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An investigation of a novel, non-invasive technique for the assessment of oxidative stress in aerobic and isometric exercise

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B.Sc. (Hons), M.Sc.(Med.Sci.)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

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Submitted: September 2010
Dedication

This work is dedicated to Dr Andy Cathcart (1978-2009), without whom it would not have been started and could not have been completed. Andy encouraged me to take on this challenge and never faltered in his support through extremely difficult circumstances. He will be remembered for his immense intelligence, enthusiasm, determination and optimism; and his indomitable spirit will continue to inspire me to greater achievement. He will be forever missed.
Abstract

The oxidative stress response to exercise is a well-established phenomenon; however, the time course of this response has not been well characterised. There is little information in the literature regarding the oxidative stress response during exercise; most authors have measured oxidative stress solely during the recovery period from exercise. There are several different invasive methods available for assessment of oxidative stress, although there is no “gold standard” technique. A novel non-invasive technique utilising laser spectroscopy to quantify expired ethane concentration has become available, but has not yet been tested in relation to exercise.

The first study described here aimed to use the laser spectroscopy technique for the first time to assess exercise-induced oxidative stress in three species: humans, horses and dogs; and to determine the utility of carbon monoxide monitoring as a means of assessment of oxidative stress. A further objective was to better characterise the oxidative stress response by the collection of data at frequent intervals during exercise and during recovery. Eight endurance-trained males performed incremental treadmill exercise to volitional exhaustion. Twelve racehorses and twelve racing greyhounds performed maximal exercise on a race track. Expired ethane concentration was measured throughout exercise in humans, and pre- and post-exercise in horses and dogs. Carbon monoxide concentration was assessed pre- and post-exercise in all species. Results indicated that the technique of laser spectroscopy was viable for use in relation to exercise in all three species. Oxidative stress was shown to increase significantly following exercise in all three species, thus supporting previous literature, and extending this finding to a trained human population for the first time. The pattern of response during incremental treadmill exercise was characterised for the first time and indicated a non-significant increase in oxidative stress in humans within 2 minutes of the onset of exercise, with the response progressively increasing alongside
increases in work rate until exercise was terminated at exhaustion. The response returned close to the resting value by 20 minutes into the recovery period. Low subject number may have contributed to the lack of significant findings during exercise. Carbon monoxide was not a useful indicator of oxidative stress in any species.

Increased functionality of the laser spectroscopy technique was investigated by pilot work in which real-time monitoring of expired ethane was attempted for the first time in relation to exercise. This allowed the observation of the oxidative stress response on a breath by breath basis. Initial tests, in which two healthy males performed incremental cycle ergometer exercise to exhaustion whilst breathing through a valve connected directly to the spectrometer, indicated that a useful output could be recorded during a prolonged period of exercise. However, the measurement of ambient ethane concentration, essential for accuracy, was not undertaken in the initial tests. Thus, further pilot work was successfully carried out in three healthy males to replicate the initial tests with concurrent ambient ethane monitoring. This pilot testing allowed development of data editing techniques. The oxidative stress response profile for incremental exercise in real-time was similar to that reported in the previous chapter. Additional tests were undertaken which illustrated that the rise in ethane output observed during incremental exercise was not simply a manifestation of the ventilatory response to exercise, rather than an indication of exercise-induced oxidative stress. This was accomplished by forcing an increase in ventilation, by imposition of an additional dead space volume during normal breathing in two individuals. This technique shows promise for more detailed characterisation of the time course of the oxidative stress response in future exercise studies via the capability for extremely high density data collection.

The main aims of the second study were to investigate the oxidative stress response throughout the entire work rate range from rest to volitional exhaustion, rather than just the
higher end of the work rate range as observed in study one; and to examine the magnitude and time course of the oxidative stress response to constant load exercise performed below and above the lactate threshold. Six healthy males performed incremental cycle ergometer exercise to exhaustion during which blood samples were collected regularly for later analysis for the presence of F2-isoprostanes. Results of the analysis were disappointing, with a high proportion of samples displaying a concentration outwith the range of the assay. However, preliminary malondialdehyde analysis suggested that the oxidative stress response may increase progressively alongside work rate throughout the entire work rate range. However, this observation is far from conclusive as it is based on data from a single subject only.

The final study was intended to investigate the effect of contraction intensity on the oxidative stress response to isometric handgrip exercise sustained to exhaustion, and to clarify the time course of the oxidative stress response during the recovery period. Due to logistical limitations, it was possible to study one contraction intensity only. Initially, pilot work was undertaken to determine the suitability of the novel non-invasive technique for ethane assessment in relation to isometric exercise, since this assessment method had not been used previously with this exercise mode. Then, six healthy males performed sustained isometric exercise at 60 % of maximal voluntary contraction until fatigue. Oxidative stress was assessed during a 30 minute recovery period via expired ethane and also via F2-isoprostanes concentration in blood collected from both the exercised arm and the non-exercised arm. This was intended to allow comparison of blood sampling site, and of the systemic oxidative stress response measured both invasively and non-invasively; however this was not possible due to poor assay results. The previous finding of a peak oxidative stress response following isometric exercise within the first 5 minutes of the recovery period was supported. Oxidative stress was assessed by ethane output for the first time in relation to isometric exercise and was found to be a viable technique; however, its use
remains to be validated against more traditional plasma markers. The potential value of non-invasive assessment was underlined by F₂-isoprostanes analysis issues.

In conclusion, the use of laser spectroscopy, including the use of real-time monitoring, appears to be a viable technique for the non-invasive assessment of exercise-induced oxidative stress, and may enhance our ability to characterise this response in future studies.
# Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dedication</td>
<td>2</td>
</tr>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>List of figures</td>
<td>20</td>
</tr>
<tr>
<td>List of tables</td>
<td>27</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>29</td>
</tr>
<tr>
<td>Author’s declaration</td>
<td>30</td>
</tr>
<tr>
<td>List of abbreviations</td>
<td>31</td>
</tr>
<tr>
<td>Chapter 1  Introduction</td>
<td>36</td>
</tr>
<tr>
<td>1.1  An introduction to oxidative stress</td>
<td>37</td>
</tr>
<tr>
<td>1.1.1  The oxygen paradox</td>
<td>37</td>
</tr>
<tr>
<td>1.1.2  Oxidants</td>
<td>41</td>
</tr>
<tr>
<td>1.1.2.1  Free radicals</td>
<td>41</td>
</tr>
<tr>
<td>1.1.2.2  Other reactive species</td>
<td>42</td>
</tr>
<tr>
<td>1.1.2.3  Formation of free radicals and reactive species</td>
<td>42</td>
</tr>
<tr>
<td>1.1.2.4  Reactivity</td>
<td>44</td>
</tr>
<tr>
<td>1.1.3  Antioxidants</td>
<td>45</td>
</tr>
<tr>
<td>1.1.3.1  Enzymatic antioxidants</td>
<td>46</td>
</tr>
<tr>
<td>1.1.3.2  Non-enzymatic antioxidants</td>
<td>48</td>
</tr>
<tr>
<td>1.1.4  Oxidative damage</td>
<td>50</td>
</tr>
<tr>
<td>1.1.4.1  Lipid peroxidation</td>
<td>51</td>
</tr>
<tr>
<td>1.1.4.2  Nucleic acid damage</td>
<td>55</td>
</tr>
<tr>
<td>1.1.4.3  Protein damage</td>
<td>55</td>
</tr>
</tbody>
</table>
1.1.5 Implications of oxidative damage

1.1.5.1 Atherosclerosis

1.1.5.2 Ageing

1.1.6 The physiological roles of reactive species

1.2 Assessment of oxidative stress

1.2.1 Direct measurement of free radicals

1.2.2 Indirect assessment of oxidative stress

1.2.2.1 Assessment of nucleic acid damage

1.2.2.2 Assessment of protein damage

1.2.2.3 Invasive assessment of lipid peroxidation

1.2.2.3.1 Malondialdehyde

1.2.2.3.2 F2-isoprostanes

1.2.2.3.3 Conjugated dienes

1.2.2.3.4 Lipid hydroperoxides

1.2.2.4 Non-invasive assessment of lipid peroxidation

1.2.2.5 Assessment of antioxidant capacity

1.3 Exercise-induced oxidative stress

1.3.1 Aerobic exercise

1.3.1.1 Maximal exercise

1.3.1.2 Submaximal exercise

1.3.1.3 Other aerobic activities

1.3.1.4 Summary of aerobic exercise-induced oxidative stress

1.3.2 Anaerobic exercise

1.3.2.1 Isometric exercise
1.3.2.2 Sprint exercise 80
1.3.2.3 Resistance exercise 81
1.3.2.4 Comparative studies 82

1.3.3 Mechanisms of exercise-induced oxidative stress 83
  1.3.3.1 Mitochondrial production of reactive species 83
  1.3.3.2 Haem proteins 85
  1.3.3.3 Activated phagocytes 86
  1.3.3.4 Xanthine oxidase 87

1.4 Objectives of current studies 89
  1.4.1 Study one 90
  1.4.2 Study two 91
  1.4.3 Study three 92

Chapter 2 Materials and methods 94

2.1 Subjects 95
  2.1.1 Exclusion criteria 95
  2.1.2 Informed consent 96

2.2 Testing procedures 96
  2.2.1 Laboratory environment 96
  2.2.2 Familiarisation 97
  2.2.3 Exercise mode 98

2.3 Measurements 99
  2.3.1 Respired air measurements 99
    2.3.1.1 Open-circuit spirometry 100
    2.3.1.2 Mass spectrometry and turbinometry 102
2.3.1.2.1 Gas sampling procedures
2.3.1.2.2 Calibration
2.3.1.2.3 Analysis

2.3.2 Ethane sampling procedures
2.3.2.1 Materials for collection and storage of samples
2.3.2.2 Ethane collection procedures
2.3.2.3 Correction for ambient ethane
2.3.2.4 Test location
2.3.2.4.1 Pilot test

2.3.3 Ethane analysis

2.3.4 Blood sampling procedures

2.3.5 Blood analysis
2.3.5.1 Measurement of malondialdehyde
2.3.5.2 Measurement of F2-isoprostanes

2.3.6 Statistical analysis

Chapter 3 Ethane and carbon monoxide responses to maximal dynamic exercise in human, equine and canine athletes

3.1 Introduction
3.1.1 Non-invasive assessment of oxidative stress
3.1.1.1 Expired and ambient ethane concentrations
3.1.1.2 Limitations of hydrocarbon measurement
3.1.1.3 Novel technique for the assessment of ethane

3.1.2 Previous exercise studies utilising ethane measurement
3.1.2.1 Previous studies in humans
<table>
<thead>
<tr>
<th>Section</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1.2.2</td>
<td>Previous studies in horses and dogs</td>
<td>128</td>
</tr>
<tr>
<td>3.1.3</td>
<td>Carbon monoxide</td>
<td>129</td>
</tr>
<tr>
<td>3.1.4</td>
<td>Aims</td>
<td>131</td>
</tr>
<tr>
<td>3.2</td>
<td>Methods</td>
<td>132</td>
</tr>
<tr>
<td>3.2.1</td>
<td>Human experiments</td>
<td>132</td>
</tr>
<tr>
<td>3.2.1.1</td>
<td>Subjects</td>
<td>132</td>
</tr>
<tr>
<td>3.2.1.2</td>
<td>Test protocols</td>
<td>132</td>
</tr>
<tr>
<td>3.2.1.2.1</td>
<td>Familiarisation</td>
<td>132</td>
</tr>
<tr>
<td>3.2.1.2.2</td>
<td>Incremental exercise test</td>
<td>133</td>
</tr>
<tr>
<td>3.2.1.3</td>
<td>Measurements</td>
<td>134</td>
</tr>
<tr>
<td>3.2.1.3.1</td>
<td>Respired air measurements</td>
<td>134</td>
</tr>
<tr>
<td>3.2.1.3.2</td>
<td>Ethane sampling procedures</td>
<td>136</td>
</tr>
<tr>
<td>3.2.1.3.3</td>
<td>Carbon monoxide sampling procedures</td>
<td>136</td>
</tr>
<tr>
<td>3.2.1.3.4</td>
<td>Heart rate measurement</td>
<td>137</td>
</tr>
<tr>
<td>3.2.1.4</td>
<td>Analysis</td>
<td>137</td>
</tr>
<tr>
<td>3.2.1.4.1</td>
<td>Determination of peak oxygen uptake</td>
<td>137</td>
</tr>
<tr>
<td>3.2.1.4.2</td>
<td>Determination of peak respiratory exchange ratio</td>
<td>139</td>
</tr>
<tr>
<td>3.2.1.4.3</td>
<td>Ethane analysis</td>
<td>139</td>
</tr>
<tr>
<td>3.2.2</td>
<td>Animal testing</td>
<td>140</td>
</tr>
<tr>
<td>3.2.2.1</td>
<td>Subjects</td>
<td>141</td>
</tr>
<tr>
<td>3.2.2.2</td>
<td>Test protocols</td>
<td>141</td>
</tr>
<tr>
<td>3.2.2.3</td>
<td>Measurements</td>
<td>141</td>
</tr>
<tr>
<td>3.2.2.4</td>
<td>Ethane analysis</td>
<td>143</td>
</tr>
</tbody>
</table>
3.2.3. Statistical analysis

3.3 Results

3.3.1 Human data
   3.3.1.1 Subject characteristics
   3.3.1.2 Ambient ethane concentration
   3.3.1.3 Ethane concentration and output at rest
   3.3.1.4 Ethane output during exercise and recovery
   3.3.1.5 Carbon monoxide concentration

3.3.2 Comparative data
   3.3.2.1 Ethane output
   3.3.2.2 Carbon monoxide concentration

3.4 Discussion

3.4.1 Human data
   3.4.1.1 Ambient ethane concentration
   3.4.1.2 Ethane concentration and output at rest
   3.4.1.3 Ethane output during exercise
   3.4.1.4 Ethane output during recovery

3.4.2 Comparative data
   3.4.2.1 Ethane output
   3.4.2.2 Carbon monoxide concentration

3.4.3 Limitations and further work

3.5 Conclusions

Chapter 4 Pilot tests investigating real-time ethane measurement

4.1 Introduction
4.2 Zero calibration tests

4.2.1 Rationale

4.2.2 Methods

4.2.2.1 Subjects

4.2.2.2 Test protocol

4.2.2.3 Measurements

4.2.2.3.1 Respired air measurements

4.2.2.3.2 Heart rate measurement

4.2.2.3.3 Ethane sampling procedures

4.2.2.4 Analysis

4.2.2.4.1 Data editing

4.2.2.4.2 Determination of peak oxygen uptake

4.2.2.4.3 Estimation of lactate threshold

4.2.2.4.4 Determination of peak heart rate

4.2.2.4.5 Ethane analysis

4.2.3 Results

4.2.3.1 Subject characteristics

4.2.3.2 Zero calibration measurement

4.2.3.3 Ethane concentration response

4.2.3.4 Ethane output response

4.2.4 Discussion

4.3 Ventilatory challenge tests

4.3.1 Rationale

4.3.2 Methods
Chapter 5  A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains

5.1  Introduction

5.1.1  Oxidative stress response to varying exercise intensity

5.1.2  Exercise intensity domains

5.1.3  Aims

5.2  Methods

5.2.1  Subjects

5.2.2  Test protocols

5.2.2.1  Familiarisation

5.2.2.2  Ramp test

5.2.2.3  Constant load tests

5.2.2.3.1  Determination of work rates

5.2.3  Measurements

5.2.3.1  Respired air measurements

5.2.3.2  Blood sampling procedures

5.2.3.3  Heart rate

5.2.3.4  Arterial oxygen saturation

5.2.4  Analysis

5.2.4.1  Determination of peak oxygen uptake
5.2.4.2 Estimation of lactate threshold 229
5.2.4.3 Determination of peak heart rate 229
5.2.4.4 Blood analysis 229
  5.2.4.4.1 MDA assay 229
  5.2.4.4.2 Isoprostane assay 230

5.3 Results 231
  5.3.1 Subjects 231
  5.3.2 Oxygen uptake responses to constant load exercise 234
  5.3.3 Oxidative stress response to incremental exercise 235
  5.3.4 Oxidative stress response to constant load exercise 240

5.4 Discussion 247
  5.4.1 Preliminary MDA analysis 247
  5.4.2 F₂-isoprostane analysis 248
  5.4.3 Study design 251
  5.4.4 Limitations and further work 251

5.5 Conclusions 252

Chapter 6 Pilot tests investigating the potential for non-invasive assessment of oxidative stress during and following isometric exercise 253

6.1 Introduction 254

6.2 Methods 254
  6.2.1 Subjects 254
  6.2.2 Test protocols 254
    6.2.2.1 Determination of maximal voluntary contraction 257
    6.2.2.2 Sustained contraction trials 258
6.2.3 Measurements

6.2.3.1 Respired air measurements

6.2.3.2 Ethane sampling procedures and analysis

6.3 Results

6.3.1 Subject characteristics

6.3.2 Ethane output response

6.4 Discussion

Chapter 7 A comparison of invasive and non-invasive means of assessment of oxidative stress during recovery from isometric exercise

7.1 Introduction

7.1.1 Isometric exercise

7.1.2 Ischaemia-reperfusion injury

7.1.3 Evidence of oxidative stress in isometric exercise

7.1.3.1 Static isometric exercise

7.1.3.2 Intermittent isometric exercise

7.1.4 Limitations in the literature

7.1.5 Assessment of oxidative stress

7.1.6 Aims

7.2 Methods

7.2.1 Subjects

7.2.2 Test protocols

7.2.3 Measurements

7.2.3.1 Respired air measurements

7.2.3.2 Ethane sampling procedures
7.2.3.3 Blood sampling procedures 280

7.2.4 Analysis 280
  7.2.4.1 Ethane analysis 280
  7.2.4.2 Blood analysis 280
  7.2.4.3 Statistical analysis 281

7.3 Results 283
  7.3.1 Subjects 283
  7.3.2 Maximal voluntary contraction 283
  7.3.3 Sustained contraction trial 285
  7.3.4 Oxygen uptake response 288
  7.3.5 Ethane response 290
    7.3.5.1 Ambient ethane concentration 290
    7.3.5.2 Ethane concentration and ethane output at rest 291
    7.3.5.3 Ethane output post-exercise 292
  7.3.6 $F_2$-isoprostane response 296
  7.3.7 Relationship between expired air and blood markers 302

7.4 Discussion 308
  7.4.1 Maximal voluntary contraction 308
  7.4.2 Sustained contraction duration 308
  7.4.3 Oxygen uptake response 309
  7.4.4 Ethane response 310
    7.4.4.1 Ambient ethane concentration 310
    7.4.4.2 Ethane concentration and ethane output at rest 311
    7.4.4.3 Ethane output post-exercise 312
7.4.5 F₂-isoprostane response

7.4.6 Relationship between expired air and blood markers

7.4.7 Limitations and further work

7.5 Conclusions

Chapter 8 General discussion

8.1 Experimental findings

8.2 Implications of findings

8.2.1 Adaptations to training

8.2.2 Antioxidant supplementation

8.2.3 Assessment of oxidative stress

8.3 Conclusions

References

Appendix A Information sheets, consent forms and questionnaires

Appendix A.1 The effect of exercise intensity on ethane production during treadmill exercise: Information sheet and consent form

Appendix A.2 A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains: Information sheet and consent form

Appendix A.3 The effect of varying the intensity of isometric exercise on oxidative stress: Information sheet and consent form

Appendix A.4 Medical questionnaire

Appendix A.5 Physical activity questionnaire
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Schematic diagram illustrating the lipid peroxidation process.</td>
<td>52</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Schematic representation of breathing assembly.</td>
<td>104</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Schematic representation of the pilot test protocol for the investigation of the expired ethane response to laboratory conditions following prior exposure to high atmospheric concentration (1.8 nmol·l⁻¹).</td>
<td>110</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Expired ethane response to laboratory conditions following prior exposure to high atmospheric concentration (1.8 nmol·l⁻¹).</td>
<td>111</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Schematic representation of the incremental exercise test protocol and sampling points for ethane and carbon monoxide (CO).</td>
<td>135</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Illustration of equipment used for the collection of expired air in equine and canine athletes.</td>
<td>142</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Ethane output prior to, during and following an incremental treadmill test to volitional exhaustion in eight subjects.</td>
<td>150</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Mean ethane output during rest, incremental treadmill exercise to volitional exhaustion, and active and passive recovery in five subjects.</td>
<td>152</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Carbon monoxide concentration at baseline and following an incremental treadmill run to exhaustion in eight subjects.</td>
<td>153</td>
</tr>
<tr>
<td>Figure 3.6</td>
<td>Mean carbon monoxide concentration at baseline and following an incremental treadmill run to exhaustion in eight subjects.</td>
<td></td>
</tr>
<tr>
<td>Figure 3.7</td>
<td>Ethane output at baseline and post-exercise for all individual equine, canine and human subjects.</td>
<td></td>
</tr>
<tr>
<td>Figure 3.8</td>
<td>Inter-species comparison of ethane output at baseline and following maximal exercise.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Schematic representation of breathing assembly for real-time measurement of ethane output.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Real-time raw ethane concentration signal from the spectrometer before and during incremental exercise to exhaustion in one male subject (pilot test one).</td>
<td></td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Real-time raw ethane concentration signal during an incremental exercise test to exhaustion in one male subject (pilot test one).</td>
<td></td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Mean ethane concentration prior to, during and following an incremental exercise test to exhaustion in two male subjects.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Mean ethane output prior to, during and following an incremental exercise test to exhaustion in two male subjects.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Responses to a ventilatory challenge in one female subject (pilot test three).</td>
<td></td>
</tr>
<tr>
<td>Figure 4.7</td>
<td>Responses to a ventilatory challenge in one male subject (pilot test four).</td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.8 Oxygen uptake response to a ventilatory challenge in one female subject (top panel) and one male subject (bottom panel).

