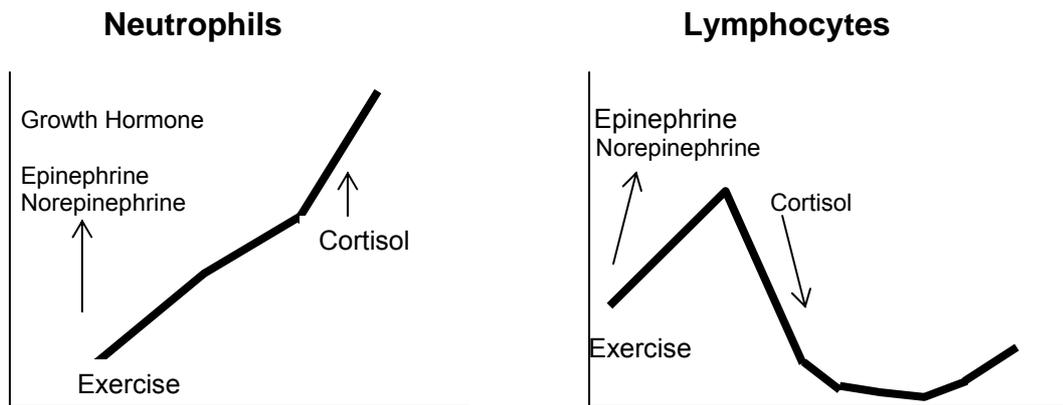


Figure 12A: The Effects of “Stress Hormones” on Neutrophils and Lymphocytes (Pedersen 1997). The initial increase in neutrophils and lymphocytes after exercise is due to increased plasma concentrations of primarily epinephrine, and to a lesser extent norepinephrine. An increased plasma concentration of cortisol will promote a further efflux of neutrophils from bone marrow, but will cause a decrease in lymphocytes, often below baseline concentrations.

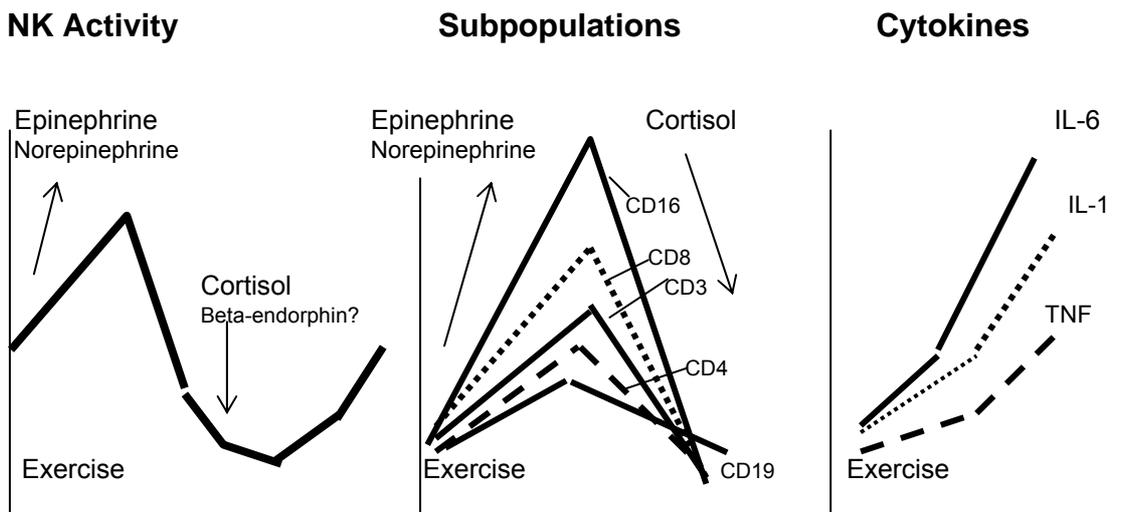


Figure 12B: The Effects of “Stress Hormones” on Natural Killer Cells, Leucocyte Subpopulations and Cytokines (Pedersen 1997). Epinephrine, and to a lesser extent norepinephrine, cause post-exercise increases in some immune cell subsets, which are then reduced by increased plasma concentrations of cortisol. Both increased catecholamines and cortisol during exercise, cause increases in inflammatory cytokines IL-6, IL-1 and TNF.

High concentrations of cortisol are known to modulate the immune response (Gabriel et al., 1992; Briggs et al., 1996; Pedersen et al., 1997; Brenner et al., 1998; Mooren and Volker 2001; Dressendorfer et al., 2002). Cortisol is a broad-spectrum immuno-suppressant which reduces the phagocytic ability of macrophages and neutrophils, chemotaxis and the expression of both Class II MHC molecules and IL-1 production by macrophages, hence CD4<sup>+</sup> cell activation (Goldsby et al., 2000). High concentrations of cortisol can prevent DNA replication in lymphocytes and cause lymphopenia, thymic involution and shrinkage of lymph nodes (Gabriel and Kindermann 1997; Pedersen et al., 1997; Brenner et al., 1998; De Boer and Noest, 1998). IL-1 and IL-2 receptors on CD4<sup>+</sup> cells are down-regulated by cortisol, affecting potential activation of B cells, NK cells and B memory cells (Brenner et al., 1998; Suzuki et al., 1999).

Corticosteroids are lipophilic and can cross the plasma membrane to bind with receptors in the cytosol (Goldsby et al., 2000). The resulting receptor-hormone complexes are then transported to the nucleus, where they bind to specific regulatory DNA sequences, either up or down-regulating transcription (Stryer 1995; Mooren and Volker 2001). Corticosteroids may induce increased transcription of the nuclear transcription factor- $\kappa$ B (NF- $\kappa$ B) inhibitor (I- $\kappa$ B), which binds to NF- $\kappa$ B in the cytosol, preventing NF- $\kappa$ B translocation into the nucleus and its activation of a number of genes, including those involved in T cell activation and cytokine production (Goldsby et al., 2000; Lewis 2001).

Plasma concentrations of cortisol increase in relation to exercise of long duration, and above moderate intensity (75%  $\dot{V}O_{2\text{ peak}}$ ) (Pedersen and Hoffman-Goetz 2000). High plasma concentrations of cortisol, (eg. post-marathon), can increase the concentrations of IL-1 and IL-6, which through negative-feedback mechanisms inhibit the production of other cytokines up to 100 fold (Suzuki et al., 1999). The increased negative-feedback inhibition may cause major perturbations in signal transduction pathways (both PIP2 and MAPK) and therefore in the production of cytokines such as IL-2, necessary for lymphocyte activation. Cortisol also affects the IL-2R $\alpha$  by inhibiting transcription of receptor DNA which further inhibits downstream signalling events (Godfrey et al., 2000; Mooren and Volker 2001).

The immuno-suppressive effects of high concentrations of cortisol have been proposed as one of the causes of the “overtraining” syndrome of athletes who undergo high volumes of training, often at high intensity and without an adequate recovery period (Friman and Wesslen 2000; Gleeson 2000b; Gleeson et al., 2002; Lakier Smith 2003). “Overtrained” athletes complain of continual fatigue, have poor sporting performances and are associated with an increased incidence of URTI and illnesses such as glandular fever and Chronic Fatigue Syndrome (Flynn et al., 1994; Friman and Wesslen 2000; Mackinnon 2000; Metz 2003).

A co-ordinated hormonal response occurs to physical activity, resulting in the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary gland (Barron et al., 1985; Brenner et al., 1998; Rhind et al., 1999). ACTH induces the production of cortisol from the adrenal cortex. Simultaneously, there is the release of epinephrine and norepinephrine from the adrenal medulla and sympathetic nerve terminals (Schedlowski et al., 1996; Ramer-Quin et al., 1997; Brenner et al., 1998) and beta-endorphins (Goldfarb and Jamurtas 1997). Arterial plasma concentrations of epinephrine and norepinephrine increase almost linearly with duration of exercise, and almost exponentially with increasing exercise intensity, when expressed relative to  $\dot{V}O_{2\text{ peak}}$  (Pedersen and Hoffman-Goetz 2000). Norepinephrine modulates cellular activity by binding to adrenergic receptors expressed on various types of cells (Schedlowski et al., 1996; Kohm and Sanders 2000). The predominant adrenergic receptor expressed on T and B lymphocytes is the  $\beta_2$  adrenergic receptor ( $\beta_2\text{AR}$ ), a G-protein-linked receptor that, when stimulated, leads to the intracellular accumulation of cAMP, activation of protein kinase A, activation of signal transduction pathways and eventual transcription of genes for IL-2 and other cytokines and receptors (Kohm and Sanders 2000).

Endurance training results in a down-regulation of  $\beta$ -adrenergic receptors on lymphocytes, making them less sensitive to epinephrine and norepinephrine (Barron et al., 1985; Schaller et al., 1999). It is possible that the training-induced down-regulation of  $\beta$ -adrenergic receptors results in a decrease in gene transcription and lymphocyte proliferation, thus contributing to training-induced immuno-suppression (Pedersen et al., 1997; Brenner et al., 1998; Schaller et al., 1999; Ronsen et al., 2001; Ronsen et al., 2002).

It is also possible that the immuno-suppressive effects of raised cortisol concentration may be lessened by cortisol receptor down-regulation after chronic endurance training (Urhausen et al., 1998). Endurance athletes training at a moderate intensity for long distances may actually undergo a down-regulation of cortisol receptors and be at less risk of immuno-suppression than athletes who train for shorter distances but at a higher intensity (Ortega et al., 1996; Urhausen et al., 1998). The immuno-suppressive effects of high volume training and high intensity training remain largely unresolved. The effects of both high intensity and high volume training on the immune response of different genders and different age groups has also not been well addressed. Clearly, further research is needed to explain the mechanisms by which training at different intensities and different volumes may effect the lymphocyte proliferative response in males and females of different ages.

Some researchers have noted significant seasonal variations in the production of cortisol (Canon et al., 1986; Brock 1987; Boctor et al., 1989; Maes et al., 1997b; Mann et al., 2000), melatonin (Brock 1987; Maes et al., 1994 ), prolactin (Maes et al., 1997b), thyroid hormones (Maes et al., 1997b) and some cell subsets (Brock 1987; Pati et al., 1987; Canon et al., 1986). The seasonal variations may affect lymphocyte proliferation and contribute to periods of reduced or enhanced lymphocyte responsiveness. Maes et al (1997a) and Garde et al. (2000) also noted seasonal variations in haemoglobin and iron, which might modulate immune function.

High intensity training can modulate immune function and result in an increased risk of infection (Hack et al., 1994; Urhausen et al., 1998; Friman and Wesslen 2000). Individuals who lead a sedentary lifestyle also appear to be at increased risk of infection, as well as chronic lifestyle diseases (Pyne 1994; Friman and Wesslen 2000). The age-related decrease in immune function has been reviewed in Section 2.9.1. and suggests that older individuals may be more at risk of developing infections and cancers (Campisi et al 2001; Anisimov 2001; Ahluwalia et al., 2001). Older individuals who are inactive are thought to produce lower concentrations of cytokines such as IL-2 and IFN (Mackinnon 1992; Pedersen 1997) which with inadequate nutrition or underlying illnesses may further compromise their immune response, increasing the risk of infections and chronic diseases (Roebathan and Chandra 1996; Beard et al.,1996; Sayer and Cooper 1997).

A compromised immune response may be partly due to iron deficiency. It is not unusual for many older people in the developed countries, especially women, to be iron deficient, usually as a result of poor nutrition (Brock 1995; Roebathan and Chandra 1996; Beard et al., 1996; Sayer and Cooper 1997). It has been reported that endurance athletes, especially distance runners, are iron deficient during times of heavy training (Clement and Sawchuk, 1984; Balaban et al., 1995; Deakin 1995; Rudzki et al., 1995; Nielsen and Nachtigall, 1998; Zhu and Haas, 1998; Friedmann et al., 2001; Schumacher et al., 2002b; Telford et al., 2002; Venkatraman and Pendergast 2002; Flynn et al., 2003). The iron deficiency found in many endurance athletes has been attributed to heel-strike haemolysis (Clement and Sawchuk 1984; Smith 1995; Telford et al., 2002), increased plasma volume (Deakin 1995), gastrointestinal micro-bleeding and low dietary iron (Deakin 1995; Nielsen and Nachtigall 1998; Rudzki et al., 1995). Many endurance athletes reduce their intake of red meat and other protein, and increase their intake of carbohydrates as a means of maintaining optimum muscle glycogen levels. However, iron deficiency manifests in reduced haemoglobin and oxygen-carrying capacity, lower numbers of erythrocytes, a reduction in ATP production through the oxidative-phosphorylation chain, and reduced training and competitive performances. The current recommendations are that athletes and older individuals need to maintain a balanced diet with an adequate iron intake to minimise the risk of iron deficiency and immuno-suppression (Beard et al., 1996; Roebathan and Chandra 1996; Nielsen and Nachtigall 1998; Friedman et al., 2001; Venkatraman and Pendergast 2002; Gleeson et al., 2004b).

### **2.11 Exercise, Training and the Immune Response**

It is increasingly accepted that regular exercise can decrease the incidence of cardiovascular disease, Type II diabetes, colorectal cancer, breast cancer and reduce obesity, high blood pressure (Cearlock and Laude-Flaws 1997; Wilmore et al., 2001a,b; Kohl 2001; Campisi et al., 2001; Wang et al., 2002). Regular, moderate intensity exercise is also thought to positively modify the immune response, while high intensity/high volume exercise is thought to decrease the immune response (Hinton et al., 1997; Pedersen 1997; Friman and Wesslen 2000). Much of the early research investigated the effects of a single bout of exercise (steady state or incremental exercise test) on immune cell subsets (Muir et al., 1984; Lewicki et al., 1988; McCarthy and Dale 1988; McCarthy et al., 1992). Early studies produced consistent findings of leucocytosis after an acute bout of exercise, and after repeated sessions of aerobic or anaerobic exercise (Muir et al., 1984; Lewicki et al., 1988; Mackinnon 1992; Nieman et al., 1993). With the advent of newer

technologies, more recent studies have investigated exercise-induced leucocytosis in more detail (Gabriel et al., 1994; Shinkai et al., 1996; Pedersen 1997; Pyne et al., 2000; Yamada et al., 2000).

The magnitude of leucocytosis in humans depends on intensity, duration and mode of exercise, concentration of epinephrine, norepinephrine, cortisol and cytokines, changes in body temperature, haemodynamics and dehydration (Nehlsen-Cannarella 1995; Brenner et al., 1998; Nieman et al., 1995a,b; Nieman 1997; Pedersen and Hoffman-Goetz, 2000; Yamada et al., 2000). Typically leucocytosis resulting from exercise (acute and chronic) is biphasic. There is initial rise in total leucocyte concentration, due to a recruitment in all lymphocyte subsets to the vascular compartment, (T cell CD3<sup>+</sup>, Th CD4<sup>+</sup>, Tc/s CD8<sup>+</sup>, NK CD16<sup>+</sup>/56<sup>+</sup>, CD3<sup>+</sup>HLA/DR, B cells CD19<sup>+</sup>), and approximately one hour after exercise, lymphocyte concentration decreases to, or below, values obtained at rest. During exercise the CD4<sup>+</sup> to CD8<sup>+</sup> ratio decreases, reflecting a greater increase in CD8<sup>+</sup> (Pedersen and Hoffman-Goetz 2000). CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes contain both CD45RO<sup>+</sup> “memory” and CD45RA<sup>+</sup> “naïve” cells. Studies have shown that the lymphocyte recruitment during exercise is primarily of CD45RO<sup>+</sup> lymphocytes, suggesting that “memory” not “naïve” lymphocytes are rapidly mobilised to the blood in response to exercise (Pedersen and Hoffman-Goetz 2000).

The total leucocyte concentration still continues to increase six hours post-exercise due to the increase in granulocytes, mainly neutrophils, and monocytes (Figure 12A). The exercise-induced, increased plasma concentrations of epinephrine, norepinephrine, and then cortisol, are the primary mechanisms responsible for the biphasic response in leucocytes following exercise (Mackinnon 1992; Pyne 1994; Brenner et al., 1998). Exercise intensity and duration, and the trained status of the individual, are responsible for the changes in epinephrine, norepinephrine and cortisol production (Pyne 1994; Pedersen et al., 1997; Brenner et al., 1998). The plasma concentrations of epinephrine, norepinephrine and cortisol decrease over twelve hours following an exercise session, with leucocytes normally at basal concentrations some 24 hours post-exercise.

NK cells, neutrophils and macrophages (components of the innate immune system), tend to be the most responsive to an single bout of exercise, whether sub-maximal, incremental or maximal in nature (Hack 1994; Shinkai et al., 1996; Peters-Futre 1997). A single bout of exercise of less than

60 minutes duration and at 60% of  $\dot{V}O_{2\text{ peak}}$  resulted in leucocytosis of a smaller magnitude compared to high intensity (>80%  $\dot{V}O_{2\text{ peak}}$ ) and/or longer duration (>60 minutes) exercise (Mackinnon 1992; Nieman 1997). Single bouts of exercise have also resulted in temporarily increased NK cell lytic activity, and a diminished lymphocyte proliferative response to mitogens (Shinkai et al., 1996; Gleeson et al., 1999; Gleeson 2000). Plasma concentrations of some cytokines (IFN- $\gamma$ , TNF, IL-1, IL-6), C-reactive protein and activated complement fragments, all of which are immuno-modulatory, have been shown to increase after single, or repeated bouts of exercise (Mackinnon 1992; Pedersen et al., 1997; Brenner et al., 1998; Shephard and Shek 1999; Gleeson et al., 1999; Gleeson 2000).

With chronic endurance training, the only consistent findings have been an elevation of NK cell activity, and suppressed neutrophil function (Hack et al., 1994; Suzuki et al., 1999; Yamada et al., 2000). Several researchers have noted decreased circulating concentrations of leucocytes in chronically trained athletes, with either haemodilution or altered leucocyte kinetics (e.g. diminished release of cells from the bone marrow) proposed as the probable cause (Gleeson et al., 1999; Gleeson 2000). There are little data about the effects of long-term endurance training on the lymphoid immune response. The findings from investigations of the effect of aerobic training on immune function, and the incidence of URTI in trained individuals has been summarised in Table 2.

Table 2: The Summary of Past Studies Investigating the Effects of Aerobic Exercise Upon Immune Function in Humans

REFERENCE	SUBJECTS	STUDY DESIGN	TRAINING	FINDINGS
Douglas & Hanson 1978	Young males. 61 trained & 126 untrained.	Group comparison – symptoms of URTI.	9 weeks of rowing (trained group).	Significantly greater frequency of URTI symptoms in trained group.
Crist et al., 1989	14 females (65 -75 yrs).	Group comparison – trained/untrained.	Aerobics (20-30 mins), 3 sessions per wk for 16 wks.	NK cytotoxic activity 33% higher in trained vs untrained.
Nieman et al., 1990	36 obese women.	Group before & after training. NK counts & URTI symptoms.	45 mins per session (walking); 5 sessions per wk for 15 wks.	No change in NK count but increase in cytotoxic activity; less URTI symptoms with training.
Nehlsen-Cannarella 1991	Mildly obese women.	Group before/after training. URTI symptoms & serum lg.	45 mins per session; 5 sessions per wk for 15 wks.	Significantly less URTI symptoms with training; 20% increase in immunoglobulins.
Heath et al., 1991	530 male & female runners.	Monitoring (log) of all subjects – URTI symptoms.	12 months distance running. Mileages b/w 400 & 1388+	Increased running mileage was significant factor for URTI.

			miles .	
Gabriel et al., 1994	171 active males, 70 females (15-68 yrs).	Cross-sectional analysis. % & counts of CD45RO+ T cell subsets.	Recreational athletes (3-4 exercise sessions per wk) compared to highly fit athletes & sedentary subjects.	Age-related increase of CD45RO+ T cell subsets in active adults.
Shinkai et al., 1995	Males; 17 older runners, 16 older untrained, 16 young untrained.	Cross-sectional analysis. Lymphocyte subsets; proliferative response to PHA; IL-2 production.	Trained group had run for 17 yrs (av); wkly average of 39+/- 22 km.	Lower lymphocyte count in runners vs controls; both older groups showed lower % of CD3+ & CD8+, higher % NK cells; higher CD4+/CD8+ ratio than young; older groups had higher % CD45RO+; PHA response sig. lower in both older groups vs young BUT older runners showed 51% greater PHA response than older controls. IL-2 lower in older groups vs young BUT higher in older trained than untrained.
Mitchell et al., 1996	Young males, trained vs untrained.	Group comparison. Lymphocyte count & response to PHA; immunoglobulins.	12 weeks aerobic cycling; 75% VO <sub>2</sub> peak for 30 mins, 3 sessions per wk; samples wk 0, 8, 12.	No change in PHA response; non-significant increase in lymphocyte count and unchanged Ig.
Gueldner et al., 1997	Older females, 25 trained vs 21 untrained.	CD3 <sup>+</sup> , CD25 <sup>+</sup> comparison between groups.	Active exercisers (walking & fitness classes) vs sedentary.	10% increase in CD25 <sup>+</sup> expression in training group.
Woods et al., 1999	Older females, 14 trained, 15 untrained.	Group comparison, T lymphocyte & NK cell count & function.	6 months aerobic walking, 3 sessions per wk; 50%-60% VO <sub>2</sub> peak.	No changes in cell counts or % CD4 <sup>+</sup> & CD8 <sup>+</sup> . Naive CD4 <sup>+</sup> cells increased after training, & memory cells decreased.
Nieman et al, 2000	21 trained elite female rowers vs 19 female untrained.	Group comparison, all leucocytes, cell count & activity; PHA proliferative response & URTI incidence; IL-1, IL-6, TNF.	2 month history of URTI; before & after samples.	Rowers had 31% higher PHA response. NK activity 1.6 fold higher in rowers. No other differences b/w groups.
Pyne et al., 2000	21 male & 20 female elite swimmers; 19 male & 6 female untrained.	Group comparison, salivary IgA, IgG & IgM & albumin ; URTI incidence.	6 month history of URTI; saliva testing pre- & post study, at 1 month, at 15 weeks, then post-study.	No significant changes in saliva Ig in swimmers or between groups; no increased incidence of URTI in swimmers; no sig diff between ill and healthy swimmers in performance; no impact of URTI on performance.
Fahlman et al., 2000	Females (70-87) 151 trained, 14 untrained.	Group comparison, CD3 <sup>+</sup> , CD4 <sup>+</sup> , CD8 <sup>+</sup> , & NK cell function.	10 weeks walking, 3 sessions week, at 70% HRR, 50 mins/day by week 3.	No between-group differences in lymphocyte function; increased NK function in TR group.
Yan et al., 2001	Males (20-39, 40-59, >60 yrs), moderate exercisers & controls.	Group comparison, CD3 <sup>+</sup> , CD4 <sup>+</sup> , CD8 <sup>+</sup> , CD16 <sup>+</sup> , CD56 <sup>+</sup> concentration & activity.	Habitual moderate exercisers over lifespan.	Age-associated decrease in CD8 <sup>+</sup> , increase in CD4 <sup>+</sup> in older controls only; no b/w group differences in leucocyte or CD3 <sup>+</sup> count; increased NK count in older exercisers compared to older controls & younger groups.

Sagiv et al., 2002	25 Males (44 – 48 yrs), 15 training, 10 controls with coronary artery disease, on $\beta$ blockers.	Group comparison, CD4 <sup>+</sup> , CD8 <sup>+</sup> count.	12 weeks training at 65-70% of HR max.	CD4 <sup>+</sup> & CD8 <sup>+</sup> count significantly increased after training; CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio significantly decreased in trained group.
Kapasi et al., 2003	Frail aged (70-85 yrs), 94 trained, 96 controls	Group comparison, CD 3 <sup>+</sup> , CD4 <sup>+</sup> count, CD25 <sup>+</sup> , CD28 <sup>+</sup> , HLA-DR activation; neopterin & TNF concentration.	32 weeks of aerobic/resistance training, 10 minute sessions every 2 hours, 5 days per week.	No change in lymphocyte subsets, activation markers & cytokine production; no differences b/w trained & control groups.
Whitham and Blannin 2003	Young males (n=21); heavy & light training groups.	Group comparison, IgG response to flu vaccination 2,4,7,10,14 days then 12 months post-vaccination.	3 weeks of training; “light” activity compared to “heavy” training (intensity not specified by authors).	No group difference in IgG 14 days post vaccination; higher IgG in “heavy” training group 12 months post vaccination.
Gleeson et al., 2004a	Elite swimmers (9 males & 2 females); controls,(7 males & 5 females)	CD3 <sup>+</sup> function (CMI multitest).	20 weeks of training; tested early season, mid-season (peak training) & late season (pre-competition taper).	No significant differences b/w groups or b/w test points for each group.
LeMaitre et al., 2004	46 patients (52-72 yrs) with chronic stable heart failure.	Pre- & post-training comparison; TNF- $\alpha$ , TNF- $\alpha$ soluble receptors 1 & 2, IL-1, IL-2, IL-6, C-2active protein.	6 weeks of cycle training or electro-stimulation, 5 days per week, 30 minutes per day.	No change in TNF- $\alpha$ , IL-1, IL-2 or IL-6; non-significant decrease in TNF receptors in cycle group.

When resting cell counts and percentages for leucocyte subsets at rest are considered, many training studies show equivocal results (Table 2). While changes in leucocyte subsets and cellular responses (e.g. neutrophil respiratory burst, NK cytolytic activity, lymphocyte cytokine production) have been reported, there is little information about the exact causes of these changes. It should be noted that changes in leucocyte concentration may be statistically significant, but not clinically significant, if leucocyte concentration remains within the normal range. If exercise-induced changes are concurrent with an increase in URTI, then statistically significant changes may well be clinically significant. Current directions in immunology of exercise research suggest that specific biochemical, signal transduction and transcriptional events play a significant role in the exercise-induced changes in leucocyte subsets, and leucocyte responses (Field et al., 1991; Pyne et al., 2000). The effects of long-term training on leucocyte, and specifically lymphocyte function, need to be examined at an intracellular level to determine how long-term aerobic/endurance training can increase, or decrease, CD4<sup>+</sup> lymphocyte numbers and mitogenic responses.

## 2.12 Summary

The decline in immune function is one of the hallmarks of normal ageing, and has been associated with the increased incidence of infections and cancers among older age groups (Pyne 1994; Cearlock and Laude-Flaws 1997; Bacurau et al., 2000; Slattery and Potter 2001; Thune and Furberg 2001; Friedenreich et al., 2001). Specifically CD4<sup>+</sup> cell function declines with age, with reductions in antigen- and mitogen-induced T cell proliferation, IL-2 synthesis and expression of high-affinity IL-2R. Thymic involution and the accumulation of “memory” CD4<sup>+</sup> cells (CD45RO<sup>+</sup>) at the expense of “naïve” CD4<sup>+</sup> cells (CD45RA<sup>+</sup>) contribute to immunodeficiency in the older individual.

As the percentage of the population over the age of 60 years continues to increase, it is becoming increasingly important to determine the factors involved in the age-related decline in immune function. With new technologies available, such as gene therapy, some factors responsible for the decline in immune function may be identified and possibly reversed. It is also valuable to determine whether the decline in immune function can be attenuated by public health interventions such as regular exercise. Interventions and strategies that modify the age-dependent decline in immune function need to be determined as a way of improving the quality of life and reducing public health costs for older individuals. There is strong evidence that regular, long-term, moderate intensity exercise improves cardiovascular and cognitive function across the age spectrum. Whether such a prescription of exercise also improves immune function in older individuals is not as clear. There is no conclusive evidence concerning long-term effects of exercise on immune function and responses in older individuals. Perhaps the age-related decline in immune function could be attenuated by a simple public health intervention such as regular aerobic exercise. The present study has attempted to resolve how long-term aerobic training affects the immune function of older men and women, and the immune function of young males (sedentary and athletes).



## 3.0 Methods

### 3.1 Experiment 1 - Males and Females Aged 65 to 75 Years

#### 3.1.1 Introduction

The purpose of Experiment 1 was to investigate whether twelve months of moderate intensity, aerobic training would increase the number of CD4<sup>+</sup> lymphocytes, and increase the responsiveness of the lymphocytes to mitogenic challenge. In Experiment 1, 14 males and 10 females aged 65 to 75 years completed twelve months of aerobic training at a moderate intensity (60% of  $\dot{V}O_{2\text{ peak}}$ ). There were three training sessions per week, each of 45 minutes duration. Sixteen age-matched untrained subjects (8 male and 8 female) acted as controls. Immunological and haematological variables were measured every four weeks.

#### 3.1.2. Subject Characteristics

##### 3.1.2.1. Recruitment and Screening

Subjects were recruited through advertisements placed in local Gold Coast newspapers. Respondents to the advertisement were initially interviewed by telephone and completed a verbal medical questionnaire. Those respondents with a history of cardiovascular disease, diabetes, asthma, orthopaedic conditions, currently taking anti-inflammatory medications, or with any other medical condition which contraindicated vigorous exercise, were excluded from further participation.

Following the initial telephone interview, those subjects identified as potentially suitable for Experiment 1 were invited to attend the laboratory on two separate occasions for further screening and familiarisation.

Each subject:

- Was provided with an information sheet setting out the details of Experiment 1 (Appendix 1);
- Completed a comprehensive medical history questionnaire (Appendix 2);
- Underwent a resting 12 lead electrocardiogram (ECG), spirometry and blood pressure measurement;
- Underwent a four site skinfold test, standing height and body mass measurements;
- Underwent a venepuncture at rest (supine) to provide a full blood count (Appendix 4).

- Was provided with a list of dates to attend the laboratory for a venepuncture for the twelve months duration of Experiment 1;

After a clear explanation of Experiment 1, including the purpose, risks and benefits of participation, the subjects underwent a detailed medical examination by a registered Medical Practitioner and provided their written consent (Appendix 3). The subjects were then further familiarised with the Lode cycle ergometer (Lode Excalibur, Holland) and other equipment to be used in the incremental and steady state exercise tests. One week later, the subjects visited the laboratory for the incremental exercise test to exhaustion on the Lode cycle ergometer under direct medical supervision. Two days after the incremental exercise test, the subjects visited the laboratory again to perform two steady state cycle exercise tests of 20 minutes duration (Monark, Sweden). The purpose of the steady state cycle exercise tests was to determine the power output and heart rates corresponding to intensities of 50% and 60% of  $\dot{V}O_{2\text{ peak}}$  for subsequent use in the twelve month training programme.

As a result of the screening process, each subject selected for Experiment 1 was:

- free from known cardiovascular and respiratory disease (as outlined in Appendix 2)
- a non-smoker
- free from metabolic diseases (as outlined in Appendix 2)
- free from any known immune dysfunction, systemic viral condition or inflammatory disease (as outlined in Appendix 2)
- not taking any medication known to interfere with the immune or exercise response
- Had haematology measurements (full blood count) within the normal ranges (Appendix 4)
- normal spirometry and a resting 12 lead ECG
- a resting blood pressure of less than 150/90 mmHg, and
- no evidence of clinically significant exercise-induced myocardial ischaemia.

No remuneration was offered to the subjects. The study was approved by the Griffith University Human Ethics Committee.

### 3.1.2.2. *Anthropometry*

Standing height was obtained using a stadiometer (Holtain, Australia) to the nearest millimetre. Body mass was measured using electronic scales to the nearest 0.05 kg (A.N.D. Mercury, Australia). To estimate adiposity, skinfold thicknesses were measured at four sites (Harpenden Calipers, UK). The measurement sites were the biceps, triceps, subscapular and suprailiac regions. The positioning for each skinfold measurement was in accordance with the procedures described in the American College of Sports Medicine Guidelines for Exercise Testing (1995). Three measurements were taken at each site with the measurement from each site totalled and then averaged to produce a sum of four skinfold sites. Percent body fat was estimated from age-specific body composition tables (Durnin and Womersely, 1974).

### 3.1.2.3. *Spirometry*

Spirometry tests were undertaken using the Schiller Spirovit spirometer (Schiller, Switzerland). Forced vital capacity (FVC), forced expiratory volume in one second (FEV<sub>1</sub>) and FEV<sub>1</sub>/FVC (%) were undertaken with the subjects standing (Burrows et al., 1983). After clear instruction and demonstration, each subject was asked to take a maximum voluntary inspiration, place his/her lips around the mouthpiece of the spirometer and blow out as "hard and fast" as possible. Three trials were undertaken with a short rest between each trial. The highest values of FEV<sub>1</sub> and FVC were recorded to the nearest 0.1 litre. All expired values were corrected to BTPS and compared with age and height predicted values (Burrows et al., 1983).

### 3.1.2.4. *Incremental Exercise Test to Exhaustion*

Each subject performed an incremental exercise test to exhaustion on an electronically braked cycle ergometer (Lode Excaliber, Holland) with the saddle height standardised for each subject. The ECG was measured from bipolar leads placed in the CM5 configuration using standard ECG electrodes (Meditrace 200, USA) and Lohmeier M607 ECG (Lohmeier, Germany) instrument. The ECG was continuously monitored throughout the incremental exercise test by a registered medical practitioner. An ECG strip was run for approximately ten seconds at the end of every minute for the duration of the incremental exercise test and during recovery.

Oxygen uptake was measured using open circuit spirometry (ExerStress, Australia). The subject breathed through a mouthpiece connected to a low resistance two-way valve (Hans Rudolph 2700,

USA). A comfortable fitted headpiece was positioned to support the two-way valve and mouthpiece throughout the incremental exercise test.

Expired air was passed into a mixing chamber via a pneumotach (Hans Rudolph, USA). The pneumotach sampled the flow rate at 100Hz. The flow rate was integrated and summed to produce an expired volume. Dried expired air was continuously sampled throughout the exercise test for percent oxygen and carbon dioxide. Percent oxygen was measured using a fast response zirconia analyser and transducer (ExerStress, Australia) and percent carbon dioxide was measured using a fast response infrared analyser and transducer (ExerStress, Australia). The analysers were calibrated immediately prior to, and after, the exercise test using standard chemically analysed gases (Linde Gases, Australia). Any drift in the analysers was corrected using standard regression analysis.

Voltage outputs from the cycle ergometer, ECG, pneumotach, oxygen and carbon dioxide analysers were relayed to a computer (Compaq Deskpro, Australia) via a 12 – bit analogue to digital board sampling at 100 Hz. Raw and calculated data were stored and displayed using custom written software (ExerStress 1.1, Australia). Data were averaged over a 30 second period. Calculated variables included oxygen uptake ( $\dot{V}O_2$ :L.min<sup>-1</sup> and mL.min<sup>-1</sup>.kg<sup>-1</sup> corrected to STPD), carbon dioxide production ( $\dot{V}CO_2$ :L.min<sup>-1</sup> and mL.min<sup>-1</sup>.kg<sup>-1</sup> corrected to STPD), ventilation ( $\dot{V}_E$ :L.min<sup>-1</sup> corrected to BTPS), respiratory exchange ratio (RER) and ventilatory equivalents for oxygen ( $\dot{V}_E / \dot{V}O_2$ ) and carbon dioxide ( $\dot{V}_E / \dot{V}CO_2$ ).

The cycling protocols for males and females had been previously determined (Morris 2001) and were stored in the Lode Workload Programmer. Each male and female subject commenced cycling at 15 watts, and at zero load respectively. After a three minute warm up period, the power was increased by 15 and 10 watts per minute for the males and the females respectively. The workload increased until exhaustion or clinical signs and symptoms prevented further exercise. Peak  $\dot{V}O_2$  ( $\dot{V}O_{2\text{ peak}}$ ), peak heart rate, peak power, peak ventilation and peak RER were determined from the average of the two highest values attained over two collection periods during the exercise test. During the exercise test, blood pressure was monitored using a standard cuff

size and mercury manometer (Baum & Co., USA). The first and fourth Korotkoff sounds were used to indicate systolic and diastolic blood pressure respectively.

#### 3.1.2.5. *Steady State Cycle Exercise Tests*

The subjects performed two steady state cycle exercise tests each, of 20 minutes duration to verify that the subsequent training intensities would correspond to a power equivalent to 50% and 60% of  $\dot{V}O_{2\text{ peak}}$  (Morris 1998). Oxygen uptake was measured using open-circuit spirometry as per section 3.1.2.4., and data were collected for one minute at rest, and then for one minute at five minute intervals throughout the test. Heart rate was monitored continuously with bipolar leads placed in the CM5 configuration (Lohmeier, Germany). An ECG strip was run during the last ten seconds of every five minute period. The subjects were allowed a 15 minute rest period, and then the steady state test was repeated with the cycle ergometer resistance set at a load estimated to be 60% of  $\dot{V}O_{2\text{ peak}}$ . The load, oxygen uptake and heart rate recorded during the 50%  $\dot{V}O_{2\text{ peak}}$  steady state test provided the initial load and training intensity for the twelve month training programme. The load was gradually increased to 60%  $\dot{V}O_{2\text{ peak}}$  after the first month of training. As steady state heart rates decreased, increments in power were made to maintain the subjects' training intensity at a heart rate corresponding to 60%  $\dot{V}O_{2\text{ peak}}$ .

#### 3.1.3. **Experimental Design**

Experiment 1 began in early January 2001 and progressed until late December. The training sessions in Experiment 1 consisted of:

- Three, 45 minute sessions per week;
- Cycling (constant load training), with intensity initially at 50% of  $\dot{V}O_{2\text{ peak}}$  for the first four weeks of training;
- Increases in exercise intensity to 60%  $\dot{V}O_{2\text{ peak}}$  with a training heart rate equivalent to that of 60%  $\dot{V}O_{2\text{ peak}}$ , as described by Stratton et al. (1994), Stachenfeld et al. (1998) and Woods et al. (1999);
- Immunological and haematological measures were taken monthly.
- Subjects were asked to keep a log of any illnesses, including any diagnosed upper respiratory tract infections (URTI)

The mode, intensity and frequency of training was adapted from Stratton et al. (1994), Stachenfeld et al., (1998), Woods et al. (1999) and Morris (1998). The training intensity of 50% of  $\dot{V}O_{2\text{ peak}}$  with corresponding heart rate was selected for the first month of training, as the subjects had been sedentary prior to volunteering for Experiment 1. The increase in training intensity to 60%  $\dot{V}O_{2\text{ peak}}$  and the corresponding heart rate occurred in the second month of training. Increments in power were made during the year to maintain the subjects' training intensity at a heart rate equivalent to 60%  $\dot{V}O_{2\text{ peak}}$ . Blood pressure was measured before and immediately after each exercise session. The subjects were weighed prior to each training session. The heart rate of each subject was monitored constantly during each training session with an ECG (CM5 placement), and heart rate recorded every ten minutes during the training session.

Cycling was selected as the mode of exercise so as to lower the risk of weight-bearing injury (Buchner 1997; Daley and Spinks 2000) and for ease of measurement and control of power output. The untrained subjects who acted as the control group continued with their daily activities and undertook no consistent aerobic exercise, such as brisk walking, jogging, cycling or swimming, or resistance training. The untrained subjects in the control group underwent the same procedures and measurements as the trained subjects in the experimental group.

#### 3.1.3.1. *Haematology*

Full blood counts were assessed with a Coulter T660 Blood Analyser (Coulter Electronics, USA). Normal blood count values are shown in Appendix 4 (Coulter 1984). The analysis provided concentrations of leucocytes, erythrocytes, haemoglobin, mean corpuscular haemoglobin and platelets, and included haematocrit and red cell volume (Deakin 1995; Gabbett et al., 2001). The Coulter T660 was unable to perform a white cell differential count, therefore the percentage of lymphocytes and other leucocyte subsets could not be recorded.

#### 3.1.3.2. *Blood Sampling Procedures.*

Blood samples were taken from each subject, in the supine position, at week 0 (pre-training), and then every four weeks through to week 52. The samples were taken between the hours of 0630

and 0900 . The subjects were fasted and rested to avoid the possibility of any immune perturbations from elevated concentrations catecholamines or cortisol. Subjects were encouraged to drink 250 to 350 mL of water at least one hour prior to blood sampling, to avoid the possibility of dehydration and its effects on blood samples.

The blood samples were taken by standard venepuncture technique, from the antecubital vein using the Vacutainer System (Becton Dickinson, Australia). The first blood sample (4 mL) was placed into a 4 mL, sterile EDTA plastic tube (Becton Dickinson, Australia) prior to analysis with the Coulter T660 Blood Analyser (Coulter Electronics, USA). The second blood sample (8 mL) was placed into sterile, glass 10ml EDTA tubes (Becton Dickinson, Australia), prior to centrifuging. Samples were stored at room temperature (21-22°C) on a roller mixer (Selby, Australia) for no longer than 20 minutes before centrifuging.

#### 3.1.3.3. *Separation of Mononuclear Cells.*

The methodology of lymphocyte proliferation assays involves the separation of mononuclear cells and then CD4<sup>+</sup> cells, determination of lymphocyte concentration and separation of cell suspension. The assays used for the analysis of IL-2R (CD25) and TfR (CD71) expression, and for quantitating intracellular Ca<sup>2+</sup> and Fe<sup>3+</sup> can best be described by the flow charts in Figures 13 & 14.

Blood from the second sample (10 mL tube) was used for this assay. Blood (5mL) was layered over 5 mL of Ficoll Histopaque 1.077 (Sigma, Australia) in a 15mL sterile centrifuge tube (Sigma, Australia) and centrifuged for 30 minutes at 400 x g, at 21°C. The supernatant was aspirated, and the opaque interface transferred to a new sterile centrifuge tube. Phosphate buffered saline (PBS) (10 mL) was added and samples were centrifuged at 250 x g for 10 minutes. The supernatant was aspirated and the cells suspended in PBS containing 5mM glucose (5mL). The sample was centrifuged at 250 x g for 10 minutes, then resuspended and washed once more. The pellet was resuspended in 0.5 mL of PBS containing glucose (5mM) and CD4<sup>+</sup> lymphocytes were separated from other leucocytes using a CD4 positive isolation kit (Dynal, Australia). A 100µL sample of lymphocyte suspension was analysed using a Coulter T660 Blood Analyser (Coulter Electronics, USA) to determine cell concentration. Cell viability was 98 ± 2% as demonstrated by the Calcein Cell Viability test (Molecular Probes, USA). Cell purity was >96% measured by flow cytometry in the forward and side scatter modes (Mooren et al., 2001a,b)

Figure 13: Flow Chart of Proliferation Assay

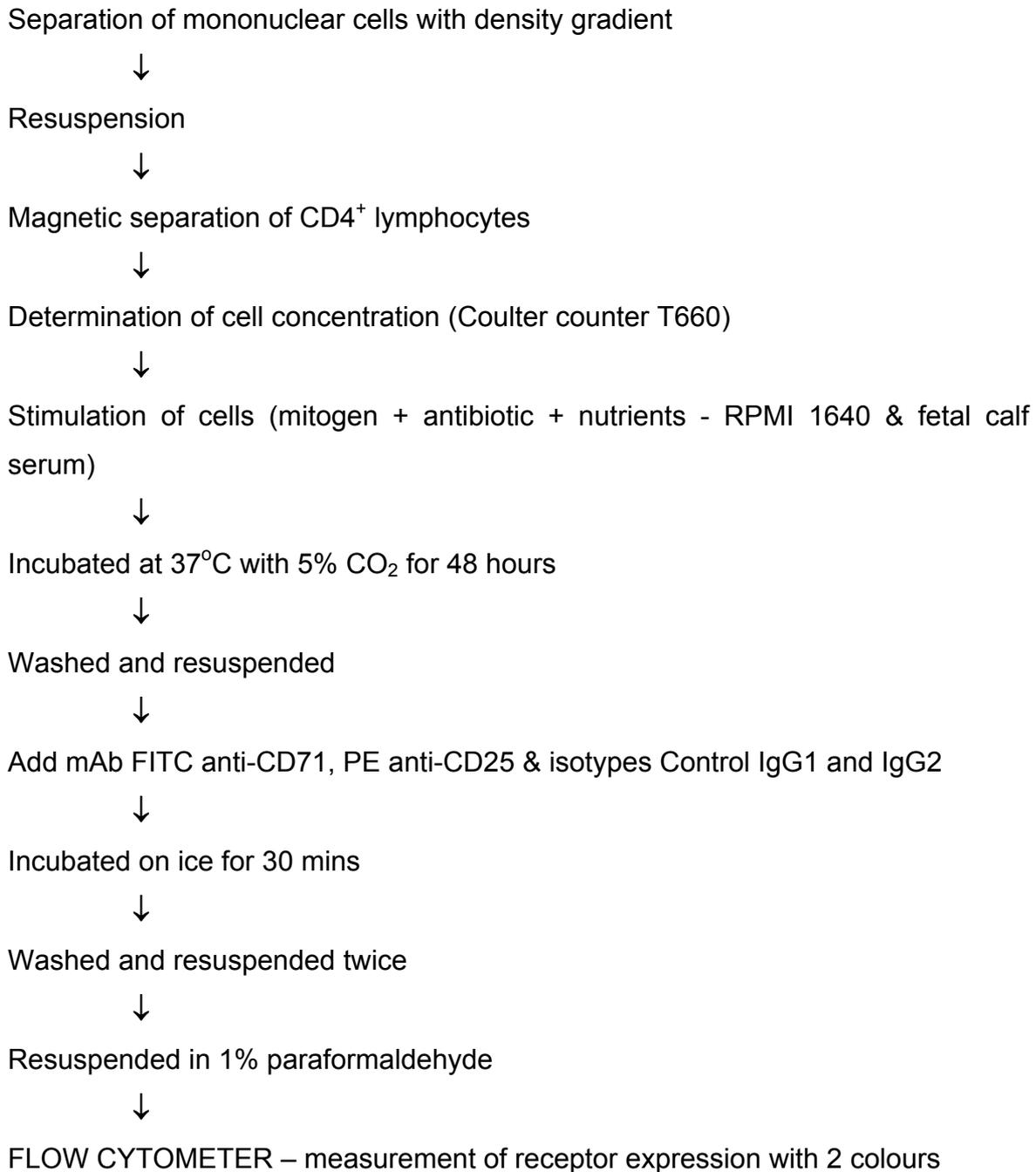


Figure 14: Flow Chart of Intracellular Calcium and Iron Assay

Separation of mononuclear cells with density gradient



Resuspension



Magnetic separation of CD4<sup>+</sup> lymphocytes



Division of cell suspension into 4 aliquots



**1)** 25% cells loaded with Fluo-3 AM (Ca<sup>2+</sup>)    **2)** 25% cells loaded with Calcein AM (Fe<sup>3+</sup>)  
 25% cells as control samples (no probe)    25% cells as control samples (no probe)



Fluo-3 sample incubated 45 mins (22°C)

Calcein sample incubated 5 mins (37°C)



Both control & probe samples processed through FLOW CYTOMETER



Thapsigargin added to Fluo-3 samples to stimulate ER Ca<sup>2+</sup> release; incubated for 5 mins at 37°C – processed through FLOW CYTOMETER

#### 3.1.3.4. *Lymphocyte Proliferation.*

A previously calculated quantity of lymphocyte suspension with a lymphocyte concentration of  $2 \times 10^6 \cdot \text{mL}^{-1}$  was removed from the centrifuge tube (Rhind et al., 1994; Gueldner et al., 1997; Ceddia et al., 1999). The lymphocyte suspension was placed in a 5mL sterile culture tube (Quantum Scientific, Australia) with 10 $\mu\text{L}$  of 10% phytohemagglutinin (PHA) (Sigma, Australia) and PBS, 10 $\mu\text{L}$  of 10% Gentamicin sulphate solution (Sigma, Australia) and sufficient RPMI-1640 (Sigma, Australia) supplemented with 20% foetal calf serum (FCS) (Sigma, Australia) to make a final volume of 1mL. The optimum concentration of PHA had been previously ascertained through reproducibility studies, and the coefficients of variation for the 10  $\mu\text{L}$  PHA incubated for 48 hours were 3.7% (percentage of positive cells in sample) and 6.1% (mean channel log fluorescence) (Section 3.3.2.) The procedure was carried out in duplicate for each sample. Culture tubes were incubated for 48 hours at 37°C with 5% CO<sub>2</sub>. The lymphocytes were then washed and resuspended in RPMI-1640.

#### 3.1.3.5. *CD25 and CD71 Flow Cytometric Analysis.*

The lymphocyte samples were divided into two aliquots in separate 5mL culture tubes (Quantum Scientific, Australia). Half of the samples had fluorescent labelled monoclonal antibodies (mAb) (Dako, Australia) added to the cell suspension. The remaining half had isotype control mAb (Dako, Australia) added. The saturating concentration of mAb and isotype control mAb had been previously established by saturation curves with reproducibility coefficients of variation of 2.1% (percentage of positive cells in sample) and 6.6% (mean channel log fluorescence) (Section 3.3.3). To load the lymphocytes with mAb, a volume of 5  $\mu\text{L}$  of mAb for each subject was suspended in a volume of 10  $\mu\text{L}$  of PBS (Sigma, Australia) for each subject in an eppendorf, and the total volume was vortexed for 30 seconds to ensure that the mAb was evenly mixed with PBS. This procedure was also performed with the isotype control mAb. 15  $\mu\text{L}$  of the mAb-PBS mix was then added to each non-control culture tube, and 15  $\mu\text{L}$  of isotype mAb-PBS mix was added to the control culture tubes. This procedure was performed in duplicate for all subjects. The expression of IL-2R was assessed using anti-CD25 mAb conjugated with Phycoerythrin (PE), and the expression of Tfr was assessed using anti-CD71 mAb conjugated with fluorescein isothiocyanate (FITC). Samples were incubated on ice and in the dark for 30 minutes. The lymphocytes were then washed twice in PBS containing 5mM glucose (0.5mL) and centrifuged at 300 x g at 4°C for 5

minutes. The supernatant was aspirated and the lymphocytes fixed in 1% paraformaldehyde (0.5mL). Samples were stored in the dark at below 4°C prior to flow cytometric analysis, which was within 30 minutes of fixation for all samples.

Flow cytometric measurements used a Facscalibur Flow Cytometer (Becton Dickinson, Australia) with an argon ion laser, which emitted at 488 nm. The instrument was calibrated using Calibrite fluorospheres (Becton Dickinson, Australia). The data analysis region was gated around lymphocytes, with controls analysed first for every sample in order to set positive and negative regions (quadrants) for fluorescence (Figure 15). The positive region was set so that less than 2% of unstimulated cells registered as positive. Receptor density was measured with mean channel log fluorescence. Fluorescence signals were displayed with logarithmic amplifiers spanning 4 log decades. Flow cytometric data files were analysed using Cell Quest Pro software (Macintosh, Australia). The percentage of lymphocytes positive for either CD25 or CD71 or both, was a measure of lymphocyte numbers showing fluorescence within the gated region (Figure 15). A minimum of 5000 cells were analysed per sample. Colour compensation settings had been previously established using a preparation of mAb-stained whole blood. B cells and T cells registered as green and red fluorescent regions respectively. Any overlap of the emission spectra of the dyes was corrected. Only quadrants 1 and 4 showed positive for red and green fluorescence.

#### FLOW CYTOMETRIC ANALYSIS

Q1 CD71 <sup>+</sup>	Q2 CD25 <sup>+</sup> /CD71 <sup>+</sup>
Q3 Auto- fluorescence	Q4 CD25 <sup>+</sup>

Figure 15: Flow Cytometric Analysis of Lymphocytes – CD25<sup>+</sup>/CD71<sup>+</sup>. CD25<sup>+</sup>/ PE appears in Quadrant 4 (Q4), CD71<sup>+</sup>/FITC in Quadrant 1 (Q1), double positive in Quadrant 2 (Q2), cell debris/autofluorescence in Quadrant 3 (Q3).

### 3.1.3.6. *Intracellular Calcium Assay:*

For each subject the lymphocytes were suspended in 980  $\mu\text{L}$  of PBS with added FCS (20 $\mu\text{L}$ ). After magnetic separation of T helper lymphocytes with CD4 positive isolation beads (Dynal, Australia), the 1mL suspension was divided into four aliquots of 250  $\mu\text{L}$ . Two aliquots were set aside for the Fluo-3 calcium assay, and two for the Calcein AM iron assay. Fluo-3 Am was prepared according to the manufacturer's directions (Molecular Probes, USA) and as per the methods of Kao (1994), Fernandez-Botran and Vetvicka (1996), and Haugland (1996). The Fluo-3 AM dyes were purchased packaged in 5 x 50 $\mu\text{g}$  lots within the same batch.

Fluo-3 AM is a derivative of the selective  $\text{Ca}^{2+}$  chelator 1,2-bis-(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), a tetracarboxylate analog of EGTA, to which various fluorophores are attached. BAPTA is pH-insensitive in the physiological pH range (7.35 – 7.45), but retains high affinity and selectivity for  $\text{Ca}^{2+}$  over  $\text{Mg}^{2+}$ . Fluo-3 AM is used extensively with intact cells because the lipophilic acetylmethyl esters of the dyes are membrane-permeable. Once inside the cell, the acetylmethyl esters are cleaved by nonspecific esterases and the  $\text{Ca}^{2+}$ -sensitive hydrophilic indicator remains inside the cell (Taylor et al., 1992; Kao 1994; Perez-Terzic et al., 1997; Haugland 1996).

To prepare the probe, 50 $\mu\text{g}$  of Fluo-3 AM was dissolved in 50  $\mu\text{L}$  of dimethyl-sulphoxide (DMSO) and divided into five aliquots. 10  $\mu\text{L}$  of Pluronic F-127 (Molecular Probes, USA) was added to each aliquot, to aid in the transport of the probe across the cell membranes. 140  $\mu\text{L}$  of RPMI 1640 was added to each aliquot, and the aliquots were then frozen at below  $-4^{\circ}\text{C}$  until needed.

To analyse the intracellular  $[\text{Ca}^{2+}]$  for each subject, two aliquots of cell suspension were allocated per subject. RPMI 1640 (250  $\mu\text{L}$ ) and FCS (6  $\mu\text{L}$ ) were added to each aliquot. Fluo-3 AM (18  $\mu\text{L}$ ) was added to one aliquot per subject, while the other aliquot remained a control sample (no probe) to establish levels of background fluorescence. This meant that flow cytometric quadrants could be adjusted to eliminate background fluorescence using the control samples (Figure 16).

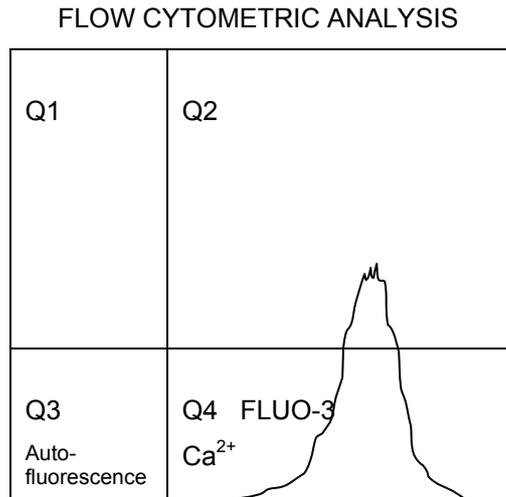


Figure 16: Flow Cytometric Analysis of Lymphocytes for Ca<sup>2+</sup>. Fluo-3 AM intensity was determined by mean channel log fluorescence in Quadrant 4. Autofluorescence was isolated in Quadrant 3 (control samples).

The Fluo-3 samples were incubated at room temperature for 45 minutes, then processed with the flow cytometer (FacsCalibur, Becton Dickinson, Australia). A control sample containing no Fluo-3 was processed first, for each subject, followed by the sample containing the probe. After the initial flow cytometric analysis, 6  $\mu\text{L}$  of thapsigargin dissolved in DMSO (100nM) (Sigma, Australia) was added to the Fluo-3 samples, and incubated for 5 minutes at 37°C to facilitate the release of ER Ca<sup>2+</sup> stores. The samples were then processed with flow cytometry to measure total Ca<sup>2+</sup> concentrations. The fluorescence increased as more intracellular Ca<sup>2+</sup> was available. The initial sample run displayed a calcium peak; the second sample run after the addition of thapsigargin, displayed a higher Ca<sup>2+</sup> peak, after the release of Ca<sup>2+</sup> from ER stores within the cell. Each assay was run in triplicate. The coefficients of variation for the Fluo-3 AM (18  $\mu\text{L}$ ) assay were 1.5%, 3.3% and 2.5% (intra-assay, Tables 15, 16 and 17) respectively (Section 3.3.4). The coefficients of variation for the assays with added thapsigargin (18  $\mu\text{L}$  of Fluo-3 AM plus 6  $\mu\text{L}$  of thapsigargin) were 0.8%, 1.9% and 1.6% (intra-assay, Tables 15, 16 and 17).

### 3.1.3.7. Calibration of Intracellular Calcium Concentrations:

The spectral properties of Fluo-3 AM enable the analysis of  $\text{Ca}^{2+}$  concentrations  $[\text{Ca}^{2+}]_i$  by flow cytometry. A standardized method of calibrating actual  $[\text{Ca}^{2+}]_i$  and intensity of fluorescence displayed by Fluo-3 AM has not yet been devised. Consequently there is reported variation in basal  $[\text{Ca}^{2+}]_i$ , and  $[\text{Ca}^{2+}]_i$  after stimulation (Kao et al., 1989; Tsien 1988; Kao 1994; Greimers et al., 1996; Berman 2000). One method to quantitate calcium ion indicators is with an intracellular calcium-sensitive electrode (Williams 1999). However, an intracellular calcium-sensitive electrode was not available for Experiment 1, nor is such an electrode suitable for a high throughput of cells from a large number of subjects.

The Coulter Epics Profile II (jointly used by the Schools of Health Science, and Physiotherapy and Exercise Science, Griffith University) was used for Experiment 2 (1999-2000). The failure of the argon laser in this instrument in December 2000, and the inability to acquire a replacement laser meant that we were obliged to use another instrument for Experiment 1 (2001 to 2002) The Facscalibur flow cytometer (Becton Dickinson, Australia) was the property of Sullivan & Nicolaidis Pathology (Taringa, Brisbane) and was used for Experiment 1. Consequently, two separate sets of  $\text{Ca}^{2+}$  calibration measurements for each instrument were performed, although the same procedure was followed for each set of data. In both cases, the calibration data yielded a similar dye dissociation constant ( $K_d$ ) of 400 nM and 450 nM respectively for the fluorescent probe Fluo-3 AM.

Two methods were used to calibrate the molar concentration of intracellular  $\text{Ca}^{2+}$  with mean channel log fluorescence. The first and simplest calibration method was by two-point calibration, involving the suspension of  $\text{CD4}^+$  lymphocytes in 1 mL of RPMI, containing 6  $\mu\text{L}$  of heat-inactivated foetal calf serum (FCS). BAPTA in the acetylmethylester form (Glycine, N,N'-[1, 2 – ethanediybis (oxy-2,1-phenylene)] bis[n-{2-[(acetyloxy) methoxy]-2-oxoethyl}]-, bis[(acetyloxy) methyl] ester, Molecular Probes, USA) was loaded into the lymphocytes to chelate free cytosolic calcium. 20  $\mu\text{L}$  of 5mM BAPTA AM in DMSO was used to provide a “minimum fluorescence” signal (i.e.  $F_{\min}$ , calcium-free signal). A separate aliquot of lymphocytes in suspension was incubated with 10  $\mu\text{L}$  of the ionophore, ionomycin (10  $\mu\text{M}$ ) (Molecular Probes, USA) to flood the cells with calcium to give a “saturating signal” or maximum fluorescence signal ( $F_{\max}$ ). The linear calibration from these two points (mean channel log fluorescence  $4.0 \pm 0.4$  to  $914.0 \pm 42.0$  in Experiment 1, mean

channel log fluorescence  $0.4 \pm 0.02$  to  $98.0 \pm 6.0$  in Experiment 2) provided an approximate quantitation of  $[Ca^{2+}]_i$  (Table 3 & 4). However, the two-point calibration method does not provide a dissociation constant ( $K_d$ ), or concentration where the probe is only half-saturated. Since others had documented saturating calcium concentrations ( $F_{max}$ ) between 10 and 40  $\mu M$  (Williams 1999; Berman 2000;), a more accurate calibration method that would provide a dissociation constant was needed.