Figure 4.9 Real-time raw ethane concentration signal from the spectrometer prior to, during and following incremental exercise to exhaustion in one male subject.

Figure 4.10 Real-time raw ethane concentration signal during an incremental exercise test to exhaustion in one male subject.

Figure 4.11 Real-time ethane concentration signal from the spectrometer during an incremental exercise test to exhaustion in one male subject.

Figure 4.12 Mean ethane concentration prior to, during and following an incremental exercise test to exhaustion.

Figure 4.13 Mean ethane output prior to, during and following an incremental exercise test to exhaustion.

Figure 5.1 Pentane production versus exercise intensity in six individuals. Subjects exercised for 20 minutes at each intensity. A post-exercise value was recorded following 20 minutes of rest. Modified from Dillard et al. (1978).

Figure 5.2 Schematic representation of the constant load test protocol and blood sampling points.

Figure 5.3 MDA response to incremental cycle ergometer exercise to volitional exhaustion in two male subjects.
Figure 5.4  F$_2$-isoprostane concentration prior to, during and following incremental cycle ergometer exercise to volitional exhaustion in six male subjects.

Figure 5.5  MDA response to constant load cycle ergometer exercise at sub- and supra-lactate threshold work rates in two male subjects.

Figure 5.6  F$_2$-isoprostane concentration prior to, during and following constant load cycle ergometer exercise at a sub-lactate threshold work rate in six male subjects.

Figure 5.7  F$_2$-isoprostane concentration prior to, during and following constant load cycle ergometer exercise at a supra-lactate threshold work rate in five male subjects.

Figure 6.1  A typical calibration plot for the handgrip dynamometer.

Figure 6.2  Experimental set-up for the recording of force during isometric contraction of the flexor digitorum muscles.

Figure 6.3  Ethane output at rest and during passive recovery from a sustained isometric contraction at 40 % maximal voluntary contraction in one male subject.

Figure 6.4  Ethane output at rest and during passive recovery from a sustained isometric contraction at 40 % maximal voluntary contraction in one female subject.

Figure 6.5  Ethane output at rest and during passive recovery from a sustained isometric contraction at 60 % maximal voluntary contraction in one male subject.
Figure 6.6  Ethane output at rest and during passive recovery from a sustained isometric contraction at 60 % maximal voluntary contraction in one female subject.

Figure 7.1  Schematic representation of the sustained contraction trial protocol.

Figure 7.2  Typical force output during a maximal voluntary contraction trial in one male subject.

Figure 7.3  Typical force output during a sustained contraction trial at 60 % maximal voluntary contraction in one male subject.

Figure 7.4  Mean oxygen uptake response of five subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

Figure 7.5  Ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in six male subjects.

Figure 7.6  Mean ethane output response of five male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

Figure 7.7  Relationship between mean ethane output and oxygen uptake in five male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.
Figure 7.8  F₂-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in the exercised and non-exercised arm in six male subjects.

Figure 7.9  Mean F₂-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in the exercised arm (EX; upper panel) and non-exercised arm (N-EX; lower panel).

Figure 7.10  Relationship between F₂-isoprostane concentration in the exercised arm versus the non-exercised arm in four male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

Figure 7.11 Panel 1. F₂-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in two male subjects.

Figure 7.11 Panel 2. F₂-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in two male subjects.

Figure 7.11 Panel 3. F₂-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in one male subject.
Figure 7.12  Mean $F_2$-isoprostane response in the non-exercised arm and mean ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

Figure 7.13  Relationship between mean ethane output and $F_2$-isoprostane concentration in the non-exercised arm in six male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.
# List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 3.1</td>
<td>Subject characteristics for study one.</td>
<td>146</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Mean ambient ethane concentration during each incremental exercise test.</td>
<td>148</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Mean resting ethane concentration and ethane output in each subject prior to an incremental exercise test.</td>
<td>149</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Subject characteristics for real-time pilot tests.</td>
<td>184</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Timing of expired air samples in relation to test phase for ventilatory challenge tests.</td>
<td>190</td>
</tr>
<tr>
<td>Table 5.1</td>
<td>Summary of methodology of previous studies relating oxidative stress to various sub-maximal exercise intensities.</td>
<td>211</td>
</tr>
<tr>
<td>Table 5.2</td>
<td>Summary of methodology of previous studies relating oxidative stress to incremental exercise to exhaustion.</td>
<td>212</td>
</tr>
<tr>
<td>Table 5.3</td>
<td>Summary of methodology of previous studies relating oxidative stress to constant load exercise.</td>
<td>213</td>
</tr>
<tr>
<td>Table 5.4</td>
<td>Subject characteristics for study two.</td>
<td>232</td>
</tr>
<tr>
<td>Table 5.5</td>
<td>Work rates measured at peak exercise and estimated lactate threshold, and calculated for sub- and supra-lactate threshold constant load exercise.</td>
<td>233</td>
</tr>
<tr>
<td>Table 5.6</td>
<td>Estimated lactate threshold and oxygen uptake responses to sub- and supra-lactate threshold constant load tests.</td>
<td>234</td>
</tr>
</tbody>
</table>
Table 5.7  F₂-isoprostane response prior to, during and following incremental cycle ergometer exercise to volitional exhaustion.  

Table 5.8  F₂-isoprostane response prior to, during and following constant load cycle ergometer exercise at a sub-lactate threshold work rate.  

Table 5.9  F₂-isoprostane response prior to, during and following constant load cycle ergometer exercise at a supra-lactate threshold work rate.  

Table 6.1  Subject characteristics for isometric pilot tests.  

Table 7.1  A summary of the methodology and main findings of previous studies of oxidative stress associated with static isometric handgrip exercise.  

Table 7.2  Subject characteristics for study three.  

Table 7.3  Maximal voluntary contraction attempts for each subject.  

Table 7.4  Sustained isometric handgrip contraction at 60 % maximal voluntary contraction.  

Table 7.5  Mean ambient ethane concentration during each sustained contraction trial.  

Table 7.6  Mean resting ethane concentration and ethane output in each subject prior to sustained isometric exercise.  

Table 7.7  F₂-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.
Acknowledgements

I would like to express my sincere and grateful thanks to the following individuals, without whom this work could not have been completed.

For academic and technical support:
Dr Andy Cathcart; Dr Carrie Ferguson; Dr Niall MacFarlane; Dr Lesley McMillan; Dr Claire Patterson; Dr Ken Skeldon; Professor Susan Ward; Mr John Wilson and Dr Cathy Wyse.

To all subjects for the time and effort given to participation in these studies; and to the Faculty of Biomedical and Life Sciences for funding this work.

To my family: my Mum and Dad, Alastair and Betty; my brother Neil; my partner Andy; and my friend Neil, for their unwavering support.

To Andy’s family, and to all my friends and colleagues, for their support and a right good laugh when needed.
Author's declaration

The experimental work presented here was conducted by myself, aided by academic and technical staff where necessary, with the exception of the experiments involving horses and dogs reported in Chapter 3, which were kindly conducted by Dr Cathy Wyse of the Division of Companion Animal Science, University of Glasgow. The written composition was my own work.

To date, one publication has been generated from this work as follows:


Rona Sutherland

13th August 2010
### List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>$^{1}$O$_2$</td>
<td>singlet oxygen</td>
</tr>
<tr>
<td>8-iso-PGF$_{2\alpha}$</td>
<td>8-iso-Prostaglandin$_{2\alpha}$</td>
</tr>
<tr>
<td>8-OhdG</td>
<td>8-hydroxy-deoxyguanosine</td>
</tr>
<tr>
<td>$\dot{\theta}_L$</td>
<td>estimated lactate threshold</td>
</tr>
<tr>
<td>$\mu\dot{V}$O$_2$</td>
<td>peak oxygen uptake</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ATPS</td>
<td>ambient temperature and pressure, saturated with water vapour</td>
</tr>
<tr>
<td>B$_f$</td>
<td>breathing frequency</td>
</tr>
<tr>
<td>BTPS</td>
<td>body temperature and pressure, saturated with water vapour</td>
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<td>C$_2$H$_6$</td>
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<td>C$<em>5$H$</em>{12}$</td>
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<td>catalase</td>
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<td>CCl$_4$</td>
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<tr>
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<td>cyclic guanosine monophosphate</td>
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<tr>
<td>-CH$_2$-</td>
<td>methylene group</td>
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<td>-C*H-</td>
<td>lipid radical</td>
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<td>CoQ$_{10}$</td>
<td>coenzyme Q$_{10}$</td>
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<td>CoQ$_{10}$H$_2$</td>
<td>ubiquinol (reduced coenzyme Q$_{10}$)</td>
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<td>copper zine superoxide dismutase</td>
</tr>
<tr>
<td>e$^-$</td>
<td>electron</td>
</tr>
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<td>ECG</td>
<td>electrocardiogram</td>
</tr>
</tbody>
</table>
EIOS  exercise-induced oxidative stress
EIPH  exercise-induced pulmonary haemorrhage
ELISA enzyme-linked immunosorbent assay
ESR  electron spin resonance
Fe$^{2+}$  ferrous ion
Fe$^{3+}$  ferric ion
FECO$_2$  fractional expired carbon dioxide concentration
FE0$_2$ fractional expired oxygen concentration
FICO$_2$ fractional inspired carbon dioxide concentration
FI0$_2$ fractional inspired oxygen concentration
GC-MS gas chromatography-mass spectrometry
GPx  glutathione peroxidase
GR  glutathione reductase
GSH  reduced glutathione
GSSG  glutathione disulphide
H$^+$  hydrogen ion
H$^*$  hydrogen radical
H$_2$CO$_3$  carbonic acid
H$_2$O  water
H$_2$O$_2$  hydrogen peroxide
HCFA  hydrocarbon-free air
HCO$_3^-$  bicarbonate ion
HLa  lactic acid
HO-1  heme oxygenase-1
HOCI  hypochlorous acid
HPLC high performance liquid chromatography
HR  heart rate
<table>
<thead>
<tr>
<th>Abbreviation</th>
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</tr>
</thead>
<tbody>
<tr>
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<td>maximum heart rate</td>
</tr>
<tr>
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<td>immunoglobulin G</td>
</tr>
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<td>lipid radical</td>
</tr>
<tr>
<td>La^-</td>
<td>lactate ion</td>
</tr>
<tr>
<td>[La^-]_a</td>
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<td>lactate dehydrogenase</td>
</tr>
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</tr>
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<td>mtDNA</td>
<td>mitochondrial deoxyribonucleic acid</td>
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<tr>
<td>MVC</td>
<td>maximal voluntary contraction</td>
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<tr>
<td>N\textsubscript{2}</td>
<td>nitrogen</td>
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<td>nicotinamide adenine dinucleotide radical</td>
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<td>reduced nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NADP^+</td>
<td>oxidised nicotinamide adenine dinucleotide phosphate</td>
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<tr>
<td>NADPH</td>
<td>reduced nicotinamide adenine dinucleotide phosphate</td>
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<td>NaOH</td>
<td>sodium hydroxide</td>
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<tr>
<td>NF-κB</td>
<td>nuclear factor-kappa beta</td>
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<td>nitric oxide</td>
</tr>
<tr>
<td>NO\textsubscript{2}^•</td>
<td>nitrogen dioxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>O</td>
<td>atomic oxygen</td>
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<tr>
<td>O\textsubscript{2}</td>
<td>diatomic oxygen</td>
</tr>
<tr>
<td>O\textsubscript{2}^•</td>
<td>superoxide radical</td>
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<td>Description</td>
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<td>--------------------------------------------------</td>
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<tr>
<td>OCl⁻</td>
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<td>hydroxyl radical</td>
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<td>ONOO⁻</td>
<td>peroxynitrite</td>
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<td>Ox-LDL</td>
<td>oxidised low density lipoprotein</td>
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<tr>
<td>PETCO₂</td>
<td>end-tidal carbon dioxide tension</td>
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<tr>
<td>PETO₂</td>
<td>end-tidal oxygen tension</td>
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<td>water vapour pressure</td>
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<td>pNpp</td>
<td>p-nitrophenyl phosphate</td>
</tr>
<tr>
<td>PO₂</td>
<td>oxygen tension</td>
</tr>
<tr>
<td>ppb</td>
<td>parts per billion</td>
</tr>
<tr>
<td>ppm</td>
<td>parts per million</td>
</tr>
<tr>
<td>PUFAs</td>
<td>polyunsaturated fatty acids</td>
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<tr>
<td>R</td>
<td>respiratory exchange ratio</td>
</tr>
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<td>R⁺</td>
<td>lipid radical or conjugated diene</td>
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<tr>
<td>RAA</td>
<td>reduced ascorbic acid</td>
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<tr>
<td>RCH₂⁺</td>
<td>ethyl or pentyl radical</td>
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<tr>
<td>RH</td>
<td>polyunsaturated fatty acid (R denotes ‘rest of molecule’)</td>
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<td>RNS</td>
<td>reactive nitrogen species</td>
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<td>RO⁺</td>
<td>alkoxy radical</td>
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<td>RONS</td>
<td>reactive oxygen or nitrogen species</td>
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<td>ROO⁺</td>
<td>peroxyl radical</td>
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<tr>
<td>ROOH</td>
<td>lipid hydroperoxide</td>
</tr>
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<td>ROOH⁺</td>
<td>hydroperoxyl radical</td>
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<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>$S_aO_2$</td>
<td>arterial oxygen saturation</td>
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<tr>
<td>SBSW</td>
<td>single beam single wavelength</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>STPD</td>
<td>standard temperature and pressure (0°C and 760 mmHg), dry</td>
</tr>
<tr>
<td>TAC</td>
<td>total antioxidant capacity</td>
</tr>
<tr>
<td>TBARS</td>
<td>thiobarbituric acid reactive substances</td>
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<td>total glutathione</td>
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<td>TH$^*$</td>
<td>tocopherol radical</td>
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<td>TH$_2$</td>
<td>tocopherol</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>$\dot{V}C_2H_6$</td>
<td>ethane output</td>
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<td>$\dot{V}CO_2$</td>
<td>carbon dioxide output</td>
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<tr>
<td>$\dot{V}E$</td>
<td>expired minute ventilation</td>
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<tr>
<td>$\dot{V}I$</td>
<td>inspired minute ventilation</td>
</tr>
<tr>
<td>$\dot{V}O_2$</td>
<td>oxygen uptake</td>
</tr>
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<td>$\dot{V}O_2$ max</td>
<td>maximum oxygen uptake</td>
</tr>
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<td>tidal volume</td>
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<tr>
<td>WR</td>
<td>work rate</td>
</tr>
<tr>
<td>XDH</td>
<td>xanthine dehydrogenase</td>
</tr>
<tr>
<td>XO</td>
<td>xanthine oxidase</td>
</tr>
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</table>
Chapter 1

Introduction
1.1 An introduction to oxidative stress

Oxidative stress has emerged as a concept having significant physiological implications. The term was first introduced in 1985 (Sies, 1985), although it was not until later that it was formally defined as an imbalance between the production of reactive species, such as free radicals, and the availability of antioxidant defences (Sies, 1991). However, the oxidative stress paradigm has been present in the literature for longer than the term itself, as the presence of free radicals in biological materials was first reported much earlier, in 1954 (Commoner et al., 1954).

A link between oxidative stress and several chronic diseases has been indicated (e.g. Jain, 2006) and, indeed, the topic has now entered the public domain as the focus of numerous health-related reports linking diet and exercise to lowered oxidative stress, with putative consequent health benefits. In general, individuals are assuming greater responsibility for their own health with the realisation that a healthy lifestyle can offer some protection against the major diseases of our time. The potential for improved health of the population, along with widespread interest in the topic, underlines this as a valuable area for further investigation.

1.1.1 The oxygen paradox

A comprehensive account of oxidative stress begins with a description of oxygen; the key molecule involved in the process. Oxygen (O) exists in ambient air as a diatomic molecule (O₂). The correct term for O₂ is dioxygen; however, it is most commonly referred to simply as oxygen in the majority of literature. It has been suggested that oxygen became available in ambient air in significant quantities over 2.5×10⁹ years ago due to the evolution of photosynthesis by blue-green algae (Graham et al., 1995). These organisms and, later, modern day plants, became capable of releasing oxygen through the process of splitting...
water. This release of oxygen over the centuries has created a present day composition of ambient air which includes approximately 21 % O$_2$. Aerobic organisms, including humans, developed the capacity to produce energy efficiently via the process of oxidation, thus taking advantage of the plentiful supply of oxygen available in the atmosphere.

The life sustaining quality of oxygen was originally discovered in the 18$^{th}$ century by Joseph Priestley (1775). He reported experiments in which he heated mercuric oxide and described the effects of the gas released:

“But to complete the proof of the superior quality of this air, I introduced a mouse into it; and in a quantity in which, had it been in common air, it would have died in about a quarter of an hour, it lived, at two different times, a whole hour, and was taken out quite vigorous;”

Humans and animals are reliant on oxygen for survival, however, paradoxically, oxygen also has well recognised toxic effects on the body (Halliwell & Gutteridge, 1984). The potential for damage from ambient oxygen arises from its propensity to convert from a relatively stable form (O$_2$) to an unstable form such as the superoxide radical (O$_2^-$).

Chemical elements each contain a specific number of electrons (e$^-$), dictated by the atomic number in a neutral atom. These are arranged in electron shells surrounding the nucleus of the atom. Within the electron shells, electrons are contained within orbitals. Atomic oxygen contains eight electrons. The inner shell, situated closest to the nucleus of the atom, has one orbital which contains two electrons. The remaining six electrons are situated in the outer shell which has four orbitals containing a maximum of two electrons each. Atoms are most stable when all orbitals in the outer shell hold the maximum number of electrons. If there are incomplete orbitals, the atom will tend to bond with other atoms or molecules.
in order to accept or share electrons. Thus, ambient oxygen tends to exist as a diatomic molecule as it shares electrons covalently with another oxygen atom.

Molecular bonding is governed by rules determining the order in which orbitals are filled. This occurs according to energy level; the orbital with the lowest energy level will be filled first. Orbitals having equivalent energy levels are filled according to Hund’s rule (Hund, 1925) such that each orbital must contain at least one electron before any orbital can contain a pair of electrons. As a result, the sixteen electrons in diatomic oxygen are arranged such that there are two unpaired electrons in separate orbitals.

Generally, atoms and molecules are most stable when there are no unpaired electrons, and the existence of two unpaired electrons in the structure of molecular oxygen makes it a keen electron acceptor. A gain of electrons, or of hydrogen, is known as the process of reduction. Whenever a reduction reaction takes place, there must also be a corresponding oxidation reaction since electrons accepted during reduction must be donated by another substance. Oxidation is defined as a loss of electrons or as a gain of oxygen. Thus, oxygen is a potent oxidising agent; it easily oxidises other substances and in the process becomes reduced.

Oxygen can accept a maximum of four electrons, in which case the final product is water (H₂O). However, according to Pauli’s exclusion principle (Pauli, 1925), electrons occupying the same orbital must have opposite spin to each other. The two unpaired electrons in diatomic oxygen have the same spin, often termed parallel spin. In order for oxygen to accept two electrons at once, the two donated electrons must both be of anti-parallel spin. A pair of electrons from another atom or molecule would not meet this criterion and, therefore, the complete reduction of oxygen tends to take place in stages with
the addition of one electron at a time. The univalent reduction of oxygen, i.e. the addition of one electron, results in the formation of the superoxide radical ($O_2^-$):

$$O_2 + e^- \rightarrow O_2^- \quad [1.1]$$

The addition of two electrons plus two hydrogen ions ($H^+$) results in hydrogen peroxide ($H_2O_2$) formation:

$$O_2^- + 2H^+ + e^- \rightarrow H_2O_2 \quad [1.2]$$

The addition of three electrons results in the formation of the hydroxyl radical ($OH^-$):

$$H_2O_2 + H^+ + e^- \rightarrow OH^- + H_2O \quad [1.3]$$

These derivatives of oxygen are highly reactive and can cause damage to body tissues.

A further one-electron reduction results in the formation of water:

$$OH^- + H^+ + e^- \rightarrow H_2O \quad [1.4]$$

The four-electron reduction of oxygen occurs in the electron transport chain of the mitochondria during oxidative phosphorylation. Cytochrome oxidase is the final enzyme in the chain and its structure is such that it can store four electrons and transfer these to molecular oxygen in a single step; thus, this process does not normally result in the formation of a large quantity of damaging species. However, it has been established (McCord & Turrens, 1994) that electrons can leak out of the chain and react with the
molecular oxygen available within the mitochondrion, thus causing the formation of potentially damaging oxygen derivatives via equations 1.1 to 1.3. This process, along with other potential mechanisms for the formation of these reactive species, will be described in more detail in a later section (section 1.3.3, page 83).

1.1.2 Oxidants

‘Oxidants’ is a collective term used to describe substances that may cause oxidative damage by oxidation reactions. Oxidants include free radicals as well as other highly reactive species which are non-radicals; all of which are typically oxygen or nitrogen based. ‘Reactive oxygen species’ (ROS) is a term that encompasses oxygen-based radicals and non-radicals, whereas reactive nitrogen species (RNS) are nitrogen-based radicals and non-radicals.

1.1.2.1 Free radicals

A free radical has been defined as any chemical species which is capable of independent existence and contains one or more unpaired electrons (Halliwell & Gutteridge, 1999). The presence of an unpaired electron causes a substance to be highly reactive and capable of oxidising a wide range of biological tissues. A free radical can be formed by the loss or gain of a single electron from a non-radical, or through the process of homolytic fission. This is a process in which breakage of a covalent bond results in the retention of one of the previously shared electrons by each atom. An example of this process is the homolytic fission of water which results in the formation of a hydrogen radical (H\(^{+}\)) and a hydroxyl radical (OH\(^{•}\)). This process can be distinguished from the ionisation, or heterolytic fission, of water in which both electrons from the shared pair are retained by the oxygen atom and the products of the reaction are a hydrogen ion (H\(^{+}\)) and a hydroxyl ion (OH\(^{-}\)). It should be
noted that the superscripted dot (•) following the chemical formula denotes the presence of an unpaired electron in an atom or molecule.

Molecular oxygen (O₂) is actually a free radical since it contains two unpaired electrons. Other examples of common free radicals are superoxide (O₂•⁻), hydroxyl (OH•), alkoxyl (RO•), peroxyl (ROO•) and hydroperoxyl (ROOH•) radicals, all of which are oxygen based. Nitric oxide (NO•) and nitrogen dioxide (NO₂•) are examples of nitrogen based free radicals.

1.1.2.2 Other reactive species

There are other chemical species in the body which function as oxidants yet are non-radicals; they do not contain unpaired electrons, however are, nevertheless, highly reactive. Examples of non-radical ROS are hydrogen peroxide (H₂O₂), hypochlorous acid (HOCl), singlet oxygen (¹O₂) and lipid peroxides. Peroxynitrite (ONOO⁻) is a non-radical ROS which could also be categorised as a non-radical RNS.

1.1.2.3 Formation of free radicals and reactive species

Free radicals and reactive species are formed by the donation or acceptance of electrons, that is, oxidation or reduction reactions. The formation of one radical tends to bring about the formation of more since any electron accepted in the formation of the initial radical must have been donated by another substance. If this substance was a non-radical initially, the loss of an electron would transform it into a radical. In addition, reactive species are, by nature, highly reactive and tend to react very quickly with other substances involving a transfer of electrons and the formation of further radicals. Specific examples of the formation of common reactive species are shown below.
Equations showing the reduction of molecular oxygen in the formation of superoxide radicals, hydrogen peroxide and hydroxyl radicals are shown above in equations 1.1, 1.2 and 1.3 respectively. However, reduction and oxidation must occur simultaneously and, therefore, in practical terms, the formation of superoxide radicals due to leakage of electrons in the mitochondria could occur as follows:

\[ 2O_2 + \text{NADPH} \rightarrow 2O_2^- + \text{NADP}^+ + H^+ \quad [1.5] \]

Hydrogen peroxide is formed via a superoxide dismutation reaction. Superoxide radicals react with each other; one superoxide radical is oxidised and the other is reduced:

\[ 2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad [1.6] \]

Hydroxyl radicals can be formed from hydrogen peroxide with the donation of an electron from a transition metal ion, generally ferrous iron or cuprous copper:

\[ H_2O_2 + Fe^{2+} \rightarrow OH^- + OH^+ + Fe^{3+} \quad [1.7] \]

Hydrogen peroxide can be utilised in the oxidation of chloride ions, to produce hypochlorous acid. This reaction is catalysed by the enzyme myeloperoxidase (MPO). In aqueous solution, hypochlorous acid partially dissociates into hypochlorite (OCl⁻) and H⁺.

\[ H_2O_2 + Cl^- \rightarrow OCl^- + H_2O \quad [1.8] \]
Nitric oxide is produced through the action of nitric oxide synthase (NOS) as shown in equation 1.9:

\[
\text{arginine} + \text{O}_2 + \text{NADPH} \rightarrow \text{NO}^\cdot + \text{citrulline} + \text{NADP}^+ \\
[1.9]
\]

Nitric oxide can be converted to nitrogen dioxide:

\[
2\text{NO}^\cdot + \text{O}_2 \rightarrow 2\text{NO}_2^\cdot \\
[1.10]
\]

Peroxynitrite is generated from the reaction of nitric oxide with the superoxide radical as shown in equation 1.11:

\[
\text{NO}^\cdot + \text{O}_2^- \rightarrow \text{ONOO}^- \\
[1.11]
\]

Alkoxyl, peroxyl and hydroperoxyl radicals along with lipid peroxides are organic molecules generally formed through the process of lipid peroxidation, to be described later (see section 1.1.4.1, page 51).

### 1.1.2.4 Reactivity

Different reactive species have varying levels of reactivity and toxicity. The most reactive cannot travel far before reacting with another molecule, causing the production of another species. For example, the hydroxyl radical is generally considered to be one of the most powerful species. It is highly reactive and reacts indiscriminately with, and causes damage to, any biological molecule. The superoxide radical is less reactive but still highly damaging since it reacts with specific targets. It is known to inactivate enzymes such as adenylate cyclase and creatine phosphokinase; thus it can detrimentally affect major metabolic pathways (McCord, 2000). The superoxide radical is also known to react with
lactate dehydrogenase (LDH), the enzyme which catalyses the conversion of pyruvate to lactate. This reaction is linked to the oxidation of the reduced form of nicotinamide adenine dinucleotide (NADH). The superoxide radical can react with NADH when it is bound to LDH to cause the formation of the $\text{NAD}^\cdot$ radical, thus interfering with the efficiency of the pyruvate to lactate conversion (Fridovich, 1986a). Superoxide can also inactivate the antioxidant enzymes catalase (CAT) and glutathione peroxidise (GPx) (Fridovich, 1986b), the functions of which will be described below (section 1.1.3.1, page 46). Hydrogen peroxide is a more stable molecule and is able to pass through cell membranes freely and diffuse some distance before further reaction; therefore, it can initiate further radical reactions and cause damage distant from its site of generation.

1.1.3 Antioxidants

Oxidants are produced persistently within the body, compelling the evolution of a wide range of antioxidant systems in an attempt to limit the damage. Antioxidants have been described as substances that can inhibit or delay substrate oxidation (Halliwell & Gutteridge, 1999). The knowledge of antioxidant function can be traced as far back as the ancient Egyptians who developed embalming methods for the preservation of cadavers using oil and plant extracts which functioned as antioxidants (Cross et al., 1987).

Antioxidants function in a number of ways to counteract the damaging effects of reactive species and each antioxidant generally acts specifically against one or more oxidants. These include removing or neutralising oxidants; converting reactive species into less damaging species; minimising the availability of substances required for the formation of reactive species, for example, transition metal ions; and protecting molecules from damage (Halliwell & Gutteridge, 1999).
1.1.3.1 Enzymatic antioxidants

Endogenous antioxidant systems can be enzymatic or non-enzymatic. Primary antioxidant enzymes are superoxide dismutase, catalase and glutathione peroxidase. The activity of all three enzymes can increase in response to increased oxidant production, for example, during exercise (Ji, 1999).

Superoxide dismutase (SOD) acts to remove superoxide radicals ($O_2^•−$) through the formation of hydrogen peroxide ($H_2O_2$) as shown previously in equation 1.6:

$$2O_2^•− + 2H^+ \rightarrow H_2O_2 + O_2 \quad [1.6]$$

This reaction will occur spontaneously and rapidly in the absence of SOD, however, the rate of reaction is substantially increased in the presence of the enzyme such that $O_2^•−$ concentration can be maintained at a low level. Spontaneous dismutation requires two molecules of $O_2^•−$, whereas enzyme-catalysed dismutation requires only one; thus the rate of spontaneous dismutation would be greatly reduced at low $O_2^•−$ concentration. The dismutation of the superoxide radical prevents its reaction with nitric oxide to produce peroxynitrite, which is more toxic than the hydroxyl radical.

In human cells, there are two SOD isoenzymes characterised by their location within the cell and by the metal ion bound to the active site: in general, copper-zinc SOD (CuZnSOD) is located in the cytoplasm and to a small extent in extracellular fluid, whereas manganese SOD (MnSOD) is found in the mitochondria (Ji, 1995). In skeletal muscle, most of the SOD activity takes place in the cytosol and is greatest in oxidative muscle fibres (Powers & Lennon, 1999).
Catalase is involved in the elimination of hydrogen peroxide:

\[ 2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2 \quad [1.12] \]

The binding of Fe\(^{3+}\) to the active site on catalase is required for catalysis of the above reaction. Catalase is widely distributed within the cell, with a high concentration found in mitochondria and in peroxisomes (Chance et al., 1979). The activity of this enzyme is again greater in more oxidative muscle fibres (Powers & Lennon, 1999).

Glutathione peroxidase also catalyses the reduction of hydrogen peroxide to water, in this case coupled with the oxidation of glutathione (GSH) to glutathione disulphide (GSSG):

\[ 2\text{H}_2\text{O}_2 + 2\text{GSH} \rightarrow \text{GSSG} + 2\text{H}_2\text{O} \quad [1.13] \]

However, NADPH may also be utilised as the reducing species:

\[ \text{H}_2\text{O}_2 + \text{NADPH} + \text{H}^+ \rightarrow 2\text{H}_2\text{O} + \text{NADP}^+ \quad [1.14] \]

For GPx to continue to function, GSH must be regenerated from GSSG; this reaction is catalysed by the enzyme glutathione reductase (GR):

\[ \text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2\text{GSH} + \text{NADP}^+ \quad [1.15] \]

Catalase and GPx can reduce lipid hydroperoxides (LH) as well as hydrogen peroxide, thus inhibiting lipid peroxidation and protecting membrane structure and function. Lipid peroxidation will be described in detail in section 1.1.4.1 (page 51).
GPx is found in both cytosol and mitochondria, with greatest activity in type I muscle fibres (Powers & Lennon, 1999). Selenium is a required part of the structure of GPx and selenium deficiency dramatically decreases GPx activity in all tissues (Ji, 1995).

Human skeletal and cardiac muscle have fairly low antioxidant activities in comparison to other organs of the body and are, therefore, at higher risk of oxidative damage (Goldfarb, 1993).

1.1.3.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants include endogenous low-molecular-mass substances, for example glutathione and ubiquinol, or may come from dietary sources and include vitamin E, vitamin C, β-carotene and various phytochemicals.

Glutathione (GSH) is located intracellularly and functions as a cofactor for the antioxidant enzyme glutathione peroxidase. It also directly scavenges hydroxyl radicals and singlet oxygen (Yu, 1994). GSH is synthesised by the liver from endogenous or dietary amino acids (Ji, 1999) and it has been shown that hepatic production may increase during prolonged exercise (Lu et al., 1990). During exercise, GSH is oxidised to GSSG in skeletal and cardiac muscle during the breakdown of hydrogen peroxide as shown in equation 1.13 and GSSG can be reduced back to GSH by glutathione reductase as shown in equation 1.15.

Ubiquinone, also named coenzyme Q_{10} (CoQ_{10}) is a component of the electron transport chain. The reduced form, ubiquinol (CoQ_{10}H_{2}), is also present in the mitochondria and in lipid membranes and functions as an antioxidant (Frei et al., 1990). Other endogenous substances known to possess antioxidant activity are uric acid, a product of nucleic acid
metabolism; and α-lipoic acid, a cofactor for pyruvate dehydrogenase, which can also be taken as a dietary supplement (Ji, 1999).

The main dietary antioxidants that have been reported to act \textit{in vivo} are vitamin E, vitamin C and β-carotene.

Vitamin E describes a group of isomers of which α-tocopherol has the most potent antioxidant activity (Goldfarb, 1993). Alpha-tocopherol is lipid soluble and is located within the lipid bilayer of cell membranes and in the inner mitochondrial membrane. It acts to scavenge superoxide, hydroxyl and lipid peroxyl radicals and may break the cycle of the peroxidation process. Although it is present at low concentration, it is difficult to deplete: when vitamin E takes an electron from a free radical, thus neutralising the radical and becoming a vitamin E radical, it can be reduced back to vitamin E by vitamin C, GSH or ubiquinol (Thérond \textit{et al.}, 2000). Acute exercise does not significantly alter vitamin E concentration (Ji, 1999). Sources of vitamin E in the diet include oils such as sunflower oil and olive oil, and foods such as whole grains and green leafy vegetables.

Vitamin C (ascorbic acid) is water soluble and present in the cytosol and extracellular fluid (Ji, 1995). It can react directly with superoxide and hydroxyl radicals in the plasma to reduce damage to erythrocyte membranes (Beyer, 1994), and acts to scavenge peroxyl radicals, hypochlorous acid and singlet O$_2$ (Sies & Stahl, 1995). On reaction with one of these compounds, vitamin C is converted to dehydroascorbic acid, and can be recycled back to ascorbic acid by GSH (Packer, 1997). Vitamin C is present in many fruits and vegetables including red peppers and citrus fruits.

Beta-carotene is a precursor of vitamin A in humans and is thus sometimes termed provitamin A. It is present in lipid membranes and acts to scavenge superoxide and peroxyl
radicals (Powers & Lennon, 1999) and singlet oxygen (Ji, 1995). Beta-carotene is found in carrots and sweet potatoes.

Finally, various phytochemicals have been reported to display antioxidant activity. Phytochemicals are substances derived from plants; they are not required for normal physiological function but may be beneficial. Included are several groups of compounds including flavonoids and carotenoids. Flavonoids include quercitin found in green tea, garlic and red wine; and catechins found in green tea and in cocoa, the major constituent of dark chocolate. Carotenoids include β-carotene, and lycopene found in tomatoes. It has been suggested that the antioxidant effect of flavonoids may not be due to direct action of the substance itself since it is thought that they are poorly absorbed, and quickly metabolised and expelled from the body. Rather, it may be that the uric acid produced during the metabolism of flavonoids may be responsible for the antioxidant action (Lotito & Frei, 2006).

The antioxidant systems described, along with others which act alongside them, are normally adequate to prevent substantial tissue damage. However, an increased production of oxidants or a reduction in antioxidant defences can disturb the balance. Oxidative stress is typically described as a condition in which there is an imbalance between oxidant production and antioxidant capacity in favour of the former, with concomitant damage to body tissues.

1.1.4 Oxidative damage

Reactive species can cause damage to a wide range of organic molecules including lipids, nucleic acids and proteins. The damage can consist of structural change in the damaged molecule which may lead to disruption in the function of the molecule.
1.1.4.1 Lipid peroxidation

Lipid peroxidation has been defined as the oxidative deterioration of polyunsaturated lipids (Tappel, 1973). Polyunsaturated fatty acids (PUFAs), found mainly in cellular and subcellular membranes, but also in lipoproteins, are those which contain at least two carbon-carbon double bonds and it is these double bonds which confer susceptibility to peroxidation. A double bond weakens the adjacent carbon-hydrogen bond making hydrogen ions vulnerable to abstraction, and consequently oxidation. Free fatty acids, and cholesterol in membranes and lipoproteins are also susceptible to oxidative damage through lipid peroxidation.