The second, more intricate method of calibration was devised by Greimers et al. (1996), who used Fluo-3 AM to measure  $[Ca^{2+}]_i$  within  $CD4^+$ ,  $CD8^+$ , and B-lymphocytes. The standard curve was based on the direct calibration of Fluo-3 fluorescence intensity with solutions of known calcium concentration (CaEGTA, C-3009 calcium calibration kit #2, Molecular Probes, USA). The  $CD4^+$  lymphocytes were transferred to a series of 11  $Ca^{2+}/EGTA$  buffers with 0-10mM EGTA and free  $[Ca^{2+}]$  ranging from 0 to 39.8  $\mu M$  (Appendix 6, Molecular Probes, USA). The solutions also contained 100 mM KCl, 10mM MOPS, pH 7.2. These solutions were prepared by the manufacturer according to the method described by Tsien (1988).

The calcium calibration buffer kit (Molecular Probes, USA) is based on the principle that when concentrations of  $Ca^{2+}$  and EGTA are very close to each other, the only free  $Ca^{2+}$  available is that which is in equilibrium with EGTA. Therefore, the  $[Ca^{2+}]_{free}$  is a function of the dissociation constant ( $K_d$ ) of CaEGTA. The calibration buffer kit provides the highest  $[Ca^{2+}]$  10mM EGTA which gives a high  $[Ca^{2+}]_{free}$  of 39.8 $\mu M$ , and a 10mM  $K_2EGTA$  solution (zero  $Ca^{2+}$ ). A precise set of fluorescence curves can be generated by varying the  $[Ca^{2+}]_{free}$  in the buffer solution. The zero  $[Ca^{2+}]$  and high  $[Ca^{2+}]$  buffer solutions are cross-diluted to produce the series of eleven solutions with the amount of total  $Ca^{2+}$  increasing by 1mM CaEGTA with each dilution (Appendix 6). The raw data (mean channel log fluorescence) appears as a sigmoid curve and is converted to a regression line (standard curve) by plotting the log of  $[Ca^{2+}]_{free}$  (X-axis) versus the  $\log[(F - F_{min}) / (F_{max} - F)]$  (Y-axis). This double log plot gives an X intercept that is the log of the  $K_d$  indicator expressed in moles/litre<sup>-1</sup>. The inverse log of the X intercept will provide a molar concentration of  $[Ca^{2+}]$ . The slope of the regression line reflects the binding of each Fluo-3 molecule with  $Ca^{2+}$ . (Figures 17 and 19).

Greimers et al. (1996) used 10  $\mu\text{M}$  A23187 ionophore (previously dissolved in DMSO, Molecular Probes, USA) to open calcium channels in the cell membranes. Thus, cells suspended in the buffers were flooded with a solution of a known  $[\text{Ca}^{2+}]$  and once in equilibrium, an intracellular measurement of  $\text{Ca}^{2+}$  could be taken. Nigericin ( $0.5 \mu\text{g}\cdot\text{mL}^{-1}$  dissolved in ethanol) was added to clamp intracellular pH. Lymphocytes were then incubated at  $37.5^\circ\text{C}$  for at least two hours before analysis by flow cytometry to obtain a stable Fluo-3 fluorescence signal. The highest  $[\text{Ca}^{2+}]$  of  $39.8 \mu\text{M}$  was enough to saturate the probe, and a maximal fluorescence ( $F_{\text{max}}$ ) was obtained. A minimal fluorescence ( $F_{\text{min}}$ ) was obtained with the zero calcium-free buffer. The 11 intermediate  $\text{Ca}^{2+}/\text{EGTA}$  buffer solutions provided graded intracellular fluorescence intensities ( $F$ ) correlated with known micromolar concentrations. The X axis log fluorescence values followed a sigmoidal curve. To obtain a regression line for calibration purposes, Greimers et al. (1996) used the formula:

$$\text{Log} [(F - F_{\text{min}})/(F_{\text{max}} - F)]$$

for the Y axis (where  $F$  was each fluorescence intensity for the 11 calcium buffers). The Y value of zero (null ratio) and corresponding X intercept provided the dissociation constant by means of calculating the anti-log of X. The X axis intercepts provided values of  $\text{Log} [\text{Ca}^{2+}]_i$  (M).

Greimers et al. (1996) then plotted their calibration data as a double-logarithmic plot, with a calculated apparent Fluo-3 dissociation constant ( $K_d$ ) for binding  $\text{Ca}^{2+}$  at  $447\text{nM}$  at  $37^\circ\text{C}$ . According to Kao et al. (1989) and Tsien (1988), the dissociation constant for Fluo-3 at  $22^\circ\text{C}$  is  $390 \text{ nM}$ , and at  $37^\circ\text{C}$ ,  $864 \text{ nM}$  (Ritter et al., 2000). The  $K_d$  for Fluo-3 is known to be temperature and pH dependent. The stability of the probe may also vary in temperatures over  $24^\circ\text{C}$ , causing dye leakage from the cell or compartmentalization within intracellular organelles (Kao 1994).

The methodology of Greimers et al. (1996) was used in Experiments 1 and 2, with the addition of an initial control sample containing lymphocyte suspension and  $6\mu\text{L}$  of FCS, but with no Fluo 3. This sample provided a background fluorescence reading, with a mean channel log fluorescence intensity of  $0.3 \pm 0.02$ . This reading should be close to an accurate  $F_{\text{min}}$ , which corresponded to a zero molar  $[\text{Ca}^{2+}]_i$ . The cell samples were centrifuged at  $200 \times g$  for 5 minutes at room temperature ( $22^\circ\text{C}$ ), and the PBS and FCS were aspirated. Eleven lymphocyte samples were duly suspended in the 11  $\text{Ca}^{2+}/\text{EGTA}$  buffers, to which was added  $10\mu\text{L}$  of  $10\mu\text{M}$  ionomycin previously dissolved in

DMSO (Molecular Probes, USA) and 6  $\mu\text{L}$  of FCS (Sigma, Australia). We also prepared one aliquot containing cell suspension and 20  $\mu\text{L}$  of 5mM BAPTA AM, to chelate available intracellular  $\text{Ca}^{2+}$  and provide a minimal fluorescence signal. The BAPTA AM molar concentration had been previously obtained by titration studies. All cell suspensions were incubated with dyes, ionomycin or BAPTA for 45 minutes at 22°. The lymphocytes were washed in PBS for 5 minutes at 200 x g to remove excess probe before flow cytometry analysis.

Two other aliquots were prepared, one containing cell suspension, 18 $\mu\text{L}$  of Fluo-3 AM, 6  $\mu\text{L}$  of FCS and 20  $\mu\text{L}$  of 10 $\mu\text{M}$  ionomycin ( $F_{\text{max}}^{\text{a}}$ ). The first aliquot provided the  $F_{\text{max}}$  fluorescence signal. The second aliquot ( $F_{\text{max}}^{\text{b}}$ ) contained cell suspension, Fluo-3 (18 $\mu\text{L}$ ), FCS and Thapsigargin (6  $\mu\text{L}$ ) (Sigma, Australia). The second aliquot was included to see if the fluorescence signal matched that of the  $F_{\text{max}}$  sample. The second aliquot was incubated without ionomycin or BAPTA, and thapsigargin was added 5 minutes prior to the end of incubation. The sample was then incubated at 37°C for a further 5 minutes, then analysed by flow cytometry. This procedure was duplicated in the laboratory on separate days, with room temperature at 22°C. The calibration procedure was performed in triplicate for each flow cytometer and the resulting data are shown in Table 3 & 4. As reported by Greimers et al.(1996), Tsien (1988) and Kao et al. (1989), we found that mean fluorescence intensity increased as a function of intracellular  $\text{Ca}^{2+}$  molarity. A typical sigmoidal curve resulted. We plotted the data as a double logarithmic plot of the known molar calcium concentration versus the ratio of maximum and minimum fluorescence (Figure 17 and 19). The data are shown as  $\text{Log} [(F - F_{\text{min}})/(F_{\text{max}} - F)]$  on the Y axis and  $\text{Log} [\text{Ca}^{2+}]_i$  (M) on the X axis (Greimers et al., 1996).

Once the  $F_{\text{max}}$  and  $F_{\text{min}}$  mean channel log fluorescence values were known, any measured fluorescence value  $F$  could be inserted in the log equation and plotted on the Y axis. The known molar  $[\text{Ca}^{2+}]$  concentrations used in calibration provided the X values for the regression line (eg. 0.017  $\mu\text{M}$  is plotted as  $\text{Log} 0.000000017$  M or  $-7.77$  on the X axis). Using the calibration line, any mean channel log fluorescence value Y will yield an X anti-log value that provides a molar concentration.

The calibration data for Experiment 1 (Facscalibur flow cytometer) yielded the X-intercept at the null ratio as equal to the log  $K_d$  indicator  $-6.4$ , corresponding to a Fluo-3  $[Ca^{2+}]K_d$  of 400 nM. The regression line indicated that  $Y = 1.1X + 6.7$ . The double logarithmic plot for Experiment 2 (Coulter flow cytometer) yielded the X-intercept at the null ratio as equal to the log  $K_d$  indicator  $-6.3$ . This corresponded to a Fluo-3  $[Ca^{2+}]K_d$  of 450 nM. The regression line for these data indicated that  $Y = 1.02X + 6.5$ . The calculated  $K_d$  's ( $-6.3$ ,  $-6.4$ ) agree with the findings of others (Haugland 1996; Greimers et al., 1996; Berman 2000).

Table 3: Experiment 1 –  $Ca^{2+}$  Calibration Data for Facscalibur Flow Cytometer (Sullivan & Nicolaidis Pathology)

Sample description	$\mu$ Molar $[Ca^{2+}]$	Mean Channel Log Fluorescence $\pm$ SD (4 Log Decades)
Autofluorescence	0	$3.0 \pm 0.2$
$F_{min}^a$ – BAPTA AM added	0	$4.0 \pm 0.4$
$F_{min}^b$ – Buffer 1	0	$4.1 \pm 0.3$
Buffer 2	0.017 $\mu$ M	$34.0 \pm 3.0$
Buffer 3	0.035 $\mu$ M	$81.0 \pm 9.0$
Buffer 4	0.070 $\mu$ M	$150.0 \pm 27$
Buffer 5	0.100 $\mu$ M	$178.0 \pm 31$
Buffer 6	0.150 $\mu$ M	$260.0 \pm 26$
Buffer 7	0.230 $\mu$ M	$330.0 \pm 37$
Buffer 8	0.350 $\mu$ M	$400.0 \pm 22$
Buffer 9	0.600 $\mu$ M	$470.0 \pm 37$
Buffer 10	1.35 $\mu$ M	$640.0 \pm 38$
Buffer 11	38.9 $\mu$ M	$837.0 \pm 34$
$F_{max}^a$ - Ionomycin added	$>38.9 \mu$ M	$914.0 \pm 42$
$F_{max}^b$ – Thapsigargin added	$>38.9 \mu$ M	$1140.0 \pm 64$

Autofluorescence sample – no probe added;  $F_{min}^a$  – BAPTA AM added to chelate  $Ca^{2+}$ ;  $F_{min}^b$  – Buffer 1, no  $Ca^{2+}$ ; Buffers 2 – 11 contained  $Ca^{2+}$  concentrations of 0.017  $\mu$ M to 38.9  $\mu$ M;  $F_{max}^a$  - 20  $\mu$ L of 10nM ionomycin added to sample;  $F_{max}^b$  - 6  $\mu$ L thapsigargin added to sample. Fluorescence emission measured in mean channel log fluorescence  $\pm$  SD.

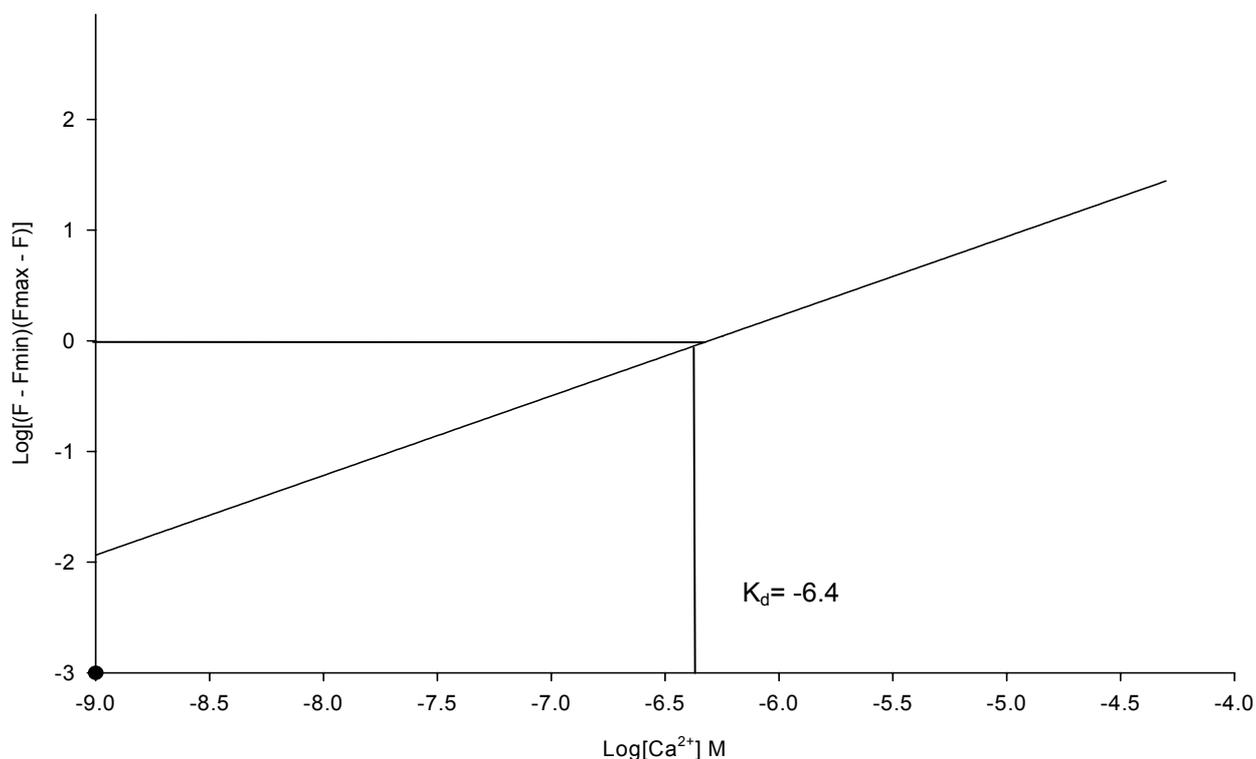


Figure 17: Experiment 1- Double Log Plot Regression for Fluo-3 AM Dissociation Constant ( $K_d$ ).

One colour calibration of Fluo-3 AM derived as a double log plot of the molar calcium concentration vs the ratio  $(F - F_{\min})/(F_{\max} - F)$ .  $F_{\min}$ ,  $F_{\max}$  and  $F$  respectively were the mean Fluo-3 channels incubated in zero free  $\text{Ca}^{2+}$ , in thapsigargin and in 11 intermediate free  $\text{Ca}^{2+}$  concentrations. The X-intercept at null ratio (-6.4) is equal to the apparent log  $K_d$  indicator and corresponds to a Fluo-3  $[\text{Ca}^{2+}] K_d$  of 400nM. Regression line  $Y = 1.1X + 6.7$ ,  $r = 0.93$ .

Although previous literature suggests that  $39 \mu\text{M}$  of free  $\text{Ca}^{2+}$  is enough to saturate Fluo-3 for a maximum signal (Haugland 1996; Williams 1999; Berman 2000), the present study found that the addition of thapsigargin, which mobilizes ER  $\text{Ca}^{2+}$  stores, provided a higher fluorescence signal than both the  $39 \mu\text{L}$  buffer, and ionomycin. The fluorescence signal achieved with thapsigargin was termed the  $F_{\max}$  signal.

### 3.1.3.8. *Intracellular Iron Assay:*

There is a little literature available reporting the measurement of intracellular iron using fluorescent probes. Due to funding and time restrictions, we were not able to devise accurate methods of quantifying molar  $[\text{Fe}^{3+}]_i$  with fluorescence intensity, as done with Fluo-3 and  $[\text{Ca}^{2+}]_i$ . It was decided to record the levels of mean channel log fluorescence every month, which would provide an estimate of the magnitude of change of available iron. Breuer et al. (1995) attempted to measure  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  by pulsing lymphocytes with radioactive-labelled transferrin ( $\text{Tf}^{55}$ ), adding iron chelators and then extracting the chelator- $\text{Fe}^{55}$  complexes with an organic solvent. While they estimated that the cytoplasmic concentration of iron fluctuated between 0.3 - 0.5  $\mu\text{M}$ , the iron-extraction methodology resulted in disruption to intracellular compartments and oxidation, confounding their results. Thomas et al. (1999) attempted to measure the thermodynamic and iron-binding kinetics of plant cells using Calcein AM, but admitted that while they were able to measure the rate of uptake of a known  $[\text{Fe}^{3+}]$  solution, they were not able to quantify intracellular  $[\text{Fe}^{3+}]$ .

Calcein AM was prepared according to the manufacturer's directions (Molecular Probes, USA) and according to the methods of Breuer et al (1995), Picard et al (1998) and Thomas et al (1999). The Calcein AM was packaged in 5 x 50  $\mu\text{g}$  containers within the same batch. 50  $\mu\text{g}$  of Calcein AM was dissolved in 50  $\mu\text{L}$  of DMSO and then divided into five aliquots. 10  $\mu\text{L}$  of Pluronic F-127 (Molecular Probes, USA) and 140  $\mu\text{L}$  of RPMI 1640 (Sigma, Australia) was added to each aliquot. The stock solutions of Calcein AM were kept frozen at below  $-4^\circ\text{C}$  until required. For each monthly assay, the fluorescent probe stock solution was diluted five-fold (1 part probe stock to 4 parts RPMI 1640). This dilution was previously calculated by repeated titration and saturation curves (Section 3.3).

Two aliquots of cell suspension per subject had previously been set aside. The lymphocytes had been suspended in RPMI 1640 (250  $\mu\text{L}$ ). Further RPMI 1640 (250  $\mu\text{L}$ ) and FCS (6  $\mu\text{L}$ ) were added to each aliquot. Calcein AM probe (3  $\mu\text{L}$ ) was added to one aliquot per subject, the other aliquot remaining a control for each subject. Background fluorescence was eliminated using the control samples and adjusting the quadrants to measure only fluorescence produced by the probe (Figure 18).

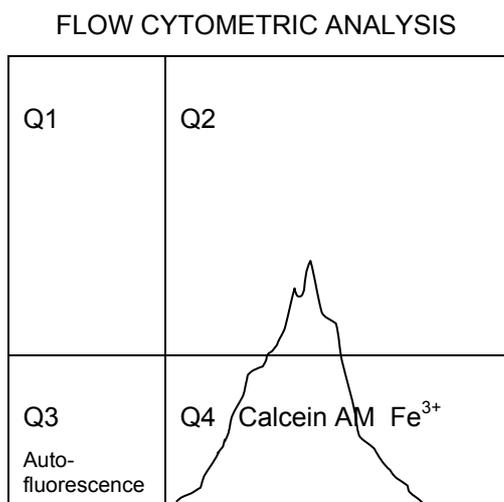


Figure 18: Flow Cytometric Analysis of Lymphocytes for Fe<sup>3+</sup>. Calcein AM intensity was determined by mean channel log fluorescence in Quadrant 4. Autofluorescence was isolated in Quadrant 3 (control samples).

The Calcein AM samples were incubated at 37°C for 5 minutes, then centrifuged at room temperature for 5 minutes at 300 x g. Half a millilitre (0.5 mL) of PBS was added to the culture tube and the cells were washed to remove excess probe and re-suspended in 0.5 mL of RPMI 1640 (Sigma, Australia). The lymphocyte samples were then processed by flow cytometry (FacsCalibur, Becton Dickinson, Australia). The Calcein AM fluorescent probe displays diminishing fluorescence as iron binds to the probe molecule. The greater the intracellular iron concentration, the less fluorescence is measured. Each sample was run in duplicate. The coefficients of variation for the intra-assay were 4.1% and 3.1% (Tables 18 and 19) (Section 3.3.5).

#### 3.1.4. Statistical Analyses for Experiment 1.

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS®) Version 9-Windows 98 package. Data from the measurement of physical characteristics, blood pressure, and incremental exercise tests, haematology analyses and flow cytometric analyses are presented as means and standard error of the mean (mean±SEM). Data from the Calcium Calibration in mean channel log fluorescence (Table 3) and the reliability and reproducibility studies (coefficients of variation Section 3.3) are presented as means and standard deviation (mean±SD). Analysis of Variance with repeated measures was used to determine significant differences between trained and untrained males and females each month and for each dependent measure. The p value for significance was set at < 0.05. A Least Significant Difference (LSD) post-hoc test with Bonferroni adjustment was used to identify the means that were significantly different (Bland 1995; Vincent 1999).

The immunological dependent variables measured were CD25 receptor expression (log fluorescence 2), CD71 receptor expression (log fluorescence 1), CD25/CD71 receptor expression (log fluorescence 1 and 2), percentage of lymphocytes positive for CD25, CD71 and double positive for both CD25 and CD71. The incremental exercise test dependent variables were  $\dot{V}O_{2\text{ peak}}$  ( $L \cdot \text{min}^{-1}$  and  $\text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ), peak power (watts), peak heart rate ( $\text{b} \cdot \text{min}^{-1}$ ), peak ventilation ( $L \cdot \text{min}^{-1}$ ) and peak RER.

The statistical analyses used were:

- Independent T tests: pre- and post-training peak incremental exercise responses.
- Analysis of Variance (ANOVA) with repeated measures: immunological and haematological data.
- LSD post-hoc test with Bonferroni adjustment.
- Multiple regression (stepwise) and Pearson correlation matrix: to investigate the relationship between leucocyte concentration and the immunological variables.

## 3.2 Experiment 2 - Males Aged 23 to 36 Years

### 3.2.1. Introduction

The purpose of Experiment 2 was to investigate whether twelve months of chronic endurance training would decrease the concentration of CD4<sup>+</sup> lymphocytes and their responsiveness to mitogenic challenge in males aged 23 to 36 years. The experimental (trained) group (n = 14) were experienced, highly trained athletes undertaking Ironman distance triathlons. These athletes competed in long-course triathlons with a swim component of 3.8 km, a cycling component of 180 km and a run component of 42.2 km. The lymphocyte function of these athletes was compared to a control group of nine age-matched untrained individuals, who were undertaking no aerobic, endurance or resistance training.

### 3.2.2. Subject Characteristics

#### 3.2.2.1. Recruitment and Screening

Trained subjects were recruited through advertisements placed in local Gold Coast newspapers and in the Queensland Triathlon Association magazine. Respondents to the advertisements were interviewed by telephone and verbally completed a medical questionnaire. Those respondents with a history of asthma, high blood pressure, recurring viral conditions (e.g. glandular fever), currently taking anti-inflammatory medications, or with any other medical condition which contraindicated vigorous exercise, were excluded from the experiment. Respondents who were not training regularly, and for long distances, were also excluded from the experiment.

Untrained subjects were recruited through advertisements placed in local Gold Coast papers and the Griffith University magazine, and also through notices placed around the campus. Nine untrained subjects aged between 23 and 36 years volunteered to participate in the experiment. Following the initial telephone interview, the trained and untrained subjects identified as potentially suitable for the experiment visited the laboratory on two separate occasions for further screening and familiarisation.

Each subject :

- Was provided with an information sheet setting out the details of the experiment (Appendix 1);
- Completed a comprehensive medical history questionnaire (Appendix 2);

- Underwent a resting 12 lead electrocardiogram (ECG), spirometry and blood pressure measurement;
- Underwent a four site skinfold test, standing height and body mass measurements;
- Underwent a resting venepuncture (supine) to provide a full blood count (Appendix 4).
- Was provided with a list of dates to attend the laboratory for a venepuncture, for the twelve months of the experiment;

After a clear explanation of the proposed experiment, subjects underwent the same screening and familiarisation procedures as used in section 3.1.2.1.

As a result of the familiarisation and screening procedures, each subject selected for Experiment 2 was:

- free from known cardiovascular and respiratory disease (Appendix 2);
- a non-smoker;
- free from metabolic diseases (Appendix 2);
- free from any known immune dysfunction, systemic viral condition or inflammatory disease (Appendix 2);
- not taking any medication known to interfere with the immune or exercise response;
- Had haematology measurements within the normal ranges (Appendix 4);
- normal spirometry and a resting 12 lead ECG;
- a resting blood pressure of less than 130/90 mmHg; and
- no evidence of clinically significant exercise-induced myocardial ischaemia.

No remuneration was offered to the subjects. The study was approved by the Griffith University Human Ethics Committee.

#### 3.2.2.2. *Anthropometry*

Subjects were measured as described in Section 3.1.2.2.

#### 3.2.2.3. *Spirometry*

Subjects were measured as described in Section 3.1.2.3.

#### 3.2.2.4. *Incremental Exercise Test to Exhaustion*

Each subject performed an incremental cycle exercise test to exhaustion on an electronically braked cycle ergometer (Lode Excaliber, Holland). Oxygen uptake was measured as described in Section 3.1.2.4.

**Trained Males:** The separate incremental cycling protocols for both trained and untrained males had been previously determined (Weston and Gabbett 2001) and were stored in the Lode Workload Programmer (Lode Excaliber, Holland). Each trained male subject commenced cycling at 60 watts and after a three minute warm up at 60 watts, the power was increased by 30 watts per minute until volitional exhaustion or clinical signs and symptoms prevented further exercise. Peak  $\dot{V}O_2$  ( $\dot{V}O_{2\text{ peak}}$ ), peak heart rate, peak power, peak ventilation and peak RER were determined from the average of the highest two values attained over two collection periods during the exercise test.

**Untrained Males:** Each untrained subject commenced cycling at 40 watts (Weston and Gabbett 2001) and after a three minute warm up at 40 watts, the power was increased by 20 watts per minute until volitional exhaustion or clinical signs and symptoms prevented further exercise. Peak  $\dot{V}O_2$  ( $\dot{V}O_{2\text{ peak}}$ ), peak heart rate, peak power, peak ventilation and peak RER were determined from the average of the highest two values attained over two collection periods during the exercise test.

#### 3.2.3. **Experimental Design**

Experiment 2 began in September 1999 and finished in September 2000. The September 1999 sampling point was termed "September A", and the September 2000 sampling point was termed "September B" (Section 4.2). The monthly venepunctures had to be arranged to accommodate major races throughout the year, and still provide blood samples every four weeks. It was also deemed more suitable to begin the experiment during a period of "aerobic base building and consolidation" in September, rather than in January during a period of hard training and racing. Since venepunctures were conducted every four weeks, the test schedule resulted in two blood sampling points in October, termed "October A" and "October B" (Section 4.2).

The subjects followed similar training regimes throughout the year but some trained under different coaches. All subjects were racing in the same events including in the Forster Ironman Triathlon in April 2000. The year was divided into specific training periods (e.g. rest/recovery; aerobic base development; pre-competition phase; race-specific phase), in which the subjects completed similar volumes and intensities of training. For long-course triathlons, the majority of training was aerobic in nature, with sessions of higher intensity (e.g. hill climbs in both cycling and running; repeated hard efforts or intervals at a high intensity, followed by recovery intervals for all disciplines; “threshold”, often known as “lactate tolerance” sessions with sustained intervals at “anaerobic threshold” for swimming, cycling and running). The high intensity training sessions were only introduced in the pre-competition and race-specific phases of the year.

Weekly training sessions, averaged over one year, involved:

- 3-4 swim sessions (averaging 12 – 16 km)
- 3-4 cycling sessions (averaging 300 – 400km)
- 3-5 run sessions (averaging 60 – 90km)

Changes in training volumes depended upon the time of year. Volumes were lower in winter (averaging 12 km swimming, 300 km cycling, 60 km running per week) and higher in summer through to autumn (averaging 16 km swimming, 400 km cycling, 100 km running per week).

To assess lymphocyte proliferative capacity, both trained and untrained subjects provided venous blood samples every four weeks. Since training volumes were high during the pre-competition and race-specific phases of the year, any training-induced immune suppression should become apparent during these periods, compared to the untrained group. A full blood count (WBC, RBC, haemoglobin concentration, mean corpuscular haemoglobin concentration, haematocrit and platelets), percentage of CD4<sup>+</sup> expressing CD25 and CD25 density, percentage of CD4<sup>+</sup> expressing CD71 and CD71 density, intracellular Ca<sup>2+</sup> concentrations and intracellular iron were measured every four weeks. Subjects were asked to keep a log of any illnesses, including diagnosed cases of URTI.

#### 3.2.3.1. *Haematology*

Blood samples were analysed for cell subsets and haematological variables as described in Section 3.1.3.1

### 3.2.3.2. *Blood Sampling Procedures.*

Blood samples were taken from each subject at week 0 (pre-training), and then every 4 weeks through to, and including, week 52. The blood samples were taken between the hours of 0630 and 0900 on the same day. The subjects were fasted and rested to avoid the possibility of any immune perturbations from elevated concentrations of catecholamines and cortisol from a previous training session (Brenner et al., 1998). Subjects were also encouraged to drink between 250 and 350 mL of water at least one hour prior to blood sampling, to avoid the effects of dehydration on cell concentrations. The blood samples were taken as described in Section 3.1.3.2.

### 3.2.3.3. *Separation of Mononuclear Cells.*

The methodology of lymphocyte separation assays is shown in Figures 13 and 14.

The lymphocyte separation procedures are described in section 3.1.3.3.

### 3.2.3.4. *Lymphocyte Proliferation.*

The lymphocyte proliferation assays were conducted as described in Section 3.1.3.4.

### 3.2.3.5. *CD25 and CD71 Flow Cytometric Analysis.*

Flow cytometric analysis was conducted as described in Section 3.1.3.5.

Flow cytometric measurements for Experiment 2 used an Epics Profile II flow cytometer (Coulter Electronics, USA) with an argon ion laser, which emitted at 488 nm. The flow cytometer was calibrated using "fluorospheres" (Coulter Electronics, USA). The data analysis region was gated around lymphocytes, with controls analysed first for every sample in order to set positive and negative regions (quadrants) for fluorescence. The positive region was set so that less than 2% of unstimulated cells registered as positive. Receptor density was measured with mean channel log fluorescence. Fluorescence signals were displayed with logarithmic amplifiers spanning 3 log decades. Flow cytometric data files were analysed using Coulter software. The percentage of lymphocytes positive for either CD25 or CD71 or both, was a measure of lymphocyte numbers showing fluorescence within the gated region. A minimum of 5000 cells were analysed per sample. Colour compensation settings had been previously established using a preparation of mAb-stained whole blood. B cells and T cells registered as green and red fluorescent regions respectively. Any overlap of the emission spectra of the dyes was corrected. Only quadrants 1 and 4 showed positive for red and green fluorescence.

### 3.2.3.6. Intracellular Calcium Assay:

The intracellular calcium assays were conducted as described in Section 3.1.3.6.

### 3.2.3.7. Calibration of Intracellular Calcium Concentrations:

Calcium concentrations were measured using the calibration techniques described in Section 3.1.3.7. Calibration data are shown in Table 4 and Figure 16.

Table 4: Experiment 2 – Ca<sup>2+</sup> Calibration Data for Coulter Epics Profile II Flow Cytometer (Griffith University)

Sample Description	$\mu\text{M} [\text{Ca}^{2+}]$	Mean Channel Log Fluorescence $\pm$ SD (3 Log Decades)
Autofluorescence	0	0.4 $\pm$ 0.02
F <sub>min</sub> <sup>a</sup> – BAPTA AM added	0	0.42 $\pm$ 0.02
F <sub>min</sub> <sup>b</sup> – Buffer 1	0	0.5 $\pm$ 0.03
Buffer 2	0.017 $\mu\text{M}$	3.6 $\pm$ 0.4
Buffer 3	0.035 $\mu\text{M}$	7.4 $\pm$ 0.5
Buffer 4	0.070 $\mu\text{M}$	14.8 $\pm$ 3
Buffer 5	0.100 $\mu\text{M}$	18.1 $\pm$ 4
Buffer 6	0.150 $\mu\text{M}$	26.4 $\pm$ 4
Buffer 7	0.230 $\mu\text{M}$	35.0 $\pm$ 3
Buffer 8	0.350 $\mu\text{M}$	42.0 $\pm$ 4
Buffer 9	0.600 $\mu\text{M}$	68.0 $\pm$ 4
Buffer 10	1.35 $\mu\text{M}$	96.5 $\pm$ 5
Buffer 11	38.9 $\mu\text{M}$	108.3 $\pm$ 5
F <sub>max</sub> <sup>a</sup> – Ionomycin added	>38.9 $\mu\text{M}$	108.0 $\pm$ 6
F <sub>max</sub> <sup>b</sup> – Thapsigargin added	>38.9 $\mu\text{M}$	134.0 $\pm$ 8

Autofluorescence sample – no probe added; F<sub>min</sub><sup>a</sup> – BAPTA AM added to chelate Ca<sup>2+</sup>; F<sub>min</sub><sup>b</sup> – Buffer 1, no Ca<sup>2+</sup>; Buffers 2 – 11 contained Ca<sup>2+</sup> concentrations of 0.017 $\mu\text{M}$  to 38.9 $\mu\text{M}$ ; F<sub>max</sub><sup>a</sup> – 20 $\mu\text{L}$  of 10nM ionomycin added to sample; F<sub>max</sub><sup>b</sup> – 6 $\mu\text{L}$  thapsigargin added to sample. Fluorescence emission measured in mean channel log fluorescence  $\pm$  SD.

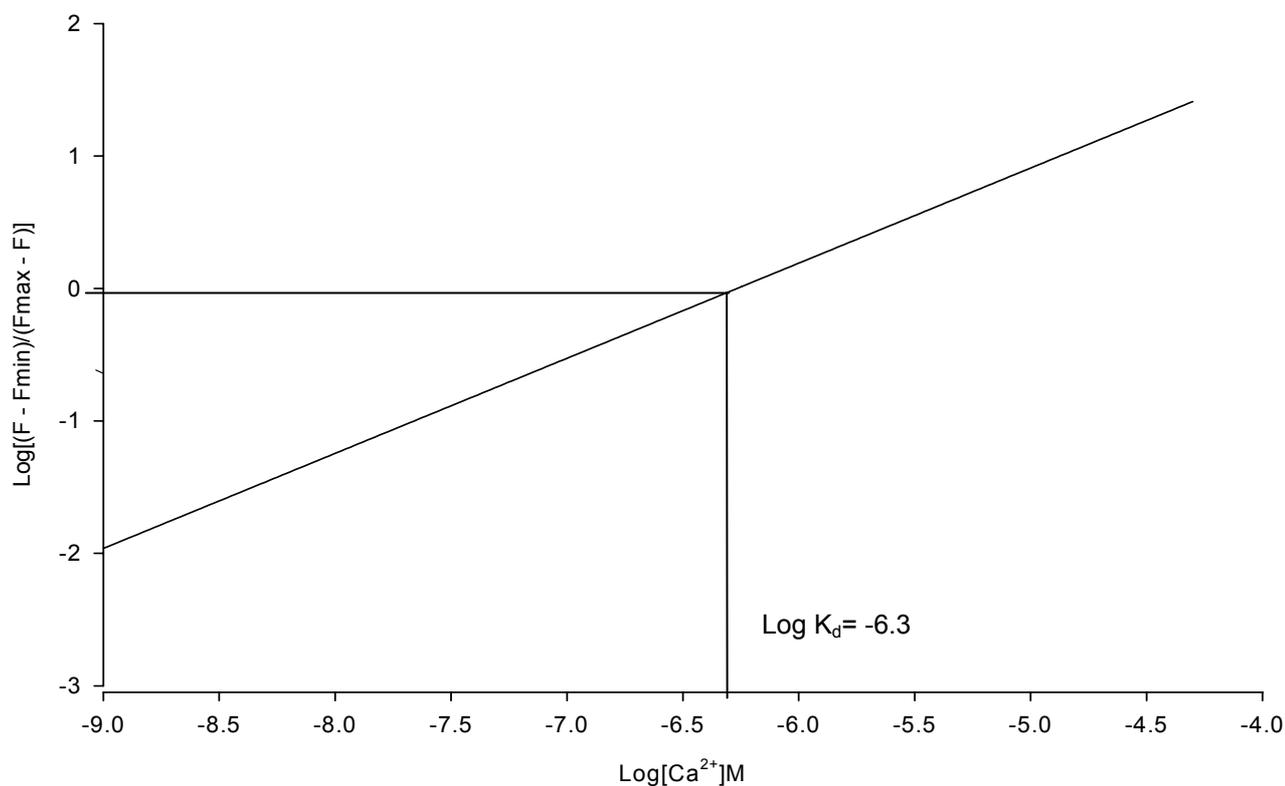


Figure 19: Experiment 2 – Double Log Plot Regression for Fluo-3 AM Dissociation Constant ( $K_d$ )

One colour calibration of Fluo-3 AM derived as a double log plot of the molar calcium concentration vs the ratio  $(F - F_{\text{min}})/(F_{\text{max}} - F)$ .  $F_{\text{min}}$ ,  $F_{\text{max}}$  and  $F$  respectively were the mean Fluo-3 channels incubated in zero free  $\text{Ca}^{2+}$ , in thapsigargin and in 11 intermediate free  $\text{Ca}^{2+}$  concentrations. The X-intercept at null ratio (-6.3) is equal to the apparent  $\text{log } K_d$  indicator and corresponds to a Fluo-3  $[\text{Ca}^{2+}] K_d$  of 450nM. Regression line  $Y = 1.02X + 6.5$ ,  $r = 0.96$ .

#### 3.2.3.8. Intracellular Iron Assay:

The intracellular iron concentration assays were conducted as described in Section 3.1.3.8.

### 3.2.4. Statistical Analyses for Experiment 2.

Statistical analyses were performed using the Statistical Package for Social Sciences (SPSS©) Version 9-Windows 98 package. Data from the measurement of physical characteristics, incremental exercise test variables, haematology analyses and flow cytometric analyses are presented as means and standard error of the means (mean $\pm$ SEM). Data from the calcium calibration in mean channel log fluorescence (Table 4) are presented as means and standard deviation (mean $\pm$ SD). Paired t-tests were used to compare pre- and post-study mean data from the incremental exercise tests for both trained and untrained groups. Analysis of Variance with repeated measures was used to determine significant differences between means for trained and untrained males each month and for each dependent measure. The p value for significance was set at < 0.05. A post-hoc test could not be performed for Experiment 2 because SPSS requires three or more groups to perform a post-hoc test (Bland 1995; Vincent 1999).

The immunological dependent variables measured were CD25 receptor expression (log fluorescence 2), CD71 receptor expression (log fluorescence 1), CD25/CD71 receptor expression (log fluorescence 1& 2), percentage of lymphocytes positive for CD25, CD71 and double positive for both CD25 and CD71. The incremental exercise test dependent variables were  $\dot{V}O_{2\text{ peak}}$  (L.min<sup>-1</sup> and mL.kg<sup>-1</sup>.min<sup>-1</sup>), peak power (watts), peak heart rate (b.min<sup>-1</sup>), peak ventilation (L.min<sup>-1</sup>) and peak RER.

The statistical analyses used were:

- Independent T tests: pre- and post-training peak incremental exercise responses.
- Analysis of Variance (ANOVA) with repeated measures: immunological and haematological data.
- Multiple regression (stepwise) and Pearson correlation matrix: to investigate the relationship between leucocyte concentration and the immunological variables.

### 3.3. Reliability and Reproducibility Tests

Reliability and reproducibility of each assay or saturation curve was determined by repeating the assay in triplicate over three days (PHA concentration, PHA incubation times, saturation curves for anti-CD25 and CD71 monoclonal antibodies and Fluo-3 AM) or duplicate (saturation curve for Calcein-AM), then calculating the coefficient of variation (CV) (%) according to Bland (1995) and Vincent (1999), where:

$$CV (\%) = \frac{\text{Standard deviation (SD)}}{\text{Mean}} \times 10^2$$

**3.3.1. CD25/CD71 Proliferation Assay.** Optimal Phytohemagglutinin concentration for isolated CD4<sup>+</sup> lymphocytes was determined with proliferative studies of 24, 48 and 72 hours duration (Coulter Epics Profile II Flow Cytometer, Griffith University). A proliferative assay was performed in triplicate to determine the optimal concentration of PHA to stimulate CD4<sup>+</sup> lymphocytes during 3 different incubation periods. The assay also determined test-re-test reliability, and was run on three separate days. A lymphocyte concentration of  $2 \times 10^6 \cdot \text{mL}^{-1}$  was used per subject per assay. The percentage of cells positive for CD25 and CD25 density was measured with mAb anti-CD25/PE; percentage of cells positive for CD71 and CD71 density was measured with mAb anti-CD71/FITC. 5000 cells per sample were analysed by flow cytometry. A stock PHA solution (5mg dissolved in 1 mL PBS) was diluted five fold (20 $\mu\text{L}$  in 80 $\mu\text{L}$  PBS, equalling 100 $\mu\text{g}$  PHA in 100 $\mu\text{L}$  PBS). 200 $\mu\text{L}$  of this working solution provided five volumes of PHA solution for reproducibility testing ie 100 $\mu\text{g}$  in 100 $\mu\text{L}$ , 50 $\mu\text{g}$  in 50 $\mu\text{L}$ , 20 $\mu\text{g}$  in 20 $\mu\text{L}$ , 10 $\mu\text{g}$  in 10 $\mu\text{L}$ , 5 $\mu\text{g}$  in 5 $\mu\text{L}$ . These volumes of PHA in PBS were added to the lymphocyte concentration prior to incubation. Sample tubes were C – control, no PHA; T – test sample, PHA added.

Table 5: Percentage of CD4<sup>+</sup> Positive for CD25 and CD71 and Receptor Density (MLF) with PHA Concentrations Incubated 24 Hours (mean± SD, n = 5)

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	0.2±0	0.2±0	0.0	1.1±0	0.1±0	1.6±0.2	0.1±0	0.0	0.1±0	0.0
Q1MLF	0.0	0.6±0.2	0.0	0.0	0.0	0.3±0	0.0	0.0	0.4±0	0.0
Q2%	0.2±0	0.2±0	0.2±0	0.0	0.1±0	0.1±0	0.2±0	0.2±0	0.2±0	0.2±0
Q2MLF	0.0	1.9±0.6	0.0	0.0	1.3±0.5	1.1±0.2	1.7±0.5	6.3±1	13±0.4	6.4±1
Q3%	1.4±0.5	8.1±1	1.8±0.5	2.4±0.6	0.1±0	1.1±0.4	1.3±0.4	3.7±0.5	1.9±0	4.9±1
Q3MLF	0.1±0	0.1±0	0.1±0	0.1±0	1.3±0.5	0.1±0	0.2±0	0.2±0	0.1±0	0.2±0
Q4%	0.2±0	1.5±0.2	0.0	1.1±0	0.1±0	0.1±0	1.2±0.2	47.1±3	0.2±0	54.9±4
Q4MLF	0.0	2.8±0.8	0.0	0.0	0.0	1.0±0	1.3±0.2	5.2±0.8	2.1±0.3	7.0±1.2

Q – Quadrant (1-4); % - % of sample cells positive; MLF – mean channel log fluorescence (intensity); CD25<sup>+</sup> /PE appears in Q4, CD71<sup>+</sup>/FITC in Q1, Double positive in Q2, cell debris/autofluorescence in Q3

Table 6: Coefficient of Variation (%) - PHA Concentrations Incubated 24 Hours

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	0.0	0.0	0.0	0.0	0.0	12.5	0.0	0.0	0.0	0.0
Q1MLF	0.0	33.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q2%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q2MLF	0.0	31.6	0.0	0.0	38.5	18.2	29.4	15.9	3.1	15.6
Q3%	35.7	12.3	27.8	25.0	0.0	36.4	30.8	13.5	0.0	20.4
Q3MLF	0.0	0.0	0.0	0.0	38.5	0.0	0.0	0.0	0.0	0.0
Q4%	0.0	13.3	0.0	0.0	0.0	0.0	16.7	6.4	0.0	7.3
Q4MLF	0.0	28.6	0.0	0.0	0.0	0.0	15.4	15.4	14.3	17.1

Table 7: Percentage of CD4<sup>+</sup> Positive for CD25 and CD71 and Receptor Density (MLF) with PHA Concentrations Incubated 48 Hours (mean  $\pm$  SD, n = 5)

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	0.5 $\pm$ 0	0.5 $\pm$ 0	0.9 $\pm$ 0.5	2.5 $\pm$ 0.5	1.2 $\pm$ 0	6.5 $\pm$ 0.2	0.5 $\pm$ 0	7.0 $\pm$ 0.8	0.2 $\pm$ 0	3.0 $\pm$ 0.5
Q1MLF	0.2 $\pm$ 0	0.2 $\pm$ 0	0.3 $\pm$ 0	0.2 $\pm$ 0	0.3 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.3 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0
Q2%	1.0 $\pm$ 0	2.0 $\pm$ 0.3	0.4 $\pm$ 0	2.0 $\pm$ 0.8	0.7 $\pm$ 0	1.7 $\pm$ 0.2	0.4 $\pm$ 0	2.2 $\pm$ 0.5	0.3 $\pm$ 0	0.1 $\pm$ 0
Q2MLF	2.8 $\pm$ 0	2.9 $\pm$ 0.5	2.9 $\pm$ 0.4	2.8 $\pm$ 0.4	2.8 $\pm$ 0.3	2.6 $\pm$ 0.3	2.1 $\pm$ 0	2.8 $\pm$ 0	2.1 $\pm$ 0	2.5 $\pm$ 0
Q3%	7.0 $\pm$ 0.5	4.1 $\pm$ 0.1	3.6 $\pm$ 0.1	6.9 $\pm$ 0.5	3.0 $\pm$ 0	3.8 $\pm$ 0.3	2.0 $\pm$ 0.1	7.2 $\pm$ 0.5	6.8 $\pm$ 0.4	1.8 $\pm$ 0
Q3MLF	0.1 $\pm$ 0									
Q4%	2.2 $\pm$ 0	66.0 $\pm$ 6	1.8 $\pm$ 0	56.0 $\pm$ 3	1.0 $\pm$ 0	70.0 $\pm$ 2	1.3 $\pm$ 0	85.0 $\pm$ 4	0.4 $\pm$ 0	76.0 $\pm$ 3
Q4MLF	1.5 $\pm$ 0.1	2.3 $\pm$ 0.5	1.4 $\pm$ 0.1	2.9 $\pm$ 0.2	1.2 $\pm$ 0.2	2.2 $\pm$ 0.2	1.2 $\pm$ 0.1	4.9 $\pm$ 0.3	1.1 $\pm$ 0.1	4.1 $\pm$ 0.2

Q – Quadrant (1-4); % - % of sample cells positive; MLF – mean channel log fluorescence (intensity) ; CD25<sup>+</sup>/PE appears in Q4, CD71<sup>+</sup>/FITC in Q1, Double positive in Q2, cell debris/autofluorescence in Q3

Table 8: Coefficient of Variation (%) - PHA Concentrations Incubated 48 Hours

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	0.0	0.0	55.6	20.0	0.0	10.1	0.0	9.4	0.0	16.7
Q1MLF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q2%	0.0	15.0	0.0	40.0	0.0	11.7	0.0	22.7	0.0	0.0
Q2MLF	0.0	17.2	13.8	14.3	10.7	11.5	0.0	0.0	0.0	0.0
Q3%	7.1	2.4	2.8	7.3	0.0	7.9	5.0	6.9	5.9	0.0
Q3MLF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q4%	0.0	4.0	0.0	5.4	0.0	3.9	0.0	3.7	0.0	3.9
Q4MLF	6.7	21.7	7.1	6.9	16.7	9.1	8.3	6.1	9.1	4.9

Table 9: Percentage of CD4<sup>+</sup> Positive for CD25 and CD71 and Receptor Density (MLF) with PHA Concentrations Incubated 72 Hours (mean  $\pm$  SD, n = 5)

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	1.6 $\pm$ 0.1	4.2 $\pm$ 0.2	1.1 $\pm$ 0.1	6.6 $\pm$ 0.2	3.1 $\pm$ 0.2	6.6 $\pm$ 0.2	0.5 $\pm$ 0	5.9 $\pm$ 0.2	0.2 $\pm$ 0.1	2.8 $\pm$ 0.2
Q1MLF	0.2 $\pm$ 0	0.2 $\pm$ 0	0.3 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0
Q2%	1.0 $\pm$ 0.3	3.2 $\pm$ 0.2	0.8 $\pm$ 0.1	3.3 $\pm$ 0.1	0.9 $\pm$ 0.2	3.4 $\pm$ 0.2	0.8 $\pm$ 0.5	4.2 $\pm$ 0.2	0.8 $\pm$ 0.5	1.1 $\pm$ 0.3
Q2MLF	2.9 $\pm$ 0.8	3.0 $\pm$ 0.8	2.8 $\pm$ 0.6	3.0 $\pm$ 1	2.8 $\pm$ 0.5	3.0 $\pm$ 0.6	2.6 $\pm$ 0.8	3.0 $\pm$ 0.8	2.8 $\pm$ 0.5	2.9 $\pm$ 0.5
Q3%	6.0 $\pm$ 0.5	5.0 $\pm$ 0.5	5.4 $\pm$ 0.5	8.3 $\pm$ 0.6	5.5 $\pm$ 0.5	5.2 $\pm$ 0.5	6.2 $\pm$ 0.5	6.3 $\pm$ 0.5	6.8 $\pm$ 0.6	6.4 $\pm$ 0.5
Q3MLF	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0
Q4%	1.8 $\pm$ 1	0.1 $\pm$ 0	0.0	14.0 $\pm$ 1.2	0.1 $\pm$ 0	21.0 $\pm$ 2	1.1 $\pm$ 0.1	65.0 $\pm$ 3	0.8 $\pm$ 0.1	9.6 $\pm$ 0.8
Q4MLF	2.6 $\pm$ 0.5	0.1 $\pm$ 0	0.0	3.6 $\pm$ 0.5	2.7 $\pm$ 0.4	3.4 $\pm$ 0.8	2.5 $\pm$ 0.2	3.7 $\pm$ 0.6	1.3 $\pm$ 0.2	2.4 $\pm$ 0.3

Q – Quadrant (1-4); % - % of sample cells positive; MLF – mean channel log fluorescence (intensity); CD25<sup>+</sup>/PE appears in Q4, CD71<sup>+</sup>/FITC in Q1, Double positive in Q2, cell debris/autofluorescence in Q3

Table 10: Coefficient of Variation (%) - PHA Concentrations Incubated 72 Hours

	C100 $\mu$ L	T100 $\mu$ L	C50 $\mu$ L	T50 $\mu$ L	C20 $\mu$ L	T20 $\mu$ L	C10 $\mu$ L	T10 $\mu$ L	C5 $\mu$ L	T5 $\mu$ L
Q1%	6.3	4.8	9.1	3.0	6.5	3.0	0.0	3.4	50.0	7.1
Q1MLF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q2%	30.0	8.3	12.5	3.0	22.2	5.9	62.5	4.8	62.5	27.3
Q2MLF	27.6	26.7	21.4	33.3	17.9	20.0	26.7	26.7	17.9	17.2
Q3%	8.3	10.0	9.3	7.2	9.1	9.6	8.1	7.9	8.8	7.8
Q3MLF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q4%	55.6	0.0	0.0	8.6	0.0	9.5	9.1	4.6	12.5	8.3
Q4MLF	19.2	0.0	0.0	13.9	14.8	23.5	8.0	16.2	15.4	12.5

#### Conclusion:

The proliferative response of CD4<sup>+</sup> lymphocytes to PHA concentrations varying from 100 to 5  $\mu$ L was assessed by the percentage of lymphocytes positive for CD25<sup>+</sup> (Q4%) and CD71<sup>+</sup> (Q1%), after incubation for 24, 48 and 72 hours. The PHA concentration of 10 $\mu$ L (10 $\mu$ g), showed the lowest CV in three assays incubated for 24, 48 and 72 hours (Q4% - 6.4%, 3.7% and 4.6% respectively; Q1% - 0%, 9.4% and 3.4% respectively). A concentration of 10 $\mu$ L of PHA was considered optimal for the proliferative studies and used for Experiments 1 and 2.

### 3.3.2. Optimal Incubation Time for PHA Concentration 10 $\mu$ g in 10 $\mu$ L of PBS.

A proliferative assay using a PHA concentration of 10 $\mu$ g in 10 $\mu$ L of PBS was performed in triplicate on three separate days to determine the optimal incubation time for CD4<sup>+</sup> lymphocytes. The assay also determined test-re-test reliability and reproducibility. The lymphocyte concentration used was 2 x 10<sup>6</sup>.mL<sup>-1</sup> per subject per assay. Samples were incubated for 24, 48 and 72 hours at 37°C. 5000 cells per sample were analysed by flow cytometry.

Table 11: Proliferation Assay for CD4<sup>+</sup> - Incubation for 24, 48 and 72 Hours.

	24hrs		48hrs		72hrs	
	C	T	C	T	C	T
Q1%	0.2 $\pm$ 0	3.3 $\pm$ 1	0.3 $\pm$ 0	3.7 $\pm$ 0.4	0.1 $\pm$ 0	0.2 $\pm$ 0
Q1MLF	0.5 $\pm$ 0.2	0.4 $\pm$ 0	0.7 $\pm$ 0.2	1.2 $\pm$ 0.2	0.5 $\pm$ 0.2	0.8 $\pm$ 0.2
Q2%	0.3 $\pm$ 0.2	22.0 $\pm$ 4	1.0 $\pm$ 0.2	26.6 $\pm$ 4	1.0 $\pm$ 0.4	4.0 $\pm$ 1
Q2MLF	1.7 $\pm$ 0.5	8.5 $\pm$ 0.2	2.6 $\pm$ 0.6	12.4 $\pm$ 2	1.4 $\pm$ 0.4	8.0 $\pm$ 4
Q3%	87.0 $\pm$ 1.4	8.0 $\pm$ 2	94.7 $\pm$ 1	4.0 $\pm$ 0.8	93.6 $\pm$ 0.8	5.2 $\pm$ 0.8
Q3MLF	0.1 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.2 $\pm$ 0	0.3 $\pm$ 0	0.3 $\pm$ 0.02
Q4%	0.5 $\pm$ 0.2	68.0 $\pm$ 5	2.0 $\pm$ 0.2	70.0 $\pm$ 3	0.8 $\pm$ 0.2	58.9 $\pm$ 6
Q4MLF	1.6 $\pm$ 0.2	4.5 $\pm$ 0.3	1.6 $\pm$ 0.2	6.8 $\pm$ 0.4	1.7 $\pm$ 0.5	4.2 $\pm$ 0.2

Control, no PHA (C); Test sample, 10  $\mu$ L PHA added. (mean  $\pm$  SD, n = 5)

Q – Quadrant (1-4); % - % of sample cells positive; MLF – mean channel log fluorescence (intensity); CD25+/PE appears in Q4, CD71+/FITC in Q1, Double positive in Q2, cell debris/autofluorescence in Q3

Table 12: Coefficient of Variation (%) for Proliferation Assay for CD4<sup>+</sup> - Incubation for 24, 48 and 72 Hours.

	24hrs		48hrs		72hrs	
	C	T	C	T	C	T
Q1%	0.0	30.0	0.0	10.0	0.0	0.0
Q1MLF	40.0	0.0	28.6	16.7	28.6	25.0
Q2%	66.6	18.2	20.0	15.0	40.0	25.0
Q2MLF	29.4	23.5	23.0	16.1	28.6	50.0
Q3%	1.6	25.0	1.0	20.0	0.8	15.4
Q3MLF	0.0	0.0	0.0	0.0	0.0	6.7
Q4%	40.0	7.4	10.0	3.3	25.0	4.3
Q4MLF	12.5	6.7	12.5	5.9	29.4	4.8

Conclusion:

The optimal incubation time for CD4<sup>+</sup> lymphocyte proliferation was found by measuring CD25<sup>+</sup> and CD71<sup>+</sup> proliferative responses to 10  $\mu$ L of PHA. The incubation period with the lowest CV for CD25<sup>+</sup> (Q4%) and CD71<sup>+</sup> (Q1%) was found to be 48 hours (CV of 3.3% and 10% respectively). An optimal incubation period of 48 hours was used for Experiments 1 and 2.

**3.3.3. Reliability and Reproducibility Assay - Saturation Curves for Monoclonal Antibodies anti-CD25 PE and anti-CD71 FITC** (Coulter Epics Profile II Flow Cytometer, Griffith University).

The saturation curves for anti-CD25 PE and anti-CD71 FITC were performed in triplicate on three separate days to determine test-re-test reliability and reproducibility. The assay was performed to ensure that lymphocytes would be fully saturated with dye during incubation. Full saturation was indicated by a steady-state of fluorescence despite an increase in the volume of monoclonal antibody. A lymphocyte concentration of  $2 \times 10^6$ .mL<sup>-1</sup> per subject was used for each assay, with 5000 cells per sample analysed by flow cytometry. Samples were incubated for 48 hours at 37°C.

Table 13: Saturation Curves for mAb anti-CD25 and anti-CD71 (mean  $\pm$  SD, n = 5)

	C1	C2	M	M1	M2	M5	M10	M15
Q1%	0.0	0.0	0.5 $\pm$ 0.05	0.7 $\pm$ 0.05	1.2 $\pm$ 0.05	2.1 $\pm$ 0.4	2.3 $\pm$ 0.3	2.5 $\pm$ 0.4
Q1MLF	0.0	0.0	0.5 $\pm$ 0	0.5 $\pm$ 0	0.6 $\pm$ 0.02	0.7 $\pm$ 0.05	0.7 $\pm$ 0.05	0.7 $\pm$ 0.04
Q2%	0.0	0.0	0.0	0.1 $\pm$ 0	0.5 $\pm$ 0	1.0 $\pm$ 0.04	1.1 $\pm$ 0.04	1.0 $\pm$ 0.04
Q2MLF	0.0	0.0	0.2 $\pm$ 0	0.2 $\pm$ 0	2.6 $\pm$ 0.4	2.7 $\pm$ 0.5	2.7 $\pm$ 0.6	2.6 $\pm$ 0.4
Q3%	98.8 $\pm$ 1	98.6 $\pm$ 1	99.2 $\pm$ 0.5	92.8 $\pm$ 2	80.0 $\pm$ 2	3.6 $\pm$ 0.6	8.8 $\pm$ 1.1	4.4 $\pm$ 0.8
Q3MLF	0.1 $\pm$ 0	0.1 $\pm$ 0	0.1 $\pm$ 0	0.2 $\pm$ 0				
Q4%	0.0	0.0	0.0	2.7 $\pm$ 0.6	18.0 $\pm$ 5	96.2 $\pm$ 2	94.5 $\pm$ 1	96.0 $\pm$ 2
Q4MLF	0.0	0.0	3.0 $\pm$ 0	4.9 $\pm$ 0.8	5.6 $\pm$ 0.6	6.3 $\pm$ 0.4	5.9 $\pm$ 0.8	6.1 $\pm$ 0.9

C1 – control sample, no PHA, no mAb ; C2 – control sample, PHA added, no mAb; M – mAb (5  $\mu$ L), no PHA; M1 – M15 – mAb added 1 to 15  $\mu$ L, PHA added.