Lipid peroxidation is a self-perpetuating process since it can be initiated by free radicals and the process itself causes the generation of further radicals. Free radical reactions in general tend to be chain reactions, since when a free radical reacts with a non-radical, the product will be a new radical molecule which can then interact with another non-radical and so on. Free radicals tend to be short-lived species due to their high reactivity and are likely to react with the first molecule which they contact. However, due to the formation of new radicals, the distance over which oxidative damage can occur is not limited to the immediate area of initial free radical generation. Thus, lipid peroxidation is a chain reaction. It occurs in three distinct stages: initiation, propagation and termination. A simplified schematic diagram of the peroxidation process is shown in figure 1.1. Equations are included for the initial stages of the process to emphasise the formation of new radicals.
Figure 1.1. Schematic diagram illustrating the lipid peroxidation process.
PUFA polyunsaturated fatty acid; R denotes rest of molecule; dashed arrows denote propagation of
the peroxidation process; shaded areas represent reaction products commonly used as markers of
lipid peroxidation. (Adapted from Dotan et al., 2004; Kneepkens et al., 1994).
The initiation stage involves the initial generation of a lipid free radical (-C\(^{\bullet}\)H-) by the abstraction of a hydrogen atom from a methylene group (-CH\(_{2}\)-) of a PUFA. This can be caused via attack by any species of sufficient reactivity, for example, the hydroxyl radical:

\[-\text{CH}_2^- + \text{OH}^{\bullet} \rightarrow -\text{C}^{\bullet}\text{H}^- + \text{H}_2\text{O}\]  \[1.16\]

The above equation could also be expressed as follows, where R denotes rest of molecule:

\[\text{RH} + \text{OH}^{\bullet} \rightarrow \text{R}^{\bullet} + \text{H}_2\text{O}\]  \[1.17\]

The propagation stage involves the production of new radicals from existing ones, by the donation of an electron to a non-radical, by the extraction of an electron from a non-radical or by a radical binding to a non-radical. The lipid radical generated in the initiation stage may react with molecular oxygen to form a peroxyl radical (ROO\(^{\bullet}\)), although at low O\(_2\) concentration two lipid radicals may react with one another to cause cross linking of fatty acid chains. These carbon-based radicals may be stabilised by molecular rearrangement resulting in the formation of conjugated dienes which can be used as a marker of lipid peroxidation. The peroxyl radicals formed at this step react with other lipid molecules to form lipid hydroperoxide (ROOH) and a further lipid radical. The process thus propagates as the newly formed lipid radical can react with oxygen or with an adjacent PUFA.

Lipid hydroperoxides may generate a variety of reaction products as shown in figure 1.1. The lipid hydroperoxide may interact with a transition metal to produce an alkoxyl radical (RO\(^{\bullet}\)) which may react with a PUFA to propagate the peroxidation process, or may undergo reaction to produce pentyl or ethyl radicals; these radicals can generate pentane or ethane respectively, which are end products of the peroxidation process. Other reaction
products include aldehydes and isoprostanes which will be described in more detail in later sections (section 1.2.2.3.1, page 64; section 1.2.2.3.2, page 66). The reaction products of the peroxidation process depend on the specific PUFA which is initially oxidised. Malondialdehyde, the most abundant aldehyde formed via lipid peroxidation, originates mainly from arachidonic acid as do the isoprostanes; pentane originates from linoleic acid and ethane from linolenic acid.

The majority of tissue damage occurs during the propagation stage. The peroxidation process is terminated by reactions between radicals to form stable molecules, or radicals are scavenged by antioxidants. An example of the termination of the peroxidation process by tocopherol (vitamin E) is shown below in equation 1.18. Tocopherol (TH₂) collides with a lipid radical (L⁺) and the tocopherol takes on the unpaired electron to become a tocopherol radical (TH⁺). The tocopherol radical can then be recycled back to tocopherol in order to scavenge further lipid radicals.

\[
L^+ + TH_2 \rightarrow LH + TH^+ \tag{1.18}
\]

Damage to lipid molecules by the peroxidation process can cause a reduction in membrane fluidity, changes in permeability and excitability, and altered function of membrane-bound enzymes (Vladimirov, 1986). In addition, the process of peroxidation results in the formation of toxic products; these may diffuse away from the site of peroxidation and cause further damage. Examples are malondialdehyde (MDA) which has been reported as a potential carcinogen (Schamberger et al., 1974) and mutagen (Bird & Draper, 1980) and isoprostanes, which can cause renal and pulmonary vasoconstriction (Praticó et al., 1995).
1.1.4.2 Nucleic acid damage

Reactive species can cause damage to DNA in the form of strand breaks in the deoxyribose-phosphate backbone of the molecule, DNA-protein cross-linking and base pair modification (Théond et al., 2000). Oxidative damage to DNA has been linked to the development of cancer (Cross et al., 1987) since oxidative lesions not repaired prior to replication can cause mutagenesis (Bjelland & Seeberg, 2003). It has been reported that mitochondrial DNA mutates at a faster rate than nuclear DNA and is less efficient at self-repair (Luft, 1995). It is at high risk of oxidative damage due to its location beside a major source of oxygen consumption and ROS production.

1.1.4.3 Protein damage

Oxidative damage to proteins can cause changes in their structure and function making them more prone to proteolysis. Proteins may be damaged indirectly via amino acid alteration or directly via denaturation, aggregation or glycation (Jenkins, 2000). Protein damage may lead to enzyme inactivation; for example, it is known that catalase and GPx are inactivated by superoxide (Fridovich, 1986a), and SOD is inhibited by H_2O_2 (Hodgson & Fridovich, 1975). Enzyme activity may also be altered by changes in membrane structure due to lipid peroxidation (Jenkins, 1988).

1.1.5 Implications of oxidative damage

Oxygen based radicals have been implicated in the pathogenesis of many major diseases including atherosclerosis, cancer, diabetes mellitus, ischaemia-reperfusion injury, rheumatoid arthritis and neurodegenerative disease (Dröge, 2002). A brief summary of the links between oxidative stress and atherosclerosis is included due to its relevance to exercise: physical inactivity is now a well established risk factor for coronary heart disease (Powell et al., 1987).
1.1.5.1 Atherosclerosis

Coronary heart disease (CHD) is the most common cause of death in the United Kingdom, affecting 21% of males and 15% of females in all age groups (Allender et al., 2006). The mortality rate from CHD is high compared to other European countries, and is highest within the UK in Scotland and Northern England (Allender et al., 2006). Most cases of CHD are caused by the development of atherosclerosis in one or more coronary arteries. Atherosclerosis is characterised by hardening and thickening of the arterial wall, due to the development of an atheroma, which eventually may progress sufficiently to cause blockage of the circulation with subsequent myocardial infarction. The development of atherosclerosis has been linked to oxidative stress (Hamilton et al., 2004) and specifically to the oxidative modification of low-density lipoprotein (LDL) cholesterol (Singh & Jialal, 2006). Under normal conditions, a regulated quantity of LDL is taken up by macrophages via the LDL receptor. However, when LDL has become highly oxidised (Ox-LDL), the structure of the molecule is modified to the extent that it is no longer recognised by the LDL receptor, and LDL is then taken up by macrophages via another set of receptors in a non-regulated manner. This leads to the presence of large quantities of Ox-LDL within macrophages, initiating the development of foam cells; an initial stage in the process of atherogenesis (Steinberg, 1997).

It has been reported that LDL becomes more susceptible to oxidation following strenuous exercise. This was shown by Liu and colleagues (1999) after a marathon race and it was noted that the effect persisted for four days following the race. Thus the effect of acute exercise on LDL oxidisability is a negative one. However, a previous study (Sanchez-Quesada et al., 1997) showed that LDL oxidisability was lower at rest in trained individuals in comparison to sedentary controls, suggesting that regular exercise may impart a protective effect.
1.1.5.2 Ageing

There is also a well acknowledged association between free radical damage and the ageing process (Cutler, 1986). The process of ageing involves changes in the body which, over time, accumulate to cause progressive functional degeneration, increased susceptibility to disease and increased probability of mortality (Dröge, 2002). The free radical theory of ageing, first proposed by Denham Harman in 1956, suggests that the accumulation of oxidative damage may be involved in the process of ageing (Harman, 1956). If the free radical theory of ageing is correct, and the ageing process is due to the effects of ROS, then a reduction in the formation of ROS or an increase in antioxidant capacity should retard the ageing process and lead to delayed mortality. Support for this hypothesis has been sought from numerous strands of research.

Initially, it was acknowledged from inter-species comparisons of longevity that an inverse correlation exists between metabolic rate and lifespan (Beckman & Ames, 1998). It was proposed that since the mitochondrial electron transport chain was a major source of reactive species, a higher metabolic rate may be consistent with a higher rate of oxidant production. It was found that some species lived longer than predicted by metabolic rate and these species generated reactive species at a lower rate per unit of mitochondrial oxygen consumption (Beckman & Ames, 1998), thus suggesting that lifespan was related more specifically to oxidant production than to metabolic rate.

Harman extended his free radical theory of ageing to suggest that the lifespan may be influenced mainly by the rate of oxidative damage to mitochondria, especially to mitochondrial DNA (mtDNA) (Harman, 1972). Due to the proximity of mtDNA to a major source of reactive species, it may be especially vulnerable to damage with potential subsequent damage to electron transport chain enzymes (Finkel & Holbrook, 2000). Evidence from skeletal muscle biopsies in young and older males has supported this view.
(Gianni et al., 2004). These authors reported greater oxidative damage to DNA and protein, compensatory upregulation of mitochondrial antioxidant enzymes and a greater frequency of mtDNA deletions in ageing muscle. However, they found no functional deficiency in the aged muscle, suggesting some redundancy in function or a capability to adapt to protect against mitochondrial damage.

It has been suggested that certain antioxidants may help to determine longevity; for example, Cutler (1991) reported a correlation between tissue concentrations of SOD, carotenoids, α-tocopherol, uric acid and lifespan in several mammalian species, including humans. This hypothesis has been corroborated by data which have shown that several species have a greater average life expectancy when antioxidants are added to the diet (Harman, 1981). For example, the worm, Caenorhabditis elegans was supplemented with synthetically produced SOD and catalase mimetics, that is, substances which function in a similar manner to these antioxidant enzymes. The mimetics were originally tested on a strain of worm which suffered from a mutation which caused accelerated ageing due to accumulation of oxidative damage. Treatment with the mimetics restored a normal lifespan to these worms. Non-mutated worms treated with the mimetics were found to have a 44% greater lifespan than untreated worms suggesting that oxidative damage may be a key factor in the determination of lifespan (Melov et al., 2000).

Another line of research has involved caloric restriction which increases lifespan in several species. For example, rats fed a calorie restricted diet lived 43% longer than control rats fed ad libitum (Sohal et al., 1994). It was proposed that this effect was due to reduction in oxidative stress in the restricted rats since it was found that there was less accumulation of oxidative damage products in major organs.
Despite the substantial amount of supporting evidence, the free radical theory of ageing has been criticised by Howes (2006) who reviewed the lack of consistent evidence of an effect of antioxidant supplementation on disease outcome in numerous large controlled trials.

1.1.6 The physiological roles of reactive species

Thus far, only the damaging effects of reactive species have been considered. However, although excessive concentrations of oxidants may be harmful, it has been suggested that more moderate concentrations may have useful physiological roles (Dröge, 2002). A well known example is the oxidative burst which occurs within phagocytic cells. Phagocytes generate many different reactive species to destroy invading pathogens as part of the immune response. This will be described further in a later section (section 1.3.3.3, page 86).

Reactive species may also function in communication; for example, the superoxide radical is useful as a signalling molecule in normal physiological processes such as cell division and proliferation (McCord, 2000); the hydroxyl radical activates guanylate cyclase, the enzyme which catalyses the production of the second messenger cyclic guanosine monophosphate (cGMP) (Mittal & Murad, 1977), which mediates, for example, relaxation of vascular smooth muscle. Nitric oxide also stimulates vascular smooth muscle relaxation and inhibits platelet adhesion (Radomski et al., 1987).

Finally, gene expression may be regulated to some extent by the redox status of the internal environment (McCord, 2000). Redox homeostasis is the situation where the production of reactive species and antioxidant capability is matched such that there is no damaging increase in oxidative species. When oxidant production does increase, for example in exercise or trauma, redox homeostasis can be restored after a brief increase in oxidant concentration. However, during this time, the increase in specific reactive species may
signal increased gene expression. For example, it has been reported that hydrogen peroxide induces the expression of the heme oxygenase-1 (HO-1) gene (Keyse & Tyrell, 1989). Finally, there is evidence that reactive species may signal adaptive changes which protect against further oxidative stress (Jackson, 1999). Nuclear factor kappa beta (NF-κB) is activated during exercise and functions to upregulate the expression of antioxidant enzymes such as SOD and catalase. It has been suggested that the activation of NF-κB may be associated with oxidative stress during exercise (Vider et al., 2001a).

1.2 Assessment of oxidative stress

A wide variety of different techniques have been employed to assess oxidative stress in vivo in previous research. Only one of these methodologies actually directly measures free radical activity; most assess oxidative stress indirectly by measuring by-products of free radical damage or by measurement of a reduction in antioxidant capacity.

1.2.1 Direct measurement of free radicals

Free radical species are difficult to measure directly in vivo due to their highly reactive and transient nature, and low steady-state concentrations. There is one technique currently available, that of electron spin resonance (ESR), which does attempt to measure these species directly. The basic premise of the technique is the detection of unpaired electrons. It is limited in that it lacks the sensitivity to detect the more highly reactive species such as $O_2^*$ and $OH^*$; however, this limitation can be overcome to some extent with the use of spin trapping techniques. These involve the addition of a molecular species to the system under study which can react with the free radical to form a more stable and longer-lived compound which can then accumulate to a concentration which can be detected by ESR. A limitation of the trapping technique is that the addition of a trapping molecule can perturb
the system under investigation, for example, by functionally neutralising the free radical so that the level of oxidant activity in the system may be reduced artificially.

ESR has not been widely used in humans in vivo to date; there are two reports in the literature of the use of ESR in relation to the assessment of exercise-induced oxidative stress (Ashton et al., 1998; Ashton et al., 1999). Ashton and colleagues (1998) reported the first use of ESR in combination with the spin trapping technique in relation to exhaustive aerobic exercise in humans. Free radical production by ESR was measured from samples of venous blood drawn prior to and following a maximal incremental test on a cycle ergometer. A post-exercise increase in free radical production was demonstrated, supported by an increased lipid peroxidation measured by malondialdehyde assay and lipid hydroperoxides. The same authors (Ashton et al., 1999) observed a similar outcome utilising an identical experimental protocol in a later study.

1.2.2 Indirect assessment of oxidative stress

Most researchers have not utilised direct measurement of free radicals, but rather have assessed oxidative stress indirectly by measuring by-products of oxidative damage. For this type of technique to be valid, it is necessary that substances measured are markers only of oxidative damage and are not produced by normal physiological processes. Most indirect techniques are invasive and rely on markers found in blood or serum samples, or in a minority of cases in muscle tissue samples (Child et al., 1999; Hellsten et al., 1996). However, there are also non-invasive techniques available which utilise samples of expired air or urine.

Although free radicals can cause damage to nucleic acids, proteins, lipids, carbohydrates and some low molecular mass antioxidants, for example ascorbic acid or uric acid, most studies investigating oxidative stress in relation to exercise have focussed on damage to
lipids via the process of lipid peroxidation. This is likely due to the relative ease of assessment. However, there are exceptions, and a small number of studies have assessed exercise-induced oxidative damage to proteins or nucleic acids.

1.2.2.1 Assessment of nucleic acid damage

Urinary 8-hydroxy-2’-deoxyguanosine (8-OHdG) is the most commonly used marker of DNA oxidation. Guanine is the most easily oxidised of the four DNA bases and repair of damage to this base results in the formation of 8-OHdG which is water-soluble and is excreted in the urine without further metabolism (Wu et al., 2004). However, this technique has been criticised since increased urinary 8-OHdG may reflect both enhanced repair as well as increased whole body DNA oxidation attributable to other sources, such as a diet high in nucleic acids and DNA released following cell death (Cooke et al., 2002). Oxidative damage to DNA may also be measured using single-cell gel electrophoresis, also known as the comet assay (Singh et al., 1988). This technique allows the measurement of single strand breakage in nuclear DNA. Damaged cells display increased migration of chromosomal DNA out of the nucleus in a shape resembling a comet; however, assessment of damage level is somewhat subjective. An additional limitation is that, again, damage is not specific to oxidation.

Few studies have investigated urinary 8-OHdG output following exercise. Elevated levels, approximately 1.3-fold above baseline, were reported 10 hours following a marathon race (Alessio & Cutler, 1990), whilst Tsai and colleagues (2001) reported an immediate increase of approximately two-fold above baseline following a 42 km run with levels remaining elevated significantly for seven days. However, some studies have reported no change, for example, following distance running (Inoue et al., 1993) or three consecutive days of moderate intensity cycling (Viguie et al., 1993). It has been suggested that DNA damage is associated with prolonged, higher intensity exercise such as marathon running.
rather than more moderate endurance exercise (Alessio, 1993). Some exercise studies have assessed DNA damage using the comet assay, again with conflicting findings (Mastaloudis et al., 2004a; Hartmann et al., 1994). Interestingly, some studies utilised both methods and reported no significant correlation between the two, perhaps reflecting differences in what each technique is measuring; in the case of 8-OHdG, possible damage to muscle and increased cell turnover, whereas the comet assay is limited to individual cells (Hartmann et al., 1998; Tsai et al., 2001).

### 1.2.2.2 Assessment of protein damage

Oxidation of amino acid side chains by reactive species results in the formation of protein carbonyls (Griffiths, 2000). The assessment of protein carbonyls as an indicator of protein oxidation has been criticised as non-specific and unreliable in vivo (Urso & Clarkson, 2003). For example, carbonyl groups may also be introduced into proteins by reaction with aldehydes during the process of lipid peroxidation (Davies et al., 1999). However, they are still considered to be a more stable marker than malondialdehyde, which has been one of the most commonly utilised markers of oxidative stress (Davies et al., 1999). Protein carbonyls have been reported to increase following cycle ergometer exercise at 70 % maximum oxygen uptake ($\dot{V}O_2$ max), and protein oxidation increased with exercise duration (Bloomer et al., 2007). The same author reported increased protein carbonyls following anaerobic exercise, both sprinting and barbell squats (Bloomer et al., 2006). However, not all studies have reported a significant change. For example, Alessio and colleagues (2000) found no change in protein carbonyls following intermittent isometric exercise. It is likely that inconsistencies in findings are due to differences in exercise protocols.
1.2.2.3 Invasive assessment of lipid peroxidation

Lipid peroxides are unstable compounds which tend to break down rapidly. Resultant products can be more stable and, therefore, can be measured in blood, urine or expired air for assessment of the extent of lipid peroxidation. There are a variety of markers available for the invasive assessment of lipid peroxidation associated with exercise; these include malondialdehyde (MDA), isoprostanes, conjugated dienes and lipid hydroperoxides. However, none of the above markers are ideal, as described in the following sections, and for this reason it has been suggested that two or more different techniques should be utilised within any study (Halliwell & Gutteridge, 1999).