Q – Quadrant (1-4); % - % of sample cells positive; MLF – mean channel log fluorescence (intensity); CD25+/PE appears in Q4, CD71+/FITC in Q1, Double positive in Q2, cell debris/autofluorescence in Q3

Table 14: Coefficient of Variation (%) for mAb Saturation Curves

	C1	C2	M	M1	M2	M5	M10	M15
Q1%	0.0	0.0	10.0	9.1	4.2	9.0	13.0	16.0
Q1MLF	0.0	0.0	0.0	0.0	3.3	7.1	7.1	7.7
Q2%	0.0	0.0	0.0	0.0	0.0	4.0	3.6	4.0
Q2MLF	0.0	0.0	0.0	0.0	15.4	18.5	22.2	15.4
Q3%	1.0	1.0	0.5	2.2	2.5	16.7	12.5	0.0
Q3MLF	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Q4%	0.0	0.0	0.0	22.2	27.8	2.1	2.2	2.1
Q4MLF	0.0	0.0	0.0	16.3	10.7	6.6	8.5	7.9

**Conclusion:**

Full saturation of the lymphocytes by monoclonal antibody was indicated by a steady-state of fluorescence in Q4MLF and Q1MLF, and a steady state of positive cells in Q4% and Q1%, despite an increase in the added volume of monoclonal antibody. The optimal concentration of mAb was considered to be the concentration showing full saturation (high percent of CD4<sup>+</sup> positive for CD25<sup>+</sup> and CD71<sup>+</sup>, and steady state fluorescence), and the lowest CV. The optimal concentration of mAb was found to be 5  $\mu$ L (M5) with a CV of 9% and 7.1% for Q1% and Q1MLF respectively, and a CV of 2.1% and 6.6% for Q4% and Q4MLF.

**3.3.4. Intra-assay Coefficient of Variation. Saturation Curves for Fluo-3 AM**

(Coulter Epics Profile II Flow Cytometer, Griffith University). The saturation curves for Fluo-3 AM were performed in triplicate to determine test-re-test reliability, thus ensuring that lymphocytes would be fully saturated with the probe during incubation. Full saturation was indicated by a steady-state of fluorescence despite an increase in the volume of Fluo-3. A lymphocyte concentration of  $2 \times 10^6 \cdot \text{mL}^{-1}$  per subject was used for each assay. 5000 cells were analysed per sample. Samples were incubated at 22°C for 45 minutes.

Table 15: Saturation Curve Run 1 (Mean Channel Log Fluorescence).

Sample	C0 $\mu$ L	P5 $\mu$ L	T5	P10 $\mu$ L	T10	P18 $\mu$ L	T18	P24 $\mu$ L	T24	P28 $\mu$ L	T28
1	1.4	8.8	10.3	10.2	16.0	12.9	24.5	10.6	27.5	14.8	29.9
2	1.3	8.2	10.5	10.2	16.2	12.8	24.2	10.8	25.5	14.3	24.9
3	1.4	8.6	10.7	10.4	15.8	13.3	24.0	10.5	25.3	10.9	25.8
4	1.3	8.7	10.2	11.0	16.1	13.1	24.6	11.0	26.1	10.6	28.0
5	1.2	8.6	10.9	10.1	15.4	12.7	24.4	11.1	25.9	11.2	25.8
CV(%)	6.2	2.3	2.9	3.9	1.9	1.5	0.8	2.8	3.4	16.1	7.4

Control (C), Probe Added (P), Thapsigargin (6 $\mu$ L) Added (T)

Table 16: Saturation Curve Run 2 (Mean Channel Log Fluorescence).

Sample	C0 $\mu$ L	P5 $\mu$ L	T5	P10 $\mu$ L	T10	P18 $\mu$ L	T18	P24 $\mu$ L	T24	P28 $\mu$ L	T28
1	0.8	8.4	10.6	10.8	16.6	11.7	25.0	11.6	25.4	12.6	26.9
2	1.1	8.0	10.9	10.1	16.1	11.9	25.6	11.0	24.9	12.3	25.8
3	1.2	8.2	10.4	10.5	16.4	12.3	26.1	11.5	25.1	12.5	27.8
4	0.9	8.3	10.8	10.7	16.2	12.8	25.9	10.7	26.5	11.9	27.1
5	1.0	8.4	10.7	10.9	15.9	12.1	26.4	11.3	26.1	11.8	26.1
CV(%)	20.0	2.4	1.9	2.8	1.9	3.3	1.9	3.6	2.7	3.3	3.0

Control (C), Probe Added (P), Thapsigargin (6 $\mu$ L) Added (T)

Table 17: Saturation Curve Run 3 (Mean Channel Log Fluorescence).

Sample	C0 $\mu$ L	P5 $\mu$ L	T5	P10 $\mu$ L	T10	P18 $\mu$ L	T18	P24 $\mu$ L	T24	P28 $\mu$ L	T28
1	0.8	8.0	10.2	10.3	16.1	11.9	24.7	10.6	25.9	13.0	27.0
2	0.7	8.2	10.5	10.2	16.0	11.8	24.6	11.1	25.8	11.9	25.9
3	0.7	7.9	10.3	10.4	15.8	12.0	25.0	10.8	25.4	13.3	25.8
4	0.8	8.0	10.2	10.5	15.5	12.3	25.6	11.0	27.0	12.0	26.9
5	0.7	8.1	10.5	10.2	15.4	12.6	25.1	11.1	25.7	12.5	25.5
CV(%)	7.1	1.2	1.9	0.9	2.1	2.5	1.6	1.8	2.3	4.9	2.6

Control (C), Probe Added (P), Thapsigargin (6 $\mu$ L) Added (T)

Inter-assay reproducibility was determined by coefficient of variation (Vincent 1999).

Conclusion:

The volume of Fluo-3 found to have full saturation and a consistently lower CV for the 3 saturation curves was 18  $\mu\text{L}$  (P18), with CV's of 1.5%, 3.3% and 2.5% respectively. The same volume with thapsigargin added (T18) also showed the consistently lowest CV of 0.8%, 1.9% and 1.6% respectively. The volume of Fluo-3 AM with the lowest CV and full saturation was 18 $\mu\text{L}$ , which was used in Experiments 1 and 2.

**3.3.5. Intra-assay Coefficient of Variation. Saturation Curves for Calcein AM** (Coulter Epics Profile II Flow Cytometer, Griffith University). The saturation curves for Calcein AM were performed in duplicate to determine test-re-test reliability. The assay was performed to ensure that lymphocytes would be fully saturated with probe during incubation. Full saturation was indicated by a steady-state of fluorescence despite an increase in the volume of Calcein AM. A lymphocyte concentration of  $2 \times 10^6 \cdot \text{mL}^{-1}$  per subject was used for each assay, with 5000 cells per sample analysed by flow cytometry. Samples were incubated for 5 minutes at 37°C.

Table 18: Saturation Curve Run 1 (Mean Channel Log Fluorescence).

Sample #	C0 $\mu\text{L}$	P1 $\mu\text{L}$	P3 $\mu\text{L}$	P5 $\mu\text{L}$	P10 $\mu\text{L}$	P15 $\mu\text{L}$
1	1.6	4.9	76.4	773.0	763.3	739.0
2	1.5	5.4	79.2	717.0	639.1	844.1
3	1.6	6.8	82.7	709.6	709.6	929.4
4	1.7	4.3	74.3	303.1	694.5	750.3
5	1.6	8.1	77.7	597.0	758.7	940.4
CV(%)	4.4	25.4	4.1	30.4	7.2	11.3

C – Control sample, no probe added ; P – Calcein AM added ( $\mu\text{L}$ )

Table 19: Saturation Curve Run 2 (Mean Channel Log Fluorescence).

Sample #	<b>C0<math>\mu</math>L</b>	<b>P1<math>\mu</math>L</b>	<b>P3<math>\mu</math>L</b>	<b>P5<math>\mu</math>L</b>	<b>P10<math>\mu</math>L</b>	<b>P15<math>\mu</math>L</b>
1	1.6	3.8	66.9	271.7	802.4	844.4
2	1.6	4.7	69.5	240.6	665.0	750.2
3	1.0	2.9	63.8	388.9	799.2	900.4
4	1.0	6.0	68.3	450.1	866.3	891.0
5	1.2	4.2	67.4	506.3	558.9	704.8
CV(%)	23.1	27.9	3.1	30.6	16.8	10.6

C – Control sample, no probe added ; P – Calcein AM added ( $\mu$ L)

Inter-assay reproducibility was determined by coefficient of variation (Vincent 1999)

Conclusion:

The volume of Calcein AM with the lowest CV but with consistent saturation was 3 $\mu$ L, and this volume was used in Experiments 1 and 2. Note that Calcein AM shows decreasing fluorescence when bound to Fe<sup>3+</sup>. High mean channel log fluorescence indicates an excess of probe.

## 4.0 Results

### 4.1 Experiment 1 - Males and Females Aged 65 to 75 Years

#### 4.1.1. General Health

During twelve months of aerobic training, the body mass and blood pressure of the Trained (TR) males and females were monitored at each training session. The pre- and post-study mean body mass and percent body fat for males and females are shown in Table 20.

TABLE 20: Body Mass and Percent Body Fat of Participating Subjects Pre- (0 months) and Post-Study (12 months)

	Untrained Males	Trained Males	Untrained Females	Trained Females
<b>Body Mass (kg)</b>				
Pre-study	82 ± 12	86 ± 12	59 ± 10	67 ± 11*
Post-study	83 ± 12	84 ± 12	59 ± 10	65 ± 10
<b>Body Fat (%)</b>				
Pre-study	26 ± 2	25 ± 1	35 ± 1	35 ± 1
Post-study	27 ± 2	24 ± 1#*	36 ± 1	34 ± 1#*

\* Significantly different from untrained group ( $p < 0.05$ ), mean±SEM

# Significantly different from pre-training ( $p < 0.001$ ), mean±SEM

While both male and female trained groups showed similar decreases in body mass over the twelve month period, there were no significant differences in pre- and post-study body mass for any of the groups. Initially there was a significant difference in body mass between the female UT and TR groups ( $p = 0.03$ ). There were no significant pre-study differences in percent body fat between male UT and TR, and female UT and TR. Percent body fat for both UT and TR male groups was significantly lower than both female groups ( $p < 0.001$ ) pre- and post-study. The male and female TR groups showed a significant reduction in percent body fat post-study ( $p < 0.001$ ), and both male and female TR groups showed significantly lower percent body fat than the male and female UT groups post-study ( $p < 0.05$ ).

#### 4.1.2 Blood Pressure

The systolic and diastolic blood pressures for UT and TR men and women were within the normal ranges (<140/90 mm Hg), for the duration of the training study. The TR groups had blood pressures measured at each training session, and the resting blood pressure measurement at the end of each month was used as the monthly average. The UT group had their blood pressures measured pre-study and post-study.

The mean pre-study systolic blood pressure was significantly higher for TR females ( $133 \pm 3$ ) compared to TR males ( $124 \pm 3$ ,  $p = 0.03$ ) in January only. Diastolic blood pressure was not significantly different between TR males and females. Although the mean systolic blood pressures of both the male and female TR groups post-study were lower than pre-study, they were not significantly so (male TR  $121 \pm 4$ , female TR  $129 \pm 4$ ). Blood pressures for the UT groups did not change significantly pre- and post-study. There were no significant differences between groups or over time, for male or female UT/TR groups, with regard to diastolic blood pressures.

#### 4.1.3. Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ )

The  $\dot{V}O_{2\text{ peak}}$  was measured pre- and post-study for UT and TR, male and female groups. There were no significant differences in  $\dot{V}O_{2\text{ peak}}$  between male UT and TR groups, and female UT and TR groups pre-study. Peak oxygen uptake, peak power, peak heart rate, peak ventilation and peak RER values attained in the incremental exercise test are shown in Table 21.

TABLE 21: Pre- and Post-Study Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ ) for Untrained and Trained Groups

Variables	<u>UT Male</u>		<u>TR Male</u>		<u>UT Female</u>		<u>TR Female</u>	
	Pre	Post	Pre	Post	Pre	Post	Pre	Post
$\dot{V}O_{2\text{ peak}}$ (L.min <sup>-1</sup> )	2.0 ± 0.01	1.9 ± 0.01	2.1 ± 0.1	2.4 ± 0.1* #	1.2 ± 0.04	1.04 ± 0.1*	1.2 ± 0.01	1.4 ± 0.01*#
$\dot{V}O_{2\text{ peak}}$ (mL.kg <sup>-1</sup> .min <sup>-1</sup> )	25 ± 2	24 ± 2	25 ± 1	29 ± 1*#	20 ± 1	18 ± 1	18 ± 1	23 ± 1*#
Power <sub>peak</sub> (W)	156 ± 5	154 ± 5	160 ± 7	186 ± 8*#	86 ± 3	83 ± 2*	89 ± 4	107 ± 4*#
Heart rate <sub>peak</sub> (b.min <sup>-1</sup> )	156 ± 4	154 ± 5*	149 ± 4	152 ± 4*	153 ± 2	149 ± 6	147 ± 7	158 ± 6
$\dot{V}_E\text{ peak}$ BTPS (L.min <sup>-1</sup> )	85 ± 5	84 ± 6	84 ± 5	99 ± 7*#	51 ± 3	46 ± 3*	52 ± 4	58 ± 3*#
RER <sub>peak</sub>	1.19 ± 0.1	1.19 ± 0.2	1.17 ± 0.2	1.17 ± 0.1	1.18 ± 0.2	1.16 ± 0.2	1.19 ± 0.1	1.17 ± 0.1

\* Post- training value significantly different from Pre-training value ( $p < 0.05$ )

# Trained group significantly different from Untrained group ( $p < 0.05$ )

Data presented as mean ± SEM; UT, untrained; TR, trained

- *Untrained males* - There were no significant differences between mean pre- and post-study peak oxygen uptake (L.min<sup>-1</sup> and mL.kg<sup>-1</sup>.min<sup>-1</sup>), peak power, peak ventilation or peak RER. There was a significant decrease in peak heart rate after twelve months ( $p < 0.05$ ).
- *Trained males* - After 12 months of training, there were significant increases in mean peak oxygen uptake in both L.min<sup>-1</sup> and mL.kg<sup>-1</sup>.min<sup>-1</sup> ( $p < 0.001$ ), peak power ( $p < 0.001$ ), peak heart rate ( $p < 0.05$ ) and peak ventilation ( $p < 0.05$ ). There was no significant change in peak RER after 12 months of training.
- *Untrained females* - There were no significant differences between mean pre- and post-study peak heart rate and peak RER. The peak oxygen uptake (L.min<sup>-1</sup>), peak ventilation and peak power showed significant decreases over the 12 months of the study ( $p < 0.05$ ).

- *Trained females* - After twelve months of training there were significant increases in peak oxygen uptake ( $L \cdot \text{min}^{-1}$  and  $\text{mL} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ ) ( $p < 0.001$ ), peak ventilation ( $p < 0.05$ ) and peak power ( $p < 0.001$ ). There were no significant changes in peak heart rate and peak RER.

#### 4.1.4. Incidence of URTI

Illnesses in UT and TR groups were reported when they occurred. The diagnoses were made by the subjects' medical practitioners. There were no significant differences in the incidence of URTI between any of the four groups. The male TR group reported two cases of URTI and one case of shingles during the twelve months of the study. The male UT group reported four cases of URTI and one case of a systemic infection resulting from a staphylococcus infection. The female TR group reported four cases of URTI. The female UT group reported one case of URTI, one case of bacterial infection (tonsillitis) and one case of unidentified abdominal pain.

#### 4.1.5. Haematology

*Note: Significant differences between TR and UT groups of each gender are marked with an asterisk at the month they occur ( \* ). Significant differences within each group between months of the year are not marked on the figures but are listed in the text preceding the figures.*

*Two blood tests occurred four weeks apart in September, the first designated September A and the second September B.*

All haematological measures remained within normal ranges (Coulter 1984) for the duration of the study, with the exception of male UT haemoglobin during January, which was lower than normal.

##### 4.1.5.1. Leucocytes

The concentration of leucocytes ( $\text{mean} \pm \text{SEM}$ ) for both men and women remained within the normal range ( $4 - 11 \times 10^9 \cdot \text{L}^{-1}$  Coulter 1984) during the twelve month study. The changes in leucocyte concentration for TR and UT male and female groups are shown in Figures 20 A, B.

There were no significant differences in pre-study mean leucocyte concentration (January) between any of the groups. Significant differences in mean leucocyte concentration were found between groups in the following months ( $p < 0.05$ ):

- Male UT was significantly higher than female UT in April ( $p = 0.046$ ), May ( $p = 0.048$ ), September B ( $p = 0.02$ ), October ( $p = 0.047$ ), November ( $p = 0.002$ )
- Male UT was significantly higher than male TR and female TR in May (male TR,  $p = 0.05$ ; female TR,  $p = 0.04$ ), June (male TR,  $p = 0.03$ ; female TR,  $p = 0.03$ ), November (male TR,  $p = 0.045$ ; female TR,  $p = 0.03$ )

Each group showed significant decreases in mean leucocyte concentration between months of the year ( $p < 0.05$ ):

- Male UT – significant decrease between November and December
- Male TR – significant decrease between April and May;
- Female TR – significant decrease between July and August.

Figure 20A: Changes in Leucocyte Concentration in Males Aged 65 - 75 Years

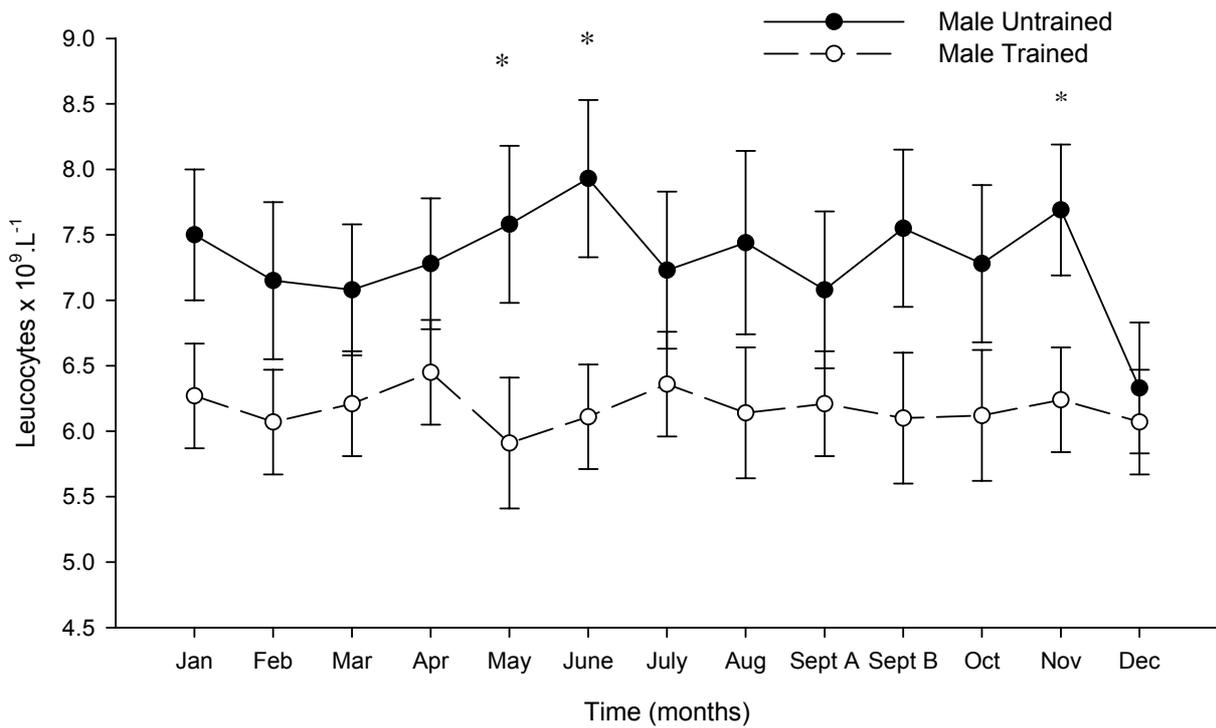
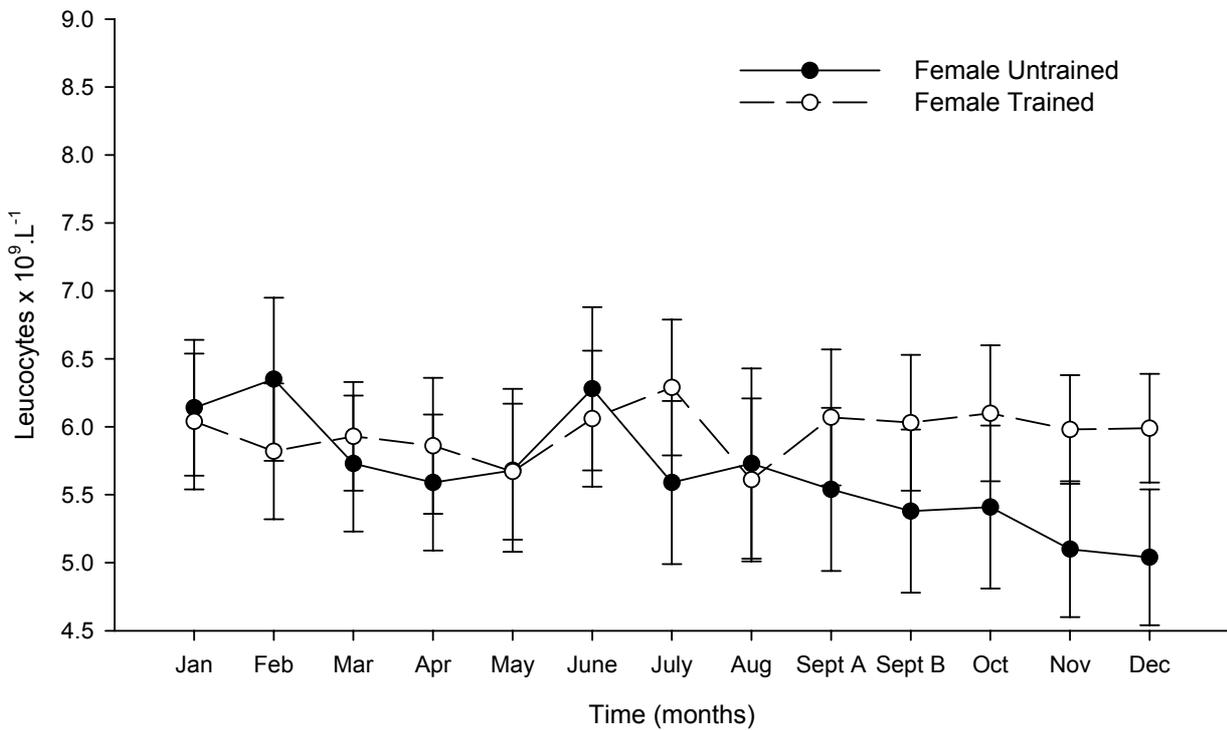


Figure 20B: Changes in Leucocyte Concentration in Females Aged 65 - 75 Years



\* significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM

#### 4.1.5.2. Erythrocytes

The changes in erythrocyte concentration (mean $\pm$ SEM) for both male and female UT and TR groups over the twelve month duration of training are shown in Figures 21 A, B. Erythrocyte concentration remained within the normal range for both male ( $4.7 - 6.1 \times 10^{12}$  cells.L<sup>-1</sup>) and female ( $4.2 - 5.4 \times 10^{12}$  cells.L<sup>-1</sup>) groups throughout the year (Coulter 1984).

The mean erythrocyte concentration in the male UT and TR groups were consistently and significantly higher than female UT and TR groups for all months of the year except June and September B ( $p < 0.05$ ). The male TR group was significantly higher than the female TR group in June ( $p = 0.01$ ). In September B, the male UT group was significantly higher than the female TR group ( $p = 0.03$ ), and the male TR group was significantly higher than the female UT ( $p = 0.018$ ) and TR ( $p = 0.005$ ) groups. Within the male TR and UT groups, and female UT and TR groups, there were no significant differences in mean erythrocyte concentration at each sampling point.

Each group showed significant changes in mean erythrocyte concentration between months of the year ( $p < 0.05$ ):

- Male UT – significant decreases between January and February, May and June, and November and December.
- Male TR – significant decreases between February and March.
- Female UT – significant increases between April and May, and May and June; significant decrease between June and July.
- Female TR – significant decreases between July and August, and September B and October.

Figure 21A: Changes in Erythrocyte Concentration in Males Aged 65 - 75 Years

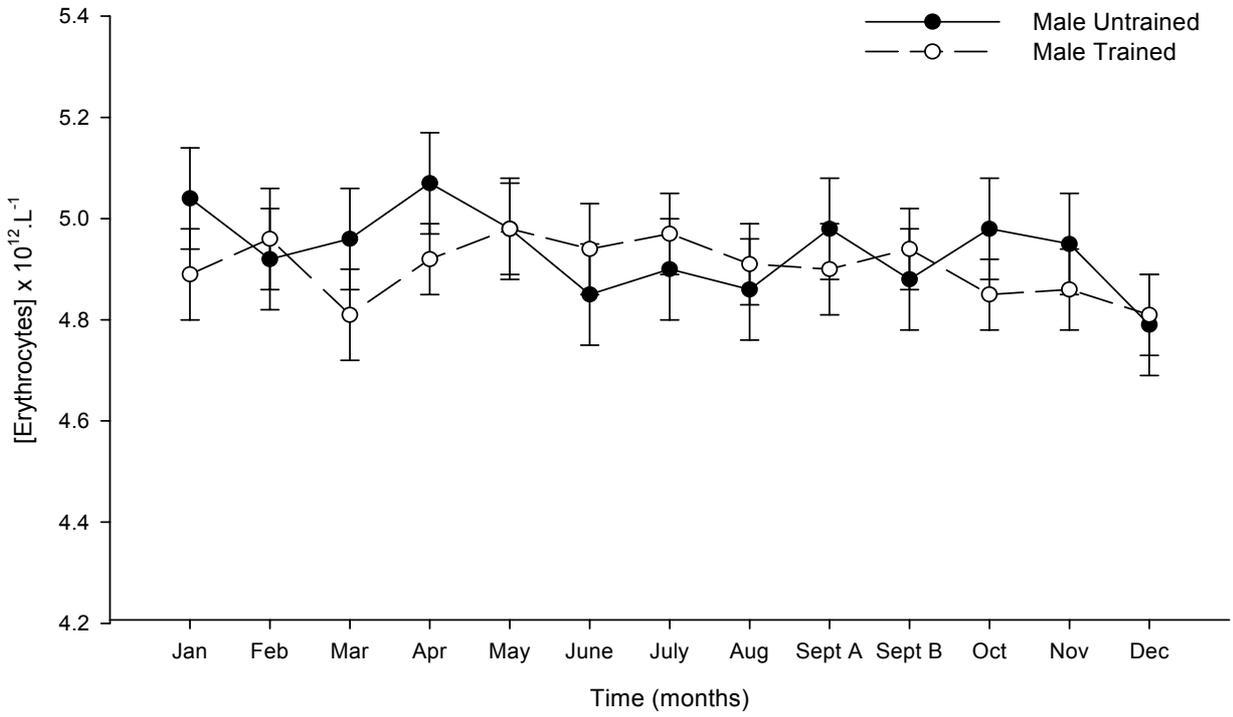
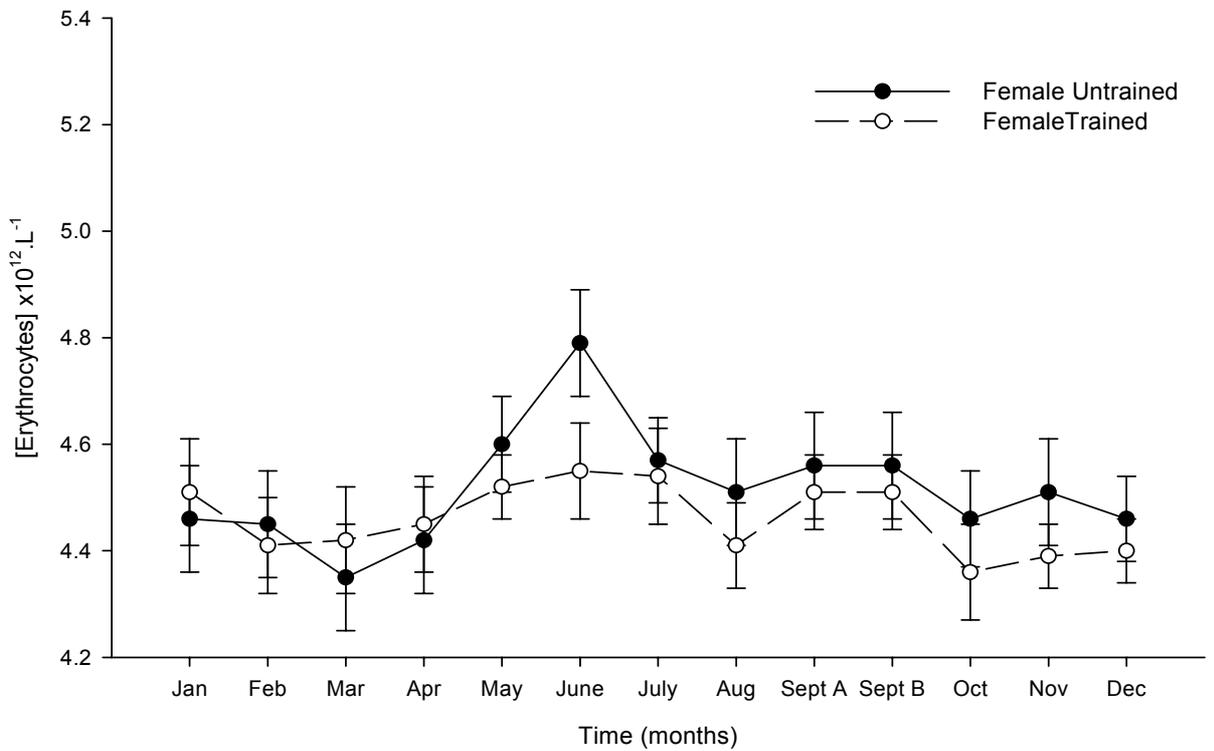


Figure 21B: Changes in Erythrocyte Concentration in Females Aged 65 to 75 Years



Error bars denote SEM

#### 4.1.5.3. Haemoglobin

The changes in haemoglobin concentration (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 22 A, B. The pre-study mean haemoglobin concentration for male TR and female UT and TR were within normal ranges (14 – 18 g.dL<sup>-1</sup> for men and 12 – 16 g.dL<sup>-1</sup> for women). The pre-study mean haemoglobin concentration of the male UT group (13.8 g.dL<sup>-1</sup>) was below the normal range but returned to the normal range in February and for the remainder of the study.

Throughout the twelve months mean haemoglobin concentration for male UT and TR groups was consistently and significantly higher than for female UT and TR groups ( $p < 0.05$ ). There were no significant differences between male UT and TR or female UT and TR groups at any sampling point.

Each group showed significant changes in mean haemoglobin concentration between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between August and September A, September B and October;  
significant decreases between July and August, September A and September B, November and December.
- Male TR – significant increase between August and September A;  
significant decreases between February and March, July and August, October and November.
- Female UT – significant increase between August and September A;  
significant decreases between July and August, September A and September B.
- Female TR – significant decrease between July and August.

Figure 22A: Changes in Haemoglobin Concentration in Males Aged 65 - 75 Years

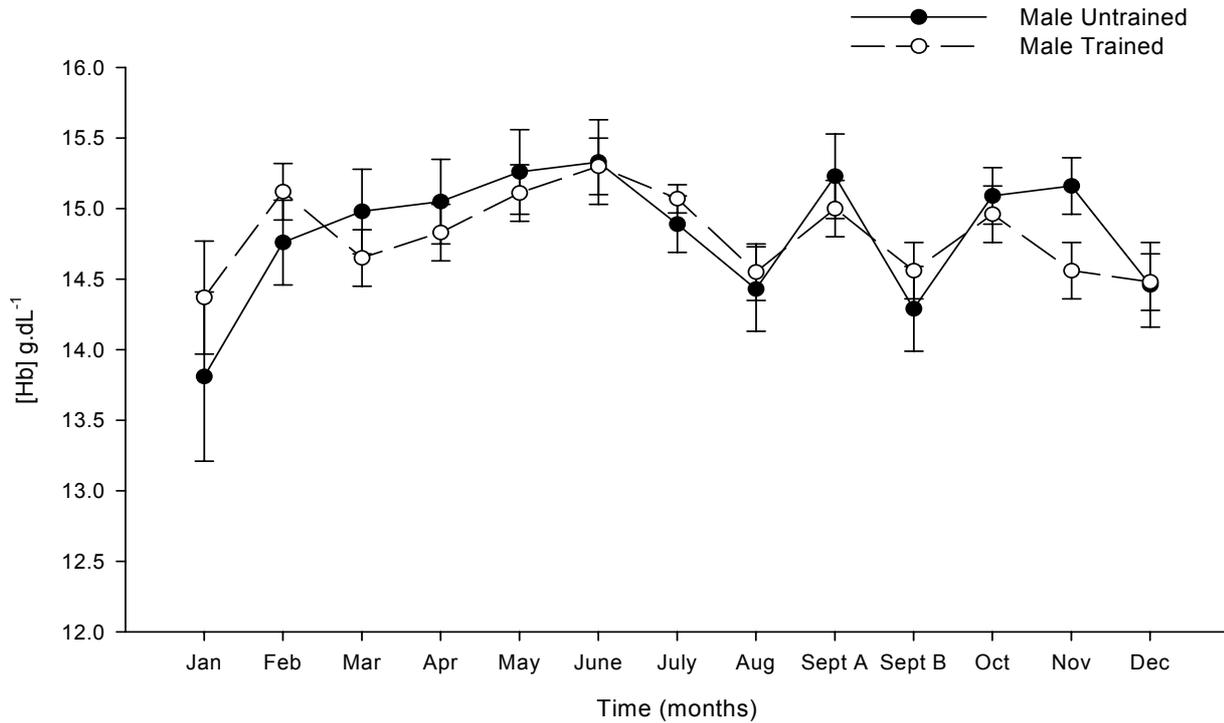
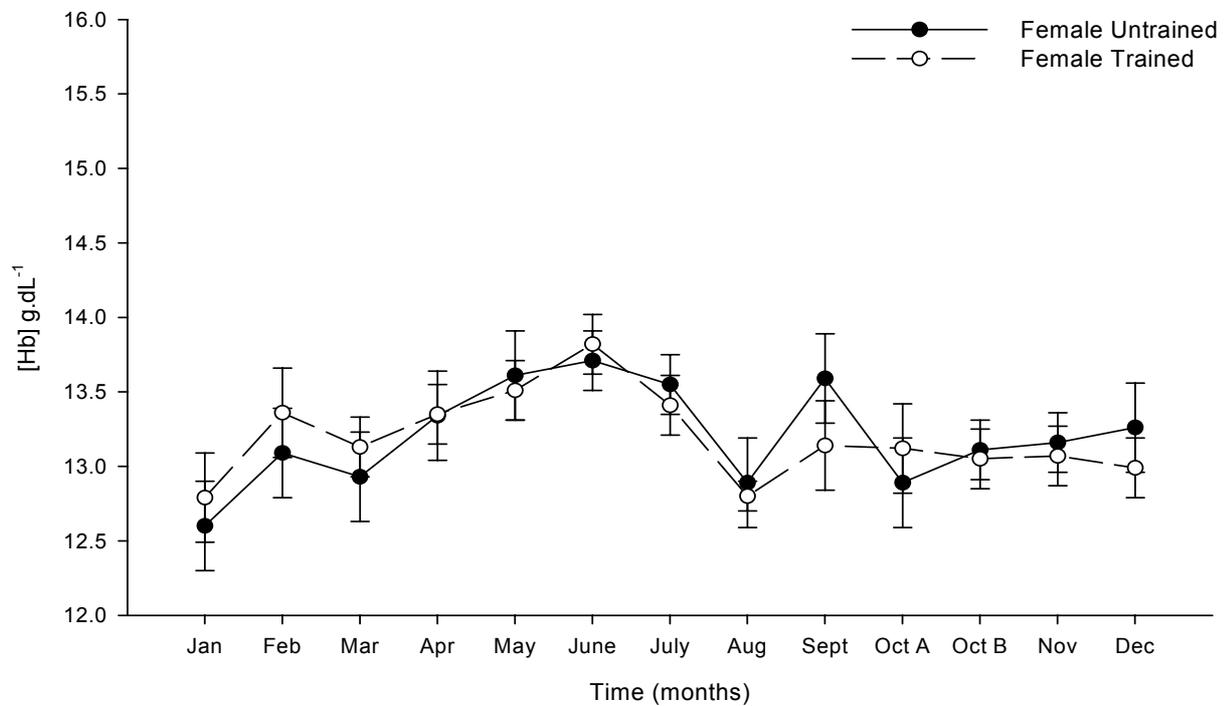


Figure 22B: Changes in Haemoglobin Concentration in Females Aged 65 - 75 Years



Error bars denote SEM

Mean corpuscular haemoglobin concentration (MCHC), the average mass of haemoglobin in a specified volume of erythrocytes, was assessed in both male and female groups. MCHC was calculated as follows:

$$\text{MCHC} = (\text{Hb}/\text{Hct}) \text{ g.dL}^{-1}. \text{ Normal range (male \& female)} = 33 \pm 1.5 \text{ g.dL}^{-1}. \text{ (Coulter 1984).}$$

The changes in MCHC (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 23 A, B. MCHC for the four groups were within the normal range (31.5 – 34.5 g.dL<sup>-1</sup>) only in June, July and October. The male group MCHC were also within normal values during January, February, March, April (UT men only), May (UT men only) and September A but not during August, September B, November and December.

Both female groups had mean MCHC below 31.5 g.dL<sup>-1</sup> during January, May, August, September B, November and December. The UT female group had mean MCHC below 31.5 g.dL<sup>-1</sup> during February and April, and the TR female group were lower during September A. There were significant differences in MCHC between male and female groups (TR and UT) in the following months:

- Male TR group MCHC was significantly higher than male UT – February ( $p = 0.047$ ), September B ( $p = 0.049$ ), October ( $p = 0.005$ )
- Male TR group was significantly higher than the female UT – February ( $p = 0.004$ ), October ( $p < 0.001$ )
- Both male UT and TR groups were significantly higher than the female TR - September A (male UT,  $p = 0.046$ ; male TR,  $p = 0.004$ )
- Female TR group was significantly higher than the female UT - October ( $p = 0.014$ )

Each group showed significant changes in MCHC between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between May and June, and September B and October; significant decreases between July and August, September A and September B.
- Male TR – significant increases between January and February, May and June, September B and October, September B and October; significant decreases between July and August, September A and B, October and November.
- Female UT – significant increases between May and June, August and September A; significant decreases between June and July, July and August, September A and B.
- Female TR – significant increases between January and February, May and June, September B and October; significant decreases between June and July, July and August, October and November.

Figure 23A: Changes in Mean Corpuscular Haemoglobin Concentration in Males Aged 65 - 75 Years

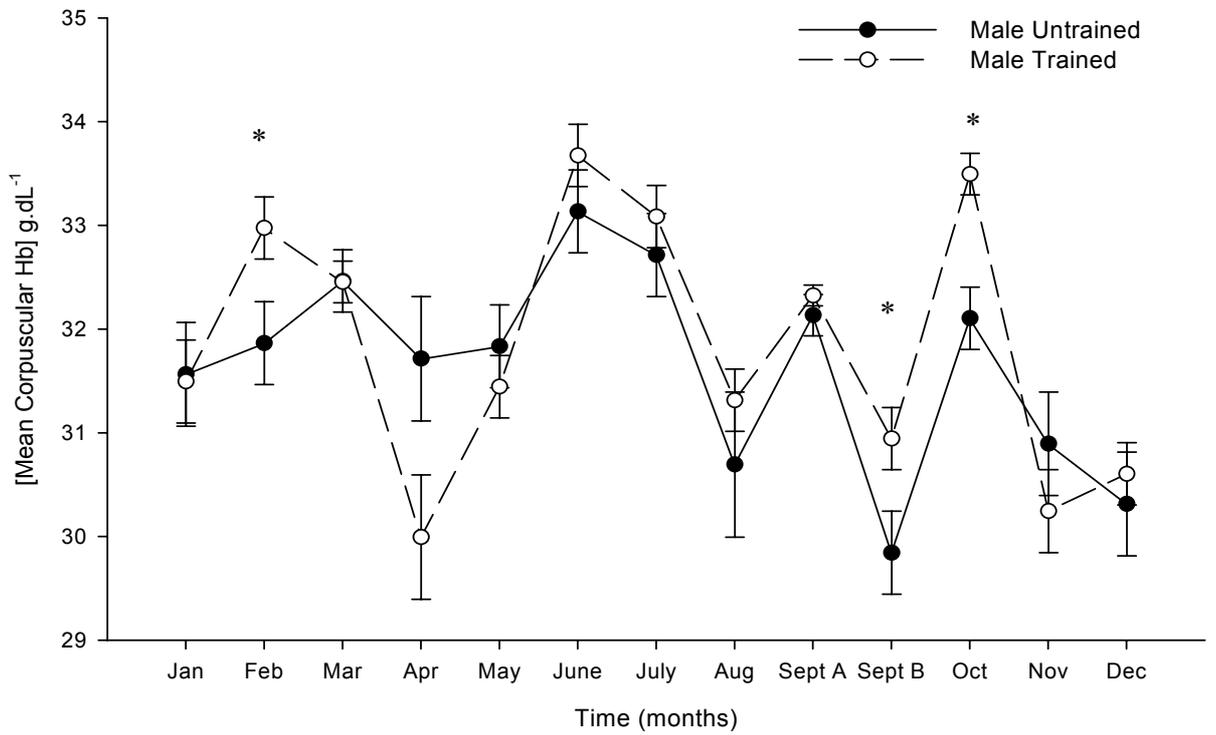
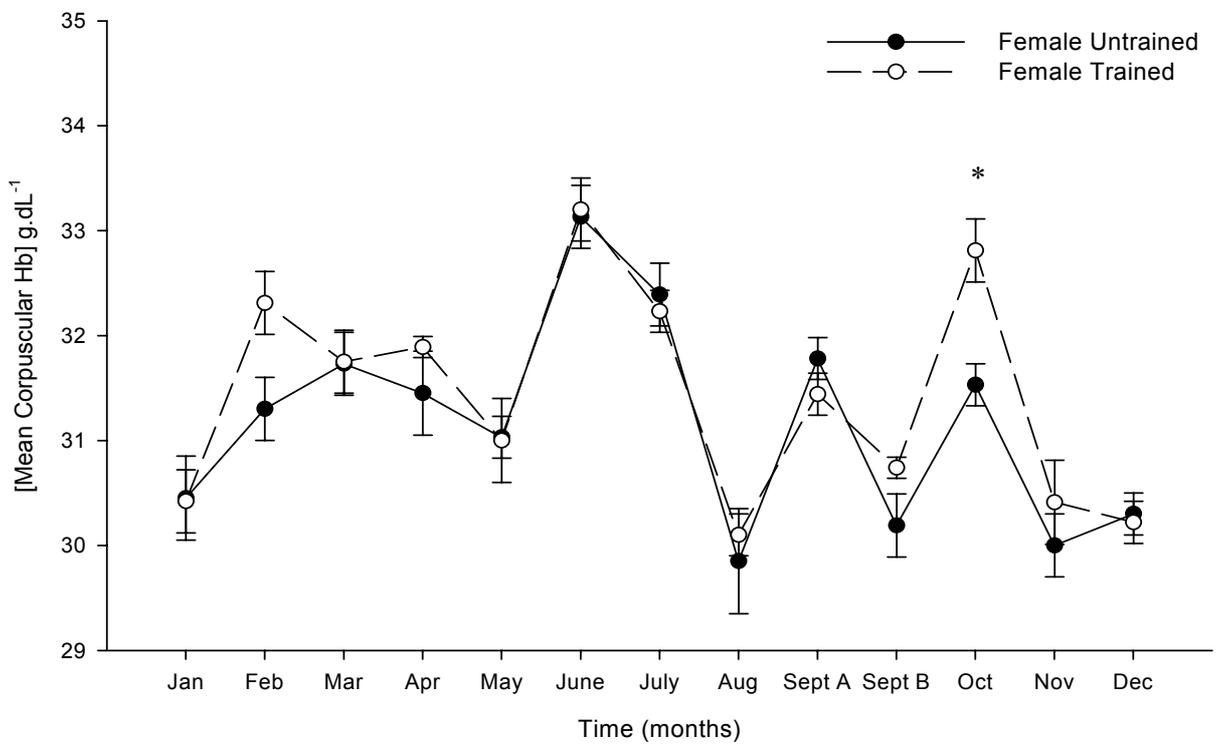


Figure 23B: Changes in Mean Corpuscular Haemoglobin Concentration in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.5.4. *Haematocrit*

The changes in haematocrit (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 24 A, B. The haematocrit for male and female groups were within the normal range (42% - 52% for males and 37% - 47% for females, Coulter 1984) for the duration of the study.

The mean haematocrit for the male UT and TR groups was consistently and significantly higher than that of the female UT and TR groups ( $p < 0.05$ ). Within genders, there were no significant differences between UT and TR groups.

Each group showed significant changes in mean haematocrit between months of the year ( $p < 0.05$ ).

- Male UT – significant increase between August and September A; significant decrease between November and December.
- Male TR – significant decrease between September B and October.
- Female UT – significant increases between August and September A, October and November; significant decrease between September B and October.
- Female TR – significant increase between August and September A; significant decreases between July and August, September B and October.

Figure 24A: Changes in Haematocrit in Males Aged 65 - 75 years

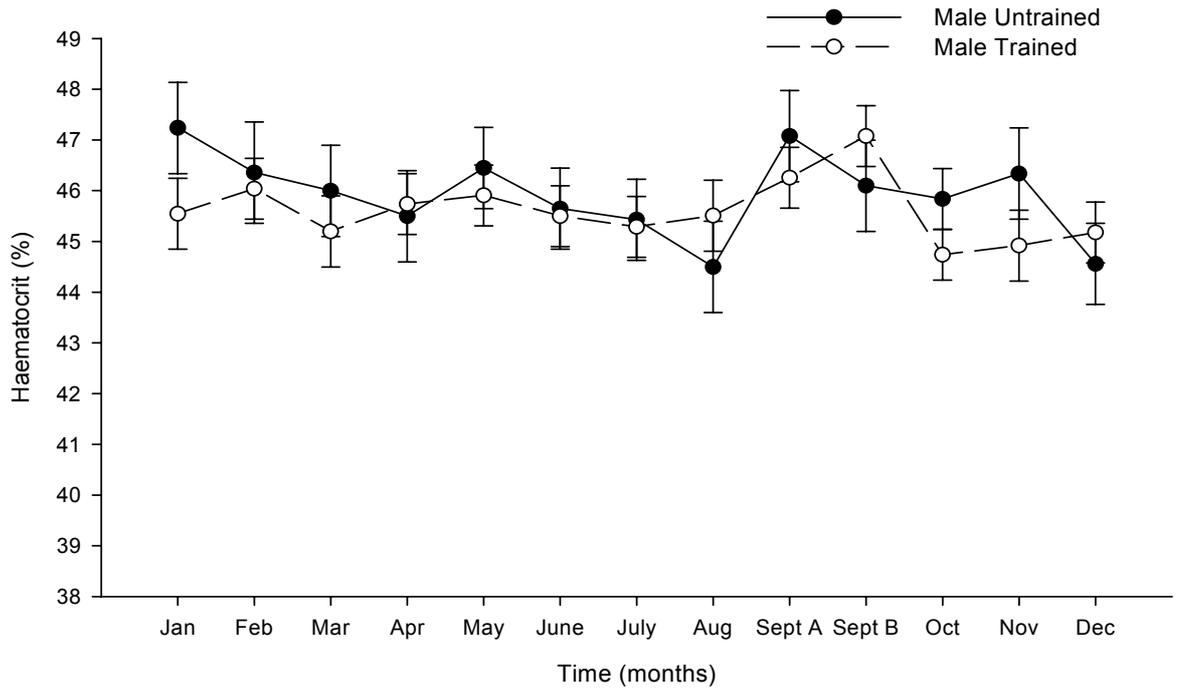
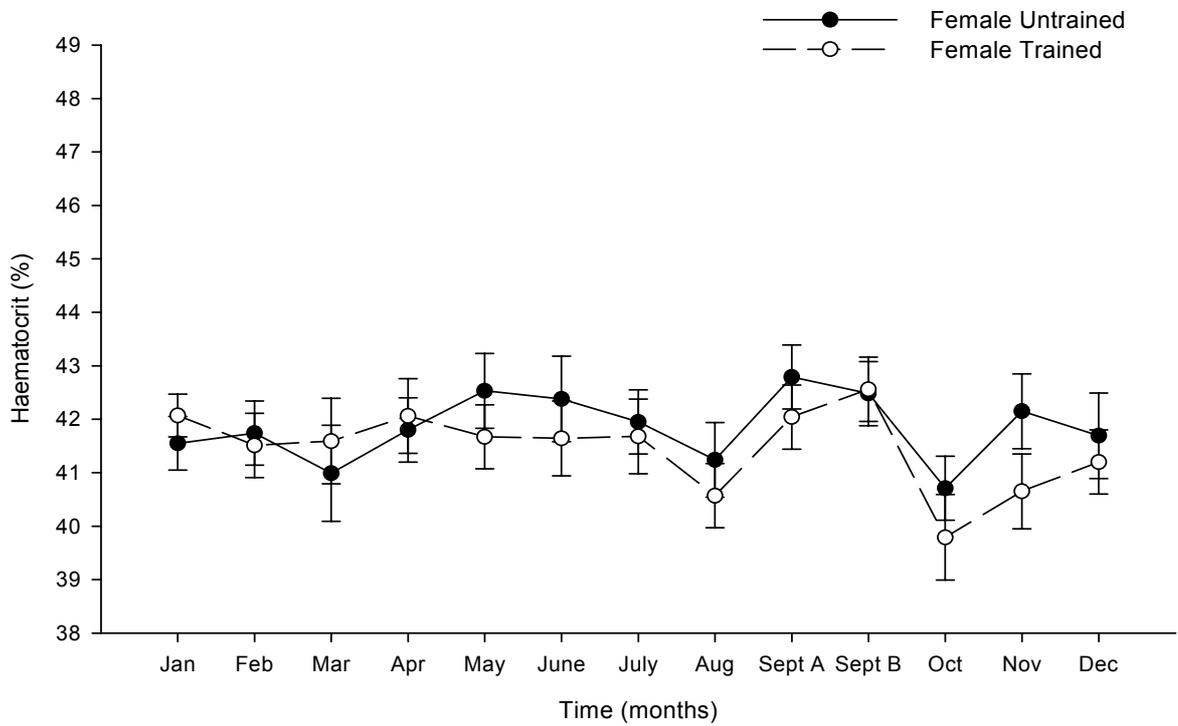


Figure 24B: Changes in Haematocrit in Females Aged 65 - 75 Years



Error bars denote SEM.

#### 4.1.5.5. Platelets

The changes in platelet concentration (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 25 A, B. The male and female UT and TR groups had values within the normal range (130 – 400 x 10<sup>9</sup>.L<sup>-1</sup>) for the duration of the study (Coulter 1984).

During January, March, April, May, June and August the mean platelet concentration of the female TR group was significantly higher than one or both male groups ( $p < 0.05$ ):

- Female UT group was significantly higher than the male UT and TR groups - January (male UT,  $p = 0.02$ ; male TR,  $p = 0.02$  )
- Female TR group was significantly higher than the TR male group – January ( $p = 0.007$ ), March ( $p = 0.037$ ), May ( $p = 0.015$ ), June ( $p = 0.03$ ), August ( $p = 0.04$ )
- Female TR group was significantly higher than the male UT – May ( $p = 0.04$ ), June ( $p = 0.03$ )

Within groups, there were significant changes in platelet concentration between months of the year ( $p < 0.05$ ):

- Male UT – significant increase between October and November;  
significant decrease between November and December.
- Female UT – significant increase between September A and September B.
- Female TR – significant increase between April and May;  
significant decrease between May and June.

Figure 25A: Changes in Platelet Concentration in Males Aged 65 - 75 Years

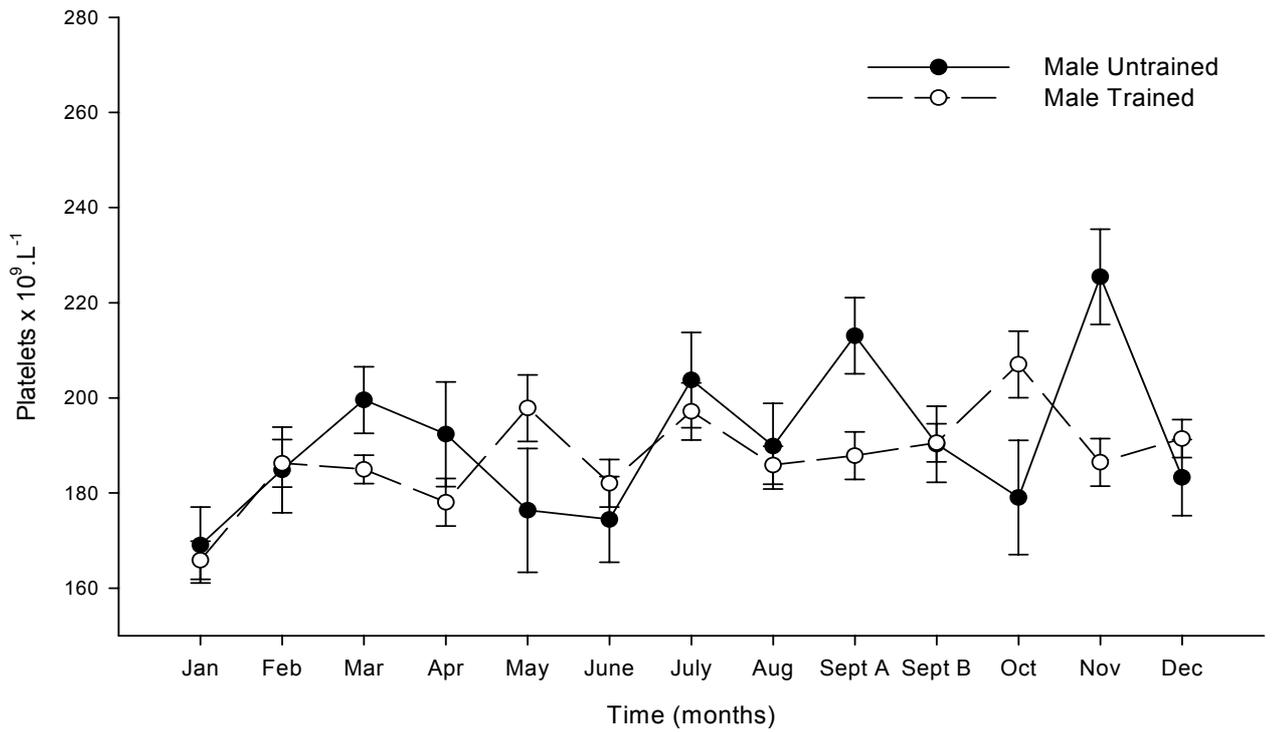
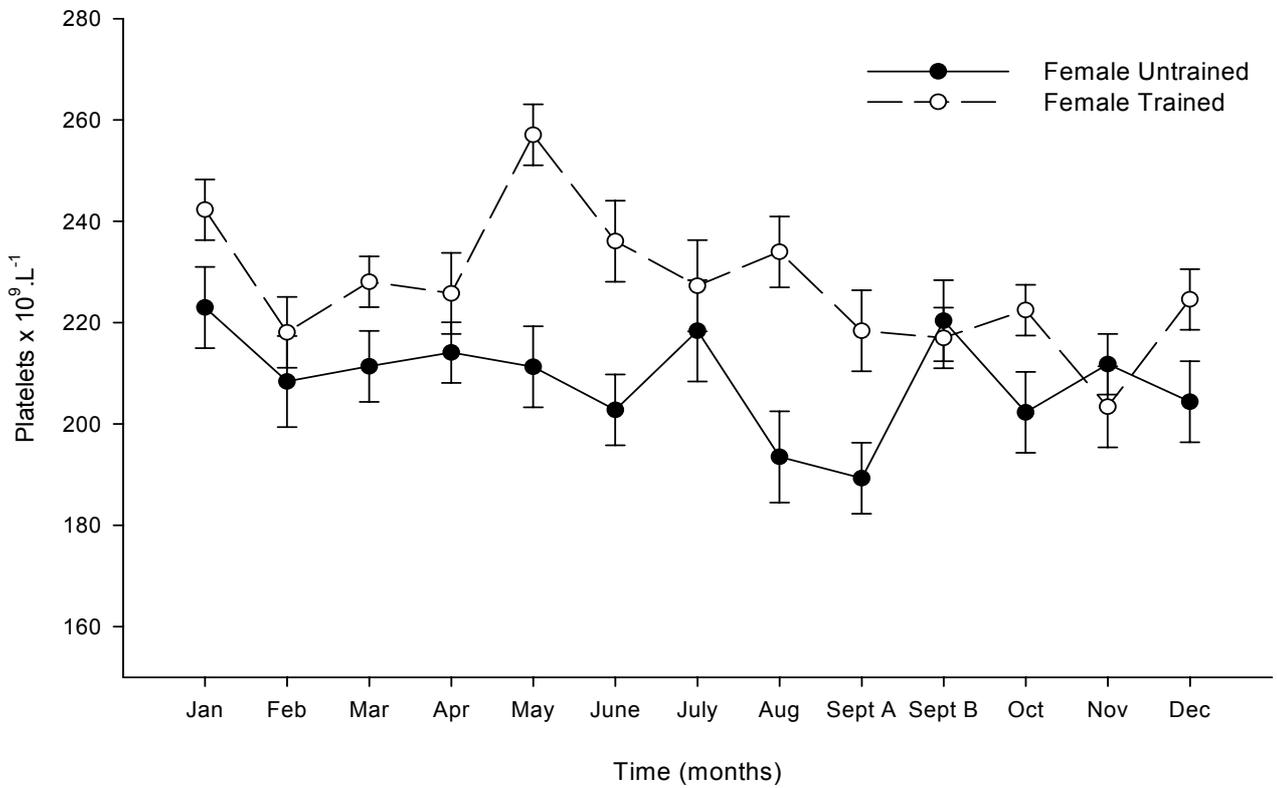


Figure 25B: Changes in Platelet Concentration in Females Aged 65 - 75 Years



Error bars denote SEM.

#### 4.1.6. Interleukin-2 Receptor (CD25) Expression and Density

The changes in the percentage of stimulated CD4<sup>+</sup> lymphocytes expressing CD25 (mean±SEM) for male and female UT and TR groups are shown in Figures 26 A, B. There were significant differences in the mean percentage of CD4<sup>+</sup> cells expressing CD25 between both TR and UT, and male and female groups ( $p < 0.05$ ):

- Male UT group had a significantly higher percentage than the male TR - January ( $p = 0.03$ )
- Male TR had a significantly higher percentage than the male UT – February ( $p = 0.002$ ), April ( $p = 0.04$ ), May ( $p = 0.001$ ), June ( $p = 0.005$ ), September B ( $p = 0.001$ )
- Male TR had a significantly higher percentage than the female UT - June ( $p = 0.017$ ), July ( $p = 0.003$ )
- Female UT group had a significantly higher percentage than the male UT – May ( $p = 0.02$ )
- Female TR group had a significantly higher percentage than the male UT group – May ( $p = 0.003$ ), August ( $p = 0.046$ )
- Female TR group had a significantly higher percentage than female UT group – July ( $p = 0.003$ )

Each group showed significant changes in the mean percentage of CD4<sup>+</sup> cells expressing CD25 between months of the year ( $p < 0.05$ ):

- Male UT – significant increase between August and September;  
significant decrease between January and February, September A and September B.
- Male TR – significant increases between January and February, August and September A, October and November;  
significant decreases between March and April, September B and October.
- Female UT – significant increase between August and September A;  
significant decrease between March and April.
- Female TR – significant increase between April and May;  
significant decrease between March and April, September A and B.