1.2.2.3.1 Malondialdehyde

MDA is formed during lipid peroxidation of PUFAs, mainly arachidonic acid and docosahexaenoic acid (Esterbauer et al., 1991). It has been the most commonly used marker of lipid peroxidation (Urso & Clarkson, 2003), however, its use has been widely criticised.

MDA has been assessed historically by the thiobarbituric acid reactive substances (TBARS) assay. Plasma or tissue samples are heated with thiobarbituric acid (TBA) at low pH and any MDA present reacts to form a coloured product (TBARS) which absorbs light at 532 nm. Following this reaction, a fluorometric or spectrophotometric assay can be carried out in order to determine the quantity of TBARS. The test is easy to use which likely explains its popularity in the literature; however, there are several limitations. Most TBARS detected may be formed by the decomposition of lipid peroxides during the acid heating stage of the assay; peroxide decomposition can be accelerated by iron salts in reagents used in the TBA test and generates RO$_2^*$ radicals which can oxidise more lipid (Gutteridge, 1986), thus over-reporting the true extent of lipid peroxidation. TBA will also react with molecules other than MDA, for example, sugars, amino acids and bilirubin and
it is not possible to distinguish between different initial reactants, therefore, TBARS may over-state the extent of MDA production. Consequently, it may be more accurate to assay MDA directly by high performance liquid chromatography (HPLC) or gas chromatography-mass spectrometry (GC-MS). It has been suggested that TBARS may overestimate the true extent of MDA production by at least ten-fold in comparison to measurement by GC-MS (Yeo et al., 1994).

Even so, other limitations remain: MDA is not solely a product of lipid peroxidation. It is also produced as a result of cyclooxygenase activity in platelets (Hamberg et al., 1975). Platelet activation is reportedly increased during exercise (Kratz et al., 2006), and many disease states associated with enhanced lipid peroxidation tend to feature persistent platelet activation (Meagher & Fitzgerald, 2000). Therefore, in such cases, it would be unclear how much of the resultant MDA was due to lipid peroxidation.

It has been suggested that the TBARS assay may be considered as a screening test to suggest the presence of lipid peroxidation, but should not be used as a convincing quantitative measure without confirmation by other markers of lipid peroxidation (Kneepkens et al., 1994). Direct assessment of MDA by HPLC is preferable.

The vast majority of exercise studies have utilised MDA as a marker of lipid peroxidation, using either technique, and results have been variable (Vollaard et al., 2005). The two studies by Ashton and colleagues (1998, 1999) utilised ESR as a direct measure of free radical production following exercise and also measured MDA by HPLC. Both methods showed a significant increase in lipid peroxidation, giving support to the use of MDA as an indirect marker.
1.2.2.3.2 F₂-Isoprostanes

F₂-isoprostanes are structurally similar to prostaglandin-F₂ and are considered to be specific and stable markers of lipid peroxidation *in vivo* (Banfi *et al.*, 2006). They are reaction products of free radical-induced peroxidation of phospholipids containing arachidonic acid. A commonly studied isoprostane is 15-Isoprostane-F₂. Only very small amounts of 15-Isoprostane-F₂ are generated by cyclooxygenase activity, the common route for production of prostaglandins from arachidonic acid; the overwhelming majority of its production is mediated by free radical activity, thus it is well suited to investigation of oxidative stress (Morrow & Roberts, 1997). F₂-isoprostanes can be measured in detectable concentrations in plasma or tissue by GC-MS or enzyme-linked immunosorbent assay (ELISA). The former may be the preferred technique since it is considered to be highly accurate, specific and sensitive (Roberts & Morrow, 2000); however, problems with ELISA regarding the presence of substances, such as proteins, in the sampled fluid which may interfere with the assay can be overcome to some extent by purification of the sample prior to assay. Isoprostanes have become more frequently used as a marker of lipid peroxidation in exercise studies in recent years (Vollaard *et al.*, 2005).

1.2.2.3.3 Conjugated dienes

The oxidation of PUFAs is accompanied by the formation of conjugated dienes; these are lipid radicals which have undergone molecular rearrangement to a more stable structure which includes a double-single-double bond configuration. The most abundant conjugated diene in human plasma is 9, 11-linoleic acid (Kneepkens *et al.*, 1994). These are formed early in the process of lipid peroxidation and their presence is generally accepted as evidence that lipid peroxidation has taken place (Recknagel & Glende, 1984). They absorb ultraviolet (UV) light in the 230-235 nm wavelength range and a measure of this UV absorbance can be used as a measure of lipid peroxidation in *ex vivo* samples of pure lipids and isolated lipoproteins. A limitation with this technique is that it is not specific for the
products of lipid peroxidation when applied to tissues or body fluids since there are other substances present which also absorb the same wavelength of UV light, for example, haem proteins. Another limitation is that formation of conjugated dienes continues *ex vivo* following sampling and therefore may not reflect the extent of lipid peroxidation *in vivo* (Dormandy & Wickens, 1987). Therefore, conjugated dienes may not be an adequate marker of lipid peroxidation *in vivo* (Jenkins, 2000). Indeed, they have been used rarely in exercise studies, and then, often in combination with another marker of lipid peroxidation (Duthie *et al.*, 1990; Kanter & Eddy, 1992; Marzatico *et al.*, 1997; Vider *et al.*, 2001b).

### 1.2.2.3.4 Lipid hydroperoxides

Conjugated dienes form lipid hydroperoxides and peroxyl radicals in the presence of O$_2$. These are unstable in the presence of transition metal ions and decompose to a variety of reaction products (Jenkins, 2000), thus are not end products of the process of lipid peroxidation. Measurement requires formation of a fluorescent or coloured reaction product or they can be assessed indirectly via MDA which is a secondary reaction product. Again this is a rarely used marker of lipid peroxidation (Vollaard *et al.*, 2005).

### 1.2.2.4 Non-invasive assessment of lipid peroxidation

Non-invasive assessment of by-products of the peroxidation process is also possible. Lipid peroxidation *in vivo* can be assessed by the measurement of volatile hydrocarbon gases such as ethane and pentane in the expired air (Kneepkens *et al.*, 1994). Both gases are terminal reaction products of the peroxidation process; ethane and pentane are derived from the peroxidation of ω-3 and ω-6 polyunsaturated fatty acids respectively (Kivits *et al.*, 1981). Justification for the use of expired hydrocarbons to assess lipid peroxidation is based on *in vitro* and *in vivo* animal studies which have shown that evolution of ethane and pentane correlate very well with other markers of lipid peroxidation, however, a direct
A comparison of exhaled hydrocarbons and other markers of lipid peroxidation has not yet been undertaken in humans in vivo (Kneepkens et al., 1994).

Both ethane and pentane can be measured by gas chromatography, however, this can be time-consuming and requires a flawless technique; for example, due to the extremely low concentrations of ethane and pentane in expired air, which are often not significantly greater than those in ambient air, it is important to avoid contamination of expired air samples with ambient air (Kneepkens et al., 1994). Potential limitations of the hydrocarbon breath test include formation of hydrocarbons from sources other than lipid peroxidation and the dependence of hydrocarbon formation on the availability of transition metal ions. These issues will be reviewed in detail in chapter 3. More recently, ethane has been measured by laser spectroscopy (Gibson et al., 2002), a technique which has several advantages including ease of use and rapid analysis times. Therefore, this technique would be well-suited to the non-invasive assessment of exercise-induced oxidative stress, however, has not been utilised previously in exercise studies.

The first usage of ethane as a marker of lipid peroxidation in vivo was reported by Riely and colleagues in 1974. They firstly demonstrated that lipid peroxidation in vitro, as measured by MDA formation, was linked to the formation of ethane. Carbon tetrachloride (CCl₄), a known oxidant, was then administered to intact mice and ethane production, not present in control animals, was observed. An early exercise study by Dillard and colleagues (1978) used pentane as a marker of lipid peroxidation to investigate the effect of exercise intensity on oxidative stress in cycle ergometer exercise. However, very few studies since have utilised either ethane (Leaf et al., 1997, 1999, 2004) or pentane (Kanter et al., 1993; Leaf et al., 1997; Pincemail et al., 1990).
Finally, the assessment of aldehydes or F2-isoprostanes in the urine are other non-invasive approaches to the assessment of lipid peroxidation in vivo, however, these would be impractical for time-dependent assessment such as the investigation of oxidative stress in exercise.

1.2.2.5 **Assessment of antioxidant capacity**

Individual antioxidants and antioxidant enzymes have been measured following exercise. The most commonly used index is the ratio of reduced to oxidised glutathione (GSH/GSSG). As described previously, reduced glutathione acts as an antioxidant and in doing so is itself oxidised. Thus the ratio of the two substances gives some indication of the oxidative insult which the reduced glutathione is helping to overcome, along with the other antioxidants present in the sample. Other antioxidants which have been studied individually include α-tocopherol and ascorbic acid (Vollaard et al., 2005), and the antioxidant enzymes superoxide dismutase, glutathione peroxidase and glutathione reductase (Urso & Clarkson, 2003).

Total antioxidant capacity (TAC) has also been used in the assessment of oxidative stress (Cao et al., 1993). This is a measure of the overall protection against oxidative stress provided by all antioxidants in the sample acting together. There are several assays available; all are based on the addition of a free radical generator to the sample of interest, followed by a measure of the sample’s ability to protect against oxidation of the antioxidants present in the sample. Oxygen radical absorbance capacity (ORAC) has been suggested as one of the better assays since the oxidation reactions are allowed to run until all the antioxidant present has been oxidised and this allows the assessment to take into account the fact that some antioxidants are faster acting than others (Prior & Cao, 1999). However, results from TAC assays do not always match those of other measures of oxidative stress (Han et al., 2000).
1.3 Exercise-induced oxidative stress

Exercise-induced oxidative stress (EIOS) refers to a condition in which the balance between oxidant production and antioxidant defences is disturbed in favour of oxidants during exercise. Numerous studies have investigated the effect of exercise on oxidative stress, however, a wide variety of exercise protocols and markers of oxidative stress have been utilised, which, to some extent, has hindered understanding in this area. Studies have included both aerobic and anaerobic exercise modalities and these will be reviewed separately.

1.3.1 Aerobic exercise

Studies which have utilised aerobic exercise can be subdivided according to the type of exercise protocol used. Some have employed an incremental exercise test to exhaustion on treadmill or cycle ergometer, some have utilised a sub-maximal constant load exercise protocol, again on treadmill or cycle ergometer, and the remainder have utilised a range of other aerobic activities including competitive endurance events. Most studies have assessed oxidative stress via markers of lipid peroxidation with only a few measuring nucleic acid or protein oxidation.

1.3.1.1 Maximal exercise

Several studies have assessed lipid peroxidation in response to maximal incremental exercise using plasma markers, and have produced varying results. Some have reported a significant increase in lipid peroxidation following exercise (Ashton et al., 1998, 1999; Bailey et al., 2001; Jammes et al., 2005; Leaf et al., 1997; Lovlin et al., 1987; Szcześniak et al., 1998; Steinberg et al., 2006, 2007; Vider et al., 2001b), whereas others have reported no significant change (Alessio et al., 2000; Hartmann et al., 1995; Jammes et al., 2004; Kretzschmar et al., 1991; Leaf et al., 1999; Niess et al., 1996; Quindry et al., 2003;
Sen et al., 1994; Sürmen-Gür et al., 1999; Viinikka et al., 1984). It should be noted that the two studies by Ashton and colleagues (1998, 1999) directly measured free radical production in addition to indirect markers of lipid peroxidation, strengthening the evidence for the reported exercise-induced oxidative stress.

Of the studies which showed no increase in lipid peroxidation following exercise, two studies did report other evidence of oxidative stress in the form of DNA damage measured 24 hours following cessation of exercise (Hartmann et al., 1995; Niess et al., 1996). Alessio and colleagues (2000) showed a non-significant increase in MDA and LH and suggested that their study lacked statistical power due to a small sample size. Sen and colleagues (1994) also reported a non-significant increase following maximal incremental exercise, but also a significant increase following 30 minutes of constant load exercise at two submaximal work rates. The constant load tests were of a longer duration than the incremental test, suggesting that exercise duration may be an important factor. This will be discussed further later (see Chapter 5). Thus, oxidative stress induced by maximal exercise has not been reported in every circumstance, but appears to be a commonly observed feature.

1.3.1.2 Submaximal exercise

Several studies have utilised a submaximal exercise protocol in the investigation of exercise-induced oxidative stress. Constant load exercise has been reported to increase oxidative stress assessed by lipid peroxidation (Alessio et al., 1997; Børshheim et al., 1999; Kanter et al., 1993; Laaksonen et al., 1999; Pincemail et al., 1990; Sen et al., 1994; Waring et al., 2003). In these studies, duration and intensity of exercise ranged from 20 minutes of cycle ergometry at 50 % \( \dot{V}O_2 \) max (Balke et al., 1984) to 90 minutes at 58 % \( \dot{V}O_2 \) max (Børshheim et al., 1999), and 30 minutes of treadmill running at 60 % \( \dot{V}O_2 \) max (Kanter et al., 1993) to 30 minutes at 80 % \( \dot{V}O_2 \) max (Alessio et al., 1997).
Some studies showed no significant increase in oxidative stress following constant load exercise (Chung et al., 1999; Kanaley & Ji, 1991; Lovlin et al., 1987; Morillas-Ruiz et al., 2005; Quindry et al., 2003; Watson et al., 2005). Two studies reported no change in oxidative stress following treadmill exercise for 30 min at 75-80 \( \dot{V}O_2 \) max (Chung et al., 1999), and for 90 minutes at 60 % \( \dot{V}O_2 \) max (Kanaley & Ji, 1991), both comparable protocols to the studies described above which did report post-exercise oxidative stress. These were the only two constant load studies which utilised female subjects and it was suggested by Chung and colleagues (1999) that this disparity in results may be due to gender. However, several other studies with male subjects have also reported no increase in oxidative stress following similar exercise protocols.

In a number of these studies, the data were inconclusive. For example, Lovlin and colleagues (1987) investigated 5 minutes of constant load exercise at both 40 % and 70 % of \( \dot{V}O_2 \) max. The data indicated an unexpected significant reduction in lipid peroxidation from baseline following exercise at the lower intensity and a non-significant reduction after the higher intensity. Therefore, although post-exercise oxidative stress was reduced, the pattern was for greater oxidative stress at higher intensity. However, again it may be that exercise duration is important and the duration here may have been too short to elicit a significant oxidative stress. Another study (Watson et al., 2005) also indicated a tendency towards reduced oxidative stress after 30 minutes of treadmill running at 60 % \( \dot{V}O_2 \) max. The resting measurement may have been spuriously elevated in both studies and the reduction in oxidative stress with exercise may simply have reflected a return towards baseline, however, this was not possible to ascertain from the available information. Both groups of authors suggested that peroxidation may have been inhibited due to increased antioxidant activity. This is substantiated to some extent by the findings of the latter study (Watson et al., 2005) in which subjects were divided into two groups; one group ate their habitual diet whilst the second group ate a diet restricted in antioxidants. The restricted
group showed significantly higher lipid peroxidation following submaximal exercise than the habitual diet group suggesting that the availability of antioxidants had a significant effect on the extent of lipid peroxidation.

Finally, two studies reported no exercise-induced increase in lipid peroxidation, however did show some evidence of other oxidative activity. Morillas-Ruiz and colleagues (2005) reported no change in lipid peroxidation and a non-significant increase in protein oxidation following 90 minutes of cycle ergometry at 70 % $\dot{V}O_2$ max. The authors suggested that the exercise intensity may not have been sufficiently high to elicit a significant oxidative stress in their trained subjects; however, a significant increase in DNA oxidation was indicated. Therefore, results were inconclusive.

The findings of Quindry and colleagues (2003) were similarly unclear. They reported no change in lipid peroxidation following 45 minutes of exercise below or above the lactate threshold. However, the authors did suggest an oxidative stress effect on the basis of neutrophilia and increased superoxide formation following supra-lactate threshold exercise. However, it is not clear that these markers are actually indicative of oxidative damage. It may be that antioxidant defences were adequate to combat the increase in superoxide generated from neutrophils such that no lipid peroxidation occurred.

Thus, overall, there is considerable ambiguity in relation to the oxidative stress response to submaximal constant load exercise.

1.3.1.3 Other aerobic activities

The third group of aerobic studies are heterogeneous in terms of exercise protocol. The findings, again, were inconsistent. Twelve previous studies have investigated oxidative stress following distance running. Most studies indicated an increase in oxidative stress at
distances of 10 km (Vasankari et al., 1995), half marathon (21 km) (Child et al., 1998, 2000; Marzatico et al., 1997), 27 km (Vasankari et al., 1995), 31 km (Vasankari et al., 1997), marathon (Hessel et al., 2000; Liu et al., 1999), 50 km (Mastaloudis et al., 2001, 2004b) and 80 km (Kanter et al., 1988; Nieman et al., 2002). Only two authors noted no change in lipid peroxidation following 21 km (Duthie et al., 1990) and marathon distance (Vasankari et al., 1997). Three studies investigated the effect of prolonged running for 2.5 hours (Dufaux et al., 1997; Steensberg et al., 2002) or 3 hours (McAnulty et al., 2003) on lipid peroxidation. Two of these studies indicated a post-exercise rise in lipid peroxidation (McAnulty et al. 2003; Steensberg et al., 2002) with no change in the other study (Dufaux et al., 1997). Exercise intensity and duration was not comparable between all three studies and this may be responsible for the discrepancy in results. For example, a significant increase in lipid peroxidation was found at an intensity of 75 % \( \dot{V}O_2 \text{max} \) for 2.5 hours (Steensberg et al., 2002), whereas no change was found at a running speed of 53-82 % of the previously estimated speed at a lactate concentration of 4 mmol·l\(^{-1}\) which was maintained for 2.5 hours (Dufaux et al., 1997). However, the finding of increased oxidative stress following distance running is fairly consistent.

Three studies used a downhill running protocol; this type of exercise involves eccentric muscle contractions which can cause muscle damage (Friden et al., 1983) which has been associated with increased free radical production (Aoi et al., 2004). A study by Maughan and colleagues (1989) found some increase in oxidative stress following 45 minutes of downhill running. Subjects with the greatest increases in creatine kinase and lactate dehydrogenase, both markers of muscle damage, also showed the greatest increase in lipid peroxidation, suggesting a potential relationship between muscle damage and oxidative stress. However, this hypothesis is not consistent with the findings of Margaritis and colleagues (1997) who reported no change in oxidative stress following triathlon competition, although muscle damage was evident as shown by an increased inflammatory
response. Duthie and colleagues (1990) also indicated muscle damage with no concomitant lipid peroxidation following a half marathon run. Sacheck and colleagues (2000) found no change in oxidative stress in trained individuals following 45 minutes of downhill treadmill running at 75 % \( \dot{V}O_2 \) max. They also found no difference between subjects consuming a low fat or high fat diet. Vitamin E bioavailability is thought to be affected by fat intake (Takanami, 2000), however, both groups of subjects appeared to have adequate antioxidant capacity to prevent exercise-induced oxidative stress. A later study by the same authors (Sacheck et al., 2003) found some increase in markers of lipid peroxidation in both young and older untrained subjects following a similar protocol as the previous study: 45 minutes of downhill running at 75 % predicted maximum heart rate (HR max). It may be that the different training status may have been responsible for the difference in findings between these two studies.