Figure 26A: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25 in Males Aged 65 - 75 Years

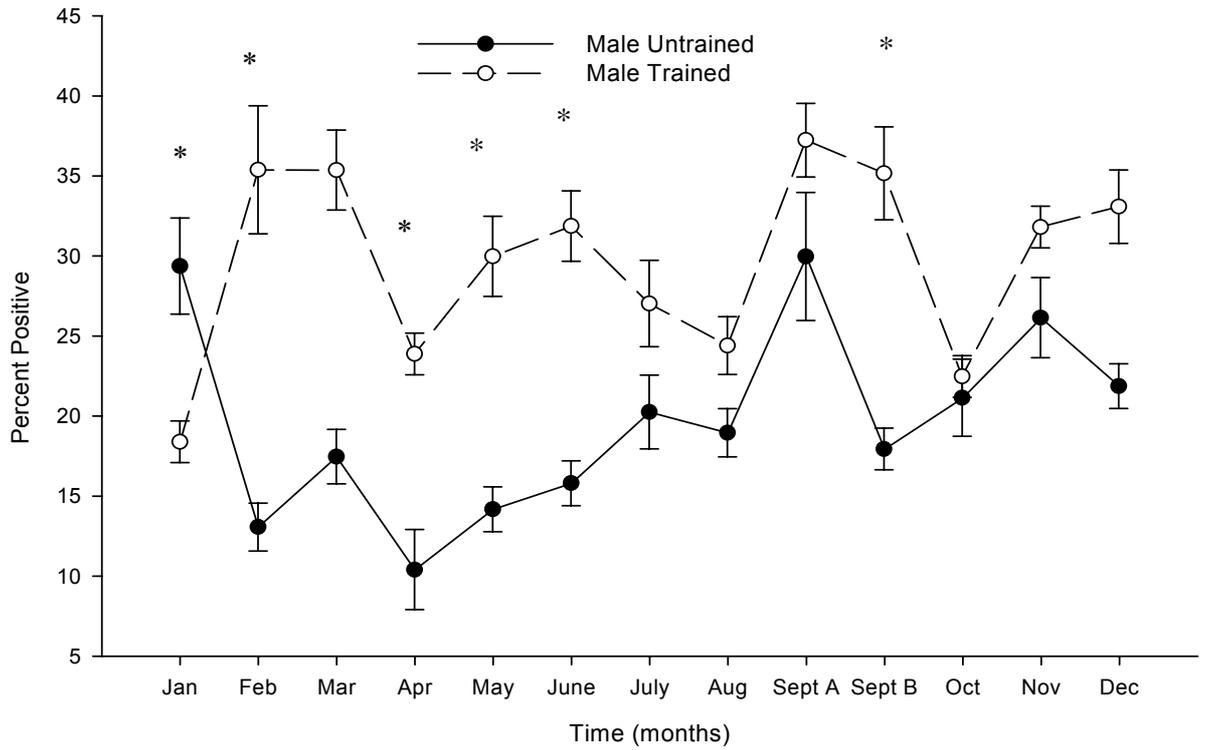
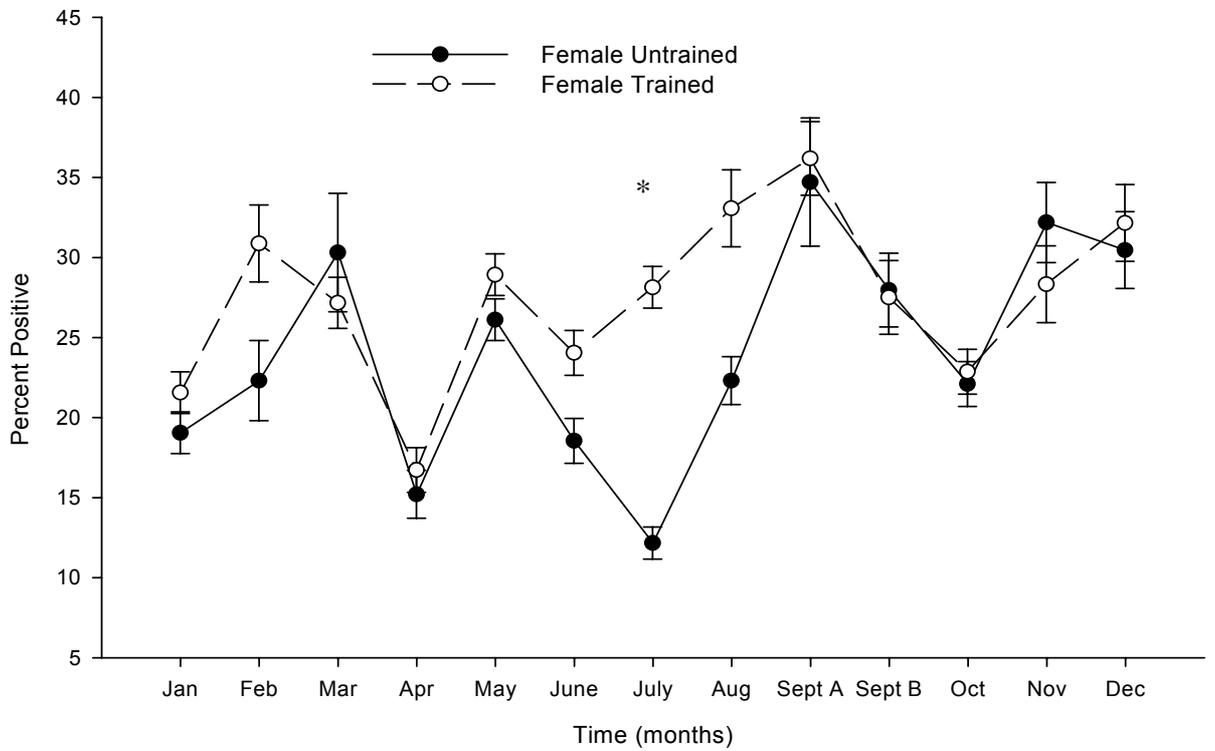


Figure 26B: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25 in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

Receptor density on the cell surface was represented by mean channel log fluorescence. The changes in CD25 density (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 27 A, B. The only significant difference between groups was found in February, where the male TR group was significantly higher than the female TR group ( $p = 0.049$ ).

Three groups showed significant changes in mean CD25 receptor density (mean channel log fluorescence) between months of the year ( $p < 0.05$ ).

- Male UT – significant increase in receptor density between August and September A; significant decreases between January and February, September A and B, October and November.
- Male TR – significant increases between August and September, November and December; significant decreases between January and February, March and April, May and June.
- Female TR – significant increases between June and July, August and September A; significant decreases between January and February, September A and B.

Figure 27A: Changes in CD25 Density on CD4<sup>+</sup> Lymphocytes in Males Aged 65 - 75 Years

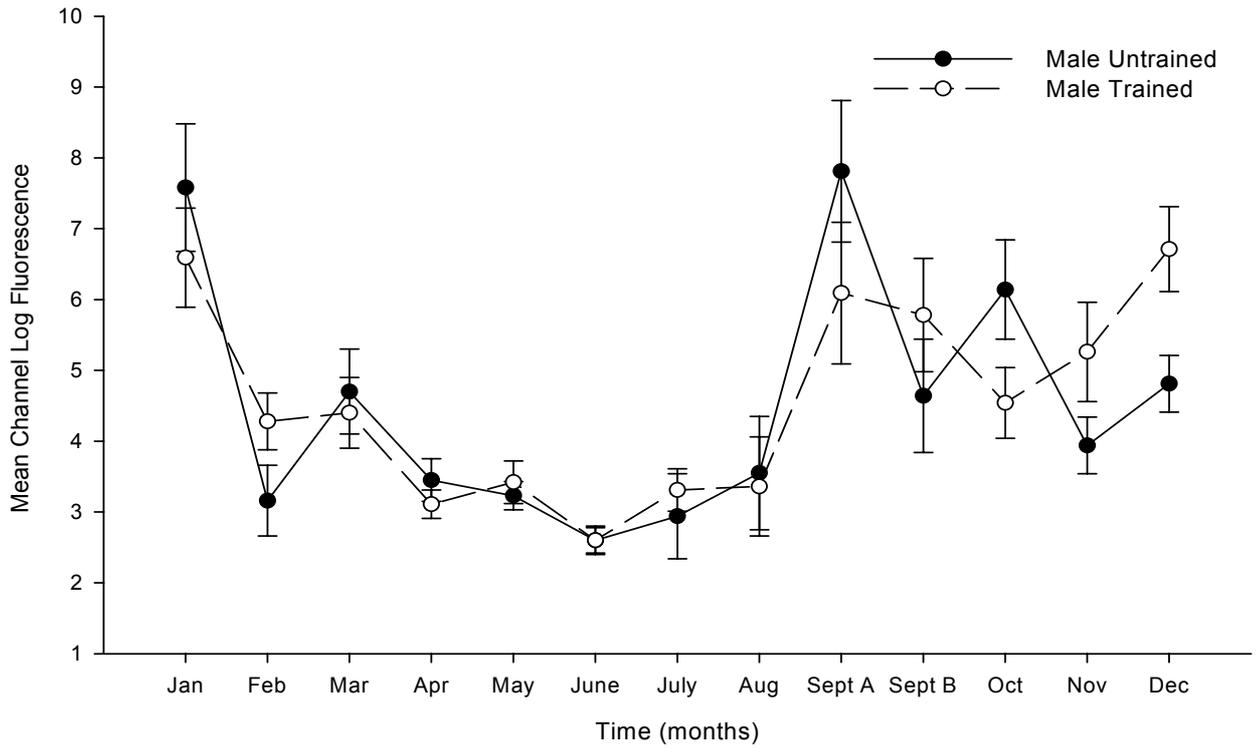
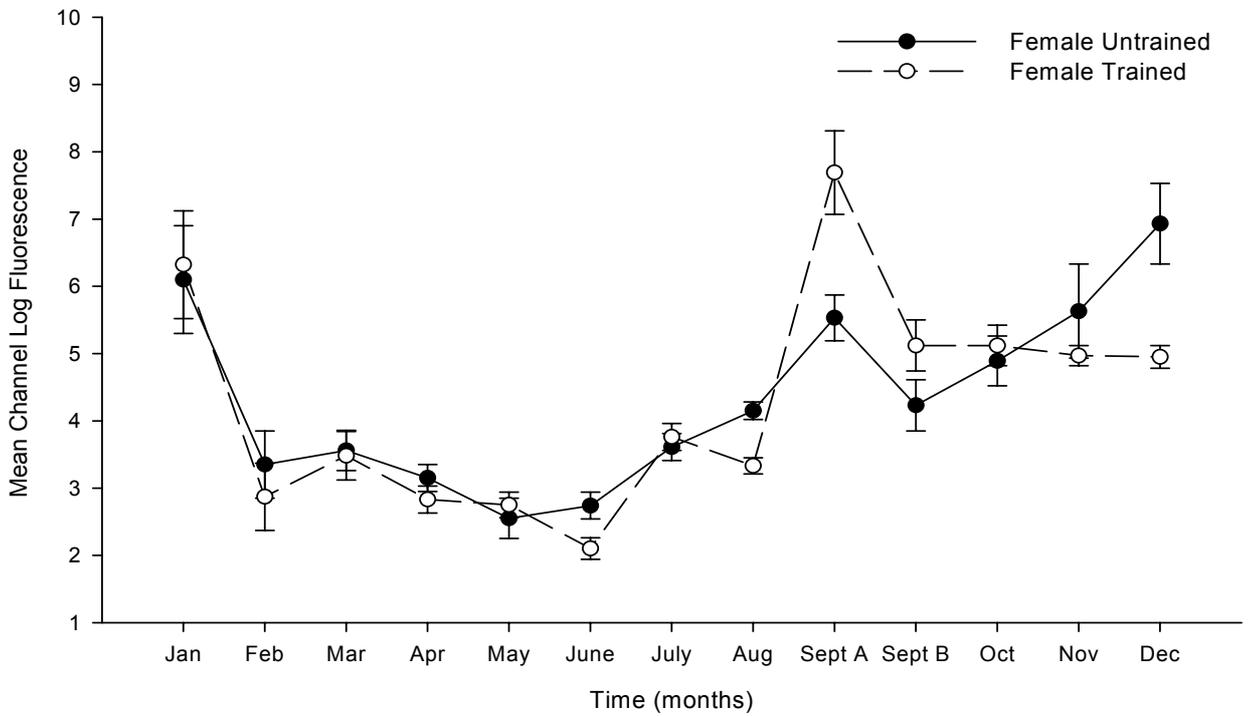


Figure 27B: Changes in CD25 Density on CD4<sup>+</sup> Lymphocytes in Females Aged 65 - 75 Years



Error bars denote SEM.

#### 4.1.7. Transferrin Receptor (CD71) Expression and Density

The changes in the mean percentage of CD4<sup>+</sup> lymphocytes expressing CD71 (mean±SEM) for male and female UT and TR groups are shown in Figures 28 A, B. There were significant differences in the mean percentage of lymphocytes expressing CD71 between the female TR, and male TR and UT groups during May, June and July ( $p < 0.05$ ):

- Male UT group showed a higher percentage than the male TR - March ( $p = 0.046$ )
- Female TR group showed a higher percentage than male TR – May ( $p = 0.035$ ), July ( $p = 0.014$ )
- Female TR group showed a higher percentage than male UT – July ( $p = 0.019$ )
- Female UT group showed a higher percentage than the male TR and female TR - September A (male TR,  $p = 0.039$ ; female TR,  $p = 0.014$ )

Each group showed significant changes in the mean percentage of lymphocytes expressing CD71 between months of the year ( $p < 0.05$ ):

- Male UT – significant decreases between March and April, June and July.
- Male TR – significant increase between September B and October.
- Female UT – significant increase between August and September A; significant decreases between January and February, September A and B, November and December.
- Female TR – significant increase between September A and B; significant decreases between March and April, June and July, July and August.

Figure 28A: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD71 in Males Aged 65 - 75 Years

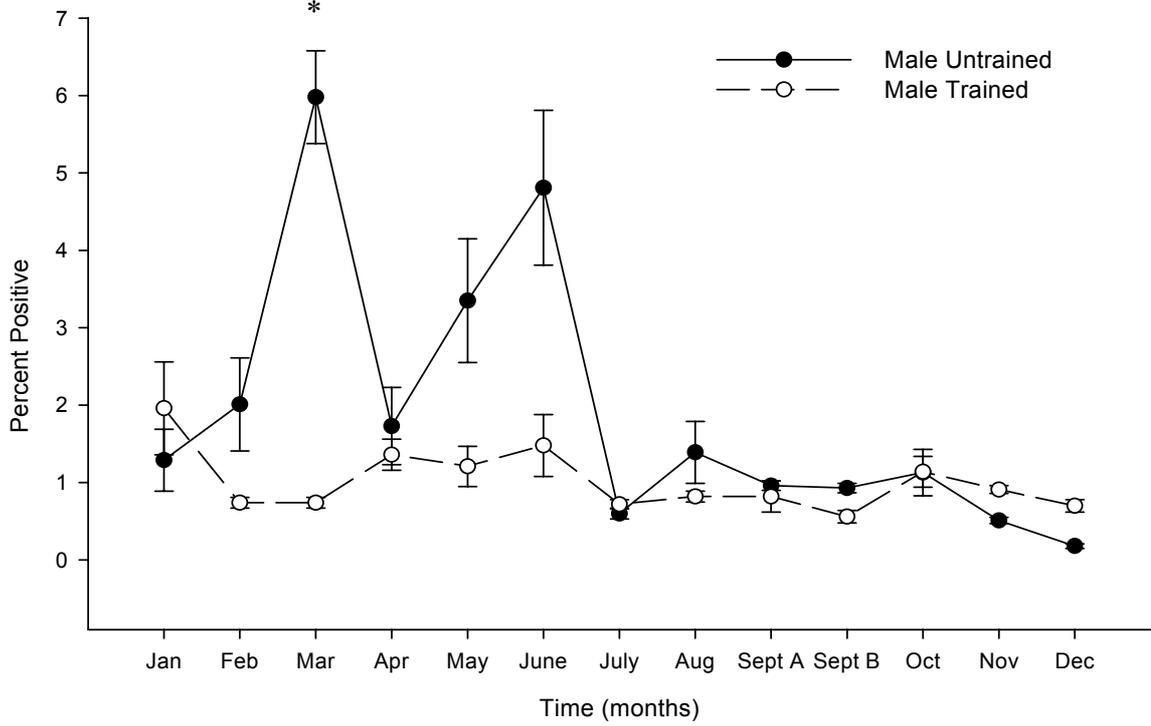
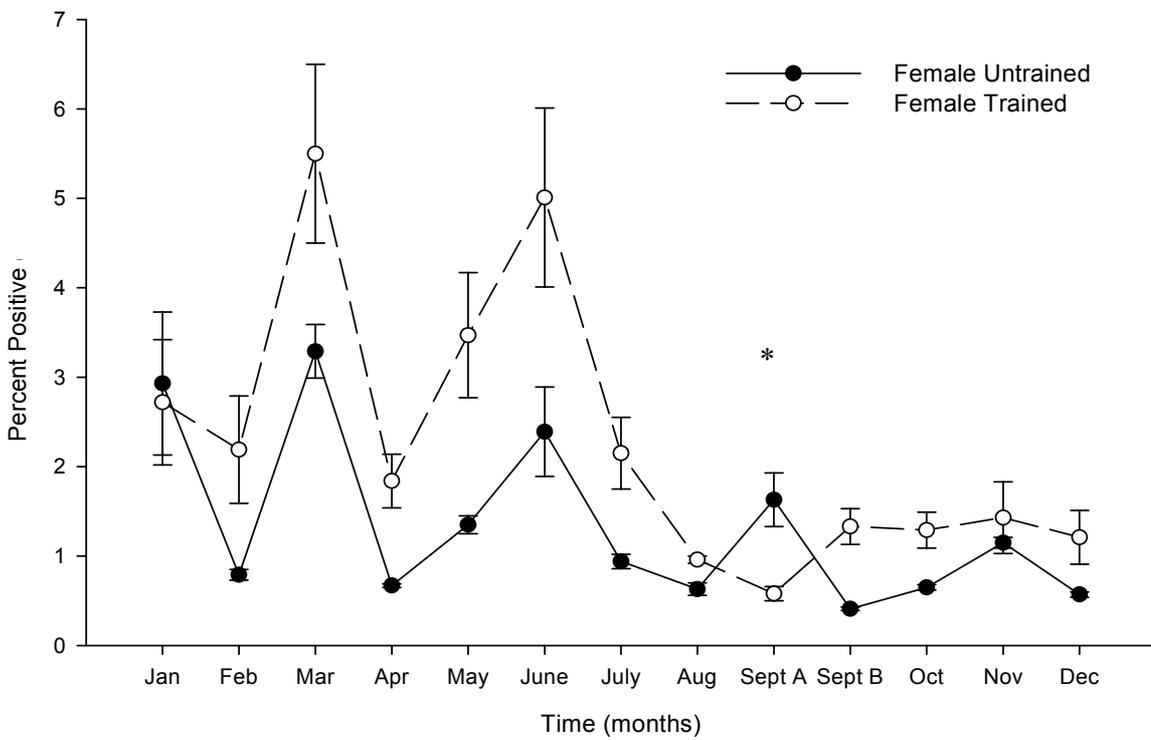


Figure 28B: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD71 in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

The density of CD71 on the cell surface was measured by mean channel log fluorescence. The changes in CD71 density (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 29 A, B.

There were significant differences in mean receptor density between groups during April, July, September, October and December ( $p < 0.05$ ):

- Male UT group showed significantly higher receptor density than the female UT and female TR – December (female UT,  $p = 0.015$ ; female TR,  $p = 0.003$ )
- Female UT group showed significantly higher receptor density than the male UT and female TR - April (male UT,  $p = 0.02$ ; female TR,  $p = 0.03$ )
- Female TR group showed significantly higher receptor density than the male UT and female UT – July (male UT,  $p = 0.044$ ; female UT,  $p = 0.045$ )
- Female TR group showed significantly higher receptor density than the male UT – October ( $p = 0.02$ )
- Female TR group showed significantly higher receptor density than the male TR - September A ( $p = 0.02$ )

Each group showed significant changes in mean receptor density between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between October and November, and November and December.
- Male TR – significant increase between September A and B.
- Female UT – significant decrease between April and May.
- Female TR – significant increase between June and July; significant decrease between July and August.

Figure 29A: Changes in CD71 Density on CD4<sup>+</sup> Lymphocytes in Males Aged 65 - 75 Years

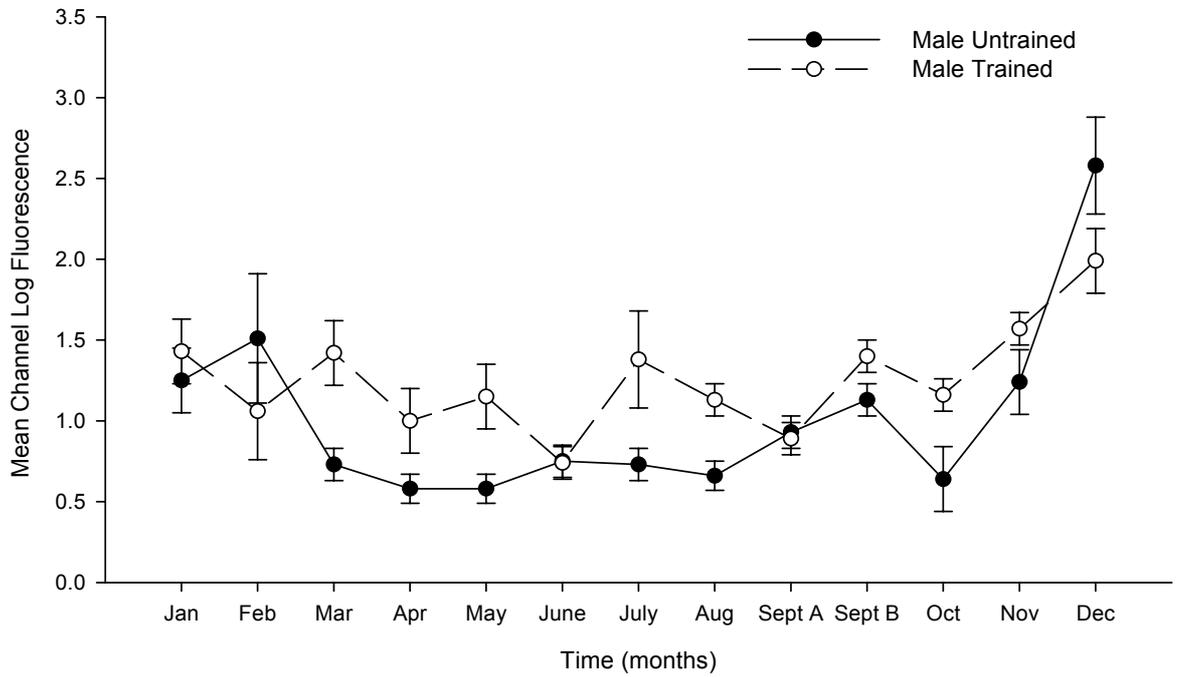
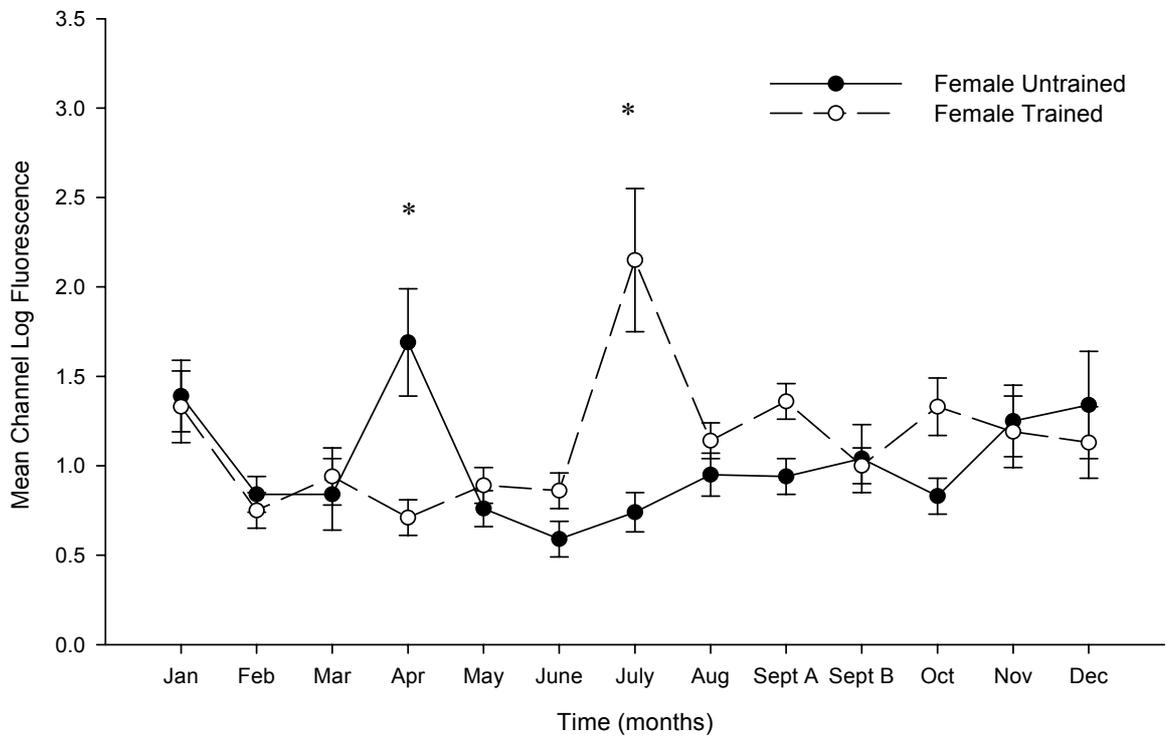


Figure 29B: Changes in CD71 Density on CD4<sup>+</sup> Lymphocytes in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.8. CD4<sup>+</sup> Lymphocytes Positive for both CD25 and CD71 - Receptor Expression and Density

The changes in CD4<sup>+</sup> lymphocytes expressing both CD25 and CD71 (mean±SEM) for male and female UT and TR groups are shown in Figures 30 A, B. Lymphocytes positive for both CD25 and CD71 are referred to as “double positive” (CD4<sup>+</sup>/CD25<sup>+</sup>/CD71<sup>+</sup>). The density of either receptor on each cell could be measured by mean channel log fluorescence in both the X (CD25) and Y (CD71) channels.

There were significant differences in the mean percentage of “double positive” CD4<sup>+</sup> lymphocytes between groups during the following months ( $p < 0.05$ ).

- Male TR group showed a significantly higher percentage of double positive cells than the male UT - December ( $p = 0.01$ )
- Male TR group showed a significantly higher percentage of double positive cells than the female UT – May ( $p = 0.02$ )
- Female TR group showed a significantly higher percentage of double positive cells than the male TR – November ( $p = 0.03$ )
- Female TR group showed a significantly higher percentage of double positive cells than the male UT - September B ( $p = 0.044$ )

Each group showed significant changes in the percentage of CD4<sup>+</sup> lymphocytes expressing the “double positive” receptors between months of the year ( $p < 0.05$ ).

- Male UT – significant increase between August and September A; significant decreases between March and April, September A and B.
- Male TR – significant increase between April and May.
- Female UT – significant increase between August and September A; significant decreases between March and April, September A and B.
- Female TR – significant increases between April and May, May and June; significant decreases between March and April, June and July, November and December.

Figure 30A: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25/CD71 in Males Aged 65 - 75 Years

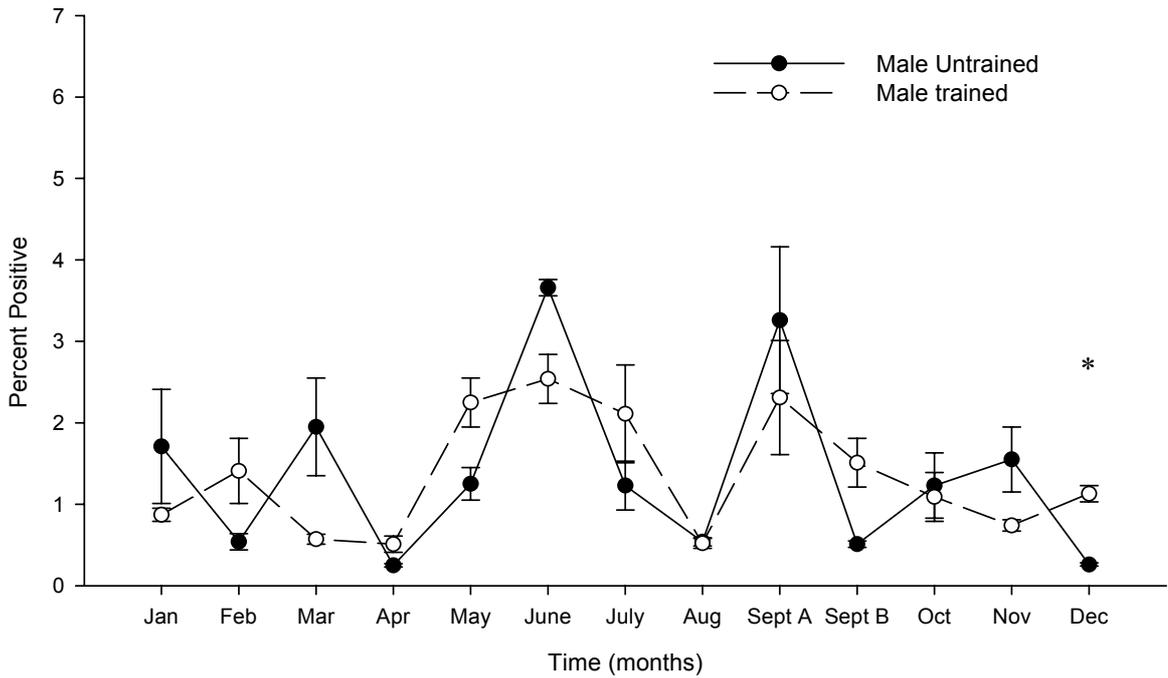
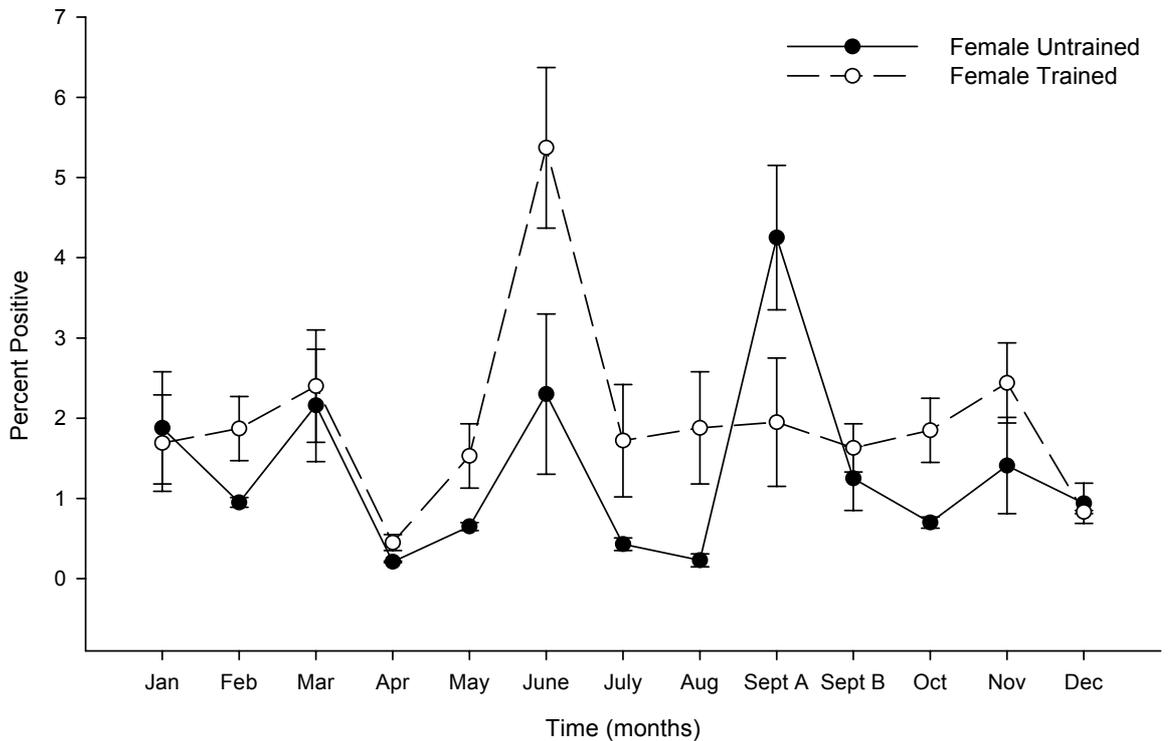


Figure 30B: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25/CD71 in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

The changes in mean CD25 density on the “double positive” CD4<sup>+</sup> lymphocytes (mean±SEM) for male and female UT and TR groups are shown in Figures 31 A, B. The only significant differences between groups occurred in May, where the male TR group showed higher CD25 density on “double positive” CD4<sup>+</sup> lymphocytes than the female UT group ( $p = 0.04$ ).

Each group showed significant changes in CD25 density on “double positive” lymphocytes between months of the year ( $p < 0.05$ ).

- Male UT – significant increase between August and September A; significant decrease between January and February.
- Male TR – significant increase between July and August; August and September A significant decreases between January and February.
- Female UT – significant increase between November and December.
- Female TR – significant increases between June and July, August and September A.

Figure 31A: Changes in CD25 Density on Double Positive Lymphocytes in Males Aged 65 - 75 Years

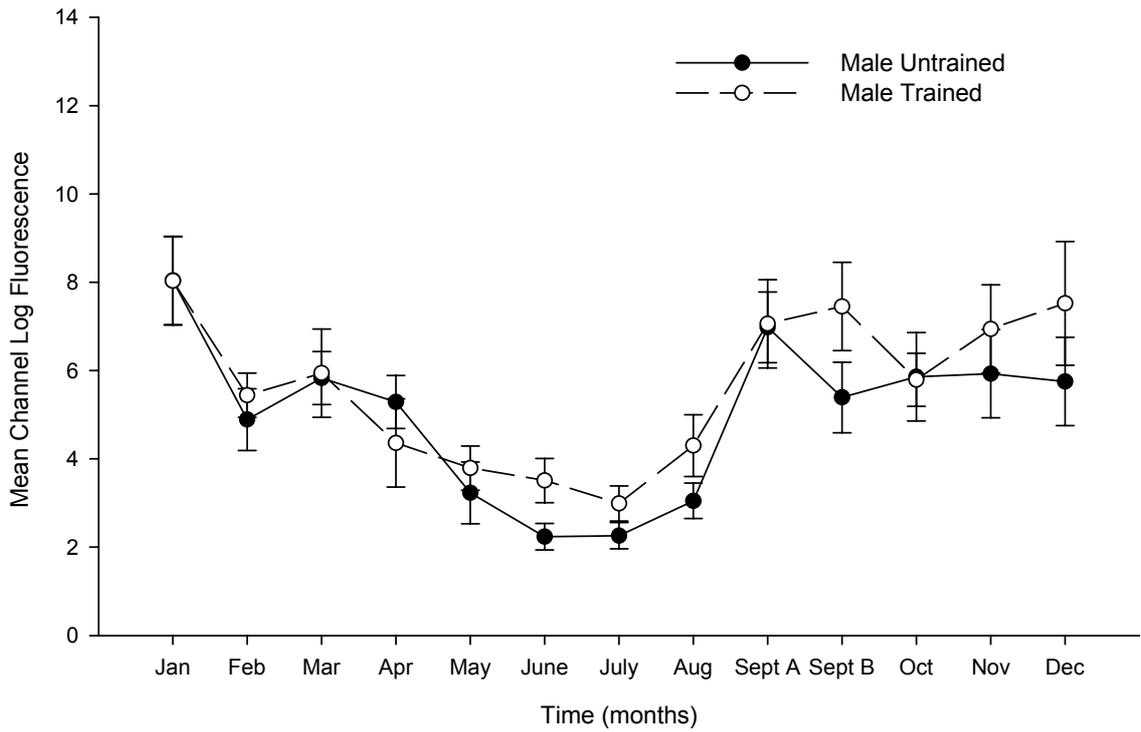
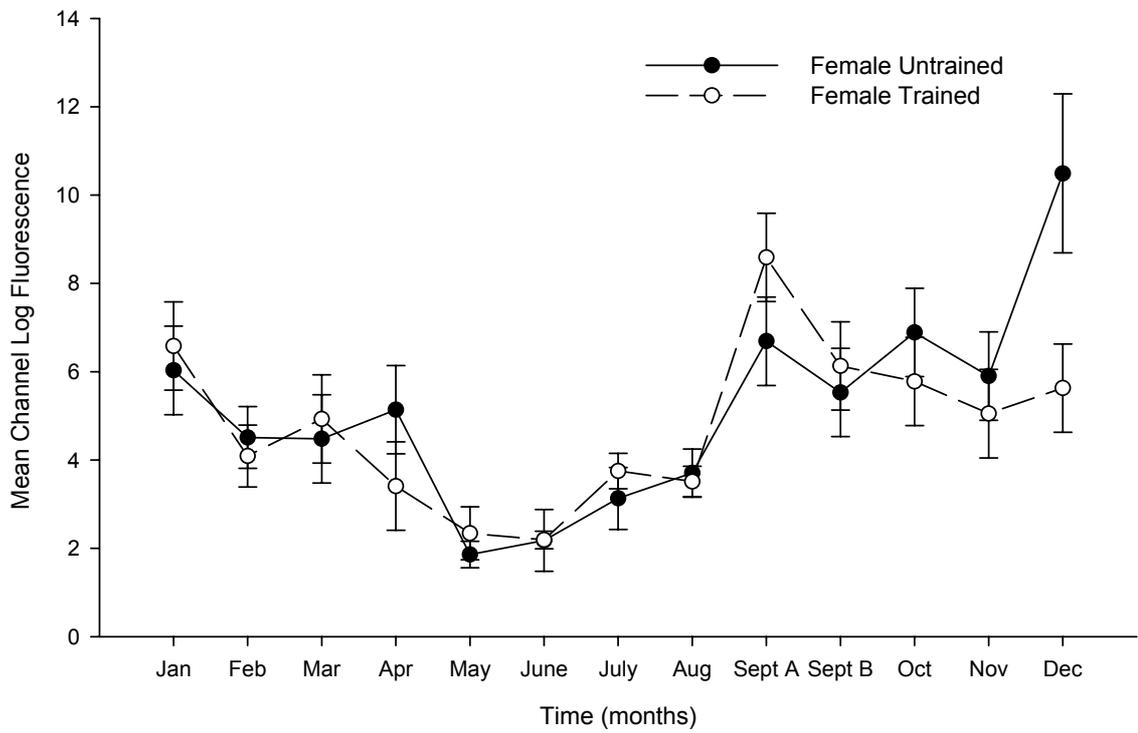


Figure 31B: Changes in CD25 Density on Double Positive Lymphocytes in Females Aged 65 - 75 years



Error bars denote SEM.

The mean changes in CD71 density on the “double positive” lymphocytes (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 32 A, B.

There were significant differences between groups during the following months ( $p < 0.05$ ):

- Male UT group showed significantly higher density than the male TR group – March ( $p = 0.001$ )
- Male TR group showed significantly higher density than the female TR group ( $p = 0.001$ )
- Male TR group showed significantly higher density than the female UT group and the female TR group – March (female UT,  $p = 0.005$ ; female TR,  $p = 0.01$ ), September B (female UT,  $p = 0.046$ ; female TR,  $p = 0.02$ )
- Male TR group showed significantly higher density than the male UT group and the female TR group – November (male UT  $p = 0.02$ ; female TR  $p = 0.014$ )
- Female UT group showed significantly higher density than the male UT group – August ( $p = 0.007$ )
- Female TR group showed significantly lower density than the male TR group – August ( $p = 0.04$ )
- Female UT group showed significantly higher density than the female TR group - August ( $p = 0.001$ ), December ( $p = 0.014$ )

Each group showed significant changes in mean CD71 density on “double positive” lymphocytes during the study ( $p < 0.05$ ):

- Male UT – significant increase between February and March;  
significant decrease between January and February.
- Male TR – significant increases between May and June, July and August, October and November;  
significant decreases between January and February, June and July.
- Female UT – significant increases between March and April, July and August;  
significant decrease between April and May.
- Female TR – significant increase between August and September A;  
significant decreases between January and February, September A and B.

Figure 32A: Changes in CD71 Density on Double Positive Lymphocytes in Males Aged 65 - 75 Years

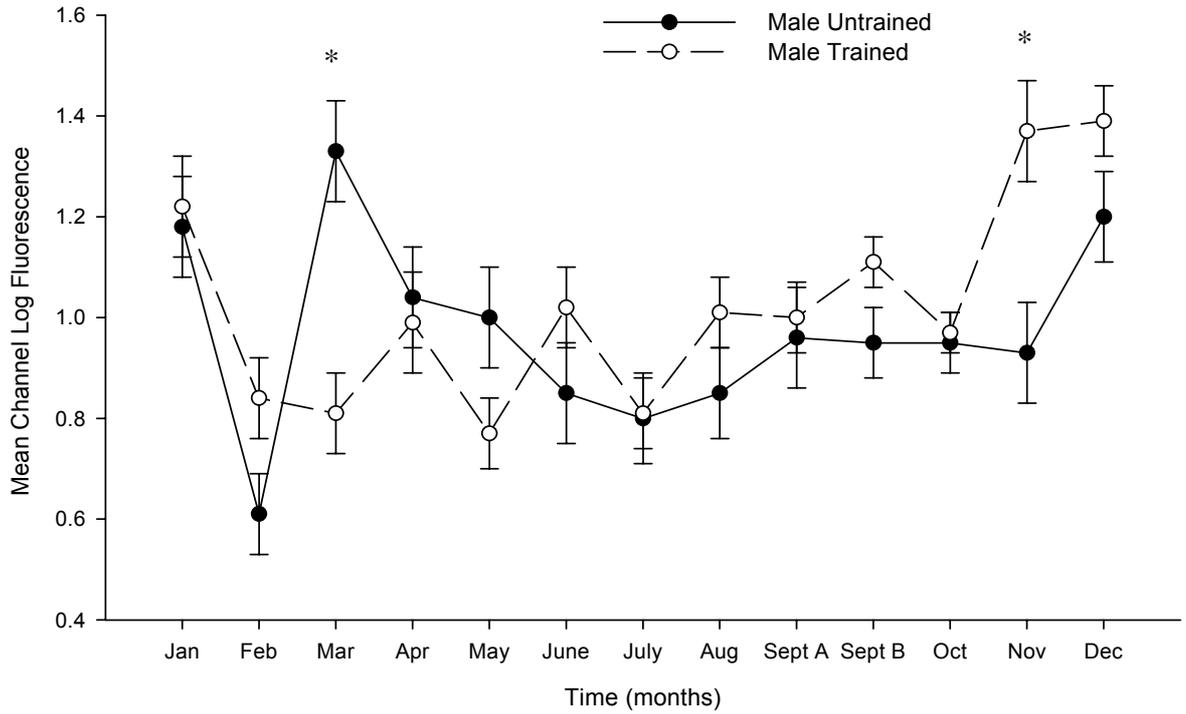
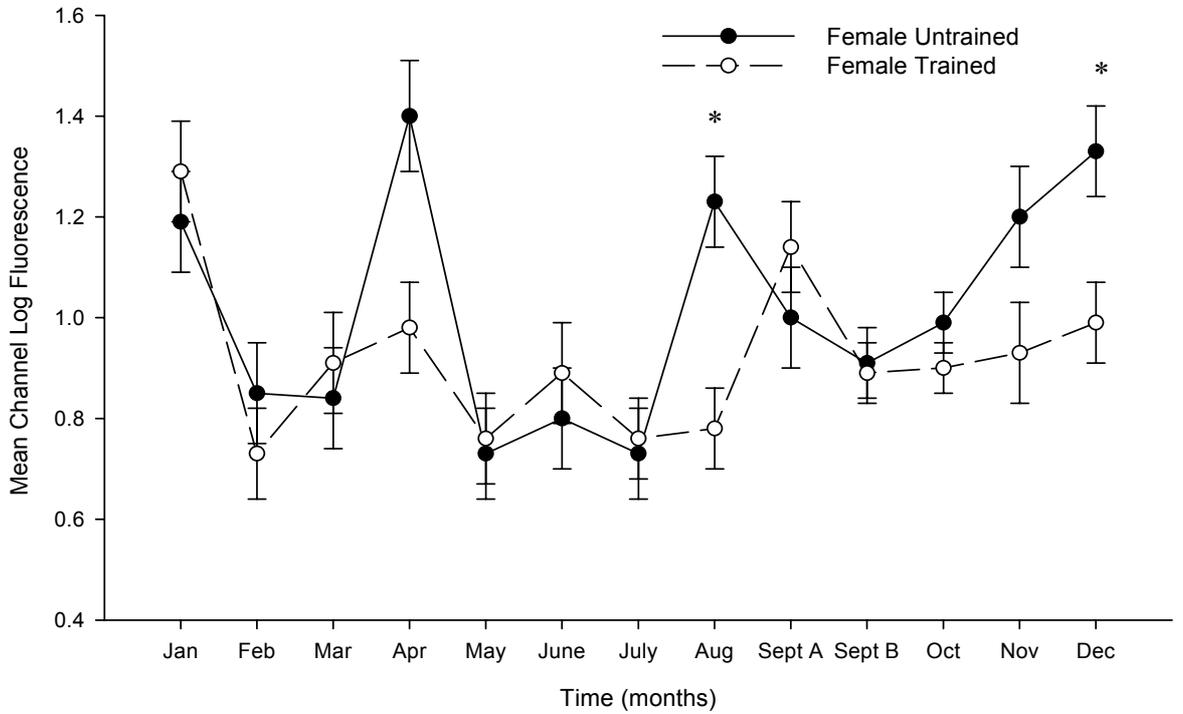


Figure 32B: Changes in CD71 Density on Double Positive Lymphocytes in Females Aged 65 - 75 Years



\* Significant difference between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.9. Intracellular Calcium

##### 4.1.9.1. Cytosolic Calcium

Cytosolic  $\text{Ca}^{2+}$  was quantified with mean channel log fluorescence and was termed  $[\text{Ca}^{2+}]_i$ . The  $[\text{Ca}^{2+}]_i$  combined with  $\text{Ca}^{2+}$  released from ER stores through the action of thapsigargin, was termed  $[\text{Ca}^{2+}]_t$ . The  $[\text{Ca}^{2+}]_i$  and  $[\text{Ca}^{2+}]_t$  were quantified using the double log regression plot shown in the Section 3.1.3.7. Changes in  $[\text{Ca}^{2+}]_i$  (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 33 A, B. The male UT and female TR groups showed the highest mean  $[\text{Ca}^{2+}]_i$  in January, with  $212 \pm 12$  nM and  $211 \pm 12$  nM respectively. The lowest recorded mean  $[\text{Ca}^{2+}]_i$  were  $20 \pm 0.5$  nM in May (male TR), and  $20 \pm 0.4$  nM in November (male TR).

There were significant differences in mean  $[\text{Ca}^{2+}]_i$  between groups, during following months, ( $p < 0.05$ ):

- Male UT group was significantly higher than female UT – February ( $p = 0.03$ )
- Male TR group was significantly higher than female UT – February ( $p = 0.03$ ), August ( $p = 0.04$ )
- Male TR group and female UT/TR groups were significantly higher than the male UT - September B (male TR,  $p = 0.01$ ; female UT,  $p = 0.014$ ; female TR,  $p = 0.014$ )
- Female UT and female TR were significantly higher than the male TR group – April (female UT,  $p = 0.001$ ; female TR,  $p = 0.007$ ), June (female UT,  $p < 0.001$ ; female TR,  $p = 0.004$ ) \*
- Female UT group was significantly higher than the male TR - September A ( $p = 0.006$ )
- Female TR group was significantly higher than the male TR – November ( $p < 0.001$ )
- Female TR was significantly higher than the female UT - December (female UT,  $p = 0.014$ )

Each group showed significant changes in mean  $[\text{Ca}^{2+}]_i$  between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between May and June, November and December; significant decreases between February and March, October and November.
- Male TR – significant increases between May and June, June and July, September A and September B;

significant decreases between February and March, July and August, September B and October, October and November.

- Female UT – significant increases between May and June, August and September A, September A and September B, November and December;  
significant decreases between July and August, October and November.
- Female TR – significant increases between May and June, June and July, September A and September B, November and December;  
significant decreases between January and February, July and August, October and November.
- The post-study  $[Ca^{2+}]_i$  in December was also significantly lower than the pre-study concentration in January for all groups.

Figure 33A: Changes in Intracellular Cytosolic  $[Ca^{2+}]_i$  in  $CD4^+$  Lymphocytes in Males Aged 65 - 75 Years

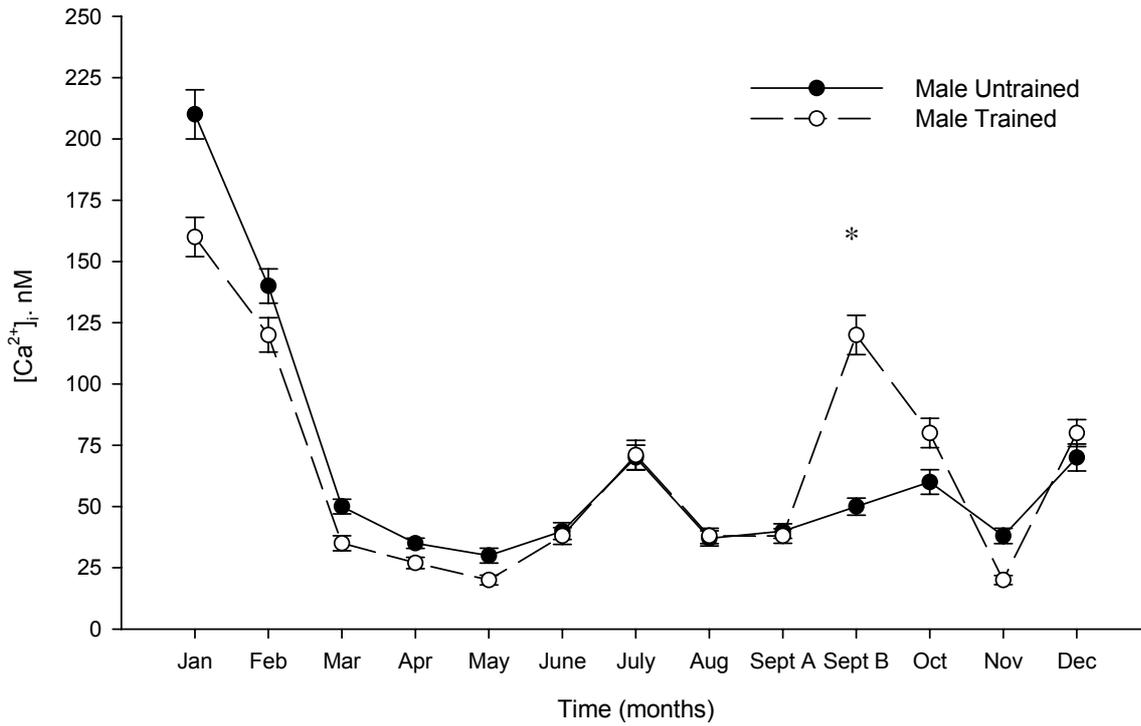
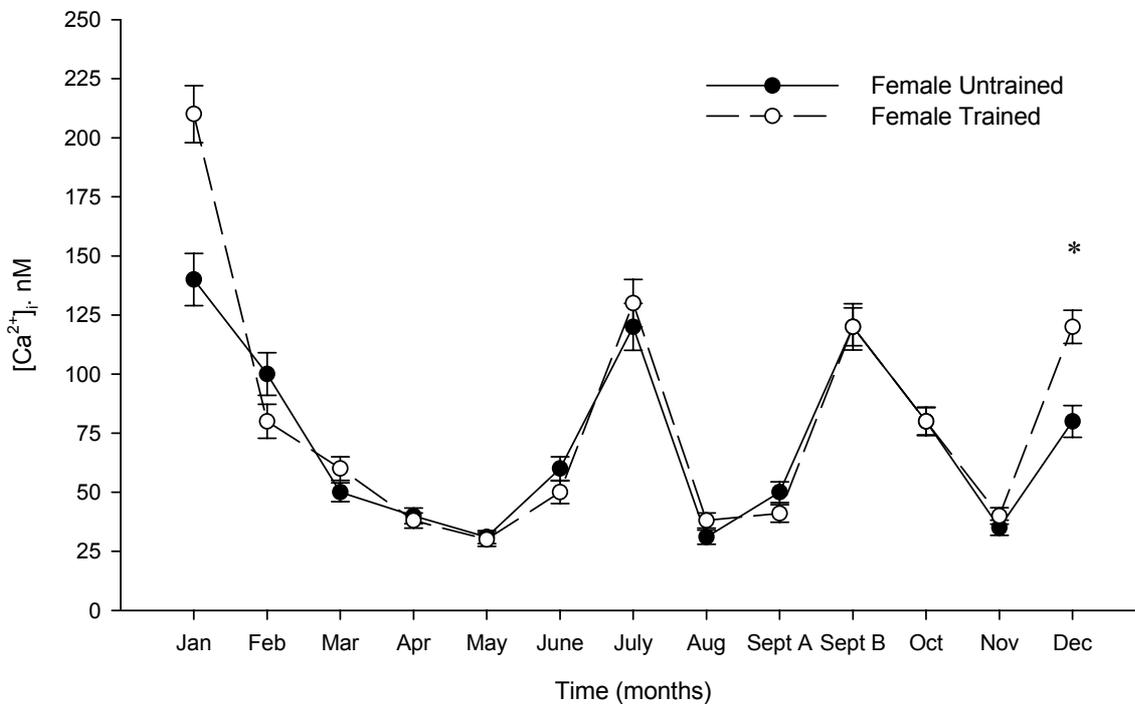


Figure 33B: Changes in Intracellular Cytosolic  $[Ca^{2+}]_i$  in  $CD4^+$  Lymphocytes in Females Aged 65 - 75 Years



\* Significant differences between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.9.2. Total Intracellular Calcium

The changes in total intracellular calcium ( $[Ca^{2+}]_t$ ) (mean $\pm$ SEM) for male and female UT and TR groups are shown in Figures 34 A, B. There were significant differences in mean  $[Ca^{2+}]_t$  between groups during the following months ( $p < 0.05$ ):

- Male UT group was significantly higher than male TR - January ( $p = 0.044$ )
- Female UT group was significantly higher than female TR – January ( $p = 0.046$ )

The highest mean  $[Ca^{2+}]_t$  for all groups occurred in January with the male UT recording a concentration of  $31 \pm 3 \mu\text{M}$ , the male TR group  $24.2 \pm 0.8 \mu\text{M}$ , the female UT group  $26.3 \pm 1 \mu\text{M}$  and the female TR group  $23 \pm 0.8 \mu\text{M}$ .

Each group showed significant changes in mean  $[Ca^{2+}]_t$  between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between September B and October, November and December;  
significant decreases between January and February.
- Male TR – significant increases between September B and October;  
significant decreases between January and February.
- Female UT – significant increases between February and March;  
significant decreases between January and February.
- Female TR – significant decreases between January and February.
- The post-study  $[Ca^{2+}]_t$  was significantly lower than the pre-study  $[Ca^{2+}]_t$  for all groups ( $p < 0.05$ ).

Figure 34A: Changes in Total Intracellular Calcium ( $[Ca^{2+}]_t$ ) in CD4<sup>+</sup> Lymphocytes in Males Aged 65 to 75 Years

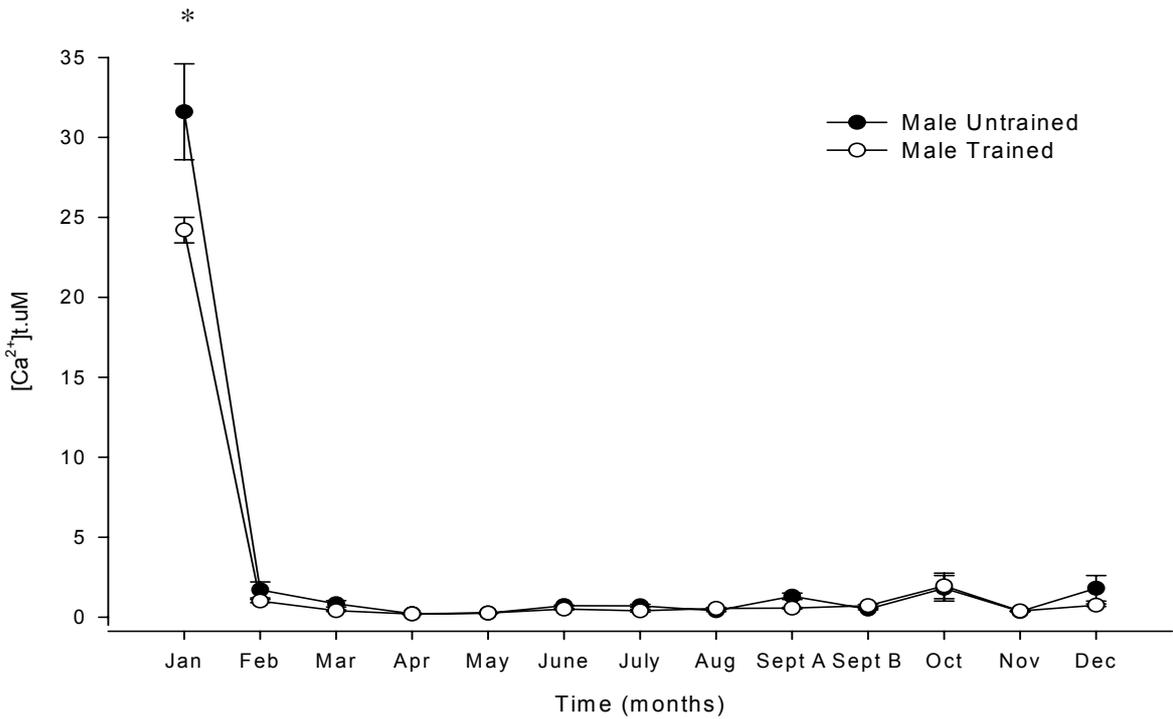
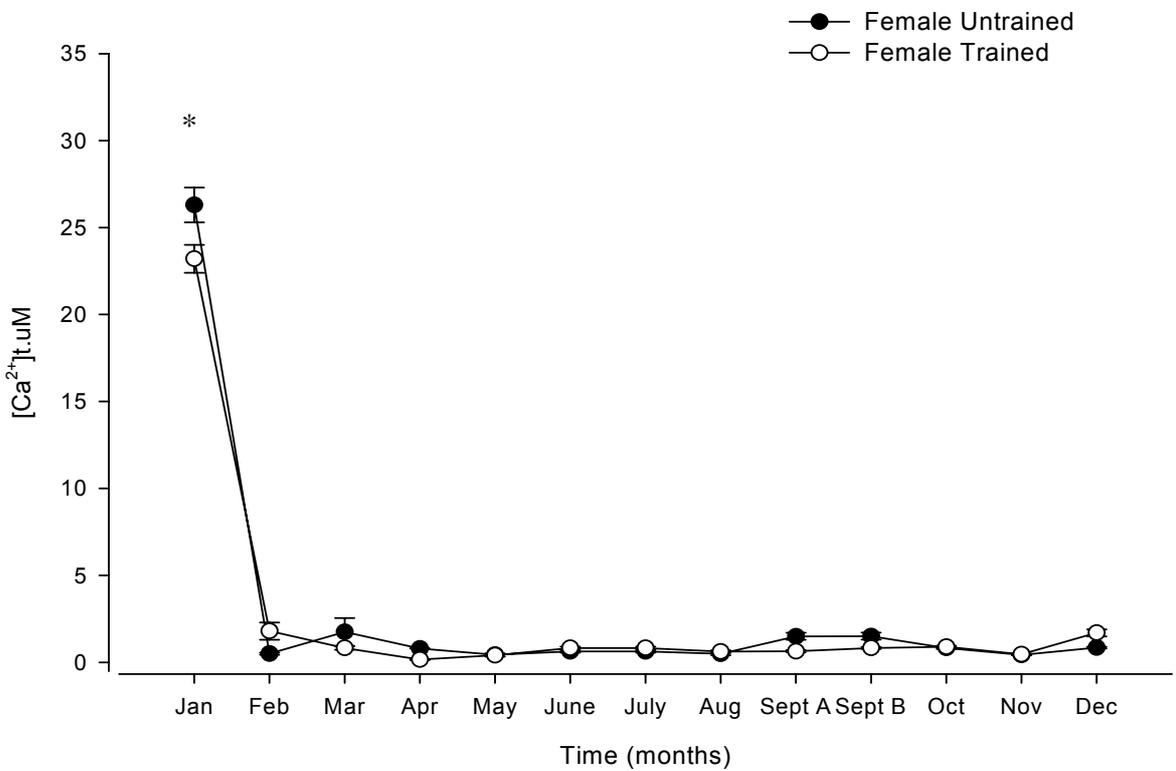


Figure 34B: Changes in Total Intracellular Calcium ( $[Ca^{2+}]_t$ ) in CD4<sup>+</sup> Lymphocytes in Females Aged 65 to 75 Years



\*Significant differences between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.10. Intracellular Iron

The changes in intracellular  $\text{Fe}^{3+}$ , estimated by mean channel log fluorescence, ( $\text{mean} \pm \text{SEM}$ ) for male and female UT and TR groups are shown in Figures 35 A, B. The mean channel log fluorescence was inversely related to the intracellular concentration of iron ( $[\text{Fe}^{3+}]_i$ ).

There were significant differences in mean channel log fluorescence between groups during the following months ( $p < 0.05$ ):

- Male UT and male TR groups showed significantly higher  $[\text{Fe}^{3+}]_i$  than the female UT group – January (male UT,  $p = 0.03$ ; male TR,  $p = 0.012$ )
- Male TR group showed significantly higher  $[\text{Fe}^{3+}]_i$  than the female TR group – January ( $p = 0.04$ )
- Female UT showed significantly higher  $[\text{Fe}^{3+}]_i$  than the male UT – July ( $p = 0.049$ ), September B ( $p = 0.016$ )
- Female UT group showed significantly higher  $[\text{Fe}^{3+}]_i$  than male TR – May ( $p = 0.025$ ), June ( $p = 0.041$ );
- Female UT group showed significantly higher  $[\text{Fe}^{3+}]_i$  than the female TR group - March ( $p = 0.013$ )
- Female TR showed significantly higher  $[\text{Fe}^{3+}]_i$  than the male UT – July ( $p = 0.017$ )
- Female TR showed significantly higher  $[\text{Fe}^{3+}]_i$  than the male TR group – April ( $p = 0.04$ ), June ( $p = 0.044$ ), July ( $p = 0.008$ )

Each group showed significant changes in mean  $[\text{Fe}^{3+}]_i$  during the study ( $p < 0.05$ ):

- Male UT – significant increases between April and May, July and August; significant decreases between January and February, June and July, August and September A, September A and B.
- Male TR – significant increases between April and May, May and June, July and August; significant decreases between January and February, June and July, August and September A, October and November.

- Female UT – significant increases between February and March, April and May, July and August;  
significant decreases between January and February, June and July, August and September A, October and November.
- Female UT - the December  $[\text{Fe}^{3+}]_i$  was significantly higher than the January  $[\text{Fe}^{3+}]_i$ .
- Female TR – significant increases between March and April, April and May;  
significant decreases between January and February, June and July, August and September A.

Figure 35A: Changes in Mean Channel Log Fluorescence (Intracellular  $[Fe^{3+}]$ ) in  $CD4^+$  Lymphocytes in Males Aged 65 - 75 Years

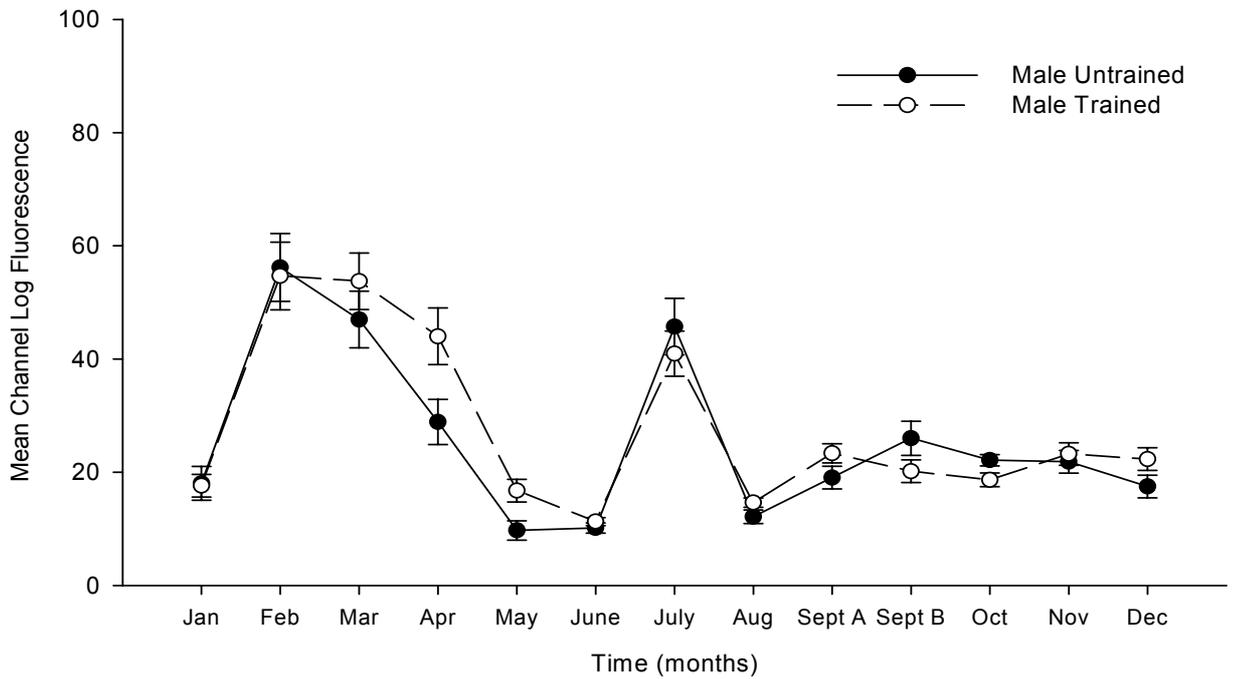
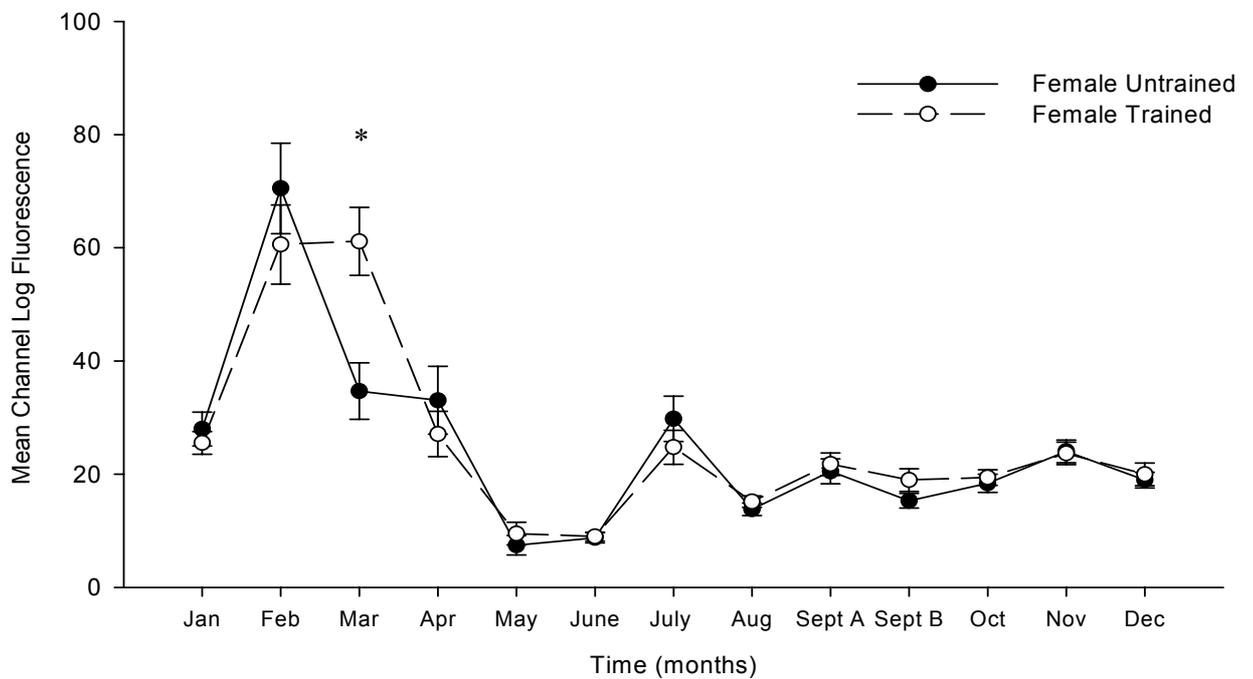


Figure 35B: Changes in Mean Channel Log Fluorescence (Intracellular  $[Fe^{3+}]$ ) in  $CD4^+$  Lymphocytes in Females Aged 65 - 75 Years



\* Significant differences between UT and TR groups for each gender ( $p < 0.05$ ). Error bars denote SEM.

#### 4.1.11. Regression Analysis

The relationships between the mean leucocyte concentration and other immunological variables for both trained and untrained groups of both genders was assessed using a stepwise multiple regression and Pearson Correlation (PC) matrix (Appendix 8.5). The multiple regression provided the strongest predictors of, and correlations with, mean leucocyte concentration. The Pearson Correlation matrix showed the strongest correlations between variables.

**Male UT:** the multiple regression summary showed that the best predictors of mean leucocyte concentration were CD71 density,  $[Ca^{2+}]_i$ ,  $[Ca^{2+}]_t$  and  $[Fe^{3+}]_i$ , with an  $r^2$  value of 0.67, and a correlation value of  $r = 0.82$ . The strongest correlations between variables were  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  ( $r = 0.87$ ); the percentage of cells positive for CD25 and CD25 density ( $r = 0.77$ ); CD25 density and  $[Ca^{2+}]_t$  ( $r = 0.66$ ); [Hb] and  $[Ca^{2+}]_i$  ( $r = -0.66$ ); [leucocyte] and CD71 density ( $r = -0.63$ ).

**Male TR:** the multiple regression summary showed that the best predictors of mean leucocyte concentration were the percentage of cells positive for CD25,  $[Ca^{2+}]_t$  and  $[Fe^{3+}]_i$  with an  $r^2$  value of 0.52, and a correlation value of  $r = 0.72$ . The strongest correlations between variables were  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  ( $r = 0.82$ ); [Hb] and CD71 density ( $r = -0.66$ ); [Hb] and CD25 density ( $r = -0.65$ ); percentage of cells positive for CD25 and those positive for CD71 ( $r = -0.67$ ); CD25 density and CD71 density ( $r = 0.60$ ).

**Female UT:** the multiple regression summary showed the best predictors of mean leucocyte concentration were CD25 density,  $[Ca^{2+}]_t$  and  $[Fe^{3+}]_i$ , with an  $r^2$  value of 0.65, and a correlation value of  $r = 0.80$ . The strongest correlations between variables were  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  ( $r = 0.57$ ); [leucocyte] and CD25 density ( $r = -0.54$ ); percentage of cells positive for CD71 and  $[Ca^{2+}]_t$  ( $r = 0.54$ ).

**Female TR:** the multiple regression summary showed the best predictors of mean leucocyte concentrations were percentage of cells positive for CD25, CD25 and CD71 density and [Hb], with an  $r^2$  value of 0.80, and a correlation value of  $r = 0.89$ . The strongest correlations between variables were  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  ( $r = 0.67$ ); [leucocyte] and CD71 density ( $r = 0.66$ ); [Hb] and CD25 density ( $r = -0.63$ ); CD25 density and the percentage of cells positive for CD71 ( $r = -0.55$ ); [leucocyte] and  $[Ca^{2+}]_i$  ( $r = 0.51$ ).

## 4.2 Experiment 2 - Males Aged 23 to 36 Years

### 4.2.1. General Health

Over the twelve month period of the present investigation there were no significant differences between mean pre- and post-study body mass, blood pressure or skinfold measurements within the UT and TR groups. The TR group had a significantly lower mean body mass, percent body fat, resting heart rate, resting systolic blood pressure and resting diastolic blood pressure compared to the UT group (Table 22).

Table 22: Pre-Study Physical Characteristics of the Participating Subjects

Variable	Untrained	Trained
Height (cm)	179 ± 2	177 ± 1
Body Mass (kg)	82.3 ± 3	73.5 ± 2 *
Age (yrs)	30 ± 2	30 ± 1
Body Fat (%)	21 ± 2	11 ± 1 *
Resting Heart Rate (b.min <sup>-1</sup> )	68 ± 3	48 ± 1 *
Resting Systolic Blood Pressure (mmHg)	123 ± 1	119 ± 1 *
Resting Diastolic Blood Pressure (mmHg)	80 ± 2	70 ± 2 *

\* Trained group significantly different from Untrained group ( $p < 0.05$ ).  
Data reported as mean±SEM

#### 4.2.2. Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ )

Trained and untrained subjects underwent an incremental test to exhaustion on a cycle ergometer as part of their initial screening and also during the month after the conclusion of the blood sampling procedures (Table 23). There were no significant differences between pre- and post-study  $\dot{V}O_{2\text{ peak}}$  ( $\text{L}\cdot\text{min}^{-1}$ ,  $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), peak power, peak heart rate and peak RER within the UT or TR groups. The TR group showed a significantly higher post-study  $\dot{V}_{E\text{ peak}}$  compared to the pre-study value. The UT group showed no significant difference between pre- and post-study  $\dot{V}_{E\text{ peak}}$ .

Table 23: Pre- and Post-Study Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ ) for Untrained and Trained Subjects

Variable	Untrained		Trained	
	Pre	Post	Pre	Post
$\dot{V}O_{2\text{ peak}}$ ( $\text{L}\cdot\text{min}^{-1}$ )	3.5±0.2	3.4±0.2	4.7±0.1*	4.8±0.1#
$\dot{V}O_{2\text{ peak}}$ ( $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ )	42±2	41±3	64±2 *	66±2 #
Power <sub>peak</sub> (W)	295±19	289±16	415±7 *	417±7 #
Heart Rate <sub>peak</sub> ( $\text{b}\cdot\text{min}^{-1}$ )	184±2	184±2	189±3	187±3
$\dot{V}_{E\text{ peak}}$ BTPS ( $\text{L}\cdot\text{min}^{-1}$ )	151±13	150±12	171±7	178±8**#
RER <sub>peak</sub>	1.27±2	1.27±3	1.27±3	1.23±2

\* Trained group (pre-study) significantly different from Untrained group (pre-study) ( $p < 0.001$ )

\*\* Trained group (post-study) significantly different from Trained group (pre-study) ( $p = 0.013$ )

# Trained group (post-study) significantly different from Untrained group (post-study) ( $p < 0.001$ )

Data presented as mean±SEM

There were significant differences ( $p < 0.05$ ) between the UT and TR groups for:

- Peak  $\dot{V}O_2$  ( $\text{L}\cdot\text{min}^{-1}$  and  $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ). The mean for the TR group was significantly higher than the UT.
- Peak power (Watts). The mean for the TR group was significantly higher than the UT.
- Peak  $V_E$  ( $\text{L}\cdot\text{min}^{-1}$ ). The post-study mean for the TR group was significantly higher than their pre-study mean. The pre-study mean  $\dot{V}_{E\text{ peak}}$  of the TR group was not statistically greater than

that of the UT ( $p = 0.06$ ). The post-study mean  $\dot{V}_{E\text{ peak}}$  of the TR group was significantly higher than that of the UT group ( $p < 0.001$ ).

#### 4.2.3. Incidence of URTI

The UT and TR groups reported seven and two cases of URTI respectively, during the study. The TR group reported one case of mild food poisoning.

#### 4.2.4. Haematology

The pre- and post-study haematology values for the UT and TR groups are shown in Table 24. Haematological indices remained within normal ranges for both groups for the duration of the study (Coulter 1984).

Table 24: Pre- and Post-Study Haematology Values for Untrained and Trained Groups

Variable	Untrained		Trained	
	Pre	Post	Pre	Post
Leucocytes (WBC x 10 <sup>9</sup> .L <sup>-1</sup> )	6.8±0.5	7.1±0.4	7.0±0.4	6.7±0.3
Erythrocytes (RBC x 10 <sup>12</sup> .L <sup>-1</sup> )	5.2±0.1	5.3±0.1	5.2±0.1	5.2±0.1
Haemoglobin (g.dL <sup>-1</sup> )	15.8±0.3	15.8±0.2	14.9±0.2*	15.1±0.2*
MCHC (g.dL <sup>-1</sup> )	33.7±0.4	33.2±0.2	32.8±0.3*	32.3±0.2*
Haematocrit (%)	47.1±0.9	47.7±0.6	45.9±0.7	47.5±0.5**
Platelets (PLT x 10 <sup>9</sup> .L <sup>-1</sup> )	210±9	238±7	180±7*	177±5*

\* Trained group significantly different from Untrained group ( $p < 0.05$ )

\*\* Post-study value significantly different to Pre-study value ( $p < 0.05$ )

Data presented as mean±SEM

*Note: In the following figures, significant differences between TR and UT groups are marked with an asterisk ( \* ) at the month they occur. Significant differences for each group between months of the year are not marked on the figures but are listed in the text preceding the figures.*

September A 1999 is the first month of the study, and September B 2000 is the final month. Two blood tests occurred four weeks apart in October, the first designated October A, the second October B.

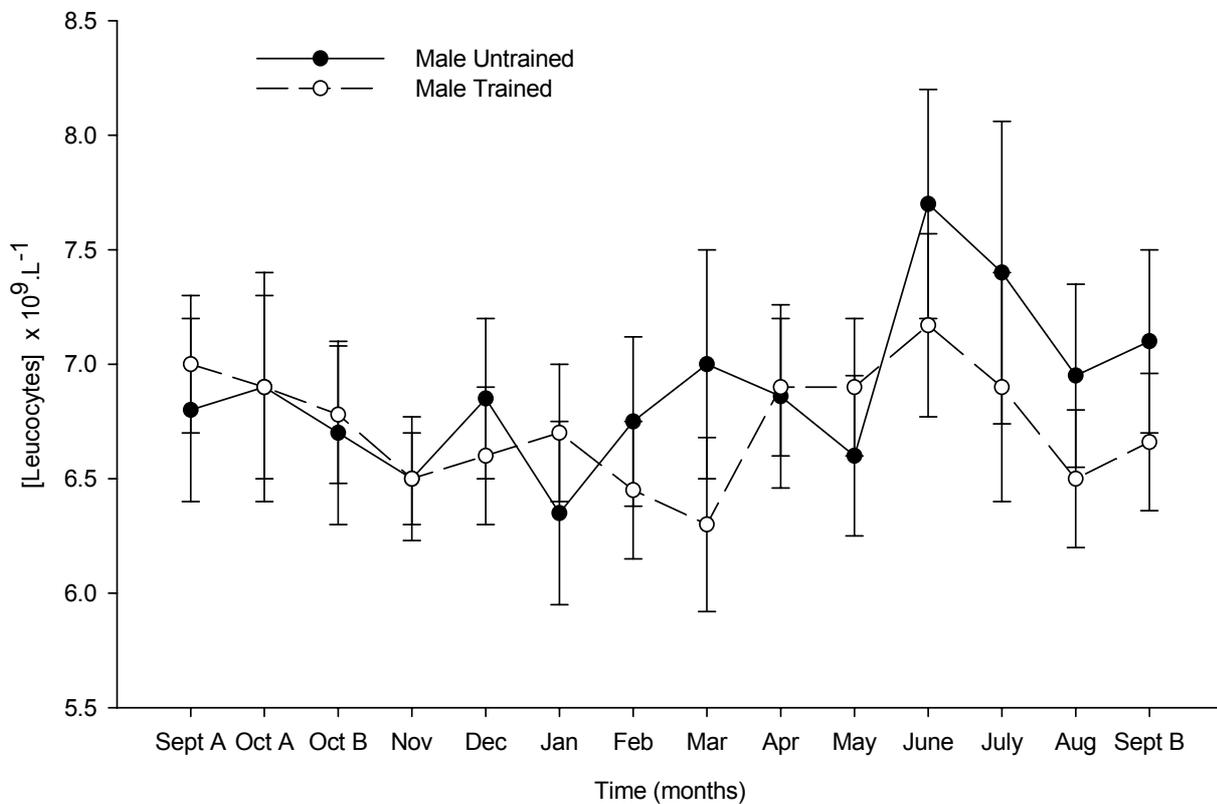
#### 4.2.4.1 Leucocytes

The changes in leucocyte concentration (mean $\pm$ SEM) in the TR and UT groups are shown in Figure 36. The mean leucocyte concentration for both groups remained within the normal range of 4 to 11  $\times 10^9.L^{-1}$  (Coulter 1984) for the duration of the study.

There were no significant between-group differences in leucocyte concentration at any of the blood sampling points during the twelve months of the study. Each group showed a significant increase between two months ( $p < 0.05$ ):

- Male UT – there was a significant increase between May and June.
- Male TR - there was a significant increase between March and April.

Figure 36: Changes in Leucocyte Concentration in Males Aged 23 to 36 Years



Error bars denote SEM.

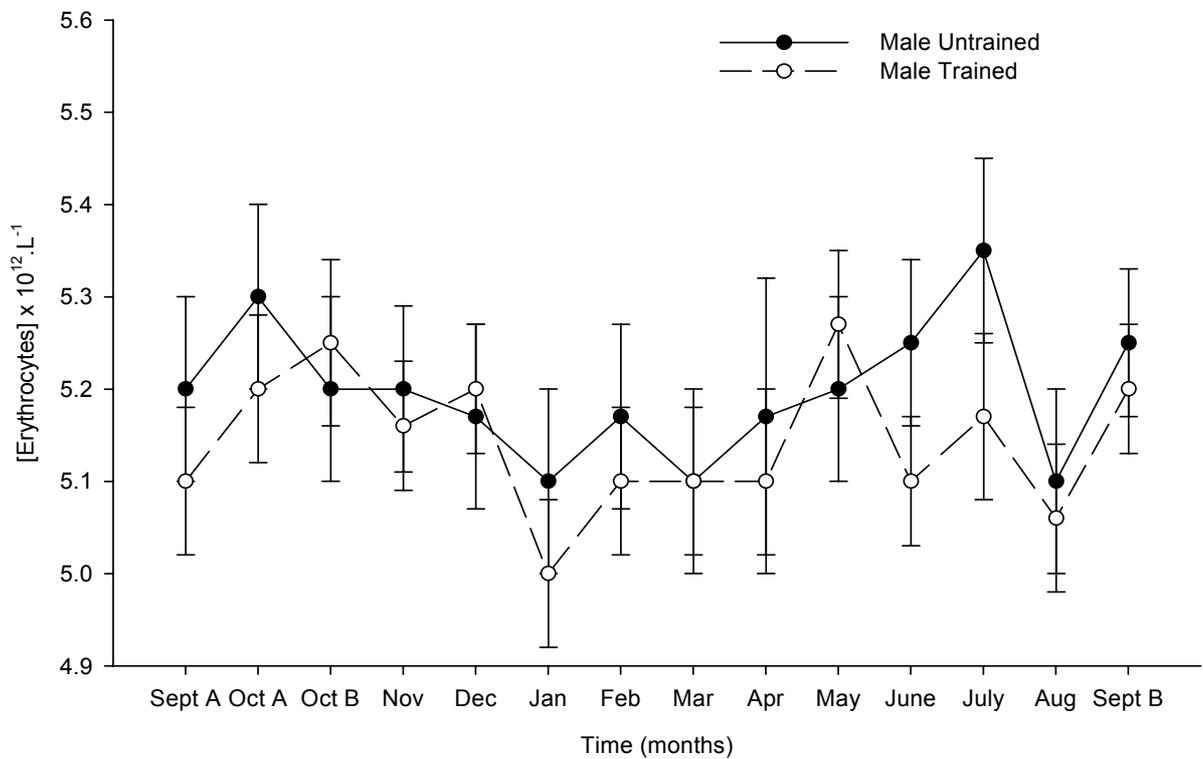
#### 4.2.4.2. Erythrocytes

The changes in erythrocyte concentration (mean $\pm$ SEM) for both UT and TR groups are shown in Figure 37. The mean erythrocyte concentration for both groups remained within the normal range of  $4.2\text{-}5.5 \times 10^{12}\cdot\text{L}^{-1}$  (Coulter 1984) for the duration of the study. There were no significant differences in mean erythrocyte concentration between UT and TR groups during the study.

Each group showed significant changes in erythrocyte concentration during the study ( $p < 0.05$ ):

- Male UT – significant increase between August and September B;  
significant decrease between July and August.
- Male TR – significant increase between August and September B;  
significant decreases between December and January, May and June.

Figure 37: Changes in Erythrocyte Concentration in Males Aged 23 to 36 Years



Error bars denote SEM.

#### 4.2.4.3. *Haemoglobin*

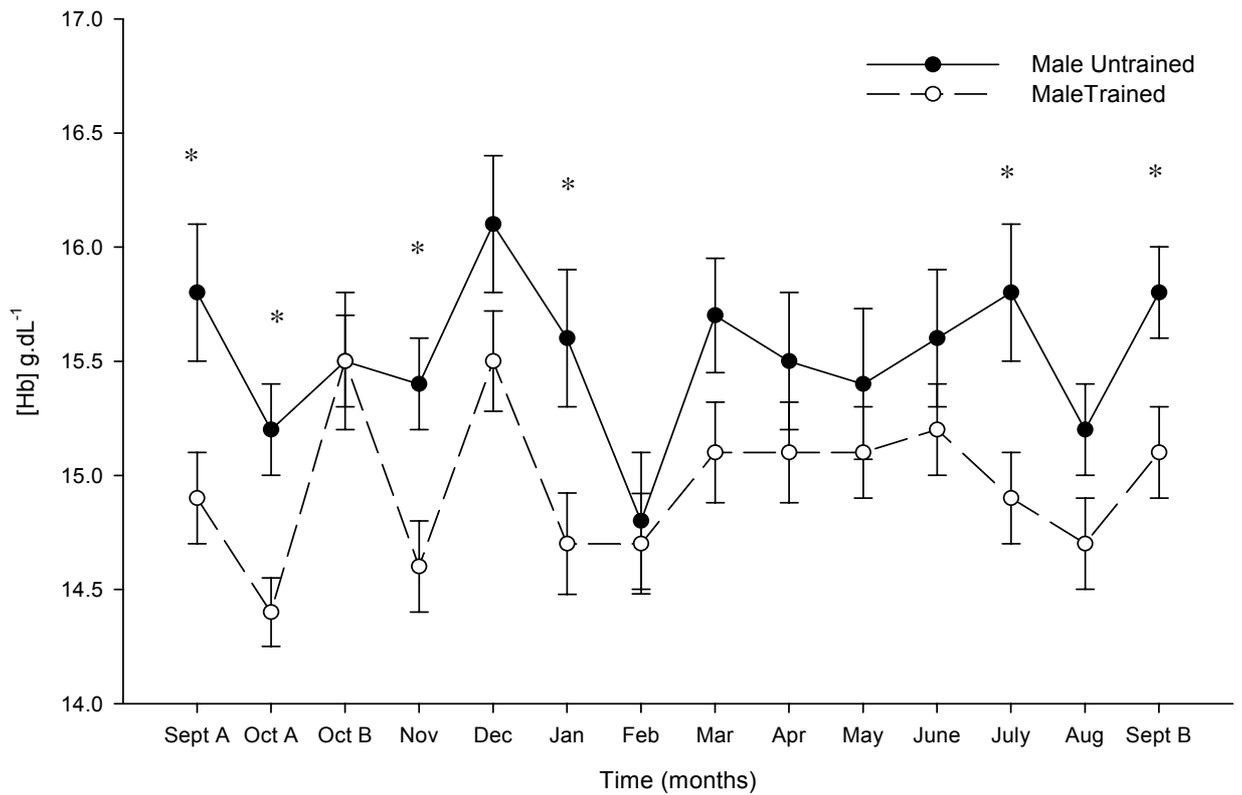
The changes in haemoglobin concentration (mean $\pm$ SEM) for the UT and TR groups are shown in Figure 38. The mean haemoglobin concentration for both groups remained within the normal range (14-16 g.dL<sup>-1</sup>; Coulter 1984). The TR group had a significantly lower mean haemoglobin concentration than the UT group during six months of the year ( $p < 0.05$ ).

- September A ( $p = 0.04$ )
- October A ( $p = 0.01$ )
- November ( $p = 0.02$ )
- January ( $p = 0.03$ )
- July ( $p = 0.04$ )
- September B ( $p = 0.04$ )

The UT group showed no significant changes in mean haemoglobin concentration between monthly sampling points. The TR group showed significant changes in mean haemoglobin concentration between the following months ( $p < 0.05$ ):

- significant increases between October A and October B, November and December.
- significant decrease between December and January.

Figure 38: Changes in Haemoglobin Concentration in Males Aged 23 to 36 Years



\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

The changes in mean corpuscular haemoglobin concentration (MCHC) (mean $\pm$ SEM) for UT and TR groups are shown in Figure 39. Mean MCHC in the UT and TR groups remained within the normal range (31.5 - 34.5 g.dL<sup>-1</sup>) except during the following months:

- October A - the TR group mean MCHC was 30.0 $\pm$ 0.3 g.dL<sup>-1</sup>
- February - the UT group mean MCHC was 31.2 $\pm$ 0.8 g.dL<sup>-1</sup> and the TR group mean MCHC was 31.3 $\pm$ 0.7 g.dL<sup>-1</sup>.

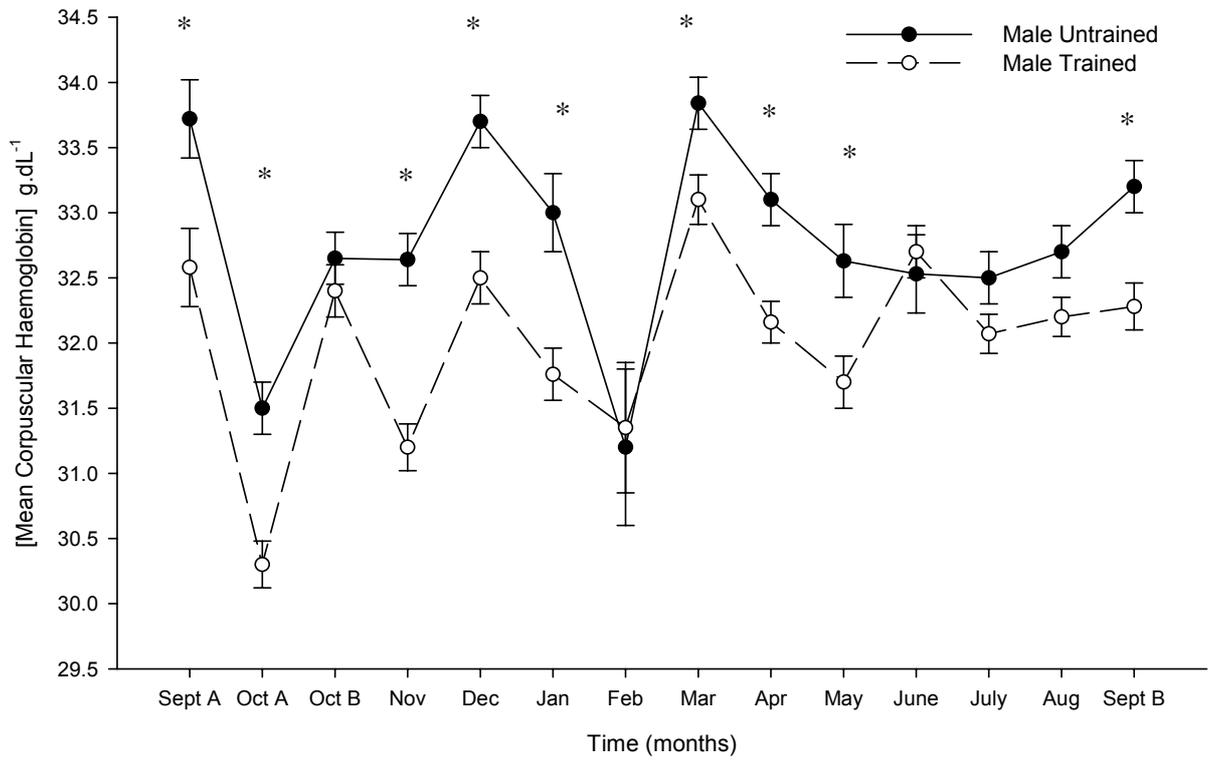
The mean MCHC in the TR group were significantly lower than the UT group during the following months ( $p < 0.05$ ):

- September A ( $p = 0.04$ )
- October A ( $p = 0.001$ )
- November ( $p < 0.001$ )
- December ( $p = 0.003$ )
- January ( $p = 0.01$ )
- March ( $p = 0.04$ )
- April ( $p = 0.002$ )
- May ( $p = 0.02$ )
- September B ( $p = 0.007$ )

Each group (UT,TR) showed significant changes in mean MCHC between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between October A and B, November and December, February and March;  
significant decreases between September A and October A, January and February, March and April.
- Male TR - significant increases between October A and B, November and December, February and March, May and June;  
significant decreases between September A and October A, October B and November, December and January, March and April, June and July.

Figure 39: Changes in Mean Corpuscular Haemoglobin Concentration in Males Aged 23 to 36 Years



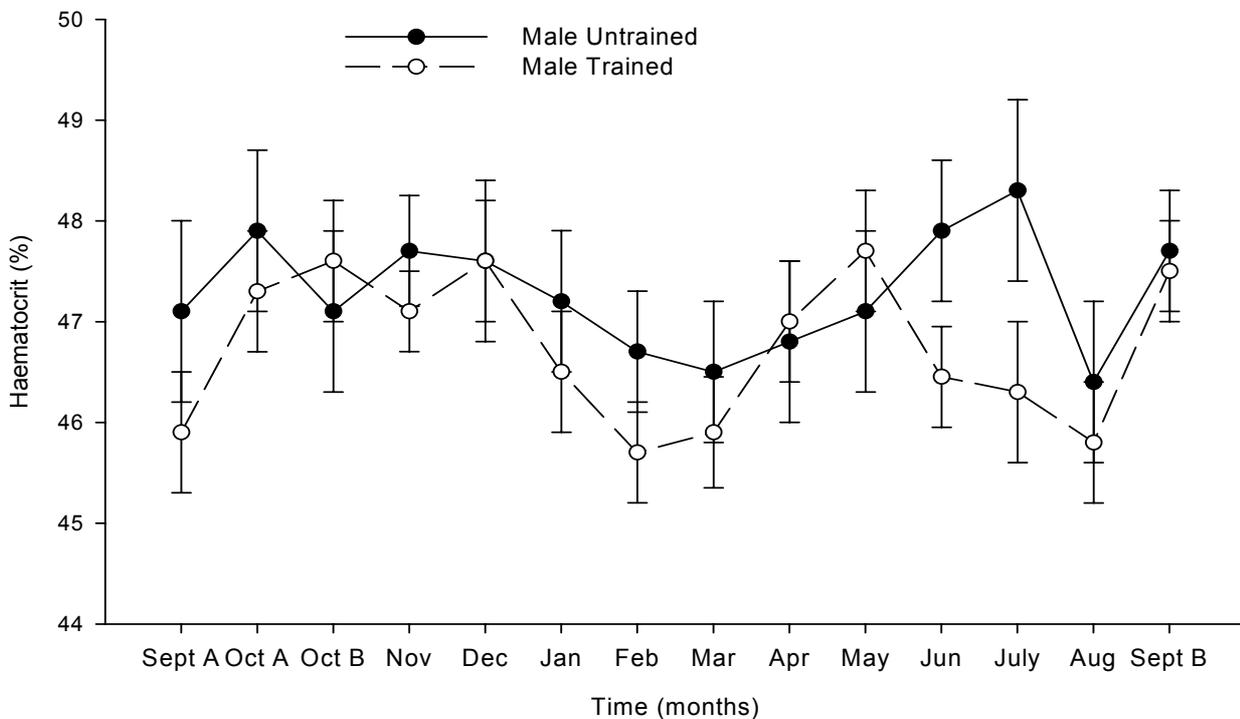
\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

#### 4.2.4.4 Haematocrit

The changes in haematocrit (mean $\pm$ SEM) for UT and TR groups are shown in Figure 40. The mean haematocrit of both groups remained within the normal range (42%-52%; Coulter 1984) during the study. There were no significant differences in mean haematocrit between groups at monthly sampling points during the study. Each group showed significant changes in mean haematocrit between months of the year ( $p < 0.05$ ):

- Male UT - significant increase between August and September B;  
significant decrease between July and August.
- Male TR - significant increases between September A and October A, March and April, August and September A;  
significant decreases between December and January, May and June.
- The September B post-test haematocrit of the TR group was significantly higher than the September A pre-test haematocrit ( $p = 0.03$ ).

Figure 40: Changes in Haematocrit in Males Aged 23 to 36 Years



Error bars denote SEM.

#### 4.2.4.5. Platelets

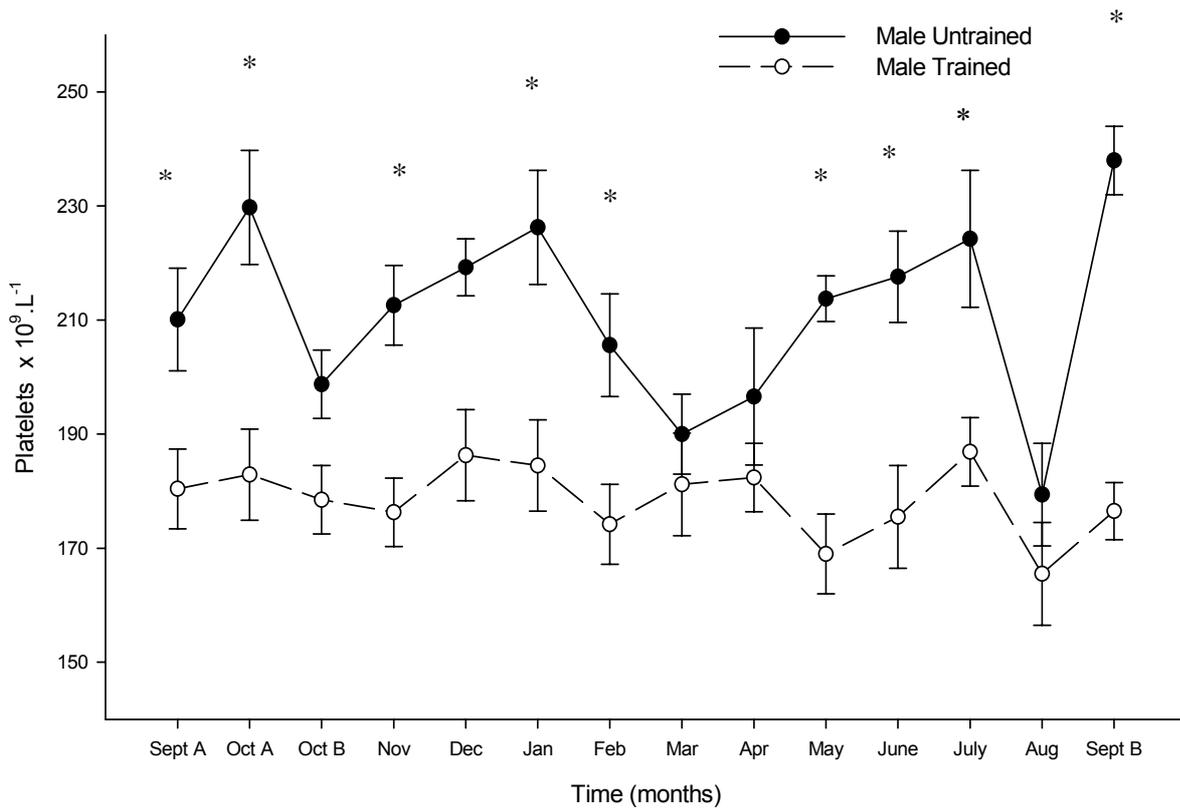
The changes in platelet concentration (mean $\pm$ SEM) for the UT and TR groups are shown in Figure 41. The mean platelet concentration of both groups remained within the normal range for the duration of the study (130–400  $\times 10^9.L^{-1}$ ). There were significant differences in mean platelet concentration between UT and TR groups throughout the year, with the UT group showing a significantly higher concentration during the following months:

- September A (p = 0.02)
- October A (p = 0.003)
- November (p = 0.002)
- January (p = 0.005)
- February (p = 0.04)
- May (p = 0.01)
- June (p = 0.001)
- July (p = 0.03)
- September B (p <0.001)

Each group (UT,TR) showed significant changes in platelet concentration between months of the year (p < 0.05):

- Male UT - significant increase between August and September B;  
significant decreases between October A and October B, July and August.
- Male TR - significant decrease between July and August.

Figure 41: Changes in Platelet Concentration in Males Aged 23 to 36 Years



\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

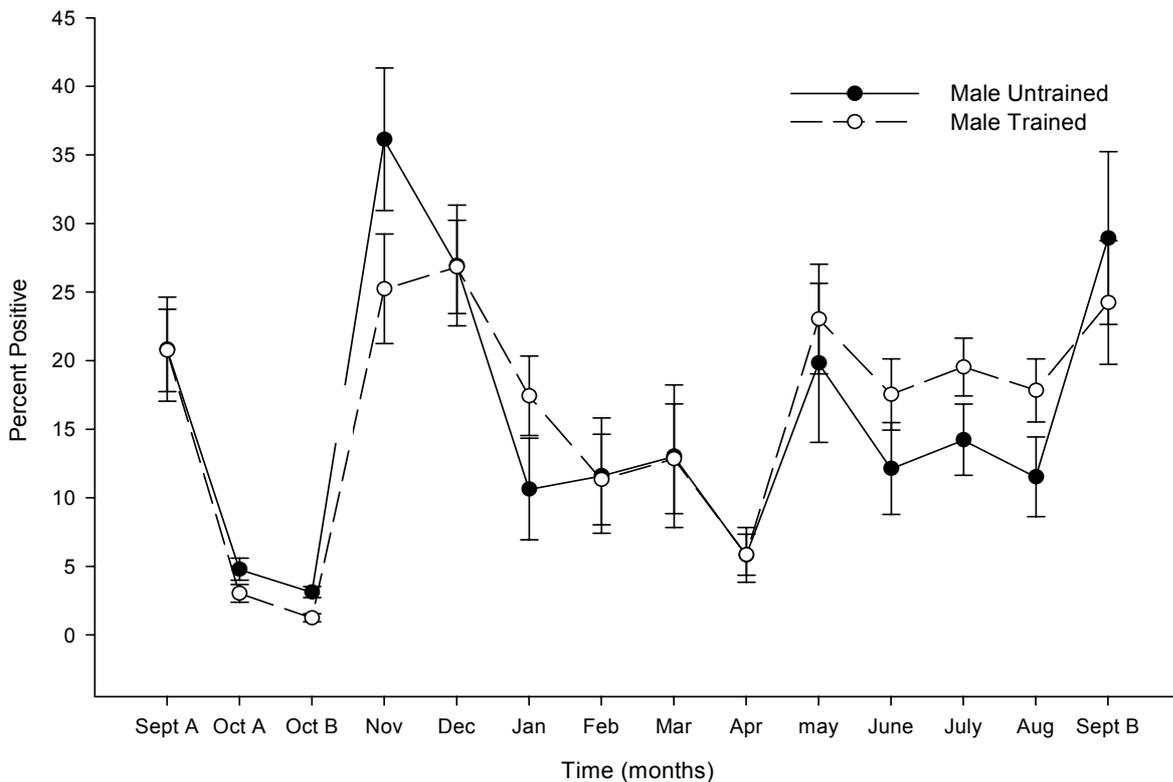
#### 4.2.5. Interleukin-2 Receptor (CD25) Expression and Density.

The changes in the percentage of CD4<sup>+</sup> lymphocytes expressing CD25 (mean±SEM) for the UT and TR groups are shown in Figure 42. There was no significant difference between TR and UT groups in the mean percentage of CD4<sup>+</sup> cells expressing CD25.

Each group showed significant changes in the mean percentage of CD4<sup>+</sup> lymphocytes expressing CD25 between months of the year ( $p < 0.05$ ):

- Male UT - significant increases between October B and November, April and May, August and September B;  
significant decreases between September A and October A, December and January.
- Male TR - significant increases between October B and November, April and May;  
significant decreases between September A and October A, October A and B, March and April.

Figure 42: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25 in Males Aged 23 to 36 Years



Error bars denote SEM.

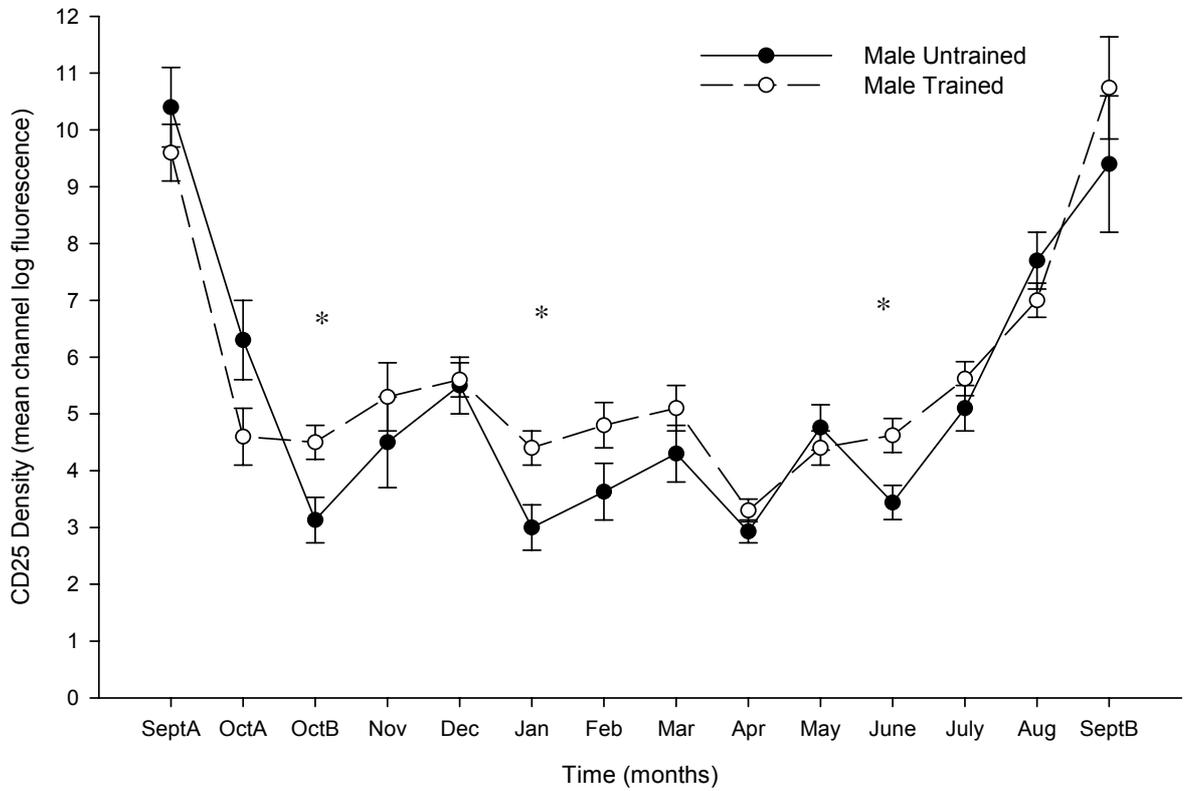
The changes in the density of CD25 expressed on the surface of CD4<sup>+</sup> lymphocytes (mean $\pm$ SEM) for UT and TR groups are shown in Figure 43. Receptor density was represented by mean channel log fluorescence. The TR group showed significantly greater mean CD25 density during the following months ( $p < 0.05$ ):

- October B ( $p = 0.02$ )
- January ( $p = 0.02$ )
- June ( $p = 0.014$ )

Each group showed significant changes in mean CD25 density on CD4<sup>+</sup> lymphocytes between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between April and May, July and August; significant decrease between October A and October B.
- Male TR – significant decreases between September A and October A, March and April.

Figure 43: Changes in CD25 Density on CD4<sup>+</sup> Lymphocytes in Males Aged 23 to 36 Years



\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

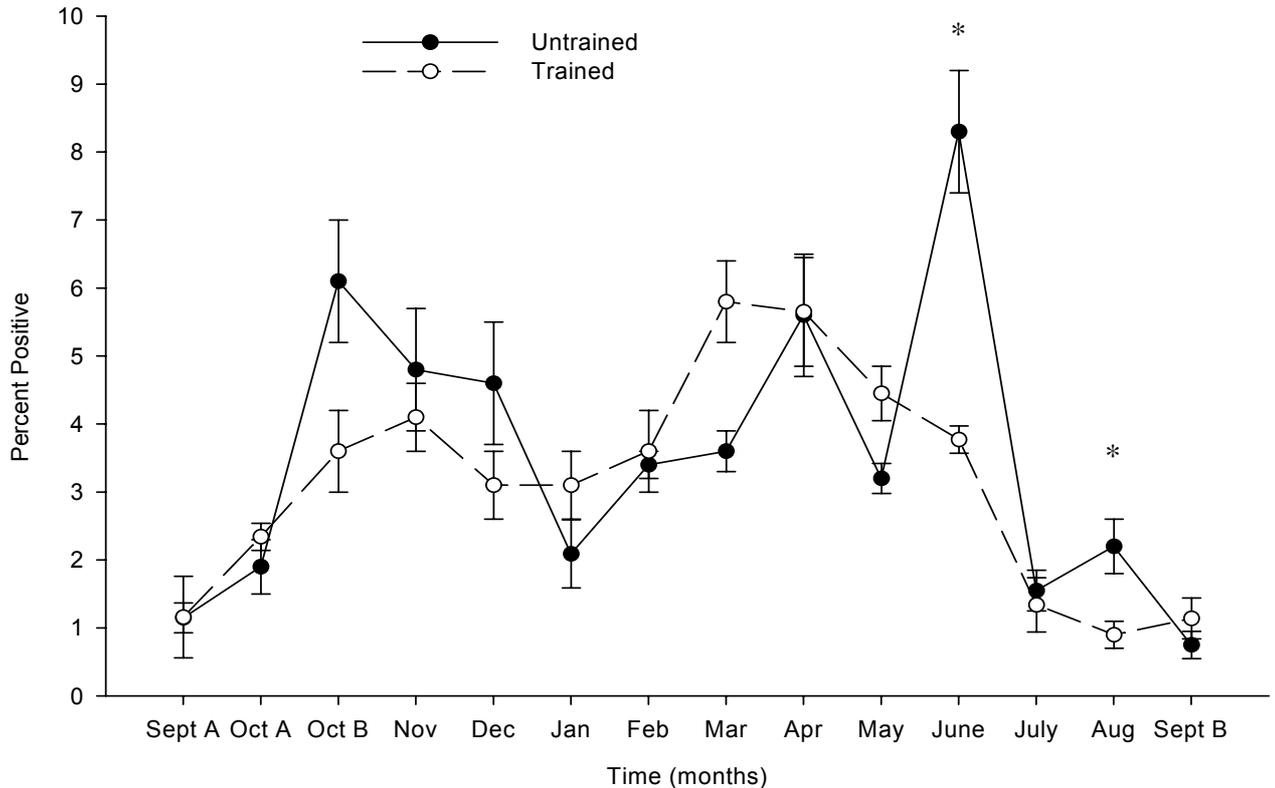
#### 4.2.6. Transferrin Receptor (CD71) Expression and Density

The changes in the mean percentage of CD4<sup>+</sup> lymphocytes expressing CD71 (mean±SEM) for both UT and TR groups are shown in Figure 44. The UT group had a significantly higher percentage of CD4<sup>+</sup> lymphocytes expressing CD71 during June ( $p = 0.001$ ) and August ( $p = 0.03$ ).

Each group showed significant changes in the mean percentage of CD4<sup>+</sup> lymphocytes positive for CD71 between months of the year ( $p < 0.05$ ):

- Male UT - significant increase between May and June; October A and October B  
significant decreases between June and July, August and September B.
- Male TR - significant decrease between June and July.

Figure 44: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD71 in Males Aged 23 to 36 Years

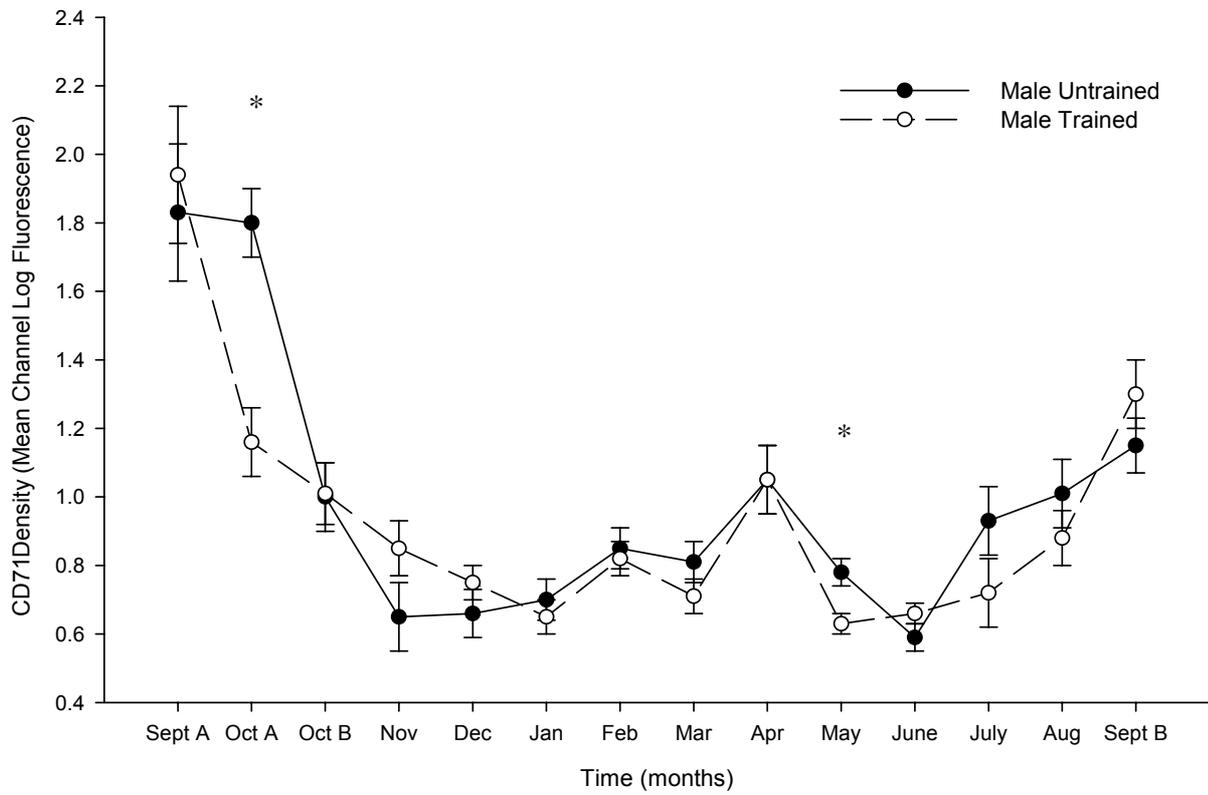


\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

The changes in the mean density of CD71 (mean $\pm$ SEM) for both groups are shown in Figure 45. The mean density of CD71 expression was denoted by mean channel log fluorescence. The UT group showed significant greater mean CD71 density than the TR group in October A ( $p = 0.014$ ) and May ( $p = 0.01$ ).

There were significant changes in mean CD71 density on CD4<sup>+</sup> lymphocytes for the UT and TR groups during months of the year ( $p < 0.05$ ):

- Male UT - significant decreases between October A and October B, October B and November, May and June.
- Male TR - significant increases between March and April, August and September; significant decreases between September A and October A, April and May.
- The TR group showed significantly lower September B post-test receptor density than September A pre-test receptor density.

Figure 45: Changes in CD71 Density on CD4<sup>+</sup> Lymphocytes in Males Aged 23 to 36 Years

\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

#### 4.2.7. CD4<sup>+</sup> Lymphocytes Positive for both CD25 and CD71 - Receptor Expression and Density

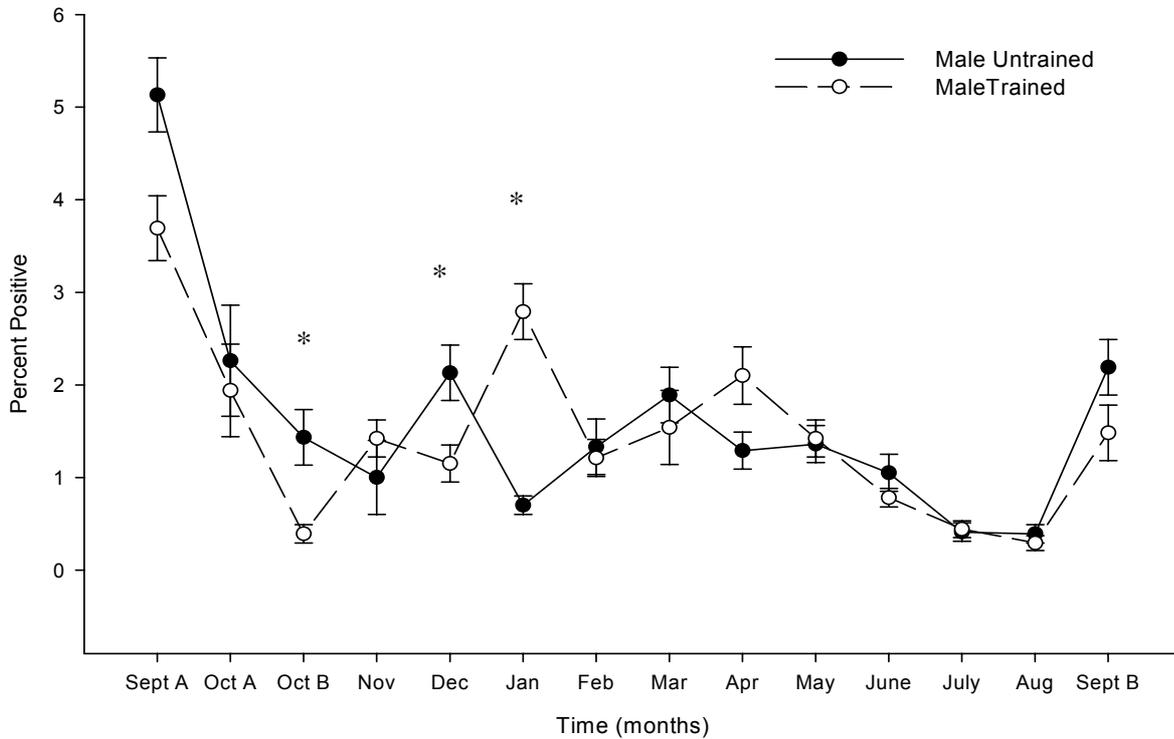
The changes in the mean percentage of CD4<sup>+</sup> lymphocytes expressing both CD25 and CD71 (mean±SEM) in the UT and TR groups are shown in Figure 46. There were significant differences between groups in the following months ( $p < 0.05$ ):

- TR group showed a significantly lower percentage of “double positive” CD4<sup>+</sup> lymphocytes than the UT group - October B ( $p = 0.013$ ), December ( $p = 0.049$ )
- TR group showed a significantly higher percentage of “double positive” CD4<sup>+</sup> lymphocytes than the UT group - January ( $p = 0.001$ )

Each group showed significant changes in the mean percentage of “double positive” CD4<sup>+</sup> cells between months of the year ( $p < 0.05$ ):

- Male UT - significant increase between August and September B;  
significant decreases between December and January, June and July.
- Male TR - significant increases between October B and November, December and January, August and September B;  
significant decreases between October A and B, January and February, May and June.
- Both the UT and TR groups showed a significantly lower post-study mean percentage of “double positive” CD4<sup>+</sup> lymphocytes compared to the pre-study mean percentage of “double positive” lymphocytes ( $p = 0.03$ ).