Some studies have utilised triathlon competition as a potential instigator of exercise-induced oxidative stress. The first of these indicated no significant increase in lipid peroxidation following triathlon competition in highly trained individuals (Margaritis et al., 1997) and the authors suggested that the lack of oxidative damage in these subjects may have been due to a protective effect provided by their training status. However, another study (Knez et al., 2007) reported significantly increased lipid peroxidation following both a half triathlon and a full triathlon, both in highly trained subjects.

Two studies have investigated the effect of intermittent exercise (Kingsley et al., 2005; Thompson et al., 2001). Both studies found a significant increase in lipid peroxidation following a 90 minute exercise protocol designed to mimic multiple sprint sports in the form of an intermittent shuttle run test (Thompson et al., 2001), or to mimic the demands of soccer match play followed by a multi-stage shuttle test to exhaustion (Kingsley et al.,
2005). Finally, one study (Maxwell et al., 1993) found no change in oxidative stress following a 60 minute period of box-stepping.

1.3.1.4 Summary of aerobic exercise-induced oxidative stress

In summary, it appears that there is an exercise-induced oxidative stress associated with aerobic exercise, however, this is not a consistent finding and it remains unclear exactly the nature of the exercise required to induce this response. Some studies have utilised very strenuous exercise and have reported no significant lipid peroxidation (e.g. Margaritis et al., 1997), whilst others have used a much lower exercise stress in terms of intensity and duration and have reported significant oxidative stress (e.g. Balke et al., 1984). Discrepancies in results are likely related to the wide variety of modes, intensities and durations of exercise, the training status of the subjects, and potentially also the time points at which samples were drawn in relation to the end of exercise.

Few studies have attempted to characterise the response in terms of exercise duration or intensity by investigating more than one intensity or duration within a single study to make comparison meaningful. Exceptions are studies by Sen and colleagues (1994) and Quindry and colleagues (2003), both of whom compared sub-lactate threshold and supra-lactate threshold constant load exercise. The former study (Sen et al., 1994) compared 30 minutes of exercise at 50 % and 77 % \( \dot{V}O_2 \) max and found a significant increase in lipid peroxidation from baseline at the lower intensity with a further rise at the higher intensity. The latter study (Quindry et al., 2003) found no change in lipid peroxidation at intensities of 10 % below and above the lactate threshold (55 and 72 % \( \dot{V}O_2 \) max respectively). This study also investigated the effect of energy expenditure on lipid peroxidation and in doing so utilised a protocol which included exercise at an intensity of 10 % below lactate threshold for two separate durations. These were 30 minutes as the standard duration for comparison with the higher exercise intensity, and also 60 minutes which was the duration
required to match the energy expenditure of 45 minutes of exercise at the higher intensity; however, no change in lipid peroxidation was seen in either trial. Thus, there is a paucity of information in this area and it would be useful to more clearly determine any effect of intensity and duration.

In some studies subjects were sedentary or untrained, whilst in others subjects were endurance trained; however, there did not appear to be any pattern in findings according to training status of the subjects. Niess and colleagues (1996) studied both trained and untrained volunteers and found that, even though there was no significant increase in lipid peroxidation following maximal exercise, lipid peroxidation was significantly lower in trained subjects in comparison to untrained subjects both at rest and post-exercise. The authors reported a significant increase in DNA damage 24 hours following the exercise session and this too was lower in trained subjects, suggesting that trained individuals may possess greater capacity to prevent oxidative stress. It has been suggested that endurance training can increase antioxidant capacity (Miyazaki et al., 2001) and it may be that trained individuals can maintain a higher relative exercise intensity without oxidative damage than untrained individuals.

The timing of post-exercise blood sampling may have an impact on the finding of significant lipid peroxidation. Some studies of incremental exercise to exhaustion which have shown a significant oxidative stress have recorded oxidative stress only at peak exercise with no further samples collected (Ashton et al., 1998, 1999; Bailey et al., 2001; Lovlin et al., 1987; Szcześniak et al., 1998). However, some studies measured oxidative stress at multiple time points during recovery. These studies have recorded the greatest effect at different time points following exercise. These have included peak exercise (Vider et al., 2001b), 5 minutes into recovery (Jammes et al., 2004; Steinberg et al., 2006, 2007) and 10 minutes into recovery (Jammes et al., 2005). Oxidative stress generally returned to
baseline within 20 to 30 minutes into the recovery period (Jammes et al., 2005; Steinberg et al., 2007; Vider et al., 2001b). Therefore, peak oxidative stress may not always occur at peak exercise, in which case, some studies which have measured oxidative stress only at peak exercise may have missed a further increase during the recovery period. For example, Alessio and colleagues (2000) may have missed a significant effect since lipid peroxidation was measured at peak exercise and not again until 1 hour into the recovery period. Other studies may have been similarly affected (Hartmann et al., 1995; Kretzschmar et al., 1991; Leaf et al., 1999; Quindry et al., 2003; Sen et al., 1994; Sürmen-Gür et al., 1999; Viinikka et al., 1984).

Another major limitation in the previous literature is the almost complete lack of oxidative stress assessment during exercise. The vast majority of studies have measured oxidative stress at baseline and then at various time points following the cessation of exercise. The few exceptions include studies which have assessed oxidative stress at lactate threshold in addition to peak exercise during maximal incremental exercise (Jammes et al., 2004, 2005; Leaf et al., 1997, 1999; Steinberg et al., 2006, 2007). Only one of these studies found a significant increase in lipid peroxidation at lactate threshold (Leaf et al., 1997). No previous studies have assessed oxidative stress at multiple time points during exercise, however, it would be of interest to determine at what point during exercise oxidative stress begins to increase.

1.3.2 Anaerobic exercise

Fewer studies have investigated exercise-induced oxidative stress associated with anaerobic exercise, although, again, studies may be subdivided according to mode and have included isometric, sprint and resistance exercise.
1.3.2.1 Isometric exercise

Isometric exercise protocols have varied: some have utilised static isometric contractions and some have utilised intermittent isometric contractions, held for a specific period or to exhaustion. The oxidative stress response to static isometric exercise has been studied by one group of authors in three separate investigations (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006). A static isometric handgrip contraction was sustained at a specified intensity, either at 50 % of maximal voluntary contraction (MVC) (Steinberg et al., 2004; Steinberg et al., 2006) or at 60 % MVC (Dousset et al., 2002) until fatigue. All three studies reported a significant rise in oxidative stress immediately following cessation of exercise. This was measured by a rise in plasma TBARS in all three studies and by a decrease in plasma reduced ascorbic acid (RAA) (Dousset et al., 2002; Steinberg et al., 2006) or erythrocyte GSH (Steinberg et al., 2004; Steinberg et al., 2006). The pattern of recovery was slightly different. In two studies (Steinberg et al., 2004; Steinberg et al., 2006), oxidative stress was reported to reach a peak, as reflected by all assessment methods, at 5 minutes of recovery with a return to baseline at 20 minutes of recovery in one study (Steinberg et al., 2006). The other study did not report recovery values at the 20 minute time point. The other study (Dousset et al., 2002) measured oxidative stress only at 20 minutes of recovery and produced some conflicting data. Plasma RAA had returned to baseline by 20 minutes of recovery similar to the later studies, however plasma TBARS indicated that peak oxidative stress occurred at 20 minutes of recovery. In summary, it appears clear that static isometric exercise at the intensities studied leads to increased oxidative stress during recovery; however, the time course of the increase is less established.

The oxidative stress response to isometric exercise has also been studied using intermittent protocols. For example, Sahlin et al. (1992) investigated isometric knee extension exercise performed at an intensity of 30 % MVC. Each contraction was sustained for 10 s followed
by 10 s relaxation for a total period of 80 minutes, or until exhaustion. Results indicated no significant increase in oxidative stress as measured by plasma MDA. It was suggested that the intensity of contraction may have been too low to elicit change in the measured variables. A threshold for oxidative stress has been indicated for aerobic exercise (Leaf et al., 1997; Lovlin et al., 1987), however, this has not been explored for isometric exercise. Three previous studies have involved intermittent handgrip exercise. Alessio et al. (2000) investigated the oxidative stress response to isometric handgrip exercise at 50 % MVC using a 45 s contraction / 45 s relaxation cycle to fatigue. There was a significant increase in lipid hydroperoxides on cessation of exercise, the level remaining high for up to one hour after exercise. Steinberg et al. (2002) reported a significant rise in lipid peroxidation immediately after intermittent isometric handgrip exercise to exhaustion. Finally, Rodriguez and colleagues (2003) found significant lipid peroxidation immediately following 60 seconds of maximal intermittent handgrip exercise in which the working muscles were made ischaemic. Thus again, the findings for intermittent isometric exercise are fairly consistent.

1.3.2.2 Sprint exercise

Oxidative stress following sprint exercise has been investigated in a small number of studies (Cuevas et al., 2005; Groussard et al., 2003; Inal et al., 2001; Marzatico et al., 1997). Two studies utilised a 30 second Wingate test as a supramaximal exercise stimulus (Cuevas et al., 2005; Groussard et al., 2003). In the former study (Cuevas et al., 2005) oxidative stress was assessed at several time points during recovery from a single Wingate sprint in trained cyclists. There was no significant change in TBARS at any time point post-exercise. GSH was reduced and GSSG/GSH ratio was increased, indicating oxidative stress, for up to 2 hours into recovery and these changes were accompanied by an increase in nuclear factor-κB (NF-κB) activation. In the latter study (Groussard et al., 2003) an increase in free radical production, measured by electron spin resonance spectroscopy, was
reported at all sampling points during recovery, however was only significantly increased at 20 minutes post-exercise. Lipid peroxidation, as measured by TBARS, was reduced following exercise, significantly so at 20 minutes and 40 minutes into recovery. The authors suggested that TBARS may not be a suitable marker of oxidative stress for high intensity exercise since the reduction could possibly reflect a clearance of MDA from the plasma, and cited other studies which had reported a similar reduction in MDA or TBARS following maximal exercise (Leaf et al., 1997; Margaritis et al., 1997; Rokitzki et al., 1994). However, one study did show an increase in MDA between 6 and 48 hours following six 150 m sprints in trained individuals (Marzatico et al., 1997). Finally, Inal and colleagues (2001) reported a decrease in GSH following a 100 m swim sprint.

1.3.2.3 Resistance exercise

The effect of resistance exercise on oxidative stress has been investigated by several authors. Increased lipid peroxidation was found 6 hours and 24 hours following three sets of strenuous whole body resistance exercise (McBride et al., 1998). Submaximal resistance exercise consisting of one circuit of ten exercises was associated with a significant increase in conjugated dienes in untrained subjects only; there was no significant change for trained subjects, again suggesting a protective effect of training (Ramel et al., 2004). MDA was not increased in either group of subjects, adding to the doubt as to the suitability of this marker. Other studies reported no change in lipid peroxidation following incremental exercise to exhaustion on a cycle ergometer followed by twenty maximal knee extensions (Sürmen-Gür et al., 1999); two hours of whole body resistance exercise (McAnulty et al., 2005b); and intermittent dumbbell squatting (Bloomer et al., 2005), although in this study protein oxidation was significantly increased post-exercise. The variety of exercise protocols has likely contributed to the inconsistency of results for this mode of exercise.
1.3.2.4 Comparative studies

Several authors have evaluated aerobic and anaerobic exercise in the same subjects (Alessio et al., 2000; Bloomer et al., 2005; Magalhães et al., 2007). Bloomer and colleagues (2005) compared 30 minutes of cycle ergometry with 30 minutes of intermittent dumbbell squatting, both performed at 70% of maximum capacity. Protein oxidation was increased in both modes, however was found to be greater for squatting. One study compared indoor climbing, which consists of static and intermittent isometric work, with treadmill running over the same duration and at the same percentage of maximal oxygen uptake for each activity (Magalhães et al., 2007). Oxidative stress was again found to be higher for the anaerobic activity. Alessio and colleagues (2000) compared treadmill running with intermittent isometric exercise both of which were performed to fatigue. Again, oxidative stress was found to be higher following isometric exercise. However, no firm conclusion can be reached since the two modes of exercise may not be directly comparable. For example, Magalhães and colleagues (2007) found that other physiological indices of exercise stress such as heart rate, blood lactate concentration and ventilation were significantly higher during climbing in comparison to running even though the mean oxygen uptake was the same.

In summary, the majority of studies have reported an increase in oxidative stress following a period of acute exercise, both aerobic and anaerobic, and this abundance of evidence seems to support the concept of exercise-induced oxidative stress regardless of the mode and volume of exercise. However, limitations in understanding still remain regarding the intensity and duration of exercise required to elicit oxidative stress. Several authors have stated that oxidative stress results particularly from high intensity exercise (Ashton et al., 1998; Cooper et al., 2002; Vincent et al., 2004), however, some studies have shown significant oxidative stress following moderate intensity exercise (Børsheim et al., 1999;
Kanter et al., 1993; Laaksonen et al., 1999). The time course of the oxidative stress response also remains to be better characterised.

1.3.3 Mechanisms of exercise-induced oxidative stress

There have been several mechanisms put forward in an attempt to explain the exercise-induced oxidative stress. The main hypotheses will be described briefly.

1.3.3.1 Mitochondrial production of reactive species

It has been widely accepted that superoxide radicals can be formed in the mitochondria due to the leakage of electrons from the electron transport chain (Ji, 1999). Oxygen is readily available within the mitochondria for use in oxidative phosphorylation and can be reduced by the leaked electrons to form superoxide radicals as follows:

\[ \text{O}_2 + e^- \rightarrow \text{O}_2^- \]  \hspace{1cm} [1.1]

It has been estimated that 1-2 % of oxygen uptake by the mitochondria results in the formation of superoxide radicals at rest (Boveris & Chance, 1973), and complex I and III have been identified as sites of leakage (Chance et al., 1979).

During exercise, oxygen uptake in active muscle can be increased by up to a factor of one hundred in maximal exercise (Meydani & Evans, 1993), and a greater flux through the electron transport chain may be expected to give rise to an increase in superoxide production. Superoxide radicals are normally scavenged by MnSOD within the mitochondria; however, an exercise-induced increase in production may overwhelm antioxidant defences and lead to oxidative stress. It has been assumed that superoxide production will increase in proportion to oxygen consumption due to a greater flux of electrons through the electron transport chain (Davies et al., 1982; Kanter, 1994; Urso &
Clarkson, 2003), however, *in vitro* evidence exists to contradict this assertion. For example, it has been reported that free radical production is actually lower at high oxygen uptakes as found in exercise compared to the lower oxygen uptakes at rest (Chance *et al*., 1979).

It has been suggested that increased oxygen uptake ($\dot{V}O_2$) during exercise may not be exclusively responsible for exercise-induced oxidative stress. Alessio and colleagues (2000) compared isometric and aerobic exercise and found that oxidative stress was higher following isometric exercise despite a lower $\dot{V}O_2$. They concluded that the source of this oxidative stress could not simply be increased mitochondrial production due to enhanced electron flux. This conclusion was supported by a study which found greater oxidative stress, as measured by electron paramagnetic resonance spectroscopy, in hypoxic exercise compared to normoxic exercise despite a lower overall $\dot{V}O_2$. The authors put forward the suggestion that decreased mitochondrial oxygen tension (PO$_2$) may trigger increased radical production during exercise since the increase in lipid peroxidation was associated with reduced arterial oxygen saturation and not increased $\dot{V}O_2$ (Bailey *et al*., 2000; Bailey, 2001). It has been suggested that the relationship between radical formation and mitochondrial PO$_2$ is U-shaped such that radical formation is highest at very low and very high PO$_2$ (Vollaard *et al*., 2005). Intracellular PO$_2$ has been shown to fall at higher work rates (above 60% of maximum work capacity) but then remain steady right up to maximal exercise (Richardson *et al*., 2001). It remains likely that superoxide production via the electron transport chain contributes towards the exercise-induced oxidative stress but may play a more minor role than originally suggested. In addition, in very high intensity exercise mitochondrial damage may cause uncoupling of electron transfer and increased leakage (Ji, 1999) contributing to the greater oxidative stress seen at higher intensity exercise (Lovlin *et al*., 1987).
A secondary source of exercise-induced free radical production may be linked to the increased release of catecholamines during exercise. One function of these substances is to increase oxidative metabolism in skeletal muscle via β-adrenergic activation, thus potentially increasing superoxide production in the mitochondrial electron transport chain. Support for this idea comes from a study in which lipid peroxidation was shown to be lower in subjects who had received the β-blocker propranolol in comparison to those who had received a placebo prior to acute cycle ergometer exercise (Pincemail et al., 1990). Catecholamines may also undergo auto-oxidation with concomitant free radical production (Ji, 1999).

1.3.3.2 Haem proteins

The haem proteins oxyhaemoglobin and oxymyoglobin may also undergo autoxidation with subsequent superoxide radical generation (Cooper et al., 2002). The iron in haemoglobin and myoglobin is in the ferrous form (Fe$^{2+}$). This can be easily oxidised to the ferric form (Fe$^{3+}$) with generation of the superoxide radical as follows:

$$\text{Fe}^{2+} + \text{O}_2 \rightarrow \text{Fe}^{3+} + \text{O}_2^- \quad [1.19]$$

This results in the formation of methaemoglobin or metmyoglobin, that is, the ferric form of each protein. These can react with peroxides to form further reactive species which may then initiate lipid peroxidation (Reeder & Wilson, 2001). The rate of haemoglobin autoxidation has a bell-shape relationship with PO$_2$ such that autoxidation is highest in the mid-range of approximately 40-80 % haemoglobin saturation (Balagopalakrishna et al., 1996). This is the opposite effect to that already described for mitochondrial PO$_2$. Arterial haemoglobin saturation is approximately 100 % during both rest and exercise, suggesting that the rate of haem autoxidation would be low. However, a lower PO$_2$ in venous and capillary blood, and a reduction in PO$_2$ during exercise may increase lipid peroxidation via
this mechanism. It has been suggested that at high exercise intensity, haemoglobin saturation can reduce further with a subsequent reduction in haem autoxidation (Vollaard et al., 2005).

### 1.3.3.3 Activated phagocytes

Several reactive species are produced and released by phagocytes such as neutrophils and macrophages during the respiratory burst; a component of the immune response. Superoxide radicals are produced *via* the action of phagocytic NADPH oxidase. This enzyme is activated when the cell is stimulated, for example by a pathogen, and O$_2$•⁻ is released into the extracellular fluid as well as into the intracellular vesicle containing the phagocytosed material. A number of reactions can then occur. Superoxide can be dismutated to produce hydrogen peroxide, which can be utilised in the oxidation of chloride ions to produce hypochlorous acid. Other highly damaging reactive species which are produced during the respiratory burst include nitric oxide, produced through the action of inducible nitric oxide synthase; hydroxyl radicals formed from the reduction of hydrogen peroxide by ferrous or cuprous ions; peroxynitrite formed by the reaction of nitric oxide with the superoxide radical; and singlet oxygen generated from hydrogen peroxide and hypochlorite.