Figure 46: Changes in CD4<sup>+</sup> Lymphocytes Positive for CD25/CD71 in Males Aged 23 to 36 Years

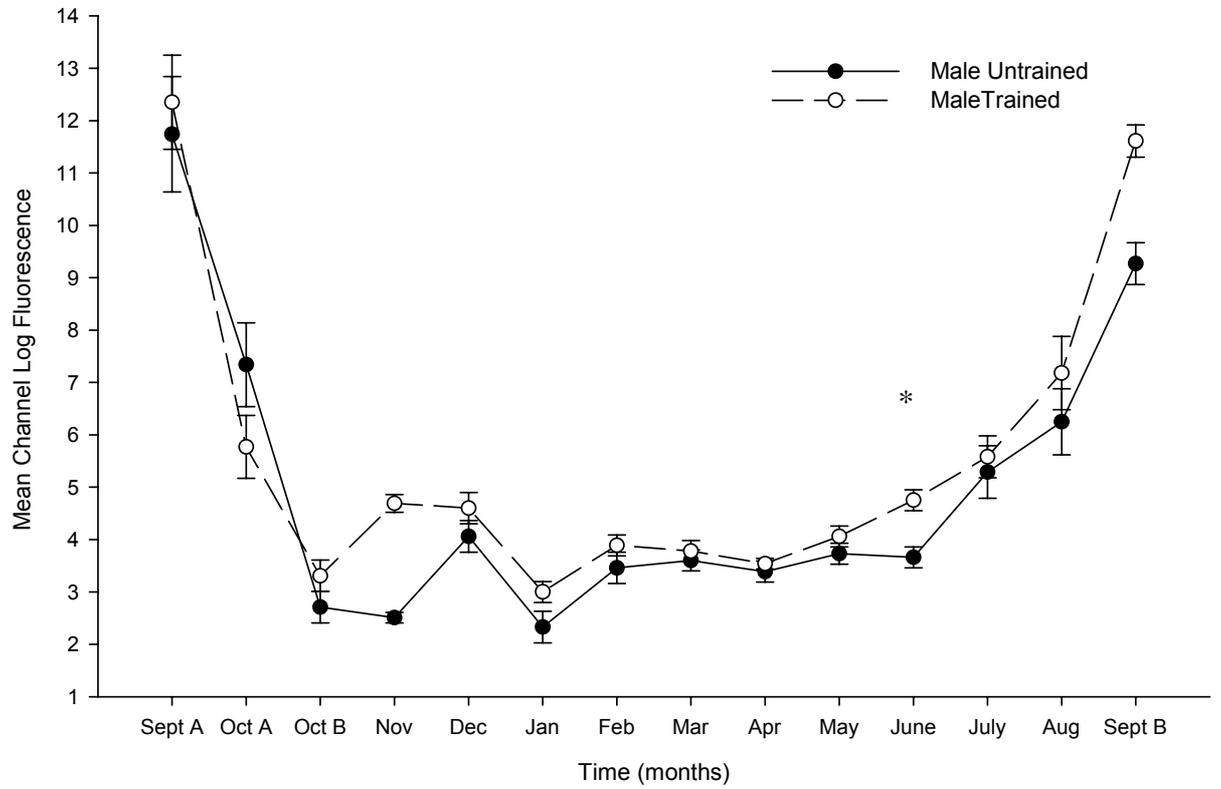


\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

The changes in mean CD25 density (mean channel log fluorescence) on the “double positive” CD4<sup>+</sup> lymphocytes (mean $\pm$ SEM) for UT and TR groups are shown in Figure 47. The TR group showed a significantly greater mean density of CD25 in June ( $p = 0.005$ ). Both UT and TR groups showed significant changes in mean CD25 density between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between January and February, June and July; significant decreases between September A and October A, October A and B, December and January.
- Male TR – significant increases between January and February, May and June, August and September B; significant decreases between September A and October A, October A and B, December and January.

Figure 47: Changes in CD25 Density on Double Positive Lymphocytes in Males Aged 23 to 36 Years



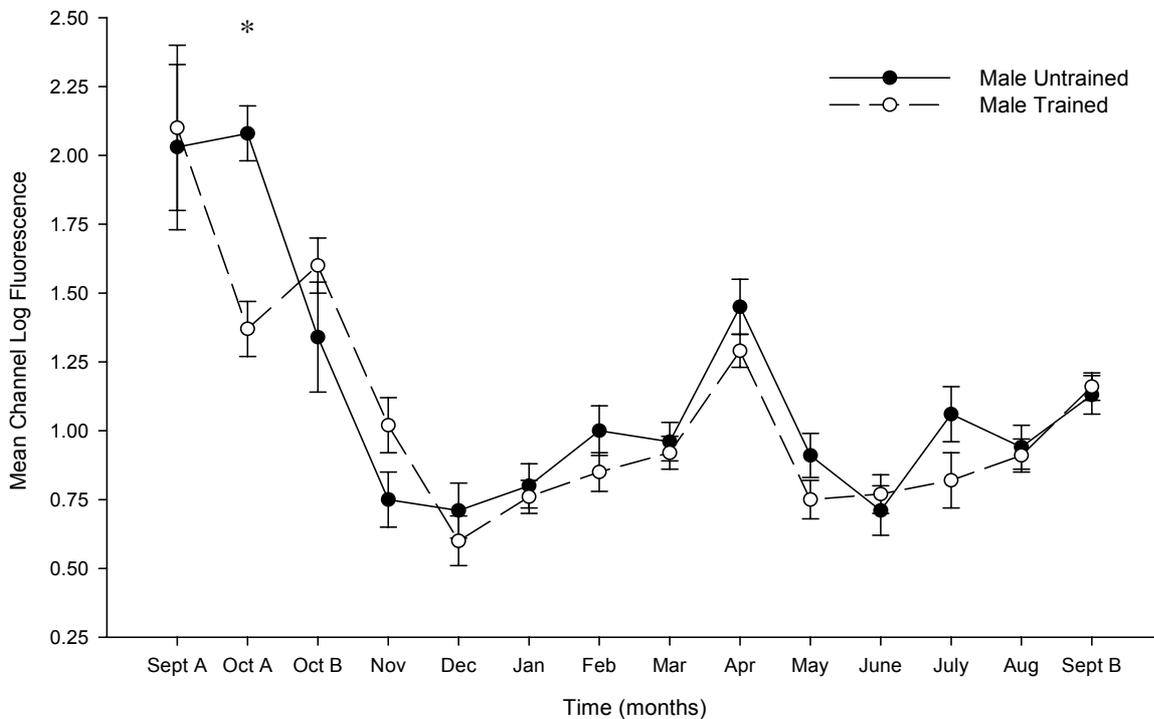
\* Significant difference between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

The changes in mean CD71 density on “double positive” CD4<sup>+</sup> lymphocytes (mean±SEM) for UT and TR groups are shown in Figure 48. The mean density of CD71 on the “double positive” CD4<sup>+</sup> lymphocytes for the TR group was significantly lower than the UT group during October A ( $p = 0.008$ ).

Each group showed significant changes in mean CD71 density between months of the year ( $p < 0.05$ )

- Male UT – significant increases between March and April, June and July; significant decreases between October A and B, April and May.
- Male TR – significant increases between March and April, August and September B; significant decreases between October A and October B, October B and November, November and December, April and May.
- The post-study mean CD71 density was significantly lower than the pre-study CD71 density, for both groups (UT  $p = 0.04$ , TR  $p = 0.009$ )

Figure 48: Changes in CD71 Density on Double Positive Lymphocytes in Males Aged 23 to 36 years



\* Significant difference between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

#### 4.2.8. Intracellular Calcium

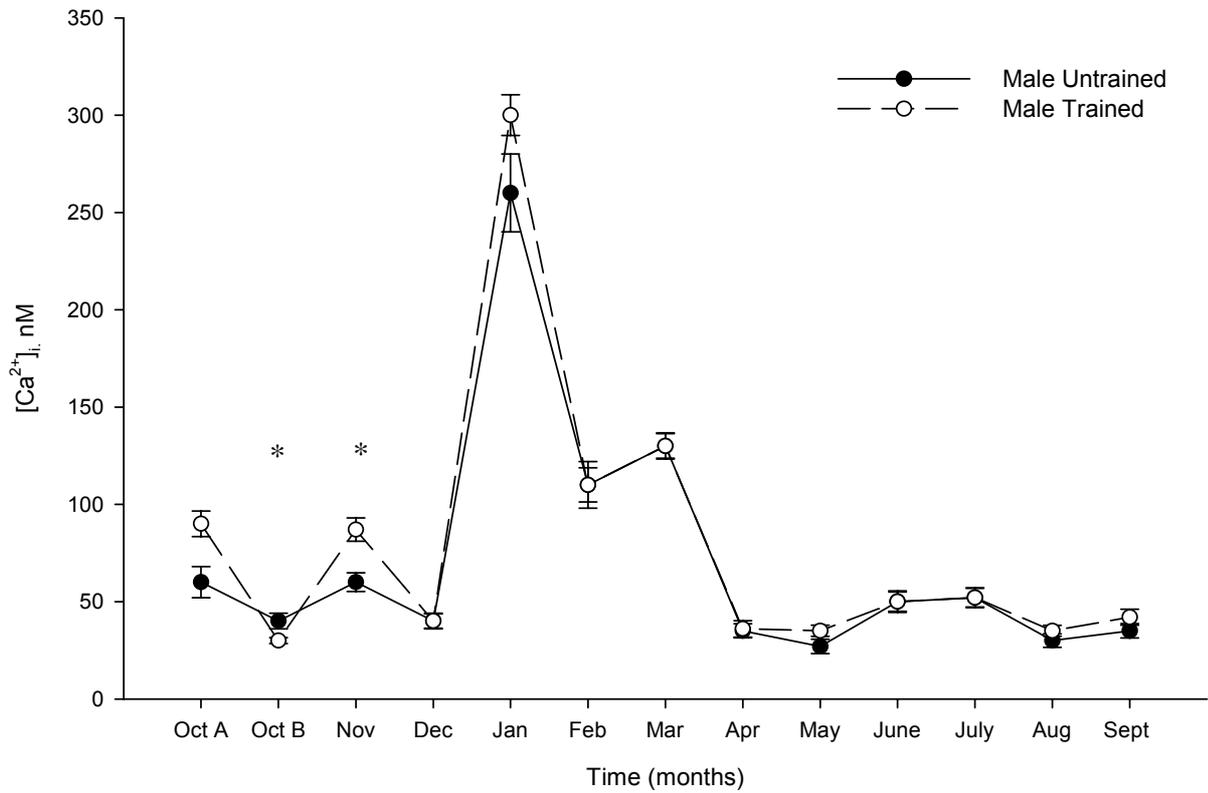
##### 4.2.8.1. Cytosolic Calcium

The changes in  $[Ca^{2+}]_i$  (mean $\pm$ SEM) for the UT and TR groups are shown in Figure 49. The UT group had significantly higher mean  $[Ca^{2+}]_i$  compared to the TR group during October B ( $p = 0.03$ ). The UT  $[Ca^{2+}]_i$  was calculated to be  $40 \pm 1$  nM compared to the TR  $[Ca^{2+}]_i$  of  $30 \pm 1$  nM. The TR group had significantly higher  $[Ca^{2+}]_i$  of  $87 \pm 5$  nM in November compared to the UT group's  $[Ca^{2+}]_i$  of  $60 \pm 3$  nM ( $p = 0.007$ ). The highest  $[Ca^{2+}]_i$  were  $263 \pm 20$  nM (UT) and  $300 \pm 12$  nM (TR) during January.

Each group showed significant changes in mean  $[Ca^{2+}]_i$  between months of the year ( $p < 0.05$ ):

- Male UT – significant increases between October B and November, December and January;  
significant decreases between October A and October B, November and December, January and February, March and April, July and August.
- The lowest recorded  $[Ca^{2+}]_i$  for the UT group was  $27 \pm 0.05$  nM was recorded in May, while the highest concentration of  $263 \pm 20$  nM was recorded in January.
- Male TR – significant increases between October B and November, December and January;  
significant decreases between October A and October B, November and December, January and February, March and April, July and August.
- The lowest recorded  $[Ca^{2+}]_i$  for the TR group was  $30 \pm 1$  nM recorded in October B, while the highest concentration of  $300 \pm 12$  nM was recorded in January.

Figure 49: Changes in Intracellular Cytosolic Calcium ( $[Ca^{2+}]_i$ ) in  $CD4^+$  Lymphocytes in Males Aged 23 to 36 Years



\* Significant difference between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

*Note: We were unable to measure  $[Ca^{2+}]_i$  in September A, as the fluorescent probe had not been delivered. The first measurement occurred in October A.*

#### 4.2.8.2 Total Intracellular Calcium

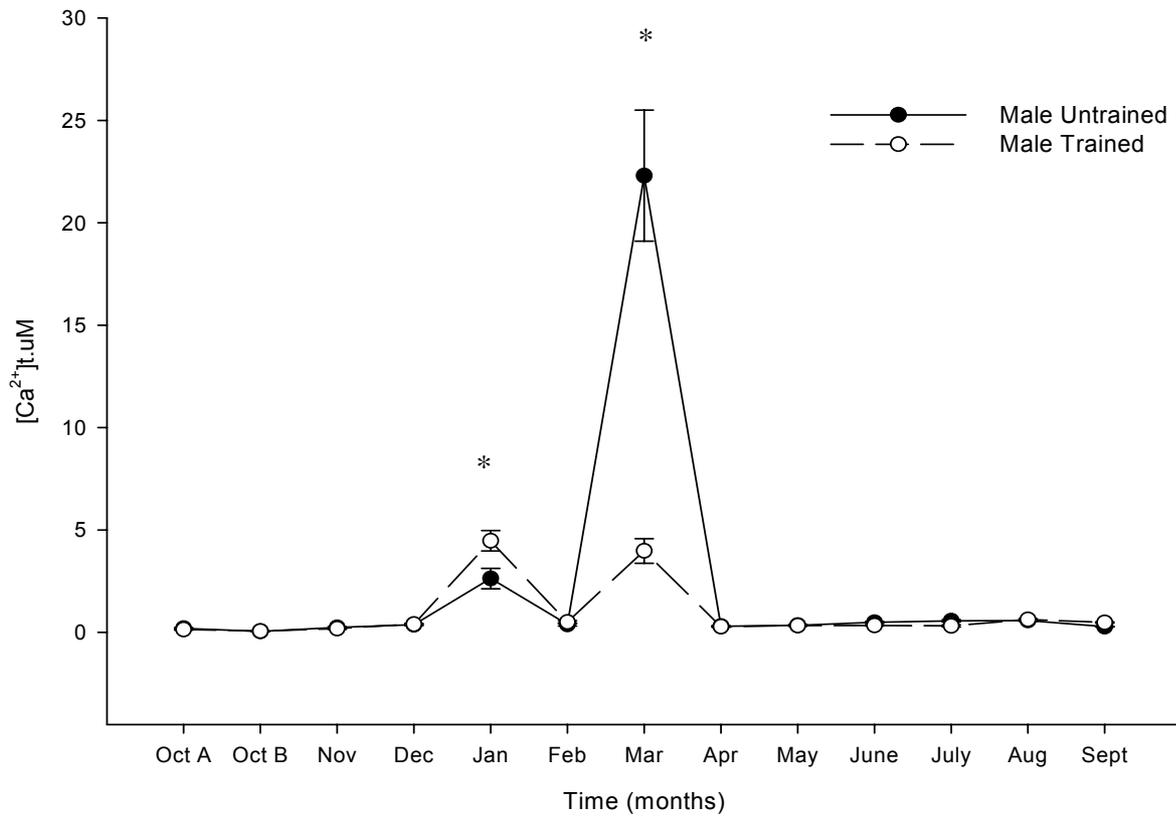
The changes in  $[Ca^{2+}]_t$  (mean $\pm$ SEM) for the UT and TR groups are shown in Figure 50. The mean  $[Ca^{2+}]_t$  was significantly higher than  $[Ca^{2+}]_i$  concentrations for both groups at all sample points during the twelve months of the study ( $p < 0.001$ ).

The male UT group showed a significantly higher mean  $[Ca^{2+}]_t$  of  $22.3\pm 3 \mu\text{M}$  in March ( $p = 0.015$ ), compared to the TR group ( $4.00\pm 0.6 \mu\text{M}$ ). The male TR group showed a significantly higher  $[Ca^{2+}]_t$  ( $4.5\pm 0.5 \mu\text{M}$ ) in January ( $p = 0.017$ ) compared to the UT group ( $2.6\pm 0.5 \mu\text{M}$ ).

Each group showed significant changes in mean  $[Ca^{2+}]_t$  between months of the year ( $p < 0.05$ ):

- Male UT - significant increases between December and January, February and March; significant decreases between January and February, March and April.
- Male UT - the lowest  $[Ca^{2+}]_t$  of  $50\pm 3 \text{ nM}$  was recorded in October A whilst the highest  $[Ca^{2+}]_t$  of  $22.3\pm 3 \mu\text{M}$  was recorded in March.
- Male TR - significant increases between December and January, February and March; significant decreases between January and February, March and April.
- Male TR - the lowest  $[Ca^{2+}]_t$  of  $56\pm 4 \text{ nM}$  was recorded in October B and the highest  $[Ca^{2+}]_t$  of  $4.5\pm 0.5 \mu\text{M}$  was recorded in January.

Figure 50: Changes in Total Intracellular Calcium ( $[Ca^{2+}]_t$ ) in  $CD4^+$  Lymphocytes in Males Aged 23 to 36 Years



\* Significant difference between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

*Note: We were unable to measure  $[Ca^{2+}]_t$  in September A, as the fluorescent probe had not been delivered. The first measurement occurred in October A.*

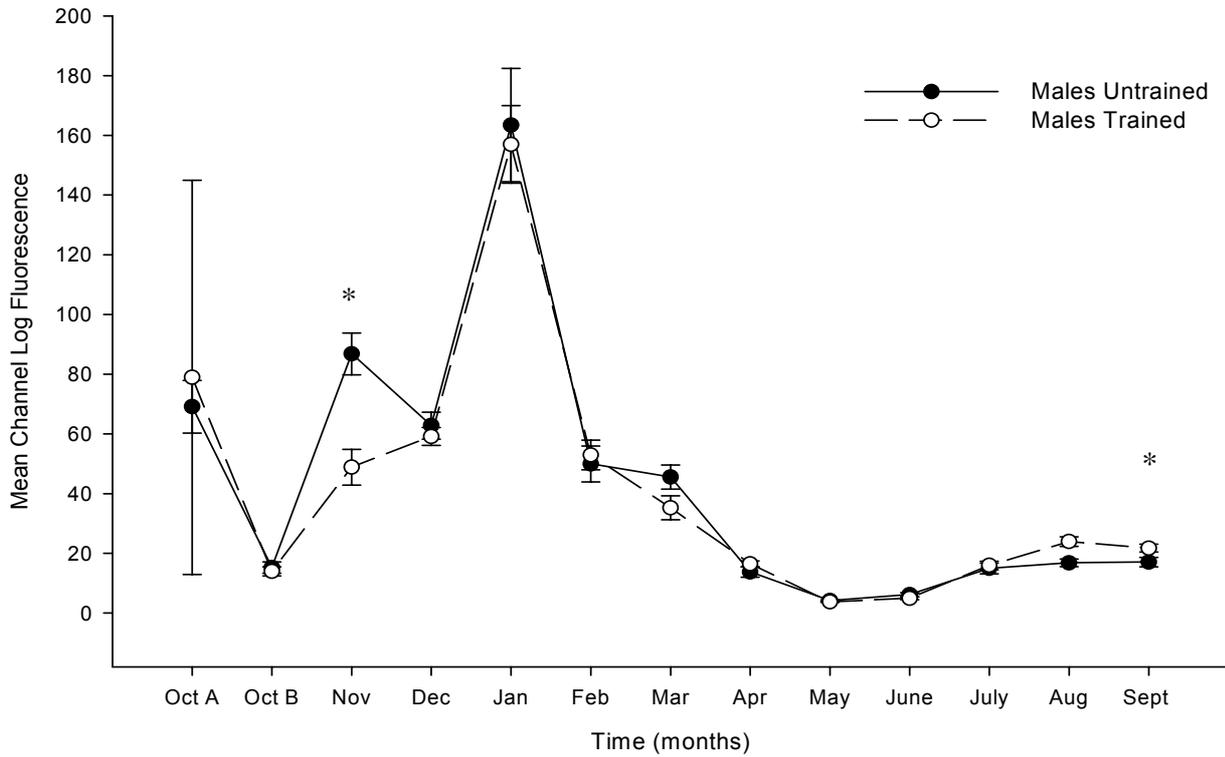
#### 4.2.9. Intracellular Iron

The changes in mean channel log fluorescence for UT and TR groups, estimating  $[\text{Fe}^{3+}]_i$ , (mean $\pm$ SEM) are shown in Figure 51. The UT group showed significantly higher mean channel log fluorescence than the TR group in November ( $p = 0.001$ ), while the TR group showed significantly higher mean channel log fluorescence than the UT in September B ( $p = 0.04$ ).

Each group showed significant changes in mean channel log fluorescence between months of the year ( $p < 0.05$ ):

- Male UT – significant decreases in  $[\text{Fe}^{3+}]_i$  between October B and November, December and January, May and June, June and July;  
significant increases between October A and B, November and December, January and February, March and April, April and May.
- Male TR – significant decreases between October B and November, December and January, May and June, June and July;  
significant increases between October A and B, January and February, March and April, April and May.
- Both TR and UT groups had significantly higher post-study  $[\text{Fe}^{3+}]_i$  compared to pre-study  $[\text{Fe}^{3+}]_i$ .

Figure 51: Changes in Mean Channel Log Fluorescence (Intracellular  $[Fe^{3+}]$ ) in Males Aged 23 to 36 Years



\* Significant differences between UT and TR groups ( $p < 0.05$ ). Error bars denote SEM.

*Note: We were unable to measure  $[Fe^{3+}]$  in September A, as the fluorescent probe had not been delivered. The first measurement occurred in October A.*

#### 4.2.10. Regression Analysis

The relationship between the concentration of leucocytes and the changes in the other immunological variables was assessed using a stepwise multiple regression and Pearson correlation (PC) matrix (Appendix 8.5) for both the TR and UT groups. The multiple regression provided the strongest predictors of, and correlations with, leucocyte concentration. The Pearson correlation matrix showed the strongest correlations between variables.

**Male UT** – the multiple regression model showed that the best predictors of mean leucocyte concentration were the percentage of lymphocytes positive for CD71,  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$ , with an  $r^2$  value of 0.73, and a correlation value of  $r = 0.86$ .

Within the correlation matrix, the strongest correlations occurred between  $[Ca^{2+}]_i$  and  $[Fe^{3+}]$  ( $r = 0.80$ ); CD25 density and the percentage of cells positive for CD71 ( $r = -0.67$ ); CD71 density and CD25 density ( $r = 0.65$ ); [leucocyte] and  $[Ca^{2+}]_t$  ( $r = 0.67$ );  $[Ca^{2+}]_t$  and the percentage of cells positive for CD71 ( $r = 0.60$ ); [leucocyte] and  $[Fe^{3+}]$  ( $r = -0.57$ ).

**Male TR** – the multiple regression model showed that the best predictors of mean leucocyte concentration were the percentage of lymphocytes positive for CD71, CD25 receptor density and  $[Fe^{3+}]$ , with an  $r^2$  value of 0.25, and a correlation value of  $r = 0.50$ .

Within the correlation matrix, the strongest correlations occurred between  $[Ca^{2+}]_i$  and  $[Fe^{3+}]$  ( $r = 0.91$ ); the percentage of cells positive for CD71 and CD25 density ( $r = -0.71$ ); CD71 density and CD25 density ( $r = 0.67$ );  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  ( $r = 0.65$ ).



## 5.0 Discussion

### 5.1 Experiment 1 - Males and Females Aged 65 to 75 Years

The principal findings of the present investigation were that long-term moderate intensity training significantly increased functional capacity in male and female subjects, and significantly increased CD25 expression in older males but not older females. Moderate intensity training did not significantly affect  $[Ca^{2+}]_i$  or  $[Fe^{3+}]_i$ . There were significant seasonal changes in CD25 expression, CD71 expression,  $[Ca^{2+}]_i$  and  $[Fe^{3+}]_i$  in TR and UT groups, suggesting that spring and autumn might be periods of decreased lymphocyte proliferation while summer was a period of increased lymphocyte proliferation and transcription. The training-induced increase in CD25 expression in older males would appear to reduce the likelihood of URTI occurring in these subjects, whereas female subjects may be more at risk of URTI during months of reduced lymphocyte proliferation. Thus moderate-intensity training appears to improve general health and well-being in older males, whereas the effects of moderate intensity training on the immune status and health of older females remains less clear.

#### 5.1.1. Subject Characteristics

The men and women who participated in Experiment 1 were free from any clinical signs of cardiovascular and respiratory disease and represented groups of untrained healthy older men and women. The body mass, percent body fat, resting systolic and diastolic blood pressure and heart rate of male and female subjects were consistent with values reported by Seals et al. (1984), Spina et al. (1993), Woods et al. (1999), Moreau et al. (2001) and Morris et al. (2002) for similarly aged individuals.

The present study did not find a significant decrease in body mass in the TR groups during the twelve month training period. The decrease in body mass over the twelve month period was 2 kg (2.3%) for males and  $2 \pm 0.5$  kg (3%) for females, and is consistent with previous findings (Seals et al., 1984; Hagberg et al., 1989; Spina et al., 1993; Woods et al., 1999; Moreau et al., 2001). Both the TR male and female groups showed a significant decrease of 1% in the percent body fat. The decrease in percent body fat was also consistent with the results of other long-term studies (Seals et al., 1984; Hagberg et al., 1989; Spina et al., 1993; Woods et al., 1999; McCole et al., 2000; Moreau et al., 2001; Wilmore et al., 2001a,b). The magnitude of the expected decrease in percent

body fat appears to depend upon the initial level of obesity of the subjects, the duration of the training study, and the intensity of the training sessions (Williams 2001). Seals et al. (1984) and Durstine et al. (2001) noted that training at a low to moderate intensity (40%-60% heart rate reserve, 50%-60%  $\dot{V}O_{2\text{ peak}}$ ) resulted in a smaller reduction in skinfold thickness than training at a higher intensity (>75% heart rate reserve, >60%  $\dot{V}O_{2\text{ peak}}$ ). It was also stated that the more obese the group, the greater the potential loss in body mass, especially when coupled with reduced calorific intake. However, McGuire et al. (2001) found that older subjects who exercised for six months at an intensity of 70 - 75% of maximum heart rate showed a reduction in both body mass and skinfold thickness.

The results of Experiment 1 suggest that the reductions in body mass (albeit statistically non-significant), and significant reductions in percent body fat, were the result of increased energy expenditure associated with the 12 month aerobic training programme. While the subjects in Experiment 1 were not grossly obese, it is possible that an increase in the number of training sessions per week and/or intensity of the training could have resulted in a significant reduction in body mass. The dilemma faced by increasing training intensity to achieve a significant reduction in body mass is the potential adverse effect on the immune responses of older individuals (Brenner et al., 1998).

#### 5.1.2. Blood Pressure

The systolic and diastolic blood pressures reported for the TR and UT groups prior to the commencement of the training programme classified them as normotensive (<140/90 mm Hg) and remained within the normotensive classification for the duration of the training programme. Prior to the training study, the systolic blood pressure of the female TR group at rest ( $133\pm 3$  mm Hg) was significantly higher than the male TR group at rest ( $124\pm 3$  mm Hg). There was no significant difference in diastolic blood pressure between male and female TR groups at rest, prior to the training study. There was no significant decrease in either systolic or diastolic blood pressures after twelve months of aerobic training, for either male or female groups, consistent with previous findings (Spina et al., 1993; Durstine et al., 2001; Daley and Spinks 2000; McGuire et al., 2001 and Fagard 2001).

Repeated bouts of exercise have been used to reduce systolic and diastolic blood pressure in older individuals, particularly hypertensive individuals. The mechanisms responsible for the resulting exercise-induced decreases in blood pressure have been well documented (Brooks et al., 1996; Fiatarone 1997; Kasch et al., 1999; Hagberg et al., 2000; Turner et al., 2000; Carroll and Kyser 2002; Chintanadilok and Lowenthal 2001; Perini et al., 2001; Mazzeo and Tanaka 2001; McGuire et al., 2001; Moreau et al., 2001) and include decreases in total peripheral resistance (Brooks et al., 1996), increased capillary density (Hagberg et al., 2000), changed plasma lipid and lipoprotein profiles (Bouvier et al., 2001), improved arterial compliance, a decrease in resting heart rate (Wannamethee et al., 2000), decreased sympathetic response (Stratton et al., 1994), decreased  $\beta$ -adrenergic receptor density (Mazzeo and Tanaka 2001), and an increase in the release of nitric oxide from endothelial cells in capillaries (Halliwell et al., 2000; Govers and Rabelink 2001; Cowley et al., 2003). As the subjects in the present study were normotensive, repeated bouts of exercise over a long duration could be expected to have a smaller effect on reducing blood pressure than if those same individuals were classified as hypertensive.

### 5.1.3. Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ )

Both the TR male and female groups showed significant increases in peak oxygen uptake both in  $L \cdot \text{min}^{-1}$  and  $mL \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$  after the training programme. While the UT male group showed no significant differences between pre- and post-study peak oxygen uptake, the UT female group showed a significant decrease in peak oxygen uptake after twelve months. The increases in  $\dot{V}O_{2\text{ peak}}$  ( $L \cdot \text{min}^{-1}$ ) for trained males (14.3%) and trained females (16.7%) in the present study are consistent with previous research (Stratton et al., 1994; Carroll et al., 1995; Stachenfeld et al., 1998; Hawkins et al., 2001; Wilmore et al., 2001a,b; Bouvier et al., 2001). The training-induced increase in  $\dot{V}O_{2\text{ peak}}$  in older individuals has been ascribed to an increase in capillary and mitochondrial density, lean muscle mass, cardiac output, stroke volume and capacity of oxidative enzymes (Maiorana et al., 2001). However, in older males, the training-induced increase in  $\dot{V}O_{2\text{ peak}}$  is likely mediated by an increase in both cardiac output and arterio-venous  $O_2$  content difference (Spina et al., 1993; Stratton et al., 1994; Wilmore et al., 2001a,b), while in older females the training-induced increase in  $\dot{V}O_{2\text{ peak}}$  is likely due to an increased arterio-venous  $O_2$  difference (Spina et al., 1993). The present study did not measure cardiac output, and therefore we can only

speculate as to whether the reported gender differences were the responsible mechanisms for the increase in  $\dot{V}O_{2\text{ peak}}$ .

The increase in arterio-venous  $O_2$  content difference in older females is suggested to result from increased skeletal muscle capillarization, mitochondrial density and a significant increase in the activity of mitochondrial enzymes (Spina et al., 1993; Brooks et al., 1996; De Vito et al., 1999; Perini et al., 2001). McCole et al. (2000) found that endurance-trained post-menopausal women did increase their cardiac output and stroke volume significantly after  $15\pm 4$  years of training. It is possible that central adaptations (i.e. CO/SV) in older females may require many years of regular aerobic training. The reasons why older women show peripheral but not central adaptations after short-term aerobic training are unclear. The influence of gender upon the time course of central adaptations could relate to differences in the blood volume response (Wilmore et al., 2001a), and increases in the activity of selected mitochondrial enzymes (Spina et al., 1993; Marcell 2003). While most training studies on older females have been short term (8 to 24 weeks), the longer duration training studies (6 to 9 months) also found that older females did not develop left ventricular hypertrophy after aerobic training, whereas older males did develop left ventricular hypertrophy (Spina et al., 1993).

The trained male and female subjects in Experiment 1 increased their peak power and  $\dot{V}O_{2\text{ peak}}$ . While the present study did not measure lean muscle mass, previous research has shown that a training-induced increase in muscle mass in older individuals is partly responsible for the increases in  $\dot{V}O_{2\text{ peak}}$  following moderate ( $40 - 60\% \dot{V}O_{2\text{ peak}}$ ) and higher intensity ( $80 - 90\% \dot{V}O_{2\text{ peak}}$ ) exercise training (Seals et al., 1984; Hagberg et al., 1989; Frontera et al., 1990; Stachenfeld et al., 1998; Turner et al., 2000; McGuire et al., 2001; Perini et al., 2001; Wilmore et al., 2001a,b; Wang et al., 2002; Marcell 2003). The adaptations of skeletal muscle to aerobic training include muscle hypertrophy, an increase in the size and number of mitochondria, mitochondrial enzymes and capillary density, all of which could contribute to an increase in oxygen extraction (Seals et al., 1984; Spina et al., 1993; Brooks et al., 1995; Perini et al., 2001; Marcell 2003). Perini et al. (2001) and Marcell (2003) noted that aerobic training in older individuals also increased neuromuscular recruitment of skeletal muscle, thus contributing to an increased exercise capacity and  $\dot{V}O_{2\text{ peak}}$ .

The present results suggest that the trained males and females did increase their peak power and  $\dot{V}O_2$ , but the mechanisms behind the significant increases in  $\dot{V}O_2$  and power were not examined.

While others (Hagberg et al., 1989; Spina et al., 1993; Morris et al., 2002) have achieved larger increases in  $\dot{V}O_{2\text{ peak}}$  using higher intensity exercise (70% to 85% of peak HR), the results of Experiment 1 suggest that twelve months of moderate intensity exercise (60% of  $\dot{V}O_{2\text{ peak}}$ ) provided sufficient stimulus to improve  $\dot{V}O_{2\text{ peak}}$  in both males and females, without the immunosuppression that has been associated with high-intensity exercise (Brenner et al., 1998) and training (Pedersen 1997; Pedersen and Hoffman-Goetz 2000).

#### 5.1.4. Training and Peak Heart Rate

A significant reduction in heart rate at rest was found in the study participants after the first six weeks of training, consistent with the results of others (Stratton et al., 1994; Zavorsky 2000; McGuire et al., 2001; Perini et al., 2001; Carroll and Kyser 2002; Yamamoto et al., 2001; Billman 2002; Morris et al., 2002). The decrease in resting heart rate with aerobic/endurance training has been variously ascribed to enhanced cardiac dilation (Stratton et al., 1994; McCole et al., 2000; Wilmore et al., 2001a,b), expansion of blood and plasma volume and enhanced baroreflex function (Convertino 1991; Zavorsky 2000), decreased sympathetic and increased parasympathetic responses (Brooks et al., 1996; Zavorsky 2000; Yamamoto et al., 2001), altered electrophysiology of the sinoatrial node and decreased  $\beta$ -adrenergic receptor number and density (Stratton et al., 1994; Zavorsky 2000). The methods of the present study do not permit us to identify the mechanisms that might have been responsible for the decrease in heart rate at rest, and during submaximal exercise. That Experiment 1 did result in a reduced heart rate at rest and during exercise, adds support that the selected exercise intensity of 60%  $\dot{V}O_{2\text{ peak}}$  did produce central adaptation in both men and women (Tate et al., 1994; Panton et al., 1996).

The training intensity of Experiment 1 was increased during the study so as to maintain a training heart rate corresponding to 60%  $\dot{V}O_{2\text{ peak}}$ . It became apparent during the course of the study that some subjects adapted to the exercise protocol faster than others. The different rates of adaptation to a fixed exercise stimulus (eg 60%  $\dot{V}O_{2\text{ peak}}$ ) have been most recently highlighted in

the HERITAGE study (Wilmore et al., 2001a,b; An et al., 2003) where older subjects showed significant variation, between and within genders, in the rate of adaptation of heart rate, stroke volume and arterio-venous O<sub>2</sub> content difference to a common stimulus. Indeed, it has been suggested that males may adapt to training more quickly than females, with faster increases in left ventricular dilation, lean muscle mass, and intrinsically higher erythrocyte number/volume and haemoglobin concentration (Schuit et al., 1999; Zavorsky 2000; Wilmore 2001; Wilmore et al., 2001b). Upon inspection, the present results suggest there is considerable variation among older individuals in the rate of adaptation to the present exercise stimuli, consistent with findings from the HERITAGE study (Wilmore et al., 2001a,b; Hagberg et al. 2002; An et al., 2001; An et al., 2003). Wilmore et al. (2001), Hagberg et al., (2002) and An et al. (2003) found that a significant percentage of the variation in cardiovascular responses to endurance training was genetic in origin. The present findings support the concept of “responders and non-responders”, and genetic variation in the trainability of older males and females (Wilmore 2001b; Hagberg et al., 2002; An et al., 2003).

#### 5.1.5. Peak Power and Ventilation

Compared to the UT groups, both male and female TR groups significantly increased peak power and peak ventilation after twelve months of training, in agreement with Seals et al., (1984), Spina et al. (1993), Stratton et al. (1994), De Vito et al., (1999), McGuire et al. (2001) and Wang et al. (2002). The significant increase in peak power in both males and females may result from an increase in muscle strength, which in turn may reflect an increased capillary-to-fibre ratio, capillary density, mitochondrial density, force production from type 1 and type 11a muscle fibres, synthesis rate of myofibrillar proteins, oxidative enzyme capacity, substrate availability and neural patterns as a result of the aerobic training programme (Meredith et al., 1989; Frontera et al., 1991; Stone et al., 1996; Bemben 1998; Daley and Spinks 2000; Frontera et al., 2000a,b). Meredith et al. (1989), Spina et al. (1993) and Evans (1999) also suggested that older individuals were able to increase their muscle glycogen content and capacity for oxidising substrates after moderate intensity aerobic training, which may also contribute to an increased power output. While the present study did not take muscle biopsies, our results suggest that training at an exercise intensity of 60%  $\dot{V}O_{2\text{ peak}}$  over an extended duration, will lead to significant increases in maximal power in older males and females.

Experiment 1 found a significant increase in peak ventilation for males (17.9%) and females (11.5%), consistent with the results of others (Seals et al., 1984; Meredith et al., 1989; Hagberg et al., 1989; Woods et al., 1999; Perini et al., 2001). Structural and functional changes occur in the lung, chest wall, respiratory muscles and vasculature with age (Johnson and Dempsey 1991), with a decrease in maximum lung capacity of up to 50% (Brooks et al., 1996; Johnson and Dempsey 1991; Daley and Spinks 2000). Peak ventilation is reduced in older individuals due to a loss of pulmonary connective tissue elasticity, an increase in the size of the alveoli and weakening of the respiratory muscles (Brooks et al., 1996; Johnson and Dempsey 1991; Daley and Spinks 2000). These changes will compromise ventilation and perfusion of the lung, particularly during exercise (Johnson et al., 1996; McClaran et al., 1999). In healthy older individuals, ventilation is not normally the limiting factor during exercise and the repeated ventilation increases that accompany moderate intensity aerobic/endurance training may increase the strength and oxygen uptake of respiratory muscles and provide opportunity for older individuals to improve their peak ventilation (Yerg II et al., 1985; Hagberg et al., 1988; Brooks et al., 1996; Habedank et al., 1998; Johnson et al., 1996; Johnson et al., 1999). The higher peak ventilation post-training may also reflect the higher venous blood lactate concentration and toleration of higher blood lactate concentration that accompanies acute and chronic exercise (Yerg II et al., 1985; Hagberg et al., 1988; McClaran et al., 1999). Peak ventilation is increased by a higher concentration of  $H^+$ , and a decreased sensitivity to high intensity exercise.

#### **5.1.6. Incidence of URTI**

There were no differences among any of the male and female UT and TR groups with the reported number of cases of URTI. The incidence of URTI in the present subjects was lower than the reported average incidence per head of population (A.I.H.W. 2000; Pyne et al., 2000; Matthews et al., 2002). The subjects had been asked to keep an accurate log of any illnesses, and diagnoses of specific infections such as URTI made by medical practitioners. The low level of reported URTI by UT and TR groups suggests that the subjects remained healthy compared to the national average (A.I.H.W. 2000), and supports our findings of normal leucocyte concentration during the 12 months of the study. The low incidence of URTI in the UT and TR groups also raises questions about the clinical utility of URTI as an indicator of the immune response.

### 5.1.7. Haematology

Haematological indices for UT and TR groups remained within the normal ranges during the year, with the exception of MCHC (Coulter 1984). Monthly changes in some indices are statistically significant but may not be clinically significant since the concentration of cell subsets remained within normal ranges. The blood counts of all UT and TR groups showed that the concentration of leucocytes remained within the normal ranges throughout the year (Coulter, 1984). While some have suggested that older individuals have reduced total leucocyte concentration at rest (Pedersen et al., 1999; Pedersen and Hoffman-Goetz 2000; Ginaldi et al., 2000), others have found total leucocyte concentration remained essentially unchanged with ageing (Rall et al., 1996; Globerson and Effros 2000; Fahlman et al., 2000; Chen et al., 1998). It has been suggested that since ageing populations have an increased percentage of “memory” CD4<sup>+</sup> and CD8<sup>+</sup> lymphocytes at rest, compared to “naïve” CD4<sup>+</sup> and CD8<sup>+</sup> cells, the total leucocyte concentration at rest remains normal (Mackinnon 1992; Gabriel et al 1993; Shinkai et al., 1995; Pedersen and Hoffman-Goetz 2000; Martinez-Taboada et al., 2002).

Twelve months of aerobic training did not significantly increase total leucocyte concentration of the female and male TR groups. These results are not consistent with the results of others who found training-induced increases in leucocyte concentration (Gabriel et al., 1994; Shinkai et al., 1995; Gueldner et al., 1997; Woods et al., 1999; Fahlman et al., 2000). In Experiment 1 we could not perform a white cell differential because the blood analyser (Coulter T660, Coulter USA) was an older model and performed only a standard blood count. Thus, different leucocyte subsets could not be measured. However, other researchers have found increases in some leucocyte subsets (e.g. NK cells) with training (Shinkai et al., 1995; Woods et al., 1999). The present results did find some significant between-group differences (male UT/TR). The UT group had a higher leucocyte concentration for three months of the year, which could be attributed to normal variation within the subject group. The results suggest that the differences between groups may not be clinically significant, since the leucocyte concentration was within the normal range for all groups (Coulter 1984).

The present study found significant month to month changes in leucocyte concentration in the male UT and TR group and female TR group, consistent with the findings of Canon et al. (1986), Pati et al. (1987), Brock (1987), Afoke et al. (1993) and Maes et al. (1997b). While the month to

month changes in leucocyte concentration may be statistically significant, the clinical significance of these changes is unclear because the leucocyte concentration was within the normal range during the study. However, it is suggested that seasonal changes in hormone production (e.g. prolactin, melatonin) and glucocorticoid concentration may be responsible for changes in leucocyte subset concentrations (Canon et al., 1986; Pati et al., 1987; Brock 1987; Maes et al., 1997b; Mann et al., 2000; Bartness et al., 2002; Demas et al., 2003). The present study did not measure hormone and glucocorticoid production. Others have found relationships between changes in total leucocyte concentration, lymphocyte concentration, lymphocyte proliferation, neuro-hormone and glucocorticoid production (Brock 1987; Maes et al., 1994, 1997b; Mann et al., 2000). For example, Brock (1987) found increases in leucocyte concentration, lymphocyte concentration and lymphocyte proliferation coincided with decreased glucocorticoid production. The changes in leucocyte concentration, neuro-hormone and glucocorticoid production may indicate a seasonal pattern of change in these variables independent of training effects (Afoke et al., 1993; Maes et al., 1997b; Mann et al., 2000; Feitosa et al., 2002a).

Some studies have investigated seasonal changes in murine and human lymphocyte proliferation. Brock (1987), Pati et al. (1987) and Canon et al. (1986) have shown increases in the PHA responsiveness of lymphocytes in February-March, June-July and September-October. These studies were all conducted in the northern hemisphere, but showed similar seasonal patterns to those patterns recorded with the subjects in Experiment 1, when the seasons should be reversed. The seasonal patterns in lymphocyte responsiveness may be due to the depression of cellular responses by elevated concentrations of glucocorticoids, melatonin and prolactin which also show seasonal variation. (Brock 1987; Boctor et al., 1989; Mann et al., 2000; Bartness et al., 2002; Demas et al., 2003). Maes et al. (1997b) analysed seasonal changes in cortisol, prolactin (PRL), thyroid stimulating hormone (TSH), testosterone, and the thyroid hormones T3 and T4, in young untrained males and females (northern hemisphere). Their findings lend some measure of support to those of Boctor et al. (1989). Both Boctor et al. (1989) and Maes et al. (1997b) found that both genders showed a decreased cortisol concentration in June, with an increased cortisol concentration in December-January, and that cortisol concentration was inversely related to leucocyte concentration. The findings of Maes et al. (1997b) are consistent with the results of Experiment 1, where leucocyte concentration was higher in June than December, albeit in different hemispheres.

During the year, the erythrocyte concentration remained within the normal ranges for all groups (Coulter 1984). The erythrocyte concentrations for UT and TR males were significantly higher than their female counterparts, and agree with the findings of others (Woods et al., 1999; Okazaki et al., 2002). There were no significant differences in erythrocyte concentration between male UT and TR groups, and female UT and TR groups, throughout the year, also consistent with the results of Convertino (1991), Morris (1998) and Okazaki et al. (2002). There were significant month to month changes in erythrocyte concentration for all groups during the year. Previous studies have noted seasonal changes in erythrocyte concentration in smokers compared to non-smokers (Kristal-Boneh et al., 1997) and in untrained males and females (Maes et al., 1995). Both groups of researchers found that erythrocyte concentration in non-smokers decreased during the northern summer and autumn, and increased during winter and spring. Maes et al. (1995) and Kristal-Boneh et al. (1997) suggested that ambient temperatures during the year affected erythrocyte concentration, with erythrocyte concentration relatively lower in summer/autumn possibly due to a warmer temperature-related increase in plasma volume. Maes et al. (1995) also suggested that climate-induced alterations in sympathetic-adrenal system activity may affect erythrocyte concentration. Climate and temperature-induced alterations in erythrocyte proliferation and maturation of cell precursors might also account for seasonal changes in erythrocyte concentrations (Maes et al., 1995). However, the monthly changes in erythrocyte concentration found in Experiment 1 were similar to those found by Maes et al. (1995) and Kristal-Boneh et al. (1997), despite the reversal of seasons in the southern and northern hemispheres. Possible explanations might include that despite more than two hundred years of European settlement in Australia, erythrocyte production and concentration have not adapted to southern hemisphere seasonal changes and continue to show the same monthly changes as European individuals in the northern hemisphere. Furthermore, the mild Gold Coast winter temperatures (averaging 21 to 23°C maximum during the day) are similar to summer European day temperatures, and the difference between the Queensland winter ambient temperatures are not that dissimilar to northern European summer temperatures.

Whether the month to month changes in erythrocyte concentration are also reflecting changes in plasma volume or addition/removal of erythrocytes from circulation cannot be determined from the methods used in Experiment 1. Plasma volume/blood volume expansion is reported to occur with

the onset of endurance training, and is explained by an increase in plasma proteins (mostly albumin) in the thoracic duct, altered vasopressin, renin and aldosterone responses (Brooks et al., 1996; Carroll et al., 1995; Green et al., 1999). It has been suggested that the increase in plasma volume in young endurance-trained athletes is responsible for the haemodilution and decrease in circulating erythrocyte and haemoglobin concentration (Green et al., 1991; Deakin 1995; Green et al., 1999; Telford et al., 2002). Furthermore, Carroll et al. (1995) also found an increased plasma volume with older subjects after six months of training. Conversely, Convertino (1991) has suggested that subjects previously exposed to endurance training may be less likely to undergo a training-induced hypervolemia. Several endurance training studies of young athletes and older males and females have failed to detect training-induced changes in blood and plasma volume (Convertino 1991; Zappe et al., 1996; Stachenfeld et al., 1998; Takamata et al., 1999; Morris 1998; Okazaki et al., 2002). In older individuals, Morris (1998) and Okazaki et al. (2002) found no change in blood volume with short term, moderate intensity endurance training. It is possible that training at a moderate intensity during Experiment 1, with three weekly training sessions, did not induce an increase in plasma volume, as indicated by the similarity in various circulating cell concentrations in UT and TR male and female groups (Green et al. 1991; Morris 1998; Okazaki et al., 2002).

The male TR, female UT and female TR groups in Experiment 1 had a haemoglobin concentration within the normal range ( $14\text{-}18\text{ g}\cdot\text{dL}^{-1}$  for males and  $12\text{-}16\text{ g}\cdot\text{dL}^{-1}$  for females, Coulter 1984) during the year. The male UT group initially had a lower than normal haemoglobin concentration of  $13.8\pm 0.6\text{ g}\cdot\text{dL}^{-1}$ . Haemoglobin concentration for the male UT and TR groups were significantly higher than those for the female UT and TR groups. The gender difference in haemoglobin concentration is consistent with previous findings (Smith 1995; Heuther and McCance 1996). At each monthly sampling point there were no significant differences in haemoglobin concentration between male UT and TR groups, or between female UT and TR groups. Our results suggest that moderate intensity exercise over 12 months had no significant effect on haemoglobin concentration.

There were, however, significant month to month changes in haemoglobin concentration for each group (male UT/TR, female UT/TR) throughout the year, consistent with the results of Maes et al. (1995) and Kristal-Boneh et al. (1997) who found higher haemoglobin concentration during the

northern hemisphere winter, and a lower concentration during summer. The studies conducted in the northern hemisphere found the seasonal changes in haemoglobin concentration were similar to changes in erythrocyte concentration and haematocrit (Maes et al., 1994, 1995; Kristal-Boneh et al., 1997). It was suggested that seasonal changes in ambient temperature, the adrenal system and long-term gene-regulated adaptations to climate in individuals of European origin may have been the responsible mechanisms (Maes et al., 1995; Chebotarev et al., 2001; Geraghty 2002; Feitosa et al., 2002a).

MacDonald et al. (1987), Neuhaus et al. (1999) and Garde et al. (2000) found seasonal variations in glycosylated haemoglobin (Hb A<sub>1c</sub>), which is directly proportional to blood haemoglobin concentration. Their results show higher Hb A<sub>1c</sub> concentrations in April-June, September and late October-November, consistent with higher haemoglobin concentrations found by Maes et al. (1995) and the present study in these months. The monthly changes in haemoglobin concentration may be related to similar changes in erythrocyte concentration and may be regulated at gene level, as part of a longstanding adaptation of biological variables to climatic and ambient temperature changes in individuals of European origin (MacDonald et al., 1987; Maes et al., 1995, 1997a,b; Garde et al., 2000).

The present study found that male and female UT and TR groups showed similar month to month changes in mean corpuscular haemoglobin concentration (MCHC), and that these changes were, as expected, similar to the monthly changes in haemoglobin concentration. Maes et al. (1997a) also found a positive correlation between monthly changes in erythrocyte, haemoglobin, MCHC and transferrin concentration, suggesting a seasonal pattern of change in iron storage proteins and erythrocyte concentration. The present results are consistent with those of Maes et al. (1995, 1997a), and add further support to the theory of a regulated relationship in the monthly changes of erythrocytes, haemoglobin concentration, MCHC and iron storage proteins (Maes et al., 1995, 1997a).

The respective haematocrits for male and female UT and TR groups remained within the normal range of 42%-52% and 37%-47% (Coulter 1984). Within each gender, there were no significant differences in haematocrit between UT and TR groups at any month, suggesting training had no significant effect on haematocrit or expansion in plasma volume. Our results are consistent with

the results of Stachenfeld et al., (1998), Morris (1998), Schumacher et al. (2002b) and Okazaki et al. (2002). In agreement with others, the higher haematocrit found in the male groups was due to the higher concentration of erythrocytes in males compared to females (Deakin 1995; Smith 1995).

There were significant month to month changes in haematocrit for male and female UT and TR groups. Our results are again consistent with the results of Maes et al. (1995), who also found decreases in haematocrit during March, August and late October, and increases during June, September and December-January. The male UT group was the only group which had a significantly lower haematocrit at the end of the study when compared to the initial haematocrit ( $p < 0.001$ ), and may be the result of a lack of training stimulus on erythropoietin production (Green et al., 1999; Smith et al., 1999). The seasonal changes in haematocrit may be due to seasonal changes in ambient temperature, or in the adrenal system which affect erythrocyte precursors, cell release and erythrocyte removal from circulation (Maes et al., 1995, 1997a). The suggestion that individuals in Australia show a similar pattern of change in erythrocyte concentration and haematocrit compared to individuals in the northern hemisphere may indicate a long-term adaptation in haematological variables that is regulated at a gene level (Maes et al. 1997a, 1995; Geraghty 2002; Feitosa et al., 2002a,b).

The seasonal changes in haematological indices suggest that there are times of the year when both inactive and active individuals are at more risk of suffering from iron deficiencies or infections (Brock 1996). The clinical implications are important in that population-based reference ranges for haematological and immunological indices do not take into account seasonal variations. Individuals may show haematological variables that appear to be within or outside the normal reference range, but due to a seasonal swing, may actually be quite normal for that individual (Afoke et al., 1993; Maes et al., 1997a; Chebotarev et al., 2001).

The groups in Experiment 1 showed normal concentrations of platelets ( $130-400$  platelets  $\times 10^9$ L Coulter 1984; Hoffbrand et al., 2001) during the year. There were no significant differences in platelet concentration between male UT and TR groups, and female UT and TR groups, consistent with other findings (Huether and McCance 1996). However, there were significant differences between genders in some months. The significant differences between genders do not seem to be training-related, and the clinical significance of these findings is unclear. There are several studies

that have examined gender differences in platelet concentration, and the effects of aerobic training (Maes et al., 1995; Wang et al., 1995; Wang et al., 1997). Wang et al. (1995, 1997) examined the effects of short-term moderate aerobic training on platelet activation in both young men and women, and found a training-induced reduction in platelet activation and adhesiveness. In the female group, the reduced platelet activation was correlated with enhanced production of nitric oxide and endothelium-derived relaxing factor. These variables were not measured in the earlier study of males (Wang et al., 1995), where the reduced platelet activation was attributed primarily to changes in lipid profiles and catecholamine production rather than increased nitric oxide production during training. It is possible that both sedentary and trained females may produce more nitric oxide and endothelium-derived relaxing factor than males, accounting for gender differences in platelet concentration in previous studies and in Experiment 1.

While there were significant month to month changes in platelet concentration in the male UT and both female UT and TR groups, the changes did not occur in the same months, suggesting normal variation within a population. The present results are not consistent with the findings of Maes et al. (1995) who investigated the seasonal changes in thrombocytes, platelet volume and plasma fibrinogen concentrations in normal young males and females, and healthy older individuals, over twelve months. The disparity between the results of Experiment 1 and the results of Maes et al. (1995) could be a reflection of variation within each subject or indeed the mildness of the Queensland winter and warmer summer temperatures.

It has been suggested that seasonal variation in platelets, plasma fibrinogen and erythrocyte concentration might result in changes in blood clotting and blood flow, thereby altering the resistance and susceptibility of patients to thrombo-embolic disorders, particularly during the northern hemisphere winter (McLaren et al., 1990; Huisveld et al., 1991; De Geus et al., 1992; Bol et al., 1993; Maes et al., 1995; Van den Burg et al., 1997). Experiment 1 found no significant differences in platelet concentrations between summer and winter, in male and female UT and TR groups and may reflect the mildness of the Queensland winter compared to the winter in northern Europe.

### 5.1.8. Interleukin-2 Receptor (CD25) Expression

The results of Experiment 1 suggest that endurance training had a positive effect on the percentage of CD4<sup>+</sup> lymphocytes expressing CD25 (CD4<sup>+</sup>/CD25<sup>+</sup>) only in the male TR group. Initially, the male TR group showed a significantly lower percentage of CD4<sup>+</sup>/CD25<sup>+</sup> lymphocytes than the UT group. The TR group then showed a significantly higher percentage of CD4<sup>+</sup>/CD25<sup>+</sup> after the onset of training, during February, April, May, June, and September B. There appeared to be no training effect on the percentage of CD4<sup>+</sup>/CD25<sup>+</sup> in the female TR group. A significantly higher percentage of CD4<sup>+</sup>/CD25<sup>+</sup> compared to the UT group occurred in July only. Is it possible that training increased CD25 expression in the female TR group during winter, but the female response to the training stimulus was slower than that of the male group?

While the results of Experiment 1 are consistent with the findings of some (Shinkai et al., 1995; Yan et al., 2001; Sagiv et al., 2002), there is a lack of agreement regarding changes in CD25 expression with ageing and gender. Sagiv et al. (2002) found significant increases in CD4<sup>+</sup> in older males after short-term moderate aerobic training. Of the five studies that investigated long-term training with older subjects (Crist et al., 1989; Shinkai et al., 1995; Gueldner et al., 1997; Woods et al., 1999; Yan et al., 2001), Shinkai et al. (1995), Gueldner et al. (1997), Woods et al. (1999) and Yan et al. (2001) investigated the impact of exercise on CD4<sup>+</sup> lymphocytes. Gueldner et al. (1997) found that older females undertaking regular exercise had a significantly higher percentage of CD4<sup>+</sup> cells expressing CD25 compared to sedentary females. Both Shinkai et al. (1995) and Woods et al. (1999) found significantly greater responsiveness to PHA and IL-2 production in trained older individuals. Yan et al. (2001) found an increased concentration of CD4<sup>+</sup> in older exercising males. However, Woods et al. (1999) found no change in the number of CD4<sup>+</sup> expressing CD25 after training, but rather a non-significant increase in the number of “naïve” lymphocytes compared to “memory” lymphocytes expressing CD25. Gabriel et al. (1993), Born et al. (1995) and Globerson and Effros (2000) suggested that increased recruitment of “memory” or activated cells acted as a compensatory response to a diminished proliferative capability, and decreased number of “naïve” CD4<sup>+</sup> lymphocytes. In Experiment 1 it is possible that regular aerobic training for twelve months increased the recruitment of “memory” CD4<sup>+</sup> cells expressing CD25, thereby enhancing the lymphocyte proliferative response in older TR males (Tharp and Preuss 1991; Gabriel et al., 1993, 1994; Born et al., 1995; Gueldner et al., 1997; Woods et al., 1999). The reasons why older females showed no effect of long-term aerobic training on the percentage of

CD4<sup>+</sup> expressing CD25 remains unclear. Whether females might respond positively to more training sessions per week or to training at a higher intensity, as has been suggested (Gueldner et al., 1997; Fahlman et al., 2000), remains to be resolved.

The present study showed significant month to month changes in the percentage of lymphocytes expressing CD25 for all four groups, with significant increases in CD25 expression during January-February (male TR), April-May (female TR), August-September A (male UT, male TR, female UT), and November (male TR). The peaks in CD25 expression in the present study are consistent with the results of Maes et al. (1994; 1997a). Possible explanations for the monthly variations include a relationship between CD4<sup>+</sup> proliferation/CD25 expression and haemoglobin (Kemp 1993; Maes et al., 1994, 1997a; Woods et al., 1999), and seasonal variations in glucocorticoids and neuro-hormones (Brock 1987; Boctor et al., 1989; Afoke et al., 1993; Maes et al., 1994; Briggs et al., 1996; Mann et al., 2000; Bartness et al., 2002; Demas et al., 2003).

The CD4<sup>+</sup> proliferative response involves not only the percentage of CD4<sup>+</sup> lymphocytes expressing CD25, but also the density of CD25 on the cell surface. The present study measured density of CD25 and found the only significant difference between groups occurred in February, where the male TR group showed greater CD25 density than the female TR group. The lack of significant difference between male UT and TR, and female UT and TR groups, suggests that twelve months of aerobic training had no significant effect on the density of CD25. To our knowledge, there have been no studies investigating training-related changes in CD25 density in older individuals. The present results raise the possibility that aerobic training may increase the recruitment of “memory” CD4<sup>+</sup>/CD25<sup>+</sup> cells, thus increasing the overall percentage of lymphocytes expressing CD25 in older individuals, rather than increasing CD25 density on each lymphocyte.

Significant decreases in CD25 density occurred during autumn (March-April) and early winter (May-June) for the male TR group only. However, all groups followed a pattern of decreasing CD25 density during March-April. Significant increases in receptor density occurred during spring (August-September A) for the male UT/TR and female TR, and the three groups also showed increases in receptor density in summer (November-December). Although other studies have suggested that winter is a time of decreased immuno-competency, the decrease in receptor density found during autumn in the present study suggests that autumn may be a season of

decreased immuno-competency for older individuals. The autumn decrease and spring-summer increase in CD25 density may be due to variations within the intracellular signalling pathway (e.g.  $\text{Ca}^{2+}$ ), or to seasonal variations in glucocorticoids, melatonin, prolactin or iron (Brock 1987; Maes et al., 1994, 1997a; Mann et al., 2000; Bartness et al., 2002; Demas et al., 2003). Increases in CD25 and percentage of  $\text{CD4}^+$  expressing CD25 occurred in early September, suggesting that spring is a period of increased lymphocyte responsiveness and cellular signalling. The present results are consistent with the studies that have investigated seasonal changes in lymphocytes in the northern hemisphere (Canon et al., 1986; Brock 1987; Pati et al., 1987; Boctor et al., 1989; Maes et al., 1994; Demas et al., 2003). These studies have shown increases in PHA responsiveness in  $\text{CD4}^+$  lymphocytes during December-January and September-October, and suggested that seasonal changes in the release of cortisol, thyroid-stimulating hormone, prolactin and luteinizing hormone could affect lymphocyte proliferation, together with genetic factors and age-related alterations to the hypothalamus (Brock 1987; Pati et al., 1987; Maes et al., 1994; Tsuchiya et al., 2002; Bartness et al., 2002; Feitosa et al., 2002a; Demas et al., 2003). The implications are that some physiological and immunological functions could be depressed for several months during the year, leading to periods of increased susceptibility to viral infections (Brock 1987; Pati et al., 1987; Maes et al., 1994).

The present study has shown that regular aerobic training may increase the percentage of  $\text{CD4}^+$  cells that express CD25 in older individuals, but not the density of receptors on the cell surface. This finding is consistent with the results of others (Shinkai et al., 1995; Woods et al., 1999; Globerson and Effros 2000; Woods et al., 2003) who found that trained older individuals showed higher concentrations of “memory” lymphocytes to maintain their immune response (Mazzeo 1993; Born et al., 1995; Woods et al., 2003). However, seasonal changes in some hormones and haematological variables may modulate the recruitment of “memory”  $\text{CD4}^+$  lymphocytes.

#### 5.1.9. Transferrin Receptor (CD71) Expression

The results of Experiment 1 suggest that aerobic training did not have a significant effect on the percentage of lymphocytes expressing CD71. The female UT group showed a significantly higher percentage of lymphocytes expressing CD71 than the male and female TR groups during only one month (September A). The female TR group showed significantly higher CD71 expression than the male TR group for two months (May, July) and the male UT group for two months (July,

December). The role of CD71 in maintaining iron status has been well documented (Neckers and Cossman 1983; Pelosi-Testa et al., 1988; Kemp 1993; Maes et al., 1997a; Skikne 1998; Schumacher et al., 2002a; Brownlie et al., 2004), but few studies have examined the effects of aerobic training or seasonal changes on CD71 expression, especially in older individuals. While it is possible that training increased CD71 expression (i.e. lymphocyte iron requirements) in the female TR group compared to the male TR group during May and July, the differences between male and female TR may also indicate variations in intracellular iron levels due to fluctuations in dietary intake of iron (Flynn et al., 2003). The subjects participating in Experiment 1 had been advised to maintain a balanced diet throughout the year, but were not asked to keep a daily record of food consumed. While none of the participating subjects were vegetarian, it was expected that there would be some variation in the dietary intake of iron and other essential minerals which could account for variations in CD71 expression.

The expression of CD71 is increased when iron is required by the cells for proliferation (Neckers and Cossman 1983; Kemp 1993; Maes et al., 1997a), and there is normally an inverse relationship between CD25 and CD71 (Neckers and Cossman 1983; Pelosi-Testa et al., 1988; Ahluwalia et al., 2001). The present study found an inverse CD25/CD71 relationship in both male groups, but the inverse relationship in the female groups was apparent only from June to December. The inverse relationship between CD71 and CD25 in males shows that changes in iron needs may be related to lymphocyte responsiveness and IL-2 production/CD25<sup>+</sup> expression, consistent with the findings of Pelosi-Testa et al. (1988), Kemp (1993) and Ahluwalia et al. (2001). Since the relationship between CD25 and CD71 in older females is not inverse throughout the year, it may be modulated by other factors such as changes in hormones and diet (Brock 1987; Kemp 1993; Maes et al., 1997a). The present results suggest that training alone is not the only variable affecting CD71 expression in older individuals.

The present results showed significant month to month changes for each group (male UT/TR, female UT/TR) in the percentage of lymphocytes expressing CD71, consistent with the results of others (Maes et al., 1997a). Maes et al. (1997a) measured monthly changes in soluble CD71 and found increases in CD71 expression during January, March, June and September. The present results suggest that iron requirements were greater during these months, possibly due to

increased lymphocyte proliferation or intracellular processes at these times (Pelosi-Testa et al., 1988; Maes et al., 1997a).

The present study found that there were significant differences between the female UT and TR groups in density of CD71 during April and July, and no significant differences between male UT and TR groups. Our findings suggest that 12 months of aerobic training had little effect on CD71 density with either gender. The differences in CD71 density between the female groups may be due to diet-related, genetic or hormonal factors (Kemp 1993; Maes et al., 1997a; Ahluwalia et al., 2001; Hagberg et al., 2002; Feitosa et al., 2002a). There were significant month to month changes in CD71 density for all the groups during the year, but none of the significant changes occurred between the same months. It seems likely that long-term aerobic training had little effect on month to month changes of CD71 density for the TR groups, and that there were variations in iron needs for each male and female group at different months of the year.

In Section 5.1.8. we found that aerobic training had more effect on the percentage of CD4<sup>+</sup> expressing CD25, rather than CD25 density. It would also seem that aerobic training has more effect on the percentage of lymphocytes expressing CD71, rather than the density of CD71 on each cell. Our results may show an increased recruitment of “memory” CD4<sup>+</sup> cells compared to “naïve” CD4<sup>+</sup> cells, which would increase the overall percentage of CD4<sup>+</sup> lymphocytes in circulation (Born et al., 1995; Woods et al., 1999; Globerson and Effros 2000; Yan et al., 2001). In times of high intracellular iron concentration in older individuals, a lower percentage of lymphocytes may express CD71, rather than the density of CD71 on each cell decreasing. Others suggest that the density of CD71 on each cell is down-regulated when intracellular iron concentration is high, and up-regulated when intracellular iron concentration is high (Neckers and Cossman 1983; Kemp 1993; Maes et al., 1997a; Gimferrer et al., 1997; Skikne 1998) but our results do not support these findings.

The difference in results between the present study and others may reflect differences in the homogeneity of participating subjects. Some examined CD71 expression in both males and females, within all age groups (Maes et al., 1997a; Skikne 1998) whereas the present study investigated CD71 expression only older men and women. It is possible that older individuals, possessing a higher percentage of “memory” lymphocytes, respond to endurance training by

recruiting higher percentages of “memory” CD4<sup>+</sup> cells positive for CD71, rather than up-regulating the concentration of CD71 receptors on the lymphocyte surface (Born et al., 1995). The present study also compared CD71 expression with haemoglobin concentrations in male and female UT and TR groups. Overall, increases in haemoglobin concentration coincided with decreases in both percentage of lymphocytes expressing CD71 and CD71 density. Conversely decreased haemoglobin concentration coincided with increased CD71 expression, supporting the negative correlation between haemoglobin concentration and CD71 expression found by previous researchers (Kemp 1993; Gimferrer et al., 1997; Maes et al., 1997a).

The present study measured the percent of lymphocytes expressing both CD25 and CD71 (CD4<sup>+</sup>/CD25<sup>+</sup>/CD71<sup>+</sup> or “double positive”), and the densities of both CD25 and CD71 on the lymphocytes. The expression of both receptors on the cell surface is an intermediate step between CD25 and CD71 expression in CD4<sup>+</sup> proliferation. We found no previous studies which examined the effect of aerobic/endurance training on this intermediate step in CD4<sup>+</sup> lymphocyte proliferation. The results of Experiment 1 showed that the monthly changes in the percentage of lymphocytes expressing both CD25 and CD71 were similar to the percentage of cells expressing only CD25, rather than those expressing CD71. High percentage of “double positive” (CD4<sup>+</sup>/CD25<sup>+</sup>/CD71<sup>+</sup>) occurred during early winter (June) for male UT/TR and female UT/TR groups, and early spring (September A) for the male UT/TR and female UT groups. Increases in the percentage of “double positive” cells, and increases in double receptor density, may imply a “speeding up” of the proliferative cycle at some times of the year when lymphocytes are more responsive to PHA (Neckers and Cossman 1983). In the present study, the increase in “double positive” lymphocytes in September A coincides with an increase CD25 expression, suggesting that early spring is a period of increased CD4<sup>+</sup> proliferation (Brock 1987; Maes et al., 1994).

The month to month changes in the percentage of “double positive” lymphocytes were similar to the month to month changes of lymphocytes expressing only CD25, which suggest the importance of the CD25 receptor as a proliferative marker and initiator of the proliferative cycle. The present results found that there was no training effect on “double positive” lymphocytes for the female UT and TR groups. However, the male TR group showed significantly higher percentage of “double positive” lymphocytes than the male UT group during spring (September A), suggesting an enhanced proliferative response during this month.

The significant month to month changes in CD25 density on “double positive” lymphocytes were similar to the month to month changes in CD25 density on “single positive” cells for all four groups. In other words, when CD25 density on the “single positive” cells increased and decreased, so did CD25 density on “double positive” cells, again indicating the importance of CD25 as a proliferative marker (Neckers and Cossman 1983). There were no significant differences between male UT/TR groups and female UT/TR groups in CD25 density on “double positive” lymphocytes, suggesting that aerobic training had no effect on CD25 density on “double positive” cells. However, the male UT group had a significantly greater CD71 density on “double positive” lymphocytes than the male TR in March, and a significantly lower CD71 density in November. The female UT group had a significantly greater CD71 density on “double positive” lymphocytes compared to the female TR group during August and December. The results suggest individual variation in iron needs within the groups, rather than an aerobic training effect on CD71 density. The similar pattern of CD71 density on “double positive” lymphocytes in all four groups throughout the year suggests seasonal iron needs may be linked to variations in lymphocyte proliferation and blastogenesis (Maes et al., 1997a). It is likely that the density of CD25 and CD71 on “double positive” lymphocytes closely follow seasonal variations in “single positive” cells, and thereby are linked with seasonal patterns and genetic regulation of immuno-competency and lymphocyte blastogenesis (Brock 1987; Maes et al., 1994, 1995, 1997a; Geraghty 2002; Feitosa et al., 2002a).

#### 5.1.10. Intracellular Calcium

There are a diversity of methods used for measuring  $[Ca^{2+}]_i$  in living cells (Tsien 1988; Kao et al., 1989; Greimers et al., 1996; Berman 2000). The present study selected the methods of Greimers et al. (1996) because these investigators used the highly sensitive Fluo-3 AM to measure  $[Ca^{2+}]_i$  within lymphocytes, and established an accurate calibration procedure that measured the dissociation constant of the fluorescent probe at the correct ambient temperature (Kao et al., 1989; Kao 1994). The present results are consistent with Griemers et al. (1996) and indicate that methods used in the present study are reliable and the results reproducible. Other investigators have measured changes in  $[Ca^{2+}]_i$  in various immune cells without calibration procedures or measuring the dissociation constant, and usually with less sensitive probes than Fluo-3 AM (Hallett et al., 1999; Berman 2000). While Fluo-3 AM is more sensitive to low nanomolar  $[Ca^{2+}]_i$  concentrations in resting cells than Indo-1 and Fura-2 (Molecular Probes, USA), most of the

investigators who used Fluo-3 AM, did so at a temperature of 37°C instead of the recommended 21°C to 24°C (Kao et al., 1989; Kao 1994).

Use of Fluo-3 AM at 37°C rather than at 21°C to 24°C changes the  $K_d$  from approximately 380 nM to 865 nM, thereby showing a higher nanomolar  $[Ca^{2+}]_i$  when the probe is half saturated (Haugland 1996). Experiments conducted at 37°C may have resulted in an inaccurate measurement of low nanomolar  $[Ca^{2+}]_i$ , producing the varied basal  $[Ca^{2+}]_i$  reported by other researchers. The manufacturers state unequivocally that Fluo-3 AM gives optimum results only when used at temperatures between 21°C and 24°C (Kao et al., 1989; Kao 1994; Thomas and Hanley 1994). Leakage of Fluo-3 from the cell or probe compartmentalization in intracellular organelles may result if Fluo-3 is used at temperatures higher than the specified 21°C to 24°C. Many studies did not calculate their own  $K_d$ , but relied upon those originally calculated by Kao et al. (1989). Other investigators who measured  $[Ca^{2+}]_i$  neglected to measure ER  $Ca^{2+}$  stores (i.e.  $[Ca^{2+}]_t$ ), which may well have contributed to  $Ca^{2+}$  flux, transcriptional processes and alterations in cellular signalling (Thastrup et al., 1990). Within the existing literature there is considerable variation in the reported concentration of transient  $[Ca^{2+}]_i$  spikes (Berman 2000; Marchi et al., 2000; Patrick et al., 2000; Alfonso et al., 2001; Mooren et al., 2001a,b; Mather and Rottenberg 2002; Berridge et al., 2003). The present study used Fluo-3 AM at 22°C during calibration measurements, reproducibility studies and testing procedures. The reproducibility of our calibration procedure and the low coefficient of variation in intra-assay (3%) and inter-assay (1%) indicate that our results are valid, accurate and therefore consistent with those of Greimers et al. (1996).

The variety of methods used to determine  $[Ca^{2+}]_i$  have created some uncertainty about the effects of training on  $[Ca^{2+}]_i/t$ . The present study found that the male TR, female UT and female TR groups showed significantly higher  $[Ca^{2+}]_i$  compared to the male UT group during September B, and the female TR group had a significantly higher concentration than the female UT group in December. It is likely that the significant differences in  $[Ca^{2+}]_i$  are related to increases in lymphocyte proliferation and intracellular signalling during those months. Indeed, the male TR and female UT/TR groups showed a significant increase in the percentage of lymphocytes expressing CD25 during September, and a high percentage of positive lymphocytes during November-December. As the significant differences occurred during one or two months of the year (for male and female respectively), it seems unlikely that aerobic training was responsible for the differences

in  $[Ca^{2+}]_i$ . Studies reporting the effects of training on  $[Ca^{2+}]_i$  in lymphocytes are few in number. Previous research found that an acute bout of intense exercise (run to exhaustion at 80%  $\dot{V}O_{2\ peak}$ ) could transiently increase  $[Ca^{2+}]_i$  in lymphocytes (Mooren et al., 2001b) with significant changes in  $Ca^{2+}$  mobilisation from intracellular stores, correlating with alterations in lymphocyte signalling 24 hours post-exercise (Mooren et al., 2001b). Significant decreases in the proliferative response of lymphocytes to PHA were noted immediately post-exercise. However, 24 hours post-exercise the PHA response and  $[Ca^{2+}]_i$  had increased compared to pre-exercise levels, suggesting that the lymphocyte proliferative response was enhanced by the acute exercise bout. However, the effects of long-term aerobic training on  $[Ca^{2+}]_i$  in lymphocytes have not been investigated until the present study.

The present study found significant month to month changes in basal  $[Ca^{2+}]_i$  in lymphocytes for both UT and TR groups during twelve months. To our knowledge, no others have conducted such a long-term investigation of aerobic training effects on  $[Ca^{2+}]_i$  in leucocytes. The highest mean  $[Ca^{2+}]_i$  occurred during January for all groups (UT/TR male/female), with concentrations varying between  $140 \pm 10$  nM (female UT) and  $212 \pm 12$  nM (male UT). The lower  $[Ca^{2+}]_i$  for all groups occurred during May, August and November, with concentrations varying between  $20 \pm 0.5$  and  $40 \pm 1$  nM, consistent with the results of others (Greimers et al., 1996; Morford et al., 1997; De la Rosa et al., 2001; Berridge et al., 2003).

There is scant information about seasonal changes or effects of aerobic training on  $[Ca^{2+}]_i$  in lymphocytes. Letellier and Desjarlais (1982) examined plasma  $[Ca^{2+}]$  over four years, and found increases in plasma  $[Ca^{2+}]$  during April-May and September-October each year. The present study found that  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  had similar peaks during September-October, but also peaked during January-February, May-July, and December. Elevated  $[Ca^{2+}]_i$  is known to drive the relocation of NFAT, transcription and lymphocyte proliferation, and has been shown to trigger apoptosis in lymphocytes and other cells (Mooren et al., 2001a,b; Suh 2002). With monthly changes in  $[Ca^{2+}]_i$  coinciding with similar changes in CD25 expression, it would seem logical to suggest a relationship between  $[Ca^{2+}]_i$  in resting lymphocytes, transcription, receptor expression and proliferation (Di Pietro et al., 1993; Fomina et al., 2000; Bautista et al., 2002). In the present study the pattern of monthly change in  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  is similar to monthly changes in CD25 expression and

leucocyte concentration. Our findings suggest a positive relationship between these variables, and support the findings of Brock (1987) and Maes et al. (1995, 1997a,b).

The  $[Ca^{2+}]_t$  was positively and significantly correlated with  $[Ca^{2+}]_i$  for all groups throughout the year, (male UT  $r = 0.87$ ; male TR  $r = 0.82$ ; female UT  $r = 0.57$ ; female TR  $r = 0.67$ ).  $[Ca^{2+}]_t$  varied from  $23 \pm 1$  to  $31 \pm 3 \mu M$  in January to lower concentrations of approximately  $0.5 \mu M$  during April and July. The magnitude of the thapsigargin-induced spikes in Experiment 1 are consistent with previous research (Burns and Lewis 1997; Berman et al., 2000; Bautista et al., 2002). There is considerable variation in  $[Ca^{2+}]_t$  induced by thapsigargin, with reported  $[Ca^{2+}]_t$  varying from 500nM to over  $10 \mu M$  (Marriott and Mason 1996; Berman 2000; Fomina et al., 2000; De la Rosa et al., 2001; Putney et al., 2001; Bautista et al., 2002). The reasons for the variations in  $[Ca^{2+}]_t$  include the use of different types of cells; use of different fluorescent probes, with shorter wavelengths and less sensitivity than Fluo-3; the measurement of calcium flux with spectrometry rather than  $[Ca^{2+}]_t$  with flow cytometry and variation in calibration procedures.

The present study found month to month changes in  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$ , which may be due to seasonality in transcriptional processes or possibly apoptosis within lymphocytes, as suggested by Kiang et al. (2003). The increases in  $CD4^+$   $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  found in Experiment 1 suggest that transcription was elevated in older individuals during the warmer months of the year (Calderwood and Stevenson 1989; Moseley 2000; Wang et al., 2000). It is tempting to suggest that the December-January peak in both  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  may relate to an increase in lymphocyte blastogenesis, lymphocytes expressing CD25 and CD25 density, and an increase in CD71 density.

The increase in  $[Ca^{2+}]_i$  and  $[Ca^{2+}]_t$  during warmer months could also be related to the manufacture and activity of heat shock proteins (HSP) and "stress-activated protein kinases" (SAPK's). The JNKs and p38MAPKs are collectively termed the "stress-activated protein kinases" because they are activated by stress-related stimuli (e.g. heat, cold, hypoxia, exercise) and are implicated in apoptosis (Moseley 2000; Fehrenbach and Northoff 2001; Suh 2002). It is plausible that cellular adaptations to higher ambient temperatures involve an increase in the transcription of HSP. HSP-70 and HSP-90 are known to act as "chaperone" proteins that facilitate the movement of hormone receptor complexes into the nucleus, and the repair and folding of cytosolic proteins (Moseley

2000; Boron and Boulpaep 2003). An increase in HSP transcription during warm months suggests an increase in transcription within the cell (Wang et al., 2000; Xiao et al., 2003). Some researchers found that exercise induces heat shock protein synthesis across a range of muscle and tissue types in both young and old age groups, with increases in  $[Ca^{2+}]_i$  (Moseley 2000; Naito et al., 2001). The present study found that there were significant differences in  $[Ca^{2+}]_i$  between male UT and TR groups during September B, and female UT and TR groups during December only, suggesting that aerobic training had little or no effect on  $[Ca^{2+}]_i$  and transcription of HSP in the TR groups.

The present results, together with the results of others (Thomas and Hanley 1994; Kiang et al., 1998; Fehrenbach and Northoff 2001; Naito et al., 2001), suggest that an increase in ambient temperature rather than repeated bouts of exercise resulted in increased transcription. Thomas and Hanley (1994) documented that a loss in stored  $[Ca^{2+}]$  from the ER had a distinct regulatory effect on the initiation of protein synthesis. Thus, the key event in the induction of the HSP synthesis may be a depletion of ER  $Ca^{2+}$  stores rather than merely an elevation of cytosolic  $Ca^{2+}$  (Thomas and Hanley 1994; Fomina et al., 2000). Kiang et al. (1998) noted an over-expression of HSP-70 resulted in significant increases in basal  $[Ca^{2+}]_i$  in unstimulated epidermoid cells, suggesting a role for HSP in the maintenance of  $[Ca^{2+}]_i$ . Kiang et al. (2003) also found that ambient heat stress increased phosphorylation of nitric oxide synthase, nitric oxide production and  $[Ca^{2+}]_i$  in lymphocytes. This process was related to an increased expression of Fas/CD95 on the lymphocyte surface, suggesting an increase in lymphocyte apoptosis with ambient temperatures greater than  $37^{\circ}C$  (Kiang et al., 2003).

In the present study, male and female UT groups showed significantly higher  $[Ca^{2+}]_t$  than the male and female TR groups in January. The higher  $[Ca^{2+}]_t$  in the UT subjects may reflect either an increase in transcription of HSP, or apoptosis, compared to the TR group (Mooren et al., 2001b; Bautista et al., 2002; Suh 2002; Kiang et al., 2003; Xiao et al., 2003). While a training effect was not apparent with  $[Ca^{2+}]_i$ , with ER stores of  $Ca^{2+}$  it is possible that the UT groups were less able to maintain lymphocyte homeostasis in the heat, resulting in an increase in intracellular signalling related to increased HSP synthesis and/or apoptosis (Moseley 2000; Fehrenbach and Northoff 2001; Kiang et al., 2003). Indeed, Fehrenbach and Northoff (2001) found that the HSP response was down-regulated in the leucocytes of endurance athletes, suggesting that regular training

caused an adaptation, a “shut down” of the HSP response. The authors suggested that trained individuals had greater “front line” cellular protection mechanisms (e.g. “anti-oxidant systems”) against reactive oxygen species, therefore “secondary repair systems” such as HSP could be down-regulated (Fehrenbach and Northoff 2001). A different theory was suggested by Liossis and Tsokos (1997), who found that subjecting T cells to repeated heat shock caused a down-regulation of the TCR/CD3 and IP3, and therefore  $[Ca^{2+}]_i$ . That is, less  $[Ca^{2+}]_i$  may be an endurance training adaptation that indicates reduced lymphocyte activation and HSP turnover.

Variations in  $[Ca^{2+}]_i$  have been previously linked to dysfunction within lymphocyte signalling pathways, for example deregulated signal transduction associated with Alzheimer’s disease, arthritis and tumour formation (Mazzeo 1993; Di Pietro et al., 1993; Whisler et al., 1996a,b; Morford et al., 1997; Mooren et al., 2001a,b). However, Hartmann et al. (1994) found no age-related alterations in basal  $[Ca^{2+}]_i$  in human lymphocytes, in agreement with our findings. While many have hypothesized that age-related reductions in  $[Ca^{2+}]_i$  may be responsible for transcriptional irregularities and some disease states (Whisler et al., 1996a,b; Morford et al., 1997; Mooren and Volker 2001), it cannot be concluded that ageing results in reduced  $[Ca^{2+}]_i$  in healthy subjects.

#### 5.1.11. Intracellular Iron

The results of Experiment 1 showed increases in mean channel log fluorescence, suggesting a reduced intracellular concentration of  $Fe^{3+}$  ( $[Fe^{3+}]_i$ ) during February and July, for all groups. Male UT and TR, and female TR groups also showed reduced  $[Fe^{3+}]_i$  during March. The lowest measurement of fluorescence (or when  $[Fe^{3+}]_i$  was the greatest), occurred in May-June for all groups. The female TR group had significantly lower  $[Fe^{3+}]_i$  than the female UT in March. While it is possible that this difference was due to aerobic training, there were no other significant differences between male UT/TR and female UT/TR groups during the year. Consequently, factors other than training may have contributed to the significant difference in  $[Fe^{3+}]_i$  between the female groups (e.g. dietary iron intake, illness, hormonal changes). However, there were significant differences in  $[Fe^{3+}]_i$  between genders during six months of the year (January, April, May, June, July, September B), with one or both female groups showing higher  $[Fe^{3+}]_i$  than one or both male groups at these times. The significant gender differences in  $[Fe^{3+}]_i$  may reflect differences in dietary iron intake during the year, or hormonal differences between genders (Kemp 1993; Maes

et al., 1997a). There are few studies that have investigated  $[\text{Fe}^{3+}]_i$  within lymphocytes (Breuer et al., 1995; Sainte-Marie et al., 1997; Thomas et al., 1999; Parrish 1999). Previous research suggests that changes in  $[\text{Fe}^{3+}]_i$  are tightly linked to changes in TfR (CD71) expression,  $[\text{Ca}^{2+}]_i$  and signal transduction (Sainte-Marie et al., 1997).

It seems reasonable to suggest that low  $[\text{Fe}^{3+}]_i$  would be sensed by IRE and result in an up-regulation of CD71 recycling and higher  $[\text{Ca}^{2+}]_i$ . Depletion of intracellular iron stores may have been caused by accelerated intracellular activity, such as transcription and DNA synthesis (Sainte-Marie et al., 1997; Picard et al., 1998). Indeed, Pelosi-Testa et al. (1988) found that lymphocyte CD71 synthesis was mediated by depletion of the regulatory intracellular  $\text{Fe}^{3+}$  pool. When CD71 synthesis was stimulated either by the addition of IL-2 or PHA, CD71 were expressed after a decrease in  $[\text{Fe}^{3+}]_i$ . The present study found periods of low  $[\text{Fe}^{3+}]_i$  coincided with high  $[\text{Ca}^{2+}]_i$  and  $[\text{Ca}^{2+}]_t$ , possibly related to increased transcription (Pelosi-Testa et al., 1988; Sainte-Marie et al., 1997). The relationship between  $[\text{Fe}^{3+}]_i$ , CD71 expression and  $[\text{Ca}^{2+}]_i$  was investigated by Sainte-Marie et al. (1997) who found that elevated  $[\text{Ca}^{2+}]_i$  resulted in an increase in CD71 recycling to the cell surface, a phenomenon which lowers the  $[\text{Fe}^{3+}]_i$  through increased binding of intracellular ferritin to its receptor. A decrease in  $[\text{Ca}^{2+}]_i$  caused a reduction in the recycling rate of CD71 (Sainte-Marie et al., 1997). The inverse relationship we found between  $[\text{Ca}^{2+}]_i$  and  $[\text{Fe}^{3+}]_i$  is consistent with the findings of Sainte-Marie et al. (1997), where increased  $[\text{Ca}^{2+}]_i$  increased the recycling of TfR and the depletion of intracellular iron pools ( $[\text{Fe}^{3+}]_i$ ).

The present study found similar month to month changes in  $[\text{Fe}^{3+}]_i$  occurred for UT and TR groups during the year, suggesting no significant effect of training on  $[\text{Fe}^{3+}]_i$ . There were significant increases in  $[\text{Fe}^{3+}]_i$  between April-May, and July-August for the male UT and TR and female UT groups, with the female TR group showing a significant increase between April-May only. Significant decreases in  $[\text{Fe}^{3+}]_i$  occurred between January-February, June- July, and August-September A for both male and female UT and TR groups. Our results lend some support to Maes et al. (1997a). Maes et al. (1997a) investigated seasonal changes in serum iron, TfR, Tf and ferritin concentrations in young males and females in the northern hemisphere and found significant increases in serum iron and Tf in March, May and between July- August, and significant decreases in serum iron and Tf in January-February, April, August and November. There is some similarity between the results of the present study and Maes et al. (1997a), which suggests that

seasonal changes in iron requirements in lymphocytes may be linked to seasonal changes in overall iron status, and lymphocyte proliferation.

The present study highlighted the relationship between  $[\text{Fe}^{3+}]_i$  and lymphocyte proliferation by the regression analysis between leucocyte concentration and the immune and haematological variables. The regression analysis showed that  $[\text{Fe}^{3+}]_i$  was one of the stronger predictors for leucocyte concentration in all groups except the female TR (male UT  $r = 0.82$ ; male TR  $r = 0.70$ ; female UT  $r = 0.80$ ). There is a paucity of data about the relationship between  $[\text{Fe}^{3+}]_i$  and other molecules within the signal transduction pathway. Emerging evidence suggests that  $[\text{Fe}^{3+}]_i$  and  $[\text{Fe}^{2+}]_i$  play an important role in iron status and DNA synthesis. While the exact mechanisms behind this relationship have not been fully defined, it appears that both  $[\text{Ca}^{2+}]_i$  and  $[\text{Fe}^{3+}]_i$  are closely involved in intracellular signalling and lymphocyte proliferation.

#### 5.1.12. Summary of Experiment 1

Peak  $\dot{V}O_2$  ( $\text{L}\cdot\text{min}^{-1}$  and  $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), peak power (watts) and peak  $\dot{V}_E$  ( $\text{L}\cdot\text{min}^{-1}$ ) increased significantly for both TR groups, but not for the UT groups. Thus, long-term moderate intensity training improves the functional capacity of older individuals, and the first experimental hypothesis was accepted. Haematological indices showed significant monthly changes over twelve months. Erythrocyte concentration, haemoglobin, MCHC, haematocrit and platelets showed decreases during autumn, late winter and late spring.

We found that the proliferative response of  $\text{CD4}^+$  lymphocytes, measured by the percentage of lymphocytes expressing CD25, was increased by aerobic training in older males but not in older females. Aerobic training did not increase density of CD25 in older males or females, suggesting that “memory”  $\text{CD4}^+$  recruitment in older males was improved by long-term aerobic training rather than receptor density. Aerobic training had no effect on CD71 expression. Thus, the second experimental hypothesis was rejected. Twelve months of aerobic training did not increase intracellular concentrations of  $\text{Ca}^{2+}$ , in older individuals of either gender and the third experimental hypothesis was rejected. Intracellular  $\text{Fe}^{3+}$  concentrations showed no training effect or gender differences. and the fourth experimental hypothesis was also rejected. Although the  $\text{CD4}^+$  proliferative response increased in older men, older women may need to exercise more often

and/or at a higher intensity to achieve the same adaptive immunological benefits as males. It is possible that aerobic training enhances the function of other leucocyte subsets (e.g. NK cells) as well as CD4<sup>+</sup>. Older women may improve the function of other leucocyte subsets with training.

Immunological variables showed significant monthly changes over twelve months. The results showed decreases in CD4<sup>+</sup>/CD25 expression during autumn, late winter and late spring, and increases in summer, early winter, and early spring. CD71 expression showed an inverse relationship with CD25 expression in males during the year, but only for the latter six months of the year in females. The percentage of “double positive” lymphocytes followed a similar pattern of change as CD25 expression. The seasonal changes indicated that autumn, late winter and late spring may be periods where individuals were more at risk of infection. The seasonal changes in [Ca<sup>2+</sup>]<sub>i</sub> were consistent with this finding, since [Ca<sup>2+</sup>]<sub>i</sub> increased at times of increased CD25 expression, indicating elevated transcriptional processes and cell proliferation. The significant increases in [Ca<sup>2+</sup>]<sub>i</sub> and decreases in [Fe<sup>3+</sup>]<sub>i</sub> during summer may indicate an increase in HSP synthesis and/or apoptosis.

## 5.2 Experiment 2 - Males Aged 23 to 36 Years

The principal findings of Experiment 2 were that the TR group showed no evidence of immunosuppression, and showed significantly higher CD25 density compared to the UT group for three months of the year. Both TR and UT groups showed seasonal variations in CD25 expression, CD71 expression,  $[Ca^{2+}]_i$  and  $[Fe^{3+}]_i$ , similar to Experiment 1 and suggesting that spring and autumn were periods of decreased lymphocyte proliferation while summer was a period of increased lymphocyte proliferation and transcription. The TR group also showed significantly lower haemoglobin and MCHC for six and nine months of the year respectively, suggesting an iron deficiency during those months. Endurance training may increase CD25 density in triathletes, thereby maintaining their immune function and general health. Significantly reduced [Hb] and MCHC in these athletes does not appear to decrease their immune function, or increase the incidence of URTI.

### 5.2.1. Subject Characteristics

The men who participated in Experiment 2 were free from any clinical signs of cardiovascular and respiratory disease, and represented untrained (UT) and highly trained (TR) healthy young men. The UT and TR groups showed no significant pre- and post-study differences in body mass, systolic and diastolic blood pressures and percent body fat. The values obtained for UT and TR group body mass, percent body fat, blood pressure and spirometry were typical of untrained and highly trained young males (O'Toole and Douglas 1995; Rudzki et al., 1995; Sleivert and Rowlands 1996; Margaritis et al., 1997; Green et al., 1999; Bentley et al., 2002). In both pre-study and post-study measurements, the TR group showed significantly lower body mass, percent body fat, resting heart rate, systolic and diastolic blood pressures compared to the UT group ( $p < 0.05$ ). Both groups were normotensive ( $<140/90$  mmHg), consistent with the findings of others (Keizer et al., 1989; Rudzki et al., 1995; Sleivert and Rowlands 1996; Margaritis et al., 1997; Green et al., 1999; Portier et al., 2001).

The TR group showed a significantly lower resting heart rate than the UT group ( $48 \pm 1$  b.min<sup>-1</sup> and  $68 \pm 3$  b.min<sup>-1</sup> respectively), and significantly lower systolic and diastolic blood pressure ( $119 \pm 1/70 \pm 2$  mmHg and  $123 \pm 1/80 \pm 2$  mmHg respectively), consistent with previous results (Roy et al., 1985; Brooks et al., 1995; O'Toole and Douglas 1995; Gute et al., 1996; Sleivert and Rowlands

1996; Bentley et al., 2002). The adaptations of the cardiovascular system to endurance training have been well documented (Gledhill et al., 1988; Roy et al., 1988; Gledhill et al., 1994; Brooks et al., 1995; Gute et al., 1996; Zavorsky 2000; McGuire et al., 2001). The mechanisms responsible for a training-induced decrease in resting heart rate include decreases in total peripheral resistance, increased capillary density, increased cardiac output, venous return and stroke volume, enhanced dilation of the left ventricle, improved arterial compliance, an increase in the release of nitric oxide and endothelial relaxing factors from endothelial cells in the capillaries, a decreased sympathetic response and decreased  $\beta$ -adrenergic receptor density, enhanced baroreflex function, increased parasympathetic response and altered electrophysiology of the sinoatrial node (Gledhill et al., 1994; Brooks et al., 1995; Hagberg et al., 2000; Zavorsky 2000; Govers and Rabelink 2001; Hawley and Stepto 2001; Hedelin et al., 2001; Mazzeo and Tanaka 2001; Yamamoto et al., 2001; Cowley et al., 2003). Gledhill et al. (1994) and O'Toole and Douglas (1995) found that Ironman triathletes showed enhanced cardiac output and stroke volume during maximal exercise, compared to untrained controls. The triathletes showed evidence of left ventricular hypertrophy and increased end-diastolic volumes, and enhanced diastolic function at rest which resulted in increased pre-load during maximal exercise (Portier et al., 2001). While we did not measure cardiac output and stroke volume, it is likely that the triathletes in the present study would have developed these physiological adaptations to endurance training. Some evidence of adaptation is provided through application of the Fick equation (i.e. the higher  $\dot{V}O_{2\text{ peak}}$  of the TR group compared to UT, with similar peak heart rates for both UT/TR, indicates an increase in stroke volume in the TR group) (O'Toole and Douglas 1995).

The TR group showed significantly higher pre- and post-study peak power on the cycle ergometer, compared to the UT group, consistent with the findings of others (Sleivert and Rowlands 1996; Hawley and Stepto 2001; Bentley et al., 2002). Endurance training results in hypertrophy of both Type IIa and Type I skeletal muscle fibres through an increase in fibre cross-sectional area and in the number of muscle fibres, an increase in capillary density, muscle citrate synthase activity and an increase in myosin heavy chain isoforms in both Type I and Type IIa muscle fibres (Meredith et al., 1989; Stone et al., 1996). Furthermore, highly trained endurance athletes may show improved patterns of neuromuscular recruitment and muscle contractility which enhance power, compared to untrained individuals (Hawley and Stepto 2001). Other factors which may contribute to increased

power in triathletes are enhanced lactate transport in Type I muscle fibres; enhanced muscle buffering capacity; the utilisation of less carbohydrate and more fat during exercise, all of which may delay the onset of fatigue (Brooks et al., 1995; Hawley and Stepto 2001; Bentley et al., 2002).

The present study found that the post-study  $\dot{V}_{E\ peak}$  of the TR group was significantly higher than their pre-study  $\dot{V}_{E\ peak}$ . While the majority of the triathletes had followed the same periodised pattern of endurance training for many years, it is possible that some of the younger triathletes had increased or modified their spring cycle training, resulting in a higher  $\dot{V}_{E\ peak}$  post-study. Pre- and post-study incremental tests were conducted in August-September 1999 and October 2000 respectively, and training volume and intensity increased through September-October, leading up to half-Ironman races in November. The periodised increase in training volume through September and October may explain the higher post-study  $\dot{V}_{E\ peak}$  for the TR group.

The pre- and post-test  $\dot{V}_{E\ peak}$  in the TR group was not significantly higher when compared to the UT group ( $p = 0.06$ ). It is possible that some of the UT subjects who were tall, with a large lung capacity, contributed to a higher mean  $\dot{V}_{E\ peak}$  than expected, thus eliminating the statistically significant difference between groups. The difference in  $\dot{V}_{E\ peak}$  between groups indicated greater ventilatory demand and incremental test performance in the TR group. Since  $\dot{V}_{E\ peak}$  is positively correlated with  $\dot{V}O_{2\ peak}$ , it was expected that endurance athletes would have higher ventilatory demand (greater  $CO_2$  production), greater lactate buffering capacity, and a higher  $\dot{V}O_{2\ peak}$  than sedentary subjects (Green et al., 1991; Johnson and Dempsey 1991; Babcock et al., 1996; Mota et al., 1999; Boussana et al., 2001). Many studies have found an increase in respiratory muscle strength and oxidative capacity with endurance training, and a training-induced change in breathing pattern are possible adaptations to increase  $\dot{V}_{E\ peak}$  in Ironman triathletes (Hagberg et al., 1988; Johnson and Dempsey 1991; Johnson et al., 1992; Babcock et al., 1996; Habedank et al., 1998; McClaran et al., 1999; Johnson et al., 1999; Eastwood et al., 2001; McMahon et al., 2002). Habedank et al. (1998), Feitosa et al. (2002b) and Weil (2003) have also suggested that a genetic factor may be implicated in the increased  $\dot{V}_{E\ peak}$  of endurance athletes, similar to the family clusters of low hypoxic responses found in untrained siblings and family members of

endurance athletes, and identical twins (Weil 2003). The HERITAGE family study has also provided evidence that major genes may account for much of the variability and trainability in ventilatory thresholds (Feitosa et al., 2002b) and endurance performance (Moore et al., 2001; Feitosa et al., 2002b; Hagberg et al., 2002; An et al., 2003).

### 5.2.2. Peak Oxygen Uptake ( $\dot{V}O_{2\text{ peak}}$ )

The present study found no significant differences between pre- and post-study  $\dot{V}O_{2\text{ peak}}$  in either the UT or TR groups. The TR group showed a significantly higher pre- and post-study  $\dot{V}O_{2\text{ peak}}$  ( $\text{L}\cdot\text{min}^{-1}$  and  $\text{mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) compared to the UT group. The present findings are consistent with those of O'Toole and Douglas (1995), Rudzki et al. (1995), Margaritis et al. (1997), O'Toole et al. (1999), Mockel et al. (2001), Bentley et al. (2002) and Jasiukeviciene et al. (2003). The present results confirm that the UT group had maintained an UT status during the year, and could be regarded as a valid control group for the TR group.

The TR group maintained a similar volume and intensity of pre-competition training in September 2000 as September 1999, and there were no significant differences between their pre- and post-study  $\dot{V}O_{2\text{ peak}}$  measurements, consistent with O'Toole and Douglas (1995). The TR group had pre-study  $\dot{V}O_{2\text{ peak}}$  values of  $4.7\pm 0.1\text{ L}\cdot\text{min}^{-1}$  ( $64\pm 2\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ) and post-study values of  $4.8\pm 0.1\text{ L}\cdot\text{min}^{-1}$  ( $66\pm 2\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ ), which are accepted values for highly trained long-course triathletes (O'Toole and Douglas 1995; Sleivert and Rowlands 1996; Bentley et al., 2002). Only one of the TR subjects raced in the "Open" or elite class, while the remainder of the subjects were regarded as well-performed "age-groupers".  $\dot{V}O_{2\text{ peak}}$  values for well trained triathletes during incremental treadmill and cycle exercise have varied from  $56\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  to  $78\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$  (O'Toole and Douglas 1995). The subjects in Experiment 2 fall into the middle of this range, with the lowest  $\dot{V}O_{2\text{ peak}}$  of  $60\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ , and the highest of  $76\text{ mL}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ . Peak  $\dot{V}O_{2\text{ peak}}$  values may vary among highly trained triathletes, depending upon the physical characteristics of the individuals, and a higher  $\dot{V}O_{2\text{ peak}}$  does not necessarily correlate with better race performance. Measurements such as anaerobic threshold and respiratory compensation threshold, which improve with higher

volume/intensity training at specific “periodised” months of the year, may be better indicators of triathlon performance than  $\dot{V}O_{2\text{ peak}}$  alone (Sleivert and Rowlands 1996; Bentley et al., 2002).

The higher  $\dot{V}O_{2\text{ peak}}$  of the TR group can be ascribed to both central and peripheral adaptations to endurance training (O’Toole and Douglas 1995). The present study did not measure either central or peripheral changes in the TR group, but central adaptations (increased cardiac output, ejection fraction, stroke volume, left ventricular hypertrophy and increased venous return at maximal exercise) have been well documented in endurance athletes (Roy et al., 1988; Spina et al., 1993; Stratton et al., 1994; O’Toole and Douglas 1995). Well documented peripheral adaptations include an increase in mitochondrial and capillary density, increased production of nitric oxide and buffering capacity of muscles, increased lean muscle mass and increased capacity of oxidative enzymes such as citrate synthase (Meredith et al., 1989; Brooks et al., 1995; Gute et al., 1996; Stone et al., 1996; Hawley and Stepto 2001).

### 5.2.3. Incidence of URTI

During the course of the study, seven cases of viral infections (URTI) occurred in the UT and only two cases were reported in the TR group. The findings from the TR group are not consistent with the results of others who documented between 1.2 to 2.0 cases of viral infection per person per year (Heath et al., 1991; Pyne et al., 2000; Nieman et al., 2000; Matthews et al., 2002). The TR and UT groups in the present study reported less than the average number of URTI per person (Pyne et al., 2000; Matthews et al., 2002), although the UT group had a higher number of URTI than the TR group. Furthermore, both groups had leucocyte concentrations that remained in the normal ranges throughout the year. Our results suggest that clinical utility of URTI as an indicator of immuno-suppression may not be accurate. The higher incidence of URTI reported by the UT group in Experiment 2 is consistent with the “J” curve, where sedentary individuals showed a higher incidence of URTI (Heath et al., 1991) compared to moderately trained individuals. The exact reasons for a higher incidence of viral illnesses in sedentary individuals remains unclear, but Mackinnon (1992), Pedersen (1997) and Nieman et al. (2000) have suggested that lack of exercise decreases the proliferative response in leucocytes, possibly by decreasing the production of cytokines such as IL-1 and IL-2.

While there is literature suggesting that athletes involved in schedules of heavy training and competition are more susceptible to URTI (Pedersen 1997; Friman and Wesslen 2000), the results of the present study continue to challenge that evidence. Urhausen et al. (1998), Nieman et al. (2000) and Pyne et al. (2000) also found that endurance athletes participating in heavy training and competition schedules did not suffer from a higher incidence of URTI compared to UT individuals. The enhanced immunocompetency in Ironman triathletes could be explained by possible training-induced adaptations, such as an increased production of proliferative cytokines (IL-2) (Nieman et al., 2000) and a decreased production of cortisol and cortisol receptors (Urhausen et al., 1998) (see Section 5.2.5.). The present study suggests that while Ironman triathletes undertake high volume training, the predominantly moderate intensity of Ironman training may be less intense than the training for Olympic distance competitions (Bentley et al., 2002), with less likelihood of high cortisol concentrations, leucopenia, reduced lymphocyte proliferation and increased URTI (Urhausen et al., 1998; Goldsby et al. 2000; Nieman et al., 2000).

#### **5.2.4. Haematology**

The haematological indices remained within the normal ranges for the duration of the study (Coulter 1984). Monthly changes in some cell subsets may have been statistically significant but not clinically significant, given that cell subset concentrations remained within normal ranges. The concentration of leucocytes in the UT and TR groups remained within the normal range throughout the year (Coulter 1984) and there were no significant differences between UT/TR groups at any blood sampling point during the study. The results suggest that training had no effect on leucocyte concentration, consistent with the results of others, who found that leucocyte concentration at rest did not differ significantly between endurance athletes and sedentary individuals (Mackinnon 1992; Nieman et al., 1995a,b; Pedersen 1997; Pedersen and Hoffman-Goetz 2000). The lack of significant difference between UT/TR groups may also suggest that the TR group did not undergo training-induced plasma volume expansion. Although blood and plasma volume were not measured in the present study, decreased cell concentrations in endurance athletes have often been attributed to expanded plasma volume (Kargotich et al., 1998; O'Toole et al., 1999; Shaskey and Green 2000; Heinicke et al., 2001; Schmidt et al., 2002). However, the present results showed no significant differences between UT/TR groups in leucocyte concentration at any monthly sampling point.

Conversely, significant month to month increases in leucocyte concentration were found in the UT and TR groups (UT May to June, TR March to April). The month to month changes in leucocyte concentration may be statistically significant but not clinically significant, since the leucocyte concentration was in the normal range during the study. Our results were not consistent with those of Maes et al. (1994), who found a significant decrease in leucocyte counts between May and August in the northern hemisphere. The results of Maes et al. (1994) included young males and females, and showed more variation in leucocyte count than the present study. The month to month changes in the present study may indicate variations in leucocyte subsets for both groups, possibly modulated by seasonal changes in cortisol concentration, prolactin concentration (Mann et al., 2000), melatonin concentration and metabolic factors (Brock 1987; Boctor et al., 1989; Maes et al., 1994).

There were no significant differences in erythrocyte concentration between UT and TR groups at any blood sampling point during the study. The present results agree with Heinicke et al. (2001) and Schumacher et al., (2002b), who also found no significant differences between the erythrocyte concentration of young male endurance athletes and sedentary subjects. However, others have reported an increased erythrocyte mass in highly trained endurance athletes, suggesting training-dependent erythrocyte formation due to erythropoietin (EPO) production, possibly from reduced arterial oxygen tension ( $\text{PaO}_2$ ) during training (Smith et al., 1999; Green et al., 1999; Schmidt et al., 2002; Spodaryk 2002). Other researchers have suggested an increase in erythrocyte mass in endurance athletes occurs through mechanical and osmotic damage to erythrocytes (i.e. heel strike haemolysis, lower concentrations of catecholamines and  $\text{PGE}_2$ ), leading to an increase in erythrocyte removal from circulation and the addition of younger erythrocytes (Smith 1995; Brenner et al., 1998; Smith et al., 1999; Shaskey and Green 2000; Spodaryk 2002). It is possible that a combination of mechanical and osmotic damage, plus loss of erythrocytes through gastrointestinal micro-bleeding (Rudzki et al., 1995; Telford et al., 2002), might be responsible for the similarity in erythrocyte concentration between TR and UT groups in Experiment 2, when the TR group was expected to have a higher erythrocyte concentration.

Blood and plasma volume were not measured in the present study, but a review of previous research provides evidence of training-induced plasma volume expansion of up to 20% with a smaller increase in erythrocyte mass, resulting in haemodilution (relatively lower erythrocyte

concentration, haematocrit and haemoglobin concentration) or “sports anaemia” (Green et al., 1999; Smith et al., 1999; Shaskey and Green 2000). However, Convertino et al. (1980) and Convertino (1991) failed to detect changes in blood and plasma volume in young athletes, suggesting that continuous moderate intensity training ( $<75\% \dot{V}O_{2\text{ peak}}$ ) did not maintain plasma volume expansion. Thus, the present results (i.e. lack of between-group difference in erythrocyte concentration) could be explained in two ways. The first suggestion is that there was no expanded plasma volume in the TR group, consistent with the findings of Convertino et al. (1991), Hoffman (1996), Morris (1998), Heinicke et al. (2001) and Schumacher et al. (2002b). The second suggestion is that if the TR group had undergone plasma volume expansion, with cell counts adjusted for plasma volume expansion, the TR group would have a higher concentration of erythrocytes than the UT group (Smith et al., 1999; Schmidt et al., 2002; Spodaryk 2002). Convertino (1991), Morris (1998) and Shaskey and Green (2000) suggested increased training intensity and duration were important stimuli for the expansion of blood volume, with consecutive days of training and a training intensity above 75% of  $\dot{V}O_{2\text{ peak}}$  producing greater hypervolemia than sessions conducted on alternative days, or at a lower intensity of 60% of  $\dot{V}O_{2\text{ peak}}$  (Convertino 1991; Morris 1998). It is possible that the continuous training protocol of two predominantly moderate intensity sessions per day, often undertaken for many years by the subjects of Experiment 2, had not increased plasma volume.

There were significant month to month changes in erythrocyte concentration for UT and TR groups, with both groups showing a significant increase between August-September B, and significant decreases between July-August (UT), December-January (TR), and May-June (TR). The results are consistent with those of Maes et al. (1995), suggesting that cellular regulation at a gene level, climatic factors, altered sympathetic and adrenal activity, and seasonal variation in the proliferation/maturation of erythrocyte precursors might affect erythrocyte concentration. The suggestions of Maes et al. (1995) do not explain the similarity of monthly variations in erythrocyte concentrations in both hemispheres. It could be suggested that many of our physiological processes have not adapted to the seasonal reversal of southern hemisphere temperatures after 200 years of settlement.

The haemoglobin concentration of the TR group was significantly lower than the UT during September A, October A, November, January, July and September B, despite the fact that erythrocyte concentration and haematocrit were similar for both groups. The consistently lower haemoglobin concentration of the TR group raises questions about potential iron deficiency rather than “sports anaemia”, which primarily reflects decreased haemoglobin through expanded plasma and blood volume (Deakin 1995; Kargotich et al., 1998). The lower haemoglobin concentration in the TR group is consistent with values found by others (Balaban et al., 1995; Rudzki et al., 1995; Zhu and Haas 1998; Smith et al., 1999; Heinicke et al., 2001).

Lower than normal haemoglobin and iron status in endurance athletes has been attributed to heel strike haemolysis (Smith 1995; Smith et al., 1999; Telford et al., 2002), ischaemic-induced gastro-intestinal micro-bleedings (Rudzki et al., 1995; Nielsen and Nachtigall 1998), inadequate iron intake (Nielsen and Nachtigall 1998; Shaskey and Green 2000), muscle damage (Buchman et al., 1998) and sweating (Rudzki et al., 1995; Hoffman 1996). The triathletes in the present study ran between 60 to 90 kilometres and cycled between 340 to 400 kilometres per week during the year. It is possible that heel strike haemolysis and gastro-intestinal bleeding may have contributed to a low haemoglobin concentration in many of the subjects. Decreased haemoglobin and ferritin can cause fatigue during training and racing, loss of oxygen-carrying capacity and aerobic performance, and immuno-suppression (Deakin 1995; Nielsen and Nachtigall 1998; Zhu and Haas 1998; Friedmann et al., 2001; Spodaryk 2002). Although none of the TR subjects reported illness during those months where haemoglobin concentrations decreased, the mean haemoglobin values during October A ( $14.4 \text{ g.dL}^{-1}$ ), November ( $14.6 \text{ g.dL}^{-1}$ ), January ( $14.7 \text{ g.dL}^{-1}$ ), February ( $14.7 \text{ g.dL}^{-1}$ ) and August ( $14.7 \text{ g.dL}^{-1}$ ) were below the optimal haemoglobin concentration for an endurance athlete of  $16 \text{ g.dL}^{-1}$  suggested by Worwood (1995) and Rudzki et al. (1995).

There were significant month to month changes in haemoglobin concentration for the TR group only, with increases between October A and B, November-December, and decreases between December-January. The changes in haemoglobin concentration could be related to changes in training intensity that are part of the “periodisation” of Ironman training. However, the monthly pattern of change for both groups is also consistent with the results of other researchers in the northern hemisphere (MacDonald et al., 1987; Neuhaus et al., 1999; Maes et al., 1995; Garde et al., 2000), lending support to the theory of an underlying genetic regulation of haematological

variables and proliferative changes (Maes et al., 1994, 1995; Geraghty 2002; Feitosa et al., 2002a; An et al., 2003). Others have suggested that seasonal changes in hypothalamic function, melatonin and glucocorticoid production may affect haemoglobin concentration (Brock 1987; Macdonald et al., 1987; Garde et al., 2000). It is interesting to note that the UT group also had month to month changes in haemoglobin concentration similar to the TR group, with the largest decrease in haemoglobin concentration occurring in February. The February blood test occurred after three of the UT subjects had recovered from viral illnesses. Indeed, the subjects who were recovering from viral illness had below normal haemoglobin concentration in February. It is well documented that iron is sequestered by transferrin in various immune cells, mostly macrophages, during times of viral and bacterial infection as a means of preventing the growth of infected cells and spread of the illness (Kemp 1993; Brock 1995; Baynes 1996; Huether and McCance 1996; Pascale et al., 1998).

The TR group had significantly lower MCHC for nine months of the year. Low MCHC is an indicator of hypochromic erythrocytes and anaemia, and when coupled with low haemoglobin concentration suggest genuine iron deficiency (Rapaport 1987; Green et al., 1999; Malczewska et al., 2001). The MCHC showed monthly changes similar to haemoglobin concentration for both TR and UT groups, again consistent with Maes et al. (1995, 1997a). The TR group reported no symptoms of viral infections during October and February when MCHC was significantly low. However, many of the TR subjects reported feeling "fatigued and flat" during October, November, January, February and April, and the training and racing performance of TR subjects with low haemoglobin may have been impaired (Zhu and Haas 1998). Several of the UT subjects were recovering from viral infections in February, and significantly low MCHC during the month of February may be due to post-viral iron sequestration.

It is possible that the decreases in haemoglobin concentration, MCHC and available iron in the blood are implicated in the minor proliferative fluctuations of the leucocytes, indicating a regulated relationship between immune cells, erythrocytes, haemoglobin concentration, MCHC and iron storage proteins (Kemp 1993; Maes et al., 1995, 1997a). An interesting observation in Experiment 2 was that a decrease in mean haemoglobin concentration preceded a fall in mean leucocyte concentration by approximately a month. Since iron is important in lymphocyte proliferation, it is possible that small fluctuations in haemoglobin concentration can influence the proliferation of

lymphocytes and total leucocyte numbers via the regulation of the labile intracellular iron pool, transferrin and TfR (Maes et al., 1997a). The question of using changes in haemoglobin concentration as a diagnostic test to identify the increased risk of infection needs to be considered further.

The present study found no significant differences between UT and TR groups in haematocrit. This finding is consistent with Convertino (1991), Balaban et al. (1995) and Schumacher et al. (2002b). There were significant month to month changes in haematocrit during the year for both groups, consistent with Maes et al. (1995) and similar to the changes in erythrocyte concentration, suggesting regulation through hormone and glucocorticoid production, and possibly at gene level (Brock 1987; Feitosa et al., 2002a).

Platelet concentrations for UT and TR groups were within the normal range (130-400 platelets x  $10^9.L^{-1}$ ) for the duration of the study (Coulter 1984; Hoffbrand et al., 2001). The TR group had a significantly lower platelet concentration than the UT group for nine months of the year. The lower platelet concentration in the TR group may be a response to training. Wang et al. (1995) found that 8 weeks of endurance training diminished platelet activity and adhesiveness in young men. Kestin et al. (1993) and Cerneca et al. (1999) also found that inhibitors of platelet activation were significantly higher in endurance athletes compared to resistance trained and sedentary individuals. This may be one way that endurance training lowers the incidence of cardiovascular events in endurance-trained individuals.

The present study found significant month to month changes in platelet concentration within UT and TR groups, consistent with the results of others and possibly due to monthly changes in plasma fibrinogen (Huisveld et al., 1991; Bol et al., 1993; Maes et al., 1995), hormone and glucocorticoid release (Maes et al., 1995) and genetic regulation (Huisveld et al., 1991; Maes et al., 1995). However, the platelet results of Experiments 1 and 2 were not consistent. The difference in age group may be responsible for the disparity between results in Experiments 1 and 2, possibly indicating more variation in platelet production and adherence with older individuals (Hoffbrand et al., 2001). The month to month changes in platelet concentration in Experiment 2 were similar to the monthly changes of erythrocytes, haemoglobin and haematocrit. The number of circulating erythrocytes is known to affect platelet activation, increasing platelet adherence by

facilitating migration of platelets towards vascular surfaces and producing ADP, enabling platelets to adhere to exposed collagen (Hue thers and McCance 1996; Boron and Boulpaep 2003). Consequently, variations in platelet concentration could be linked to variations in erythrocyte concentration. Others have also suggested that seasonal variation in platelets, plasma fibrinogen and erythrocyte concentration might result in changes in blood clotting and blood flow, thereby altering the susceptibility of subjects to thrombo-embolic disorders (Huisveld et al., 1991; Bol et al., 1993; Maes et al., 1995).

#### 5.2.5. Interleukin-2 Receptor (CD25) Expression

The present study found no significant differences between groups in the percentage of CD4<sup>+</sup> lymphocytes expressing CD25, suggesting that chronic endurance training had no effect on the percentage of lymphocytes expressing CD25. These results are in agreement with those found by Moyna et al. (1996a,b) and Mitchell et al. (1996). Few studies have documented the effects of long-term endurance-type training on lymphocyte proliferation. Some researchers have found that trained young males show significantly higher percentages of cells expressing CD25 (Kono et al., 1988; MacNeill et al., 1991; Shinkai et al., 1995), whilst others have merely documented lower circulating numbers of lymphocytes in endurance athletes (Gleeson et al., 1999).

The present study found there were significant month to month changes in the percentage of lymphocytes expressing CD25 for both UT/TR groups. The changes indicate decreases in the percentage of CD4<sup>+</sup> lymphocytes expressing CD25 during late spring and early autumn, and increases during early summer and winter, consistent with the results of Canon et al. (1986), Brock (1987) and Maes et al. (1994) and Mann et al. (2000) who noted low periods of T cell blastogenesis and the percentage of T cells positive for CD25 in April and October (northern hemisphere), and higher proliferative responses in early September and January. Early studies by Brock (1987) noted that the blastogenic and proliferative responses of lymphocytes in mice showed similar seasonal fluctuations over a period of two years. The mechanisms underlying monthly/seasonal changes may be related to seasonal demands for iron, and to seasonal variations in glucocorticoids, melatonin and hypothalamic activity (Brock 1987; Kemp 1993; Maes et al., 1994; Mann et al., 2000).