An increase in circulating neutrophils has been reported following exercise and is thought to be associated with an increase in catecholamine and cortisol concentrations in the circulation (Pyne, 1994). There is an immediate and transient rise in circulating neutrophil count which is likely to be due to increased catecholamine concentration which can increase demargination of neutrophils from the vascular endothelium. A delayed increase in neutrophils, several hours after the cessation of exercise is likely due to increased cortisol concentration which induces the release of mature neutrophils from bone marrow.
These neutrophils may then be activated to produce reactive species by exercise-induced muscle damage or by the presence of other reactive species (Meydani & Evans, 1993). They migrate to the site of damage, for example, the working muscles, and release reactive species. This is akin to the acute phase inflammatory response designed to remove any debris associated with muscle damage or invading pathogens, however, the release of reactive species by the activated neutrophils may cause secondary oxidative damage (Ji, 1999).

Several studies have investigated production of reactive species by neutrophils following exercise, however results have been inconsistent with some reporting an increase, some a decrease and some no change (Suzuki et al., 1996). It was suggested that this variability may have been due to differences in the intensity and duration of exercise studied, and to the sampling points chosen. A regular finding was that of a delayed response following the end of short-term exercise, for example, Hack and colleagues (1992) reported increased neutrophil activation 24 hours following the cessation of a 20 minute bout of exhaustive exercise. However, production of reactive species by neutrophils tended to be evident quickly following prolonged exercise, for example, Hessel and colleagues (2000) measured an increase in free radicals generated by neutrophils 10 minutes into recovery following a marathon race. Thus it takes time for neutrophils to migrate and become activated suggesting that this may not be a major source of oxidative stress in short-term exercise, however, may contribute during the recovery period and during more prolonged exercise (Ji, 1999).

1.3.3.4 Xanthine oxidase

Another potential source of free radical production is the xanthine oxidase pathway. Xanthine oxidase (XO) is an enzyme which catalyses the oxidation of hypoxanthine to xanthine and xanthine to uric acid as part of the process of adenosine catabolism. During


the course of these reactions, O$_2$ is utilised as an electron acceptor and becomes reduced with concomitant formation of superoxide radicals and hydrogen peroxide. Most oxidation of hypoxanthine and xanthine in vivo is actually catalysed by xanthine dehydrogenase (XDH) which transfers electrons to NAD$^+$ rather than O$_2$ with no free radical production, however, xanthine dehydrogenase can be converted to the more active oxidase form, XO, under circumstances of ischaemia or inflammation.

The xanthine oxidase pathway has been recognised as a major source of free radical production in ischaemia-reperfusion injury (Downey, 1990). This can occur following a period of tissue ischaemia, for example following organ transplantation or myocardial infarction. On restoration of the circulation to the hypoxic tissue, oxidative damage can occur and this may be due partly to free radical production by neutrophils activated by cytokines released by the damaged tissues (Babior, 2000). However, XO is generated in the damaged tissue and may contribute to further oxidative damage. During ischaemia, the cell must rely on anaerobic sources of adenosine triphosphate (ATP) resynthesis. At rest, ATP stores will decline very slowly due to the availability of creatine phosphate, however, once this is depleted, the cell must rely on anaerobic glycolysis alone which cannot maintain an adequate rate of ATP resynthesis. Therefore, adenosine will accumulate and will be broken down to hypoxanthine, which will be available for use as a substrate by XO on reperfusion (Walker, 1991).

At high exercise intensity, at or above $\dot{V}O_2$ max, skeletal muscle may be subject to hypoxia due to inadequate blood supply (Packer, 1997), for example, this may be the case in isometric exercise during which blood flow to the exercising muscle may be reduced or eliminated by the effect of increased intramuscular pressure on the blood vessel walls. It has been suggested that exercise under these conditions may activate XO (Hellsten, 1994). Xanthine oxidase has been shown to increase after strenuous exercise (Hellsten et al.,
1996; Viña et al., 1995) and eccentric exercise (Hellsten et al., 1997). In addition, studies utilising allopurinol, a XO inhibitor, have indicated that markers of oxidative stress were decreased in subjects taking allopurinol, suggesting that XO may contribute to free radical production following exercise (Heunks et al., 1999; Viña et al., 2000). However, production of reactive species by XO occurs several hours after the cessation of exercise (Koyama et al., 1999). Xanthine oxidase requires O$_2$ as an electron acceptor and therefore, could only function following reoxygenation of the hypoxic tissue. Thus, this mechanism is likely to be insignificant during exercise, and may contribute more to oxidative stress during recovery from exercise (Vollaard et al., 2005). Under aerobic conditions, XDH will catalyse the breakdown of adenosine metabolites and therefore, the xanthine oxidase pathway may only apply to anaerobic exercise such as isometric, eccentric or sprint exercise (Ji, 1999).

In summary, the exact sources of exercise-induced oxidative stress remain to be more firmly elucidated. It is likely that a number of mechanisms act together to produce the overall effects measured following exercise and that different mechanisms will contribute in different proportions to different modes of exercise.

### 1.4 Objectives of current studies

The overall aims of the current studies were to investigate the use of a novel non-invasive technique for the assessment of oxidative stress, and to better characterise the oxidative stress response to both maximal incremental and submaximal aerobic exercise and to isometric exercise.
1.4.1 Study one

While non-invasive assessment of oxidative stress in exercise studies is desirable from the point of view of subject comfort, there is scant information in the literature concerning its use in this context. This may be partly due to the time-consuming nature of traditional hydrocarbon measurement. However, a novel technique for the detection of ethane gas has become available (Gibson et al., 2002); this has not been used previously for exercise studies and is well-suited for this purpose due to ease of use and rapid analysis time. Oxidative stress has been measured previously in horses and dogs in relation to performance (Chiaradia et al., 1998; Marshall et al., 2002), however, previously this has not been measured non-invasively. Therefore, the current study was a means of testing the utility of this novel technique in three species.

A major limitation of previous exercise studies has been the lack of sampling during exercise, since the methodological design of most studies have included only pre-exercise and post-exercise samples. Some studies did include sampling at a single time point during exercise, at the lactate threshold (Jammes et al., 2004, 2005; Leaf et al., 1997, 1999; Steinberg et al., 2006, 2007), and of those which showed a significant rise in oxidative stress either immediately following or later in recovery from exercise, only one study reported a significant increase in oxidative stress at the lactate threshold (Leaf et al., 1997). In three studies which assessed lipid peroxidation at the lactate threshold, peak exercise and at several time points during recovery (Jammes et al., 2004, 2005; Steinberg et al., 2006), all showed the peak response to occur early in recovery, rather than at peak exercise, and, in fact, in two studies the increase in oxidative stress at peak exercise was not significant (Jammes et al., 2004, 2005). Therefore, it remains unclear if oxidative stress rises during incremental exercise to exhaustion, or only in the post-exercise period. The previous study which did indicate a rise in oxidative stress at one time point during exercise (Leaf et al., 1997) utilised ethane as a marker of lipid peroxidation, and it was the
intention of the current study to extend this finding by the assessment of ethane at regular time points throughout incremental exercise to exhaustion.

Thus, the main aims of this study were to utilise a novel non-invasive technique for the assessment of lipid peroxidation in three species: human, canine and equine; and to better characterise the oxidative stress response to exhaustive incremental exercise by the high density collection of data throughout the exercise period.

1.4.2 Study two

The first objective of this study was to extend the findings of the previous study by again measuring oxidative stress throughout an incremental exercise test to exhaustion, but this time utilising a cycle ergometer protocol in order to assess the entire range of exercise intensities between rest and maximal exercise. In the first study a treadmill protocol was utilised which prevented the examination of the lower end of the work rate range.

In addition, the intention was to examine the oxidative response throughout constant load exercise to gain some appreciation of the time course of the response in terms of its onset and any effect of accumulated duration. Several studies have investigated the response to constant load exercise; however, none have measured oxidative stress during the exercise period, only during the recovery period. It was also of interest to investigate the effect of exercise intensity on the oxidative stress response throughout exercise. Few studies have examined oxidative stress at more than one intensity in the same group of subjects in order to meaningfully compare any difference in response (Dillard et al., 1978; Quindry et al., 2003; Sen et al., 1994). Findings have been inconsistent and again have been limited to baseline and post-exercise time points. Therefore, it was proposed to study the response to exercise below and above the lactate threshold since marked physiological differences would be expected in the body’s response to each intensity.
Finally, it was intended to assess oxidative stress by the novel ethane technique, but also by a more traditional plasma marker of oxidative stress in order to compare the novel technique to one of the more reliable markers of lipid peroxidation.

Therefore, the main aims of this study were to investigate the time course of the oxidative stress response throughout incremental exercise to volitional exhaustion, and during constant load exercise at two intensities, below and above the lactate threshold; and to compare the findings of the novel ethane technique to a more traditional method of lipid peroxidation assessment.

1.4.3 Study three

The first aim of this study was to investigate the oxidative stress response to static isometric exercise at several exercise intensities. Very few studies have examined this mode of exercise, although findings have been fairly consistent (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006). A rise in oxidative stress has been found following static isometric exercise to exhaustion; however, previous studies have examined a limited range of intensities, 50 % or 60 % of maximum voluntary contraction. Therefore, an objective of the current study was to investigate a wider range of exercise intensities and to determine if an intensity threshold for oxidative stress exists.

The time course of the recovery period was also of interest since previous reports have been inconsistent; therefore, it was intended to collect data at a greater number of time points than in previous studies. The mechanisms of oxidative stress in isometric exercise remain unclear (Bloomer & Goldfarb, 2004). Isometric exercise has been associated with tissue de-oxygenation and it is likely that hyperaemia associated with the cessation of exercise may contribute to exercise-induced oxidative stress. However, it remains to be determined whether increased oxidant production is restricted to early reperfusion of the
mitochondrion, as a result of increased O$_2$ flux, or has a late component associated with neutrophil infiltration and activation.

The final aim was to compare systemic and local responses to isometric handgrip exercise by obtaining samples for analysis both from the venous outflow of the working muscle and from the opposite arm to give an estimate of systemic oxidative stress. Oxidative stress has been assessed typically using plasma markers. It was intended to investigate the use of non-invasive assessment of oxidative stress during isometric exercise by utilising the ethane breath test. The successful correlation of ethane output with a traditional plasma marker of lipid peroxidation, assessed systemically, may indicate the potential for non-invasive assessment in this mode of exercise.

Therefore, the main aims of this study were to study the effect of exercise intensity on the oxidative stress response to static isometric exercise; to examine the time course of recovery; to compare local and systemic oxidative stress responses; and to compare the systemic response assessed by both invasive and non-invasive markers.
Chapter 2

Materials and methods
2.1 Subjects

2.1.1 Exclusion criteria

Subjects in all studies were required to be in good health at the time of testing and were asked to complete an approved medical questionnaire (see Appendix A.4) to assess their suitability. Individuals were excluded if they had any pre-existing medical condition which could increase the risk associated with strenuous exercise.

Potential subjects were excluded from taking part in any study if they were currently taking any antioxidant supplement, and were asked to consume their normal diet for the duration of their involvement in the study. Breath hydrocarbons are not affected by prior food consumption: ethane and pentane output were not influenced for up to six hours following consumption of a standard meal when compared to the fasting state (Zarling et al., 1992), however, it has been reported that hydrocarbon excretion, specifically pentane, may be reduced by both vitamin E (Van Gossum et al., 1988) and β-carotene supplementation (Gottlieb et al., 1993). Potential subjects were also excluded if they used recreational or performance-enhancing drugs, tobacco or prescription medication.

Other inclusion or exclusion criteria pertinent to each individual study are described in the relevant chapters.

Several other criteria for participation in individual tests in any study were specified as follows: no alcohol consumption within the 48 hour period prior to any test; no strenuous exercise in the 24 hour period prior to any test; no caffeine intake for 4 hours prior to a test; and no food consumption for 2 hours prior to a test.
2.1.2 Informed consent

All studies involving human volunteers were approved by the appropriate ethics committee of the Faculty of Biomedical and Life Sciences at the University of Glasgow. Subjects in all studies were asked to read an information sheet relating to the specific study and to provide written, informed consent prior to participation. The relevant information sheets and consent forms can be seen in Appendix A.1.

2.2 Testing procedures

2.2.1 Laboratory environment

The laboratory was arranged to provide a non-intimidating and comfortable environment for the subject. Monitoring equipment was placed out of view of the subject wherever possible and all audible alarms were switched off to minimise distraction. Background music was used to take the subject’s attention away from other extraneous noise, but was at low enough volume not to interfere with communication between subject and investigator. Ambient temperature was regulated at 22 °C for all experiments in study two which were carried out in an air-conditioned laboratory (Pina et al., 1995). The other studies took place in an alternative location due to the necessity for a low ambient ethane concentration. The alternative laboratory did not have an air-conditioning facility and therefore, ambient temperature was recorded at the start and end of each experiment and did not vary outwith 20-24 °C. Ambient temperature, as a mean of all values measured at the start and end of each test, was 20.9 °C in study 1 and 20.1 °C in study 3.
2.2.2 Familiarisation

Subjects in all studies underwent an initial familiarisation visit to the laboratory in order to minimise any anxiety associated with testing. If the subject is anxious, this may give rise to physiological responses, such as hyperventilation, which may obscure the responses under investigation.

During this visit, the subject was shown round the laboratory environment and introduced to all personnel who would be present at testing sessions. The purpose of each piece of equipment was explained and specific measurement procedures were demonstrated. The subject was familiarised with the gas exchange equipment; this involved checking that the mouthpiece and nose-clip could be worn comfortably whilst remaining airtight. Subjects were encouraged to breathe through the equipment for several minutes in order to become accustomed to it. Due to the interference of this equipment with communication, the subject was instructed to communicate with the investigator during testing sessions with the use of hand signals. The “thumbs up” signal was used to indicate that all was well; the “thumbs down” signal was used to indicate that there was a problem and the subject was encouraged to point to the site of the problem so that the investigator could take remedial action.

Test protocols were described in detail and, where possible, the subject was given the opportunity to perform a trial run. Finally, the subject was encouraged to ask any questions before providing written informed consent. Other familiarisation procedures specific to each study are described in the relevant chapters.

The weight and height of subjects in all studies were measured during the subject’s first visit to the laboratory (Avery Weigh-Tronix, Birmingham, UK; Leicester Height Measure, Invicta Plastics Ltd., Leicester, UK).
2.2.3 Exercise mode

Three different modes of exercise were utilised across the three studies reported. The oxidative stress response associated with aerobic exercise was investigated in the first two studies, although in the first study treadmill exercise was used, whereas the second study utilised a cycle ergometer with work rate control. In the third study, isometric exercise was provoked with the use of a handgrip dynamometer. All pieces of equipment are described in the relevant chapters.

Treadmill exercise was chosen as the testing mode for human subjects in the first study in order to allow comparison of maximal exercise responses amongst equine, canine and human athletes.

A major advantage of treadmill use for maximal exercise testing is that most individuals are familiar with walking and running; however, it is more difficult to accurately quantify work rate on the treadmill in comparison to the cycle ergometer since it is dependent on body mass during walking and running. A number of protocols are available for maximal exercise testing on the treadmill; one of the most commonly used is the Balke protocol (Balke, 1954) which utilises a constant speed of walking or running with an increase in the slope of the treadmill at regular intervals throughout the test. This is a useful design since it eliminates the problem of increasing speed through the walk-run transition with concomitant individual variation in metabolic cost, and allows an approximately linear increase in work rate over time. The protocol utilised in study one was a modified Balke protocol incorporating a faster test speed to ensure that test duration was 8-17 minutes which is considered to be optimal (Buchfuhrer et al., 1983).

The cycle ergometer was chosen as the mode of exercise in the second study since the primary aim was to investigate the oxidative stress response to exercise over the entire
tolerable work rate range. A limitation of the first study was that the treadmill protocol utilised a fairly high initial work rate, and therefore, oxidative stress was not assessed at the lower end of the work rate range. The use of an electromagnetically-braked cycle ergometer allowed very small (1 W) increments in work rate from an initial resting level, and also permitted changes in work rate to be made independently of pedalling cadence. Thus, this was more practical than using a friction-braked model which relies on the subject pedalling at a constant cadence throughout the test in order to maintain a given work rate.

The aim of the third study was to investigate the oxidative stress response to isometric exercise, and this was done using a handgrip dynamometer. The choice of isometric mode was influenced by previous literature in the area; although few studies have explored oxidative stress in relation to isometric exercise, the majority have utilised handgrip dynamometry as a testing mode, thus supplying some data for comparison. In addition, the study design required sampling the venous outflow of the working muscle; this was more straightforward for handgrip exercise which utilises muscles in the forearm than for, for example, isometric knee extension exercise which would have required access to the venous outflow of the quadriceps muscle group.

2.3 Measurements

2.3.1 Respired air measurements

Ventilatory and pulmonary gas exchange variables were measured either by open-circuit spirometry utilising the Douglas bag method (Consolazio et al., 1963), or by mass spectrometry and turbinometry. Studies one and three involved assessment of oxidative stress \textit{via} expired ethane; this required a test location with a low ambient ethane
concentration. Since the mass spectrometer was located in a laboratory with a comparatively high ambient ethane concentration (2.5 nmol·l⁻¹), with respect to a previously reported range of 68 to 726 pmol·l⁻¹ (Dumelin et al., 1978; Sexton & Westberg, 1984; Knutson et al., 1999), the Douglas bag method was used in these studies.

2.3.1.1 Open-circuit spirometry

Mixed expired air was collected in Douglas bags for determination of ventilation, oxygen uptake and carbon dioxide output. The subject wore a nose-clip and breathed through a mouthpiece attached to a low resistance two-way non-rebreathing valve (Hans Rudolph 2700, Kansas City, USA). The breathing valve was connected to a Douglas bag (Cranlea, Birmingham, UK) via a length of wide-bore flexible plastic tubing. In study one, the subject wore a head-support (Hans Rudolph 2726, Kansas City, USA) to bear the weight of the breathing valve throughout a continuous exercise protocol. In study three, the breathing valve was supported by an adjustable clamp attached to a floor stand. In this latter series of experiments, the subject was allowed to take the mouthpiece out between samples and this was found to be the most practical arrangement.

A Servomex 1440C gas analyser (Servomex Group Limited, East Sussex, UK) was used for the measurement of fractional expired oxygen (O₂) and carbon dioxide (CO₂) concentrations. This analyser consists of two discrete units, one for the measurement of oxygen concentration which utilises a paramagnetic oxygen transducer and one for the measurement of carbon dioxide, based on a single beam single wavelength (SBSW) infrared transducer.

The measurement of oxygen concentration with this analyser is based upon the paramagnetic property of oxygen; that is, oxygen is attracted to a magnetic field. Most other gases do not display this property and this allows for a high specificity of analysis.
The measuring cell within the unit contains two nitrogen-filled glass spheres set out in a dumb-bell arrangement and suspended within a magnetic field. The sample gas is introduced into the cell and any oxygen present will be attracted to the magnetic field and will cause rotation of the dumb-bell arrangement. A mirror is situated on the dumb-bell arrangement, equidistant from the two spheres, and rotation of the dumb-bell is sensed by a light beam projected on to the mirror and reflected onto a pair of photocells. Differences in the reflected light reaching each photocell gives a measure of rotation and a current is produced to move the dumb-bell arrangement back to its original position. This current is directly proportional to the oxygen concentration of the sample gas.

The Servomex analyser utilises photometric determination of carbon dioxide concentration; this relies on the principle that different gases absorb specific wavelengths of light and this property can be used to measure the concentration of a specific gas in a gas mixture. An infrared light source is focussed onto a photometric detector situated at the far side of the sample cell through which the sample gas flows continuously. The SBSW transducer arrangement allows a single light source to be used along with a single optical filter. The filter allows the passage of light of the correct wavelength for absorption by the gas of interest. The amount of light reaching the detector, i.e. the absorbance, gives a measure of the concentration of the gas of interest in the sample.

The analyser was calibrated according to the manufacturer’s instructions prior to every test. For calibration, 100 % N₂ was used to zero the analyser; ambient air was used as a span gas; and a mixture consisting of 17.9 % O₂, 5.04 % CO₂ with a balance of N₂ was used as a test gas. The oxygen unit is accurate to ± 0.1 % and functions within an operating range of 0-25 % O₂, whilst the carbon dioxide unit is accurate to ± 1 % of full scale and was set to operate from 0-8 % CO₂.
Expired air volume was measured using a Harvard dry gas meter (Harvard Apparatus, Massachusetts, USA) which was calibrated prior to each test using a 3 litre syringe (Hans Rudolph MA5530, Kansas City, USA). Expired air temperature was measured using a digital thermometer (Kane-May KM330, Comark Ltd., Hertfordshire, UK) with the probe positioned inside the inlet tube of the volume meter.

### 2.3.1.2 Mass spectrometry and turbinometry

In study two, respiratory gases were analysed by mass spectrometry in which the concentration of a gas of interest within a gas mixture can be determined based on its mass to charge ratio. The gas sample is ionised by electron bombardment and then accelerated through a magnetic field. Ions are deflected according to both the mass and charge of the ion; lighter ions undergo greater deflection as do ions with more charge. Distinct gas species can be distinguished from one another by extent of deflection. The concentration of a specific species of interest can be determined as electrical current proportional to the number of ions detected. The quadrupole spectrometer used in this study can separate ions by mass to charge ratio so that only one species may pass to the detector at a time.

### 2.3.1.2.1 Gas sampling procedures

The subject wore a nose-clip and breathed through a mouthpiece connected to a low dead space breathing assembly which included a low resistance ($< 1.5 \text{cmH}_2\text{O} \cdot \text{l}^{-1} \cdot \text{s}^{-1}$ at $3 \text{l} \cdot \text{s}^{-1}$) turbine flow transducer (Interface Associates Inc., Laguna Niguel, CA, USA). The turbine assembly consisted of an impeller housed within a plastic casing. The impeller rotates in opposite directions in response to inspiratory and expiratory air flow. Light emitting diodes are housed within the outer casing of the turbine assembly; these transmit infra-red light across the path of the impeller. As the impeller rotates, the light beams are broken and this is detected by internal phototransistors which produce electrical current proportional to flow rate. Thus, inspiratory and expiratory flow could be determined. A saliva trap was
placed between the mouthpiece and the turbine assembly in order to prevent contamination and any potential subsequent decrement in performance of the transducer.

A gas sample line was connected from the breathing assembly to a quadrupole mass spectrometer (QP9000, Morgan Medical, Kent, UK) for determination of O₂, CO₂ and N₂ concentrations. Expired air was continuously sampled at a flow rate of 1 ml·s⁻¹ and gas concentrations were determined at 20 ms intervals.

The breathing assembly was suspended by an adjustable chain at a comfortable height for the subject. A schematic representation of the breathing assembly is shown in figure 2.1.
Figure 2.1. Schematic representation of breathing assembly.
Components are not drawn to scale.
2.3.1.2.2 Calibration

The turbine transducer and mass spectrometer were calibrated prior to every test according to the manufacturer’s instructions.

A volume calibration was carried out using a 3-litre calibration syringe (Hans Rudolph, Kansas City, MO, USA). Air was drawn in fully and expelled fully from the syringe through the turbine assembly ten times in each direction. The calibration was accepted within a mean volume range of 2.995-3.005 l. The procedure was then repeated to check the calibration.

A single point gas calibration was carried out with a known gas mixture of 10.38 % O₂, 7.06 % CO₂ and 81.62 % N₂. A test gas mixture of 20.42 % O₂, 2.01 % CO₂ and 76.64 % N₂ was then introduced into the system. The calibration was accepted if agreement between the actual test gas concentrations and the readout from the mass spectrometer agreed within one decimal place. The gas calibration was repeated immediately after each experiment to check for drift. The data were considered to be accurate if the calibration values agreed within 1 %; otherwise the test data would have been discarded. No data were required to be discarded for this reason.

The delay time between the onset of flow through the breathing assembly and the production of output by the spectrometer was measured prior to each experiment. The calibration syringe was filled with a high CO₂ concentration gas mixture which was then rapidly expelled through the breathing assembly using a low dead space solenoid valve to ensure a square wave input (Beaver et al., 1973). The delay time was noted and this procedure was repeated. A difference of up to 20 ms between the two measurements was accepted; otherwise the procedure was repeated until a reproducible value was obtained.
2.3.1.2.3 Analysis

As the subject exhales into the breathing assembly, a small volume of the expired air is drawn into the gas sample line connected to the mass spectrometer. There is a physical delay before this gas is analysed due to the transit time required from mouth to the analyser and also due to the response time of the analyser. This delay was measured during calibration procedures as described above (section 2.3.1.2.2, page 105). The remainder of the expirate passes through the turbine transducer and produces instantaneous signals of flow rate and volume. The signals from both volume transducer and mass spectrometer are amplified, passed through a digital to analogue converter and passed to the computer where they are time aligned.

Ventilation and pulmonary gas exchange variables were determined on a breath by breath basis using the gas exchange algorithms of Beaver et al. (1973).

2.3.2 Ethane sampling procedures

Expired air was analysed for the presence of ethane gas in studies one and three. General sampling and analysis procedures are described below.

2.3.2.1 Materials for collection and storage of samples

Sample bags used for the collection and storage of expired air to be analysed for hydrocarbon gases should be constructed from materials which do not release hydrocarbon gases, as this could present a source of contamination. In addition, the sample bag should be impermeable to hydrocarbon gases to prevent leakage, and all seals and connections must be airtight to avoid contamination by ambient air. Ambient air ethane concentration has been reported in the range of 68-726 pmol·l\(^{-1}\) by various authors (Dumelin et al., 1978; Sexton & Westberg, 1984; Knutson et al., 1999), while expired air ethane concentration at rest in healthy subjects has been recorded as 25 pmol·l\(^{-1}\) (Knutson et al., 2000). Therefore,
a concentration gradient for ethane would tend to exist in the direction of the collected expired air sample.

Tedlar bags (SKC Limited, Dorset, UK) are made of polyvinyl fluoride which is known to have low permeability to gases. Kneepkens et al. (1994) have suggested that Tedlar bags will not contaminate breath samples with hydrocarbons and can be stored for over 48 hours with no significant loss of hydrocarbons. Drury et al. (1997) observed no significant change in pentane concentration within a 1 litre Tedlar bag following 24 or 48 hours of storage. However, pentane concentration did increase by 30% following 72 hours of storage. Knutson et al. (2000) found a significant increase in pentane concentration within a 3 litre Tedlar bag only after 24 hours of storage. The increased hydrocarbon concentration within the bag was suggested to be due to gradual inward diffusion of ambient hydrocarbons. Gas mixtures containing a known concentration of ethane with a balance of nitrogen were stored in Tedlar bags prior to analysis and showed approximately 10% alteration in concentration over a period of one week (Skeldon et al., 2005). Tedlar bags were used in the current studies for the transfer and transient storage of expired air samples, and all samples were analysed within 24 hours of collection.

All other equipment which was used for collection or storage of expired air samples, including mouthpiece, nose-clip, tubing and Douglas bags, was checked prior to each experiment to ensure that ethane concentration was not abnormally high with the potential to mask any change in ethane concentration due to the exercise.

### 2.3.2.2 Ethane collection procedures

Expired air, collected in Douglas bags as previously described (section 2.3.1.1, page 100) was sampled to determine ethane concentration. A 5 litre sample of expired air was
transferred from each Douglas bag into a Tedlar bag immediately following the end of each test.

In order to correct for the presence of ambient ethane in expired air samples, a sample of ambient air was drawn into a 5 litre capacity Tedlar bag, using a hand operated pump, in the middle of each period of expired air collection.

All samples were analysed, following calibration of the spectrometer, within 24 hours of collection (Kneepkens et al. 1994).

2.3.2.3 Correction for ambient ethane

Many studies have utilised a period of breathing hydrocarbon-free air prior to sample collection in order to eliminate the effect of ambient hydrocarbons and thus ensure reproducibility of results. As previously stated, ambient air can contain a relatively high concentration of ethane; this can vary markedly depending on the location of the air supply to the laboratory in relation to parking areas or other sources of ethane pollution (Habib et al., 1999). This technique is generally used in protocols involving single or few samples and would be impractical in exercise studies due to the lengthy duration of testing. In the current studies, the concentration of ethane in the expired air has been calculated as the difference between the measured concentration in the expired air sample and the background concentration in the ambient air. It has been verified that this method also produces consistent results (Cheng & Lee, 1999). In the current studies, ambient ethane concentration was monitored throughout each experiment and was found to be in the range 58-326 pmol·l⁻¹. This is lower than, or comparable to, values reported in previous studies (Dumelin et al., 1978; Sexton & Westberg, 1984; Knutson et al., 1999).
2.3.2.4 Test location

The location of testing was chosen from several potential locations with reference to environmental ethane concentration. Ambient air was sampled from several locations prior to the start of the study and was found to vary from 2 ppb (89 pmol·l\(^{-1}\)) to approximately 400 ppb (18 nmol·l\(^{-1}\)) in different locations. A location with a low ambient ethane concentration was chosen and background ethane was monitored throughout each test to take account of fluctuations, as described above.

Subjects were required to be present in the laboratory for at least 30 minutes prior to collection of the first baseline expired air sample in order to allow time for equilibration with environmental ethane. Subjects arrived at the laboratory having come from a known high ethane environment, such as rooms and laboratories which had been tested previously for ambient ethane, or from an unknown environment. Therefore, it was considered prudent to allow a period of time during which the subject could wash out any excess residual ethane from the body. A 30 minute period for equilibration prior to baseline sampling has previously been utilised (Phillips et al., 2000), however, no empirical evidence was provided to support the use of this time period. Therefore, a pilot test was conducted to determine if 30 minutes was suitable.

2.3.2.4.1 Pilot test

A pilot test was conducted on one subject to determine the time taken for expired ethane to stabilise following arrival at the laboratory from a known high ethane environment. The subject spent the two hours immediately prior to the test in an environment with an ambient ethane concentration of 1.8 nmol·l\(^{-1}\), sampled two minutes prior to the subject leaving this environment to attend the testing session. This ambient concentration is 2.5-27 times higher than values previously reported in the literature (Dumelin et al., 1978; Sexton & Westberg, 1984; Knutson et al., 1999) as described above.
The subject spent two minutes travelling in the open air to reach the laboratory. Upon arrival, the subject was seated and remained so throughout the test. Without delay, a mouthpiece was inserted, a nose-clip was attached, and a two minute sample of expired air was collected into a Douglas bag after a one minute period of familiarisation with the gas collection equipment. Therefore, the collection of the first expired air sample began just 4 minutes after the subject had left the high ethane environment. Subsequent samples were collected as shown in figure 2.2. A sample of ambient air was collected at each time point to correct for ambient ethane concentration.

![Figure 2.2: Schematic representation of the pilot test protocol for the investigation of the expired ethane response to laboratory conditions following prior exposure to high atmospheric concentration (1.8 nmol·l⁻¹).](image-url)
Samples were analysed for ethane concentration and corrected for ambient ethane as described in section 3.2.1.4.3 (page 139). A plot of expired ethane concentration against time is shown in figure 2.3. Several data points are missing for the following reasons: the samples at minutes 5 and 15 were analysed, however, the concentrations measured were not credible and were omitted; for the minute 20 sample the Douglas bag was inadvertently opened prior to analysis and thus the sample was discarded; for the minute 50 sample ambient ethane concentration was greater than expired ethane concentration.

![Figure 2.3](image.png)

Figure 2.3. Expired ethane response to laboratory conditions following prior exposure to high atmospheric concentration (1.8 nmol·l⁻¹).
The pattern of response indicated that initial expired ethane concentration was higher than expected, approximately 38 pmol·l$^{-1}$, in comparison to the previously reported 25 pmol·l$^{-1}$ (Knutson et al., 2000), up to 10 minutes after arrival at the laboratory. After 25 minutes in the laboratory, expired ethane concentration had fallen to approximately 13 pmol·l$^{-1}$ and remained fairly stable until the end of sampling at 60 minutes after arrival.

Since the ambient ethane concentration of the previous environment was higher than previously reported values, it was considered to be unlikely that a subject would present himself for testing having come from an environment with higher ambient ethane than was tested in this pilot study. Therefore, it was made a requirement of each test that the subject should remain at rest in the laboratory for 30 minutes prior to the onset of sampling to ensure that any excessive ethane from the subject’s previous environment was washed out of the body, in order to assist in the measurement of a stable baseline value.

2.3.3 Ethane analysis

Expired ethane has been measured traditionally by gas chromatography (GC), alone or alongside mass spectrometry (GC-MS). Ethane is present in expired air in quantities at the parts per billion (ppb) level. In order to measure a single sample at this level using gas chromatography, the sample would require to be pre-concentrated which is a time-consuming procedure, typically requiring at least 30 minutes per sample.

Expired ethane concentration was measured in two of the current studies using a novel technique, that of ultra-sensitive laser absorption spectroscopy, developed by the Optics Department at the University of Glasgow (Gibson et al., 2002). The spectrometer is sensitive to 0.1 ppb of ethane and can measure a sample in approximately 2 seconds (Skeldon et al., 2005).
The spectrometer utilises a mid-infrared laser which operates at a wavelength of 3.4 µm at which ethane is absorbed without interference from other gases abundant in expired air, such as water vapour and carbon dioxide. The laser light passes through a beam splitter which directs it into two channels: the sample cell used for determination of ethane concentration from the gas sample of interest; and the reference cell which contains a known concentration of ethane (500 ppm) and is used for calibration. Sample bags are attached to the inlet port of the spectrometer and the sample gas is drawn through the sample cell at a standard flow rate of 5 l·min⁻¹. Ethane absorption is measured by a photodetector from which the ethane concentration of the sample gas is derived. Sampling is continuous and ethane concentration is updated once per second.

The spectrometer is calibrated continually against a known ethane standard of 500 ppm contained within its reference cell. In addition, the zero reference is checked, typically at intervals of 60 seconds, by introducing hydrocarbon-free nitrogen gas, which has an ethane concentration of less than 0.1 ppb, into the sample cell. The duration of this procedure is typically 12 seconds.

The accuracy of the spectrometer has been verified within 10% against empirical ethane concentrations in the range of 0 to 250 ppb (Skeldon et al., 2006). The same authors are currently undertaking a comparison of the spectrometer’s performance in relation to GC-MS.

The spectrometer is currently in use as a measurement tool in a number of life science and clinical settings, including the measurement of expired ethane in lung cancer patients (Skeldon et al., 2006), in ischaemia-reperfusion injury during organ transplantation (Skeldon et al., 2005) and pre and post radiation treatment (Skeldon et al., 2005).
Ethane data from the current studies are expressed as concentrations, i.e. ppb or pmol·l\(^{-1}\), in order to assist comparison with previously published data. However, this does not facilitate inter-individual comparisons, and therefore, the expression of ethane output as a rate, corrected for body weight, i.e. pmol·kg\(^{-1}\)·min\(^{-1}\), is also used (Risby & Sehnert, 1999).

### 2.3.4 Blood sampling procedures

Blood was sampled from an indwelling cannula placed in an antecubital vein. A tourniquet was placed around the upper arm in order to increase pressure inside the vessel of interest to facilitate the process of cannulation. Skin was cleaned with an alcohol swab prior to insertion of a sterile 20G intravenous cannula with injection port (Biovalve 106.10, Vygon, Cirencester, UK). Once in place, the cannula was attached to a three way tap connected to a 10 cm length of narrow flexible plastic tubing (BD Connecta Plus 3 394995, Oxford, UK). This sample line was filled with 0.9 % sodium chloride (NaCl) (FPE 1307, Baxter Healthcare Ltd., Norfolk, England). The cannula was secured in place using a sterile, self-adhesive polyurethane dressing (Dermafilm, Vygon UK Ltd., Cirencester, UK).

The sampling procedure involved an initial withdrawal of 2-3 ml of fluid in order to clear the NaCl solution from the sample line. Following this, a blood sample of approximately 6 ml was withdrawn and was immediately transferred into a plastic tube coated with lithium heparin (BD Vacutainer 367885, Oxford, UK). The tube was inverted gently eight times, according to manufacturer’s instructions, to prevent clotting, and was then placed immediately on ice. Finally, the sample line was flushed with 2-3 ml of NaCl in order to prevent clotting and maintain patency of the line. All fluid withdrawal and insertion was achieved using a 10 ml sterile hypodermic syringe (BD Plastipak 302188, BD, Oxford, UK). On withdrawal of the cannula at the end of the test, pressure was applied to the puncture site in order to minimise the risk of haematoma development. Descriptions of sampling times for each study are included in the relevant chapters.
2.3.5  Blood analysis

Following each test, blood samples were centrifuged at 3000 g at 4 °C for 15 minutes. Immediately thereafter, samples were stored on dry ice while the plasma was divided into aliquots of approximately 500 μl. Subsequently, samples were stored at -80 °C until further analysis which took place within 17-20 months of testing. Specific assay procedures for the measurement of markers of lipid peroxidation are described below.

2.3.5.1  Measurement of malondialdehyde

In study two, described in chapter 5, plasma samples from tests with the first subject were analysed initially for the presence of malondialdehyde (MDA) in order to determine if a response was present prior to continuing with the remainder of the experimental trials. This was done using a spectrophotometric assay (Bioxytech MDA-586, Oxis Research, Portland, OR, USA). The basis of the assay is that MDA in the plasma sample reacts with a chromogenic agent, Nmethyl-2-phenylindole, to produce a coloured product which can be detected at a wavelength of 586 nm. The assay is specific for MDA, since under the hydrochloric acid conditions of the assay there is little absorbance at this wavelength from the most common alternative aldehydic product of lipid peroxidation (Erdelmeier et al., 1998).

Standard solutions of MDA at concentrations of 0 μM, 0.5 μM, 1.0 μM, 2.0 μM, 3.0 μM and 4.0 μM were prepared and analysed to produce a standard curve from which the unknown concentrations of MDA in the plasma samples could be determined. Analysis of standards and samples was carried out in triplicate.

2.3.5.2  Measurement of F₂-isoprostanes

In studies two and three, described in chapters 5 and 7, plasma samples were analysed using a commercial enzyme-linked immunosorbent assay