The results of Experiment 1 also confirm the month to month variations in percentage of cells expressing CD25, since the older individuals showed increases of CD25-positive lymphocytes during September and November-December, and decreases during April and October. It is interesting that the percentage of lymphocytes expressing CD25 in the 65 – 75 year age group was generally higher than that of the younger men, suggesting an increased recruitment of “memory” lymphocytes in response to PHA (Gabriel et al., 1994; Gueldner et al., 1997). Previous studies have suggested that older individuals had decreased numbers of lymphocytes responsive to PHA compared to younger individuals (Shinkai et al., 1995). The present study did find a difference between age groups when examining the receptor density. The CD25 density in older subjects did not increase with training, but did increase in young, trained individuals.

The results of Experiment 2 show considerable variation of up to three-fold in the percentage of lymphocytes expressing CD25 within each group, but less variation with receptor density. The magnitude of monthly changes in the percentage of lymphocytes expressing CD25 was similar for all subjects, but some subjects consistently demonstrated a lower percentage of cells expressing CD25 throughout the year compared to others in the group (e.g. variations between 1% and 19% CD4<sup>+</sup>/CD25<sup>+</sup>). Conversely, there were other subjects who consistently demonstrated relatively higher percentages expressing CD25 (e.g. variations between 15% to 60% CD4<sup>+</sup>/CD25<sup>+</sup>). The concept of high/low individual physiological “set-points” has been mentioned by Afoke et al. (1993), Maes et al. (1994,1997a,b), Paglieroni and Holland (1994), Hagberg et al. (2002) and Geraghty (2002). They suggested that there may be a genetic component involved with the regulation of many biological variables such as haematological indices, CD25 and CD71, and the results of Experiment 2 would lend some measure of support to this theory. Several of the subjects in both the TR and UT groups had consistently low CD25 expression compared to other subjects, which suggests that those with the low CD25 expression are more at risk of contracting illnesses. Indeed, these same subjects in the UT group reported URTI through the year. The genetic basis of variation in receptor expression is worth further research, since individuals who express lower numbers of CD25 throughout the year may be more at risk of contracting illnesses, and have less capacity to activate other immune cells.

There were significant differences between UT and TR groups in CD25<sup>+</sup> density, with the TR group showing a significantly higher CD25<sup>+</sup> density for three months of the year (October B,

January and June). A higher density of CD25<sup>+</sup> indicates that the lymphocytes have greater ability to bind IL-2, proliferate and activate NK and B cells further downstream. In both UT and TR groups, the changes in receptor density followed a similar month to month pattern to the percentage of cells expressing CD25 (Brock 1987; Maes et al., 1994). There was a decrease in receptor density in late spring, an increase in early summer, a decrease in autumn and an increase over winter. Decreases in CD25 density (TR group) cannot be attributed to increases in training intensity (September-October) or the Forster Ironman triathlon in April, since the UT group also showed similar decreases in density. Of more interest is the significantly higher CD25 density of the TR group in October B, January and July, suggesting that lymphocytes in the TR group were more responsive in these months and better able to activate other lymphocytes. The present results suggest that the TR group had a greater proliferative response than the UT for at least three months of the year, and lend support to Bury et al. (1996), Ferrandez et al. (1996) and Nieman et al. (2000), who found that endurance TR athletes had a greater CD4<sup>+</sup> proliferative response than UT. The results of the present study challenge previous hypotheses that endurance athletes undertaking large volumes of training have lymphocytes less responsive to mitogenic stimulation (Nieman et al., 1995c). Indeed, a greater density of CD25 implies lymphocytes which are better able to respond to mitogens or antigens, and proliferate.

Other studies have also suggested that chronic endurance exercise is not immuno-suppressive (Petrides et al., 1997; Urhausen et al., 1998; Hoffman-Goertz et al., 1999; Pyne et al., 2000; Gleeson et al., 2004a). Some have suggested that after prolonged exercise, there is a reduction in glucocorticoid sensitivity by mature lymphocytes due to a down-regulation of low affinity Type II glucocorticoid receptors in the cytosol of lymphocytes (Cohen and Duke 1984; Petrides et al., 1997). Perhaps this down-regulation of cortisol receptors acts as an adaptation to continual endurance training, to avoid the immuno-suppressive effects of continual high plasma concentrations of cortisol (Urhausen et al., 1998).

The results of Experiment 2 provide some guidance to athletes undertaking chronic endurance training, and continue to challenge suggestions of exercise-induced immuno-suppression with chronic training. The reasons for the apparent lack of CD4<sup>+</sup> cell suppression may well involve the interaction of a number of complex hormonal adaptations to chronic training (Feitosa et al., 2002a). The production of ACTH and the immuno-suppressive glucocorticoid, cortisol, might be

reduced after chronic training, as well as a down-regulation of the glucocorticoid receptors in a manner similar to the down-regulation of  $\beta$ -adrenergic receptors associated with chronic endurance training (Schaller et al., 1999; Kohm and Sanders 2000). Other researchers have noted that endurance athletes show symptoms of reduced function of the hypothalamic-pituitary-thyroid (HPT) and hypothalamic-pituitary-adrenal (HPA) axes (lower plasma cortisol and ACTH) after years of chronic training (Barron et al., 1985; Loucks et al., 1988; Duclos et al., 1998; Urhausen et al., 1998; Ronsen et al., 2001; Ronsen et al., 2002). Urhausen et al. (1998) and Fry et al. (1998) found there was a HPA dysregulation and reduced ACTH and cortisol production during chronic training, expressed as an impaired response of pituitary hormones to additional high intensity, endurance training and resistance training bouts. These results suggested a lack of adrenal cortical response to exercise, which implies a reduction in possible immuno-suppressive effects from cortisol.

The hypothesis of a dysfunctional HPA axis response goes some way towards explaining why less cortisol may be produced after years of high volume endurance training. Eight of the fourteen triathletes who volunteered for the present study had competed in Ironman distance triathlons for over five consecutive years, two had competed in such events for ten consecutive years, with the remainder of subjects ( $n = 4$ ) training for, and racing at, this distance for more than two years. Even though the subjects had complained of "fatigue and feeling flat" during the year, they reported significantly fewer viral infections than their sedentary counterparts. Since the density of  $CD4^+/CD25^+$  in the TR group was actually greater than the UT group, it is not unreasonable to suggest that they have adapted to high volumes of endurance training by producing less ACTH and cortisol. Due to financial constraints, the present study did not measure ACTH and plasma cortisol concentration, but in hindsight these measurements would have been useful. The present results are consistent with those of Pyne et al. (2000) and Gleeson et al. (2004a). Pyne et al. (2000) investigated the incidence of URTI and monitored salivary immunoglobulins in elite swimmers. They found no significant differences in salivary IgA concentration in the swimmers compared to healthy UT subjects, when both groups were monitored from April to September (southern hemisphere). The UT group actually showed significantly lower IgA concentration than the swimmers, and reported more cases of URTI. Pyne et al. (2000) did not find evidence of a significant association between URTI and competitive performance, with swimmers who reported viral symptoms performing just as well as their healthy team members. A similar study by Gleeson

et al. (2004a) assessed the impact of five months of training on T lymphocyte function in elite swimmers. Despite transient T lymphocyte suppression immediately after a training session, due to temporary T cell subset redistribution, the T lymphocyte responses were not suppressed by extended periods of elite-level training during the five months of the study.

While weekly Ironman distance training involves large volumes for each component of the event (12-16 km swim, 340-400 km cycle, 60-90 km run), the predominant training intensity remains mostly moderate (i.e. between 60% and 85% of maximal heart rate) compared to higher intensity training for shorter distance races. The cortisol release-response to exercise intensity has been well documented (Cohen and Duke 1984; Tabata et al., 1990; Petrides et al., 1997; Pedersen 1997; Inder et al., 1998; Urhausen et al., 1998; Hoffman-Goertz et al., 1999), with higher intensity exercise significantly increasing blood concentrations of cortisol (Tabata et al., 1990; Hoffman-Goertz et al., 1999). Thus high volume but more moderate intensity Ironman training may result in a lower plasma concentration of cortisol, thereby decreasing the risk of leucopenia and infection.

#### **5.2.6. Transferrin Receptor (CD71) Expression**

The UT group showed significantly higher percentages of CD4<sup>+</sup> lymphocytes expressing CD71 in June and August, compared to the TR group, with no significant differences between groups for the remainder of the year. These results do not agree with those of Heinicke et al. (2001), Malczewska et al. (2001) and Spodaryk (2002), who found that CD71 expression was significantly higher in endurance trained cyclists, runners, swimmers and triathletes compared to an UT group. They suggested that iron needs of the athletes were greater than those of sedentary individuals. Despite the significantly lower haemoglobin concentration and MCHC found in the TR group for three and nine months of the year, the lack of between-group difference in CD71 expression does not suggest a training-induced iron deficiency.

One possibility was that the TR group maintained intracellular iron concentrations (therefore low CD71 expression) despite low haemoglobin concentration, enabling unimpaired lymphocyte proliferation, as proposed by Kemp (1993), Erikson et al. (1997) and Nielsen and Nachtigall (1998). Research has suggested there may be a critical level of iron depletion in ferritin stores, iron carrying proteins and [Fe<sup>3+</sup>]<sub>i</sub> before lymphocyte proliferation is affected (Kemp 1993; Erikson et al., 1997). Furthermore, moderate iron deficiency has not been implicated in clinical immune

suppression (Kemp 1993; Hoffman 1996; Mast et al., 1998; Beard 2000; Boron and Boulpaep 2003).

The results of the present study suggest an inverse relationship between CD25 and CD71 expression, consistent with the results of other researchers (Neckers and Cossman 1983; Skikne et al., 1990; Kemp 1993; Maes et al., 1995; Maes et al., 1997a; Gimferrer et al., 1997; Ahluwalia et al., 2001), and with the results of the male groups in Experiment 1. The inverse relationship between CD25 and CD71 expression is reflected in month to month changes in the percentage of lymphocytes expressing CD25 and CD71. Pelosi-Testa et al. (1988), Sainte-Marie et al. (1997) and Ahluwalia et al. (2001) have documented the close relationship between CD71, CD25 and lymphocyte activation, where CD25 expression is high provided intracellular iron is freely available. Low intracellular iron results in reduced CD25 expression and increased CD71 expression (Neckers and Cossman 1983; Gimferrer et al., 1997; Sainte-Marie et al., 1997). The month to month variations in CD71 expression in the present study were consistent with those of Maes et al. (1997a), who measured soluble CD71. The similarity between the findings of Experiment 2 and Maes et al. (1997a) suggests that monthly variations in CD71 and CD25 may be inherent physiological and immunological adaptations within Caucasians.

The TR group showed significantly lower CD71 density during only two months of the year, suggesting there was no training effect on CD71 density. The results are not consistent with those of Heinicke et al. (2001) and Spodaryk (2002), and suggest that intracellular iron reserves were adequately maintained within lymphocytes, despite low haemoglobin concentration and MCHC in the TR group (Kemp 1993; Erikson et al., 1997). Both UT and TR groups followed a similar pattern of month to month change in CD71 density. CD71 density did show an inverse relationship with CD25 density during the year. The months where CD71 density increased for both groups (September A, April, September B) coincided with periods of decreased CD25 density, suggesting that spring and autumn may be periods of reduced immuno-competency and greater iron needs (Brock 1987; Maes et al., 1994; Maes et al., 1997a; Mann et al., 2000).

The UT group showed significantly higher percentages of "double positive" cells during October B and December, while the TR group showed significantly higher percentages during January. The results suggest no training effect on the percentage of CD4<sup>+</sup>/CD25<sup>+</sup>/CD71<sup>+</sup> lymphocytes. The

between-group differences may be related to changes in iron requirements and lymphocyte proliferative responses during the year (Brock 1987; Kemp 1993; Maes et al., 1997a). The month to month changes in the “double positive” cells are similar to monthly changes in the percentage of cells expressing CD71. The density of CD25 receptors on the “double positive” cells did not have an inverse relationship with the density of CD71 on the same cells. CD4<sup>+</sup>/CD25<sup>+</sup>/CD71<sup>+</sup> showed high densities in September A in both UT and TR groups, with CD4<sup>+</sup>/CD25<sup>+</sup> density also high in September for both groups, consistent with other researchers who found September to be a period of increased lymphocyte blastogenesis and proliferation (Brock 1987; Maes et al., 1994, 1997a,b).

#### 5.2.7. Intracellular Calcium

The results of Experiment 2 suggested that chronic endurance training had little effect on basal [Ca<sup>2+</sup>]<sub>i</sub> in CD4<sup>+</sup> lymphocytes. There were significant differences between TR and UT groups in October B and November only. As with the older subjects in Experiment 1, there was a significant increase in [Ca<sup>2+</sup>]<sub>i</sub> for both UT and TR groups during December-January, but with the younger age group this peak in [Ca<sup>2+</sup>]<sub>i</sub> was partially sustained during February-March. The between-group similarity lends some support to Hoffman-Goetz et al. (1999), Pedersen and Hoffman-Goetz (2000) and Mooren et al. (2001b), in that while an exercise bout may transiently increase [Ca<sup>2+</sup>]<sub>i</sub>, the concentration and lymphocyte proliferative response returns to normal, or is even enhanced, within 24 hours. Thus, chronic training may not result in disturbed Ca<sup>2+</sup> homeostasis or apoptosis in resting lymphocytes, as suggested by Phaneuf and Leeuwenburgh (2001).

There were significant month to month changes in [Ca<sup>2+</sup>]<sub>i</sub> for the UT and TR groups, during the year. While the present results partly agree with those of Letellier and Desjarlais (1982), who found increased serum Ca<sup>2+</sup> concentrations during April-May and September-October for four years, we found that [Ca<sup>2+</sup>]<sub>i</sub> is significantly increased in young individuals during the hottest months of the year, irrespective of endurance training. A sustained increase in [Ca<sup>2+</sup>]<sub>i</sub> in activated lymphocytes is known to drive gene transcription for IL-2 and CD25, through the translocation of NFAT and an increase in CRAC channels (Fomina et al., 2000; Glotzer et al., 2000). It is possible that the month to month changes in [Ca<sup>2+</sup>]<sub>i</sub> are related to seasonal changes in lymphocyte proliferation (Brock 1987), transcription for CD25 (Brock 1987; Maes et al., 1994), HSP and SAPK's (Moseley 2000; Naito et al., 2001; Kiang et al., 1998) or lymphocyte apoptosis (Phaneuf and Leeuwenburgh 2001; Kiang et al., 2003).

The present study found that  $[Ca^{2+}]_i$  increased during months when CD25 expression increased, suggesting an increase in transcription and proliferation (Thomas and Hanley 1994; Moseley 2000; Wang et al., 2000; Fehrenbach and Northoff 2001). It is also plausible that cellular adaptations to higher ambient temperatures involve an increase in the transcription of HSP and SAPK's (Kiang et al., 1998; Fomina et al., 2000; Hoth et al., 2000). HSP-70 and HSP-90 are known to act as "chaperone" proteins that facilitate the movement of hormone receptor complexes into the nucleus (Moseley 2000; Boron and Boupaep 2003), and the repair and folding of cytosolic proteins. An increase in HSP transcription during warmer months suggests an increase in transcription within the cell. Other researchers found that exercise training increases  $[Ca^{2+}]_i$  and induces HSP synthesis across a range of muscle and tissue types in both young and old age groups. While the present study measured  $[Ca^{2+}]_i$  in lymphocytes, no significant difference was found between UT and TR groups for most of the year, suggesting that endurance training had not increased  $[Ca^{2+}]_i$  or enhanced transcription (Moseley 2000; Naito et al., 2001). However, Kiang et al. (2003) found that heat stress increased nitric oxide production and  $[Ca^{2+}]_i$  in lymphocytes, through an increased phosphorylation of nitric oxide synthase. This process was related to an increased expression of Fas/CD95 on the lymphocyte surface, suggesting an increase in lymphocyte apoptosis with heat stress (Suh 2002; Kiang et al., 2003).

Experiment 2 found that  $[Ca^{2+}]_t$  was positively correlated to  $[Ca^{2+}]_i$  for the TR group only, throughout the year ( $r = 0.65$ ). There were significant differences between UT and TR group  $[Ca^{2+}]_t$  during the warmer months, in contrast to  $[Ca^{2+}]_i$  during these months. As in Experiment 1, the UT group showed a significantly higher  $[Ca^{2+}]_t$  than the TR group during the hottest time of the year (February-March) and this may be due to an greater increase in HSP to maintain homeostasis, and/or increased apoptosis in the UT group (Fomina et al., 2000; Berridge et al., 2003; Kiang et al., 2003). Fehrenbach and Northoff (2001) found that endurance athletes had a down-regulated HSP response to heat stress, and suggested that regular endurance training caused a relative "shut down" of HSP production with an enhanced "front line" response of antioxidants to heat-related reactive oxygen species. A different theory was suggested by Liossis and Tsokos (1997), who found that subjecting T cells to repeated heat stress caused a down-regulation of the TCR/CD3, PIP2 and IP3, and therefore  $[Ca^{2+}]_t$ . That is, less  $[Ca^{2+}]_t$  may be an endurance training adaptation that indicates reduced lymphocyte activation and HSP turnover. It

would be worthwhile to investigate these theories further by examining the nature of gene regulation and HSP production within the immune cells of TR and UT individuals.

#### 5.2.8. Intracellular Iron

The results of Experiment 2 showed significant differences in mean channel log fluorescence between UT and TR groups, during November and September B. The UT group had significantly lower  $[\text{Fe}^{3+}]_i$  in November, but higher  $[\text{Fe}^{3+}]_i$  in September B. The differences may be related to endurance training, or may reflect between-group differences in dietary iron intake through the year (Kemp 1993; Maes et al., 1997a; Gleeson et al., 2004b). The present study found an inverse relationship between the  $[\text{Ca}^{2+}]_i$  and  $[\text{Fe}^{3+}]_i$  during the year, as in Experiment 1, and consistent with the findings of Cheung et al. (1988), Pelosi-Testa et al. (1988) and Sainte-Marie et al. (1997). Sainte-Marie et al. (1997) found that an increase in  $[\text{Ca}^{2+}]_i$  within lymphocytes resulted in the increased recycling of TfR from the lymphocyte cytoplasm to the membrane, lowering  $[\text{Fe}^{3+}]_i$  through the increased binding of intracellular ferritin to its receptor. Thus an elevation of  $[\text{Ca}^{2+}]_i$  during lymphocyte proliferation and increased transcription results in a decrease in  $[\text{Fe}^{3+}]_i$ . The inverse relationship between  $[\text{Ca}^{2+}]_i$  and  $[\text{Fe}^{3+}]_i$  suggests that increased periods of cell proliferation and transcription are indicated by increased  $[\text{Ca}^{2+}]_i$  and decreased  $[\text{Fe}^{3+}]_i$  (Maes et al., 1994; Sainte-Marie et al., 1997; Maes et al., 1997a). In fact, high  $[\text{Fe}^{3+}]_i$  for the TR group coincided with a significant increase in the percentage of lymphocytes expressing CD25, and an increase in CD25 density. The UT group also showed increased  $[\text{Fe}^{3+}]_i$ , percentage of lymphocytes expressing CD25 and CD25 density during the same months.

The present study found that high  $[\text{Fe}^{3+}]_i$  occurred in late October, when both TR and UT groups had higher haemoglobin, erythrocyte, and leucocyte concentrations, coupled with decreasing CD71 density, suggesting a reduced need for intracellular iron (Kemp 1993; Maes et al., 1997a). In November, when  $[\text{Fe}^{3+}]_i$  was lower for both groups, haemoglobin, erythrocyte and leucocyte concentrations decreased, while CD71 expression increased. The depletion of intracellular iron stores may be due to increased intracellular activity such as transcription and receptor expression (Brock 1987; Pelosi-Testa et al., 1988; Picard et al., 1998).

### 5.2.9. Summary of Experiment 2

The numbers of leucocytes and responses to PHA of CD4<sup>+</sup> lymphocytes showed no negative effect from large volumes of endurance training, and the first experimental hypothesis was rejected. The endurance athletes in the present study were able to adapt to large volumes of consistent endurance training and participate in long-course events, without showing signs of immuno-suppression. Experiment 2 found no significant differences between UT/TR groups in leucocyte and erythrocyte concentration, and haematocrit. The TR group showed a significantly lower haemoglobin concentration and MCHC than the UT group for six and nine months of the year respectively, suggesting a genuine iron deficiency in endurance athletes rather than “sports anaemia”. There was no evidence of immuno-suppression in the TR group, in terms of low leucocyte concentration and CD25 expression. The TR group had a significantly higher CD25 density than the UT group during three months of the year, which suggesting an enhanced response to PHA. Chronic endurance training did not cause an increased incidence of URTI in the TR group. The UT group reported a higher incidence of URTI. Therefore the second experimental hypothesis was rejected.

Intracellular and total Ca<sup>2+</sup> concentrations followed a similar seasonal pattern to that documented in Experiment 1. Increases in [Ca<sup>2+</sup>]<sub>i</sub> corresponded to increases in CD25 expression. There was an inverse relationship between [Ca<sup>2+</sup>]<sub>i</sub> and [Fe<sup>3+</sup>]<sub>i</sub>. There was no evidence of decreased [Ca<sup>2+</sup>]<sub>i</sub> with endurance training, and the third experimental hypothesis was rejected. There was also no evidence of increased [Fe<sup>3+</sup>]<sub>i</sub> with endurance training, and the fourth experimental hypothesis was also rejected.

Experiment 2 supported the findings of Experiment 1 in that the same seasonal variations occurred in haematological indices, and with CD25 expression. There were increases in CD4<sup>+</sup>/CD25<sup>+</sup> expression in summer and winter, with decreases during autumn and spring, with an inverse relationship between CD71 expression and CD25 expression. Thus, there appeared to be times of the year when both groups might be more vulnerable to infections. The seasonal changes in haemoglobin and MCHC may contribute to periods of training fatigue in endurance athletes.

## 6.0 Review of Hypotheses

### 6.1 Experiment 1- Males and Females Aged 65 to 75 Years

Experiment 1 investigated the effects of twelve months of moderate aerobic training on the proliferative response of CD4<sup>+</sup> lymphocytes in men and women aged 65 to 75 years. The experimental hypothesis was that:

**Moderate aerobic training for twelve months would increase CD4<sup>+</sup> lymphocyte proliferation in males and females aged 65 to 75 years**

To quantify CD4<sup>+</sup> cell proliferation, the following specific experimental hypotheses (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>) were proposed for the various dependent measures.

H<sub>1</sub>: Moderate aerobic training for twelve months will significantly increase peak oxygen consumption, peak power and peak ventilation and in males and females aged 65 to 75 years.

ACCEPT

H<sub>2</sub>: Moderate aerobic training for twelve months will significantly increase the proliferative response of CD4<sup>+</sup> lymphocytes to Phytohemagglutinin in males and females aged 65 to 75 years, as assessed by the expression of CD25 and CD71 receptors.

REJECT

H<sub>3</sub>: Moderate aerobic training for twelve months will significantly increase the intracellular concentration of Ca<sup>2+</sup> in CD4<sup>+</sup> lymphocytes in males and females aged 65 to 75 years.

REJECT

H<sub>4</sub>: Moderate aerobic training for twelve months will significantly increase the intracellular concentration of Fe<sup>3+</sup> in CD4<sup>+</sup> lymphocytes in males and females aged 65 to 75 years.

REJECT

## 6.2 Experiment 2 – Males Aged 23 to 36 Years

Experiment 2 investigated the effects of long-term endurance training on the proliferative response of CD4<sup>+</sup> lymphocytes in males aged 23 to 36 years. The experimental hypothesis was that:

**Endurance training for twelve months will decrease CD4<sup>+</sup> lymphocyte proliferation in males aged 23 to 36 years**

To quantify CD4<sup>+</sup> cell proliferation the following specific experimental hypotheses (H<sub>1</sub>, H<sub>2</sub>, H<sub>3</sub>, H<sub>4</sub>) were proposed for the various dependent measures.

H<sub>1</sub>: Endurance training for twelve months will significantly decrease the concentration of leucocytes in males aged 23 to 36 years.

REJECT

H<sub>2</sub>: Endurance training for twelve months will significantly decrease the proliferative response of CD4<sup>+</sup> lymphocytes to Phytohemagglutinin in males aged 23 to 36 years, as assessed by the expression of CD25 and CD71 receptors.

REJECT

H<sub>3</sub>: Endurance training for twelve months will significantly decrease the intracellular concentration of Ca<sup>2+</sup> in CD4<sup>+</sup> lymphocytes in males aged 23 to 36 years.

REJECT

H<sub>4</sub>: Endurance training for twelve months will significantly decrease the intracellular concentration of Fe<sup>3+</sup> in CD4<sup>+</sup> lymphocytes in males aged 23 to 36 years.

REJECT

## 7.0 Conclusions

The results of Experiment 1 showed that twelve months of moderate intensity aerobic training significantly increased the aerobic capacity of older males and females, consistent with the results of many other researchers. Moderate intensity exercise is being increasingly prescribed to halt the effects of ageing, and the decline in functional capacity. Training three times per week for twelve months at an intensity of 60% of  $\dot{V}O_{2\text{ peak}}$  stimulated a physiological response resulting in an improved functional capacity. It is possible that increasing the number of training sessions per week, the duration or intensity of sessions may have increased the aerobic capacity further, but the risk of causing injury to the subjects or decreasing their immune response through higher intensity training precluded this option. However, increasing the number or duration of moderate intensity training sessions after 12 months would certainly increase the aerobic capacity of older subjects.

CD25 expression increased in older males with training. It is possible that older females needed more training sessions per week to show a positive response, or perhaps training sessions at a higher intensity, and this theory is worthy of further investigation. Furthermore, significant monthly changes in CD25 expression were found during spring and autumn, and increases in summer and early winter, indicating that autumn, late winter and late spring may be periods where individuals were more at risk of succumbing to infections. The monthly changes in  $Ca^{2+}$  were consistent with this finding, since  $Ca^{2+}$  increased during times when the percentage of lymphocytes positive for CD25 increased, indicating increased transcriptional processes and cell proliferation. There were significant increases in  $Ca^{2+}$  during summer which were not related to training, but could be ascribed to increased transcription of HSP or possibly apoptosis. These findings suggest a regulatory mechanism for maintaining cellular homeostasis during hotter months.

The results of Experiment 2 showed no evidence that chronic endurance training caused a decreased immune response in the subjects, or increased cases of URTI. The TR group had a significantly higher density of CD25 receptors than the UT group during three months of the year, suggesting that endurance training enhanced the response of  $CD4^+$  lymphocytes. Experiments 1 and 2 found similar seasonal variations occurring in haematological variables and CD25 expression. The results suggest a genuine iron deficiency in endurance athletes, rather than

“sports anaemia”. The seasonal changes in haemoglobin may contribute to periods of training fatigue in endurance athletes, and extra dietary iron intake or iron supplements may be beneficial at these times. Intracellular and total  $\text{Ca}^{2+}$  concentrations showed similar seasonal increases to Experiment 1, corresponding to increases in the percentage of lymphocytes positive for CD25 and in CD25 density, and ambient temperatures.

There appeared to be times of the year when both UT and TR groups might be more vulnerable to infections (spring and autumn). The numbers of leucocytes and responsiveness of  $\text{CD4}^+$  lymphocytes showed no negative effect from large volumes of endurance training, suggesting that endurance athletes are able to adapt to large volumes of training and participate in long-course events year after year, without showing signs of immuno-suppression. Possible explanations for this include a down-regulation of the HPA axis and reduction in cortisol production, and an increase in some immune cell receptors, and possibly cytokines. Endurance athletes usually follow a periodised training programme with “off season” recovery training of low/moderate intensity and low volume. However, the present results suggest that Ironman triathletes have adapted to moderate to high volumes of training at a low/moderate intensity during the “off season” without an increase in URTI. The normal training practice of “aerobic base” building during the “off season” may include higher volumes of training than was previously thought acceptable. Further recommendations for endurance athletes include a balance of high intensity, tempo and moderate intensity training sessions during the “pre-competition” and “competition” phases to increase performance; adequate cross training; adequate rest and recovery sessions; regular full blood counts and iron status tests. Further investigation of the relationship between increased  $[\text{Ca}^{2+}]_i$ , HSP turnover, apoptosis and cellular homeostasis in the heat could provide valuable information to athletes, as many endurance and team sporting activities are conducted during the warmer months of the year.

The present study suggests that long-term moderate aerobic training has health benefits for older individuals, improving functional capacity in males and females, and improving the  $\text{CD4}^+$  proliferative response in older males. Furthermore, long-term endurance training in young triathletes does not appear to be immuno-suppressive, but rather maintains the  $\text{CD4}^+$  proliferative response and may result in adaptations worthy of further research.

## 8.0 Appendices

### 8.1 Appendix 1 – Subject Information Sheet. Experiment 1 Males and Females Aged 65 to 75 Years. Research Project – THE EFFECT OF LONG-TERM AEROBIC TRAINING ON T HELPER LYMPHOCYTE PROLIFERATION IN MEN AND WOMEN AGED 65-75 YEARS.

Dear Project Participant,

Thank you for responding to our advertisement. For 12 months we will be training and monitoring a group of men and women aged 65 to 75 years, in an effort to improve their cardiovascular fitness and their immune response. Previous research has indicated that long-term, regular moderate exercise not only reduces the risk of cancers, cardiovascular disease, osteoporosis and high blood pressure, but may also increase the activity and number of some specific immune cells called lymphocytes, which fight specific bacteria and viruses. However, there are only a few studies that have trained older people for over 6 months. The emphasis with this project is to look at the long-term prospects and health of both men and women aged 65-75 years, in an effort to reduce chronic diseases of old age.

This project involves 12 months of moderate training in our laboratory at Griffith University. The sessions are 3 times per week (Monday, Wednesday and Friday), for about 50 minutes duration (only 40 minutes of exercise). The training is done on exercise bikes coupled to an E.C.G. to monitor heart rate. Blood pressures and weight are monitored daily, and the sessions are supervised by myself (a physiologist) or a trained 3<sup>rd</sup> year student. Once a month, all subjects come in on a Saturday morning to donate 14mls of blood (2 tubes), which is processed to show a full blood count, iron status and T cell numbers and activity. This means that your health is monitored for a full year, as well as you undertaking a supervised exercise program with other people. Previous research participants have all shown reduced resting heart rates, lowered blood pressure and many have lost considerable weight! We emphasise that as a volunteer you may drop out if you feel like it, but we would like to have people that are keen to train for most of the year.

**We also need a similar number of people who are not very active and don't want to train, but would like to come in for the blood test once per month to monitor their health.** This comparison group (control subjects) are equally important, because we need to compare the trained and control subjects to ascertain if the training is having the desired effect. I have enclosed a basic medical questionnaire for you to read and fill out. If you could post this back to me OR phone me (if interested in participating), I will contact you to arrange a meeting at the university. I can then show you all the equipment and conduct a basic medical screening (e.g. height, weight, blood pressure and resting 12 lead E.C.G.) We have had great success training previous groups, and I look forward to hearing from you,

Thank you,

**Sue Broadbent**, School of Physiotherapy and Exercise Science, Griffith University, Parklands Drive, Parklands, 4215 Ph: 55315892, 0413053351 or (Uni) 55948281

**Subject Information Sheet – Experiment 2 Males Aged 23 to 36 Years.****Research Project – THE EFFECT OF LONG-TERM ENDURANCE TRAINING ON T HELPER LYMPHOCYTE PROLIFERATION IN MEN AGED 22-36 YEARS.**

Dear Project Participant,

Thank you for responding to our advertisement. For 12 months we will be monitoring a group of male long course triathletes to assess the effects of chronic training on their immune response. Some previous research has indicated that long-term, large volumes of training may reduce the activity and number of some specific immune cells called lymphocytes, which fight specific bacteria and viruses. However other research has not proved this. The emphasis with this project is to look at the long-term effects of your Ironman training, and see how it impacts on your blood count, iron status, T helper cells and intracellular calcium and iron levels. We also need to assess a group of untrained men in your age group, who are doing no regular exercise or resistance training, and compare the results.

This project involves you training and racing over 12 months. Once a month, both training and non-training subjects come in on a Friday or Saturday morning to donate 10mls of blood (1 tube), which is processed to show a full blood count, iron status and T cell numbers and activity. This means that your health is monitored for a full year. We emphasise that as a volunteer you may drop out if you feel like it, but we would like to have people that are dedicated to train and race, and donate blood, for the year. You will receive a copy of all your results.

**We also need a similar number of people who are not very active and don't want to train, but would like to come in for the blood test once per month to monitor their health.** This comparison group (control subjects) are equally important, because we need to compare the trained and control subjects to ascertain if the training is having a negative effect. I have enclosed a basic medical questionnaire for you to read and fill out. If you could post this back to me OR phone me (if interested in participating), I will contact you to arrange a meeting at the university.

Both the training and control group need to participate in an incremental cycle test to volitional exhaustion, to measure Maximum oxygen uptake. This test is valuable for the triathletes because it is a measure of your aerobic capacity, and can be used as a basis for training intensities, rather than heart rates. I can show you all the equipment and conduct a basic medical screening (eg height, weight, blood pressure and resting 12 lead E.C.G.). The  $\dot{V}O_2$  max test will take place 1 week later. This type of long-term immunology research has not been done before with athletes and is very important for athletes, coaches and non-sporting people. I look forward to hearing from you,

Thank you, Sue Broadbent,

School of Physiotherapy and Exercise Science

Griffith University – Gold Coast, Parklands Drive, Parklands, 4215 Ph: 55315892, 0413053351 or (Uni) 55948281

## 8.2 Appendix 2 – GRIFFITH UNIVERSITY Medical History Questionnaire

Name:-----

Address:-----

-----

Phone:( )------(W)

Phone:( )------(H)

Age:-----

DOB:-----

*Please read the following questions very carefully*

*If you have any difficulty please advise the medical Practitioner*

1. **Family history.** Indicate if any of your immediate Family (parents, brothers, sisters, grandparents) have experienced any of the following, the age at which diagnosis occurred, and their relationship to you.

High blood pressure-----

High cholesterol-----

Stroke-----

Diabetes-----

Cancer-----

2. **Personal medical history.** Include symptoms that apply to you.

- Pain or discomfort in the chest following exercise, eating or exposure to cold.

- Frequent heart papitations or flutter

- Pain in lower lungs when walking or climbing

Stairs

- Unusual shortness of breath

- Very poor exercise tolerance

- Frequent dizziness

- Chronic cough

- Frequent colds or flu

- Frequent headaches

- Frequent aches or pains in the joints

- Frequent backache

- Other current symptoms that exercise may effect.

3. Are you presently experiencing, or have you ever been treated by a doctor for any of the following **allergies** such as Hayfever, Eczema or other rashes?

- Yes

- No

- Details-----

4. **Lungproblems** (Asthma/Emphysema/Bronchitis /Shortness of breath/Other)

- Yes

- No

Details-----

5. **Heart problems** (Rheumatic fever/chest pain/ Palpitations/Ankle swelling/Other)

- Yes

- No

Details-----

6. **Blood Pressure Problems**

- Yes

- No

Details-----

7. **Cholesterol Problems**

- Yes

- No

Details-----

8. **GutProblems**(Ulcer/Abdominal pain/Diarrhoea/ Constipation/ Hernia/ Other)

- Yes

- No

Details-----

9. **Unexplained Weight Loss**

- Yes

- No

Details-----

10. **Urinary Problems** (Burning/ Difficulty with control of urine)

- Yes

- No

Details-----

11. **Blood Loss** (Vomit/ Sputumn/ Bowel action/ Urine)

- Yes

- No

Details-----

12. **Easy Bruising**

- Yes
- No

Details-----

13. **Endocrine Problems** (Diabetes/ Thyroid/ Other)

- Yes
- No

Details-----

14. **Fitting, Fainting, Blackouts,** Muscle Weakness, Loss of Consciousness, Loss of Sensation

- Yes
- No

Details-----

15. **Headaches**

- Yes
- No

Details-----

16. **Sight or Hearing Problems**

- Yes
- No

Details-----

17. **Nervous Conditions**

- Yes
- No

Details-----

18. **Bone or Joint Injury**

- Yes
- No

Details-----

19. **Other Joint Problems**

- Yes
- No

Details-----

20. **Work Related Injuries**

- Yes
- No

Details-----

21. Are you exposed to a **noisy or dusty environment**?

- Yes
- No

Details-----

22. **How often do you take over the counter medications** such as aspirin etc?

- Daily                      Occasionally
- Weekly                    Never

23. **Medication.** Are you taking any medication prescribed by your Doctor or another Health Care Provider? If so, list details ie type of drugs, dosage etc

-----

-----

-----

24. **Sleeping Patterns** How many hours do you sleep on average per night?-----Hours

25. Do you ever have trouble falling asleep?

- Yes
- No

-Occasionally

26. **Smoking Status**

- Never smoked

- Quit smoking more than 10 years ago

- Quit smoking less than 10 years ago

- Currently smoke (number of years)-----

27. If currently smoking, how many cigarettes per day?

-----

28. **Physical Activity.** How many times per week do you exercise for at least 20 – 30 minutes?

- Do not have a regular program

- Once per week

- 2 – 3 times per week

- 4 – 5 times per week

- more than 5 times per week

29. **Alcohol Consumption.** In the past 2 weeks how many days did you consume an alcoholic beverage.

- Did not drink in past 6 months

- Did not drink in past 2 weeks

- 1 – 2 days

- 3 – 4 days

- 5 – 7 days

- 8 – 10 days

- 11 – 14 days

30. In the past 2 weeks how many drinks **ON AVERAGE** per day.

- Did not drink in past 6 months

- Did not drink in past 2 weeks

- 1 – 3 drinks

- 4 – 6 drinks

- 7 or more drinks

### 8.3 Appendix 3 - Informed Consent Form – Experiments 1 and 2.

(to be initialled at every paragraph, and signed and witnessed on the final page)

Chief Investigator: Ms Sue Broadbent

Phone: 07)55 315892 or 0413 053351

Supervisors: Professor Greg Gass, Dr Norman Morris

07)55 528921

#### **Project Title**

The Effects of 12 Months of Endurance Training on Lymphocyte Proliferation in Males and Females Aged 65 to 75 years, and Young Males Aged 23 to 36 Years.

#### **1. The Study**

##### The Purpose of the Study

This study aims to 1) evaluate the effects of 12 months of moderate, aerobic training on the ability of T Helper lymphocytes to divide and mount a normal immune response in older individuals, and 2) evaluate the effects of 12 months of chronic, endurance training on the same cells and cell functions in young males. T Helper lymphocyte proliferation can be assessed by measuring the number of Interleukin-2 and Transferrin receptors on the cell membrane, and how well they respond to a stimulant or mitogen. Intracellular calcium and intracellular iron concentrations are also involved in lymphocyte division, and we will be assessing these as well. This study will provide valuable information about the effects of long-term training on the lymphocyte immune response.

-----

##### Your Involvement

The study will run for 12 months, with blood samples taken every month for all subject groups. There will be 2 groups for each age division (1 training group and 1 non-training group). All subjects will be required to undergo 2 incremental cycling tests to exhaustion ( $VO_{2max}$  tests) at the beginning and end of the study. For the 65 to 75 year age division, the initial incremental test will provide an estimate of your training intensity (50% and 60% of maximum heart rate), for your training sessions. The training sessions are of 45 minutes duration, with a 5 minute warm-up at low resistance, 37 minutes of training at a moderate intensity, then a 3 minute warm-down period at low resistance. The training sessions are to be undertaken 3 times per week (Monday, Wednesday and Friday).

Monthly blood samples will be taken between 6am and 9am – 14 mls only will be taken. You must be rested and fasted (water can be taken prior to testing). The blood will be taken from you in the supine position.

-----

## **2. Risks**

- The taking of venous blood from a vein at the front of the elbow is a very safe procedure, and the person who performs this will be certified to do so. The sampling will only take several minutes.
  - All venous blood-taking will follow strict sterile procedures.
  - The blood sample is taken before any exercise to minimise effects on the lymphocytes. You must not do any exercise or eat breakfast before the blood sample is taken.
- 

## **3. Benefits**

To You:

From this study, you will find out the current state of your immune system – your lymphocyte concentration and how your disease fighting capacity responds to regular exercise. You will undergo an initial medical screening including a 12 lead ECG, blood pressure reading, skin-fold measurement, and spirometry (lung function) test. You will receive a monthly full blood count to monitor all cell concentrations and your iron status. The 65 to 75 years training group will be able to participate in a fully supervised, 12 month exercise program where weight and blood pressure are monitored at each training session. The 23 to 36 years training group will be able to provide blood test results and immune response results to coaches.

To Others:

When the study is completed and the results analysed, it will be possible to provide information on the effects of long-term training on the immune response to other individuals in your age group, medical practitioners and coaches involved in many sports. Currently there is limited information about the direct impact of training on specific aspects of immune function. There is limited information about how much exercise is enough to promote a healthy immune response, and how much training is too much. Moderate endurance training may increase immune function and lower the risk of infections and some chronic diseases like cancer.

-----

**4. Confidentiality**

Your records will be kept secure and confidential. Only myself and my supervisor will see your name and results together. Your results will be coded with a number and letter, and will be collated, analysed and reported in this manner.

No other person will have access to your data except yourself and the investigators. The results of the study will be published and your information will only appear combined with other participants, so that no personal identification in any published work is possible.

-----

**5. You As A Volunteer**

- It is important that you freely volunteer to be part of this study. You should understand what will be required. If you wish to know more about the procedures, please contact one of the investigators.
- If you wish to discontinue as a subject then you should feel free to do so. There will be no consequences if you decide to withdraw – this is your right at any time.

-----

**6. Any Matter of Concern**

If any aspect of the study or your participation gives you concern, then please don't hesitate to contact one of the chief investigators. Their names and phone numbers appear on the front of the information sheet.

-----

**7. Feedback**

You will receive monthly copies of the full blood count. You will receive written feedback about your personal yearly results, and the overall group results, at the completion of the testing period. You will also receive copies of each incremental test you complete. There will be opportunities to ask questions during the test sessions. ECG results can be forwarded to your GP upon request. We appreciate your co-operation with this study, and will be happy to provide further information if you are interested.

Signature -----

Witness-----

Date-----

## 8.4 Appendix 4 - Normal Haematology Values

(Coulter T660 Blood Analyser, Coulter Electronics, U.S.A.).

WBC x 10 <sup>9</sup> .L <sup>-1</sup>	Male 4 – 11	Female 4 – 11
RBC x 10 <sup>12</sup> .L <sup>-1</sup>	Male 4.7 – 6.1	Female 4.2 – 5.4
Hb g.dL	Male 14 - 18	Female 12 - 16
Hct (%)	Male 42 - 52	Female 37 - 47
MCH pg	Male 27 - 31	Female 27 - 31
MCHC g.dL	Male 33 ± 1.5	Female 33 ± 1.5
Platelets x 10 <sup>9</sup> .L <sup>-1</sup>	Male 130 - 400	Female 130 - 400

## 8.5 Appendix 5 – Pearson Correlations and Stepwise Multiple Regression Model Summaries

### Pearson Correlation Matrix – Experiment 1 Untrained Males

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb	
PC	Leuc	1.000								
	IL2%	-.118	1.000							
	IL2den	-.239	.772	1.000						
	Cal	-.146	.288	.386	1.000					
	Thap	-.286	.551	.663	.868	1.000				
	TfR%	.293	-.432	-.275	-.133	-.115	1.000			
	TfRden	-.627	.251	.146	.398	.414	-.403	1.000		
	Iron	-.260	-.278	-.195	.354	.106	.110	.011	1.000	
	Hb	.175	-.300	-.355	-.660	-.579	.360	-.426	.020	1.000
Sig.	Leuc									
	IL2%	.351								
	IL2den	.215	.001							
	Cal	.317	.170	.096						
	Thap	.171	.025	.007	.000					
	TfR%	.165	.070	.182	.333	.354				
	TfRden	.011	.204	.317	.089	.080	.086			
	Iron	.196	.179	.261	.117	.365	.360	.486		
	Hb	.175	.159	.117	.007	.019	.113	.073	.474	

### Regression Model Summary – UT Males

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.879 <sup>a</sup>	.773	.318	.3237
2	.875 <sup>b</sup>	.766	.437	.2940
3	.858 <sup>c</sup>	.737	.473	.2845
4	.830 <sup>d</sup>	.689	.466	.2863
5	.819 <sup>e</sup>	.671	.506	.2753

- Predictors: (Constant), MUTIRON, MUTTFRDEN, MUTIL2DEN, MUTTFPER, MUTHB, MUTCAL, MUTIL2PER, MUTTHG
- Predictors: (Constant), MUTIRON, MUTTFRDEN, MUTIL2DEN, MUTTFPER, MUTCAL, MUTIL2PER, MUTTHG
- Predictors: (Constant), MUTIRON, MUTTFRDEN, MUTTFPER, MUTCAL, MUTIL2PER, MUTTHG
- Predictors: (Constant), MUTIRON, MUTTFRDEN, MUTTFPER, MUTCAL, MUTTHG
- Predictors: (Constant), MUTIRON, MUTTFRDEN, MUTCAL, MUTTHG
- Dependent Variable: MUTLEUC

Key: MUT (Male UT)

IRON ([Fe<sup>3+</sup>]i)

TFRDEN (CD71 density)

IL2DEN (CD25 density)

TFPER (CD71 percent positive)

IL2PER (CD25 percent positive)

CAL (Ca<sup>2+</sup>]i)

THG ([Ca<sup>2+</sup>]t)

HB (Haemoglobin concentration)

LEUC (Leucocyte concentration)

Pearson Correlation Matrix – Experiment 1 Trained Males

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb	
PC	Leuc	1.000								
	IL2%	-.392	1.000							
	IL2den	-.093	.155	1.000						
	Cal	.009	-.222	.528	1.000					
	Thap	-.131	-.319	.541	.815	1.000				
	TfR%	.255	-.666	-.078	.120	.266	1.000			
	TfRden	-.040	.032	.597	.339	.160	-.307	1.000		
	Iron	.451	.263	-.169	.041	-.230	-.350	.050	1.000	
	Hb	-.124	.230	-.650	-.301	-.265	-.023	-.661	.117	1.000
Sig.	Leuc									
	IL2%	.092								
	IL2den	.382	.306							
	Cal	.488	.233	.032						
	Thap	.335	.144	.028	.000					
	TfR%	.201	.007	.399	.348	.189				
	TfRden	.448	.459	.016	.129	.301	.154			
	Iron	.061	.193	.291	.447	.225	.121	.436		
	Hb	.343	.225	.008	.159	.191	.470	.007	.352	

Regression Model Summary – TR Males

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.811 <sup>a</sup>	.658	-.025	.1621
2	.811 <sup>b</sup>	.658	.180	.1450
3	.811 <sup>c</sup>	.657	.315	.1326
4	.808 <sup>d</sup>	.653	.405	.1235
5	.766 <sup>e</sup>	.587	.380	.1260
6	.719 <sup>f</sup>	.517	.355	.1286
7	.696 <sup>g</sup>	.484	.381	.1260

- a. Predictors: (Constant), MTRIRON, MTRCAL, MTRHB, MTRIL2PER, MTRTFPER, MTRTFRDEN, MTRTHG, MTRILDEN  
 b. Predictors: (Constant), MTRIRON, MTRHB, MTRIL2PER, MTRTFPER, MTRTFRDEN, MTRTHG, MTRIL2DEN  
 c. Predictors: (Constant), MTRIRON, MTRHB, MTRIL2PER, MTRTFRDEN, MTRTHG, MTRIL2DEN  
 d. Predictors: (Constant), MTRIRON, MTRIL2PER, MTRTFRDEN, MTRTHG, MTRIL2DEN  
 e. Predictors: (Constant), MTRIRON, MTRIL2PER, MTRTHG, MTRIL2DEN  
 f. Predictors: (Constant), MTRIRON, MTRIL2PER, MTRTHG  
 g. Predictors: (Constant), MTRIRON, MTRIL2PER  
 h. Dependent Variable: TRLEUC

Key: MTR (Male TR)  
 IRON ([Fe<sup>3+</sup>]i)  
 TFRDEN (CD71 density)  
 IL2DEN (CD25 density)  
 TFPER (CD71 percent positive)  
 IL2PER (CD25 percent positive)  
 CAL (Ca<sup>2+</sup>]j)  
 THG ([Ca<sup>2+</sup>]t)  
 HB (Haemoglobin concentration)  
 LEUC (Leucocyte concentration)

Pearson Correlation Matrix – Experiment 1 Untrained Females

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb	
PC	Leuc	1.000								
	IL2%	-.484	1.000							
	IL2den	-.542	.453	1.000						
	Cal	.261	-.339	.270	1.000					
	Thap	.110	.299	.511	.572	1.000				
	TfR%	.427	.080	-.057	.092	.537	1.000			
	TfRden	-.351	.000	.456	-.063	-.165	-.157	1.000		
	Iron	.403	-.157	-.087	.303	-.102	-.007	.065	1.000	
	Hb	-.047	-.067	-.347	-.282	-.489	-.144	-.365	-.258	1.000
Sig.	Leuc									
	IL2%	.047								
	IL2den	.028	.060							
	Cal	.191	.129	.186						
	Thap	.361	.161	.037	.021					
	TfR	.073	.397	.427	.382	.029				
	TfRden	.120	.499	.059	.419	.295	.305			
	Iron	.086	.305	.389	.157	.370	.491	.417		
	Hb	.439	.414	.123	.175	.045	.319	.110	.198	

Regression Model Summary – UT Females

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.903 <sup>a</sup>	.815	.445	.3095
2	.892 <sup>b</sup>	.795	.508	.2915
3	.884 <sup>c</sup>	.782	.564	.2745
4	.866 <sup>d</sup>	.750	.571	.2721
5	.845 <sup>e</sup>	.715	.572	.2719
6	.804 <sup>f</sup>	.646	.529	.2853

- a. Predictors: (Constant), FUTIRON, FUTTFPER, FUTIL2DEN, FUTCAL, FUTHB, FUTTFRDEN, FUTIL2PER, FUTTHG
- b. Predictors: (Constant), FUTIRON, FUTIL2DEN, FUTCAL, FUTHB, FUTTFRDEN, FUTIL2PER, FUTTHG
- c. Predictors: (Constant), FUTIRON, FUTIL2DEN, FUTCAL, FUTHB, FUTIL2PER, FUTTHG
- d. Predictors: (Constant), FUTIRON, FUTIL2DEN, FUTCAL, FUTIL2PER, FUTTHG
- e. Predictors: (Constant), FUTIRON, FUTIL2DEN, FUTIL2PER, FUTTHG
- f. Predictors: (Constant), FUTIRON, FUTIL2DEN, FUTTHG
- g. Dependent Variable: FUTLEUC

Key: FUT (Female UT)

IRON ([Fe<sup>3+</sup>]i)

TFRDEN (CD71 density)

IL2DEN (CD25 density)

TFPER (CD71 percent positive)

IL2PER (CD25 percent positive)

CAL (Ca<sup>2+</sup>]i)

THG ([Ca<sup>2+</sup>]t)

HB (Haemoglobin concentration)

LEUC (Leucocyte concentration)

Pearson Correlation Matrix – Experiment 1 Trained Females

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb
PC Leuc	1.000								
IL2%	-.171	1.000							
IL2den	.390	.291	1.000						
Cal	.506	-.158	.381	1.000					
Thap	.095	.219	.347	.672	1.000				
TfR%	-.038	-.307	-.545	-.041	.099	1.000			
TfRden	.659	.210	.402	.478	.050	-.273	1.000		
Iron	-.121	.035	-.115	.138	.216	.286	-.193	1.000	
Hb	.189	-.241	-.631	-.333	-.362	.489	-.160	-.071	1.000
Sig. Leuc									
IL2%	.288								
IL2den	.094	.168							
Cal	.039	.303	.100						
Thap	.379	.236	.123	.006					
TfR%	.451	.154	.027	.447	.373				
TfRden	.007	.246	.087	.049	.435	.183			
Iron	.347	.454	.354	.326	.239	.172	.263		
Hb	.268	.214	.010	.133	.112	.045	.300	.409	

Regression Model Summary – Trained Females

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.913 <sup>a</sup>	.833	.499	.1310
2	.913 <sup>b</sup>	.833	.599	.1172
3	.912 <sup>c</sup>	.832	.665	.1071
4	.910 <sup>d</sup>	.828	.705	.1005
5	.892 <sup>e</sup>	.795	.692	.1026

- Predictors: (Constant), FTRIRON, FTRIL2PER, FTRCAL, FTRHB, FTRTFPER, FTRTFRDEN, FTRIL2DEN, FTRTHG
- Predictors: (Constant), FTRIRON, FTRIL2PER, FTRCAL, FTRHB, FTRTFPER, FTRTFRDEN, FTRIL2DEN
- Predictors: (Constant), FTRIRON, FTRIL2PER, FTRCAL, FTRHB, FTRTFRDEN, FTRIL2DEN
- Predictors: (Constant), FTRIL2PER, FTRCAL, FTRHB, FTRTFRDEN, FTRIL2DEN
- Predictors: (Constant), FTRIL2PER, FTRHB, FTRTFRDEN, FTRIL2DEN
- Dependent Variable: FTRLEUC

Key: FTR (Female TR)

IRON ([Fe<sup>3+</sup>]i)

TFRDEN (CD71 density)

IL2DEN (CD25 density)

TFPER (CD71 percent positive)

IL2PER (CD25 percent positive)

CAL (Ca<sup>2+</sup>]i)

THG ([Ca<sup>2+</sup>]t)

HB (Haemoglobin concentration)

LEUC (Leucocyte concentration)

Pearson Correlation Matrix – Experiment 2 Untrained Males

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb	
PC	Leuc	1.000								
	IL2%	-.143	1.000							
	IL2den	.100	.410	1.000						
	Cal	-.444	-.098	-.140	1.000					
	Thap	.665	-.111	-.246	-.057	1.000				
	TfR%	.231	-.180	-.670	-.285	.595	1.000			
	TfRden	-.054	-.194	.646	.007	-.362	-.512	1.000		
	Iron	-.572	.094	-.253	.800	-.177	-.189	-.161	1.000	
	Hb	.235	.376	.249	-.040	.096	-.030	-.088	-.035	1.000
Sig.	Leuc									
	IL2%	.312								
	IL2den	.366	.073							
	Cal	.050	.370	.316						
	Thap	.005	.353	.199	.424					
	TfR%	.213	.269	.004	.161	.012				
	TfRden	.428	.253	.006	.491	.101	.031			
	Iron	.016	.375	.191	.000	.273	.259	.292		
	Hb	.210	.093	.195	.446	.372	.460	.383	.452	

Regression Model Summary – Untrained Males

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.904 <sup>a</sup>	.817	.525	.2348
2	.903 <sup>b</sup>	.815	.599	.2157
3	.898 <sup>c</sup>	.807	.642	.2040
4	.896 <sup>d</sup>	.803	.679	.1930
5	.874 <sup>e</sup>	.764	.659	.1989
6	.855 <sup>f</sup>	.731	.650	.2015

- a. Predictors: (Constant), UTHB, UTFFPER, UTIRON, UTIL2PER, UTTHG, UTFFRDEN, UTCAL, UTIL2DEN
- b. Predictors: (Constant), UTHB, UTFFPER, UTIRON, UTIL2PER, UTTHG, UTCAL, UTIL2DEN
- c. Predictors: (Constant), UTHB, UTFFPER, UTIRON, UTIL2PER, UTTHG, UTCAL
- d. Predictors: (Constant), UTHB, UTFFPER, UTIL2PER, UTTHG, UTCAL
- e. Predictors: (Constant), UTFFPER, UTIL2PER, UTTHG, UTCAL
- f. Predictors: (Constant), UTFFPER, UTTHG, UTCAL
- g. Dependent Variable: UTLEUC

Key: UT (Male UT)  
 IRON ([Fe<sup>3+</sup>]i)  
 TFRDEN (CD71 density)  
 IL2DEN (CD25 density)  
 TFFPER (CD71 percent positive)  
 IL2PER (CD25 percent positive)  
 CAL (Ca<sup>2+</sup>]i)  
 THG ([Ca<sup>2+</sup>]t)  
 HB (Haemoglobin concentration)  
 LEUC (Leucocyte concentration)

## Pearson Correlation Matrix – Experiment 2 Trained Males

	Leuc	IL2%	IL2den	Cal	Thap	TfR%	TfRden	Iron	Hb	
PC	Leuc	1.000								
	IL2%	-.112	1.000							
	IL2den	-.011	.479	1.000						
	Cal	-.342	-.046	-.238	1.000					
	Thap	-.439	.246	.007	.649	1.000				
	TfR%	-.212	-.295	-.713	.153	.130	1.000			
	TfRden	.268	-.078	.658	-.228	-.367	-.444	1.000		
	Iron	-.290	-.025	-.183	.906	.483	-.041	-.111	1.000	
	Hb	.152	.073	-.041	-.453	-.150	.260	-.157	-.414	1.000
Sig.	Leuc									
	IL2%	.351								
	IL2den	.485	.041							
	Cal	.116	.439	.206						
	Thap	.058	.199	.491	.006					
	TfR%	.234	.153	.002	.301	.329				
	TfRden	.177	.395	.005	.217	.099	.056			
	Iron	.157	.466	.265	.000	.040	.444	.353		
	Hb	.302	.402	.445	.050	.305	.184	.296	.070	

## Regression Model Summary – Trained Males

Model	R	R Square	Adjusted R Square	Std. Error of the Estimate
1	.793 <sup>a</sup>	.629	.037	.2334
2	.793 <sup>b</sup>	.628	.195	.2134
3	.724 <sup>c</sup>	.524	.116	.2235
4	.670 <sup>d</sup>	.449	.104	.2250
5	.634 <sup>e</sup>	.401	.135	.2211
6	.498 <sup>f</sup>	.248	.023	.2351

- a. Predictors: (Constant), TRIRON, TRIL2PER, TRTFRDEN, TRHB, TRTFRPER, TRTHG, TRIL2DEN, TRCAL
- b. Predictors: (Constant), TRIRON, TRIL2PER, TRTFRDEN, TRHB, TRTFRPER, TRIL2DEN, TRCAL
- c. Predictors: (Constant), TRIRON, TRTFRDEN, TRHB, TRTFRPER, TRIL2DEN, TRCAL
- d. Predictors: (Constant), TRIRON, TRTFRDEN, TRHB, TRTFRPER, TRIL2DEN
- e. Predictors: (Constant), TRIRON, TRTFRDEN, TRTFRPER, TRIL2DEN
- f. Predictors: (Constant), TRIRON, TRTFRPER, TRIL2DEN
- g. Dependent Variable: TRLEUC

Key: UT (Male TR)

IRON ([Fe<sup>3+</sup>]<sub>i</sub>)

TFRDEN (CD71 density)

IL2DEN (CD25 density)

TFPER (CD71 percent positive)

IL2PER (CD25 percent positive)

CAL (Ca<sup>2+</sup>]<sub>i</sub>)

THG ([Ca<sup>2+</sup>]<sub>t</sub>)

HB (Haemoglobin concentration)

LEUC (Leucocyte concentration)

**8.6 Appendix 6 - Components of Graded Calcium Buffer Solutions** (Molecular Probes, USA).

1. Zero Free  $\text{Ca}^{2+}$  Buffer: 10 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
2. 0.017  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 1 mM CaEGTA, 9 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
3. 0.038  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 2 mM CaEGTA, 8 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
4. 0.065  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 3 mM CaEGTA, 7 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
5. 0.100  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 4 mM CaEGTA, 6 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
6. 0.150  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 5 mM CaEGTA, 5 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
7. 0.225  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 6 mM CaEGTA, 4 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
8. 0.351  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 7 mM CaEGTA, 3 mM EGTA in 100 mM KCl, 30 mM MOPS pH 7.2
9. 0.602  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 8 mM CaEGTA, 2 mM EGTA in 100 mM KCl, 30 Mm MOPS pH 7.2
10. 1.35  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 9 mM CaEGTA, 1 mM EGTA in 100 mM KCl, 30 Mm MOPS pH 7.2
11. 38.9  $\mu\text{M}$  Free  $\text{Ca}^{2+}$  Buffer: 10 mM CaEGTA in 100 mM KCl, 30 mM MOPS pH 7.2



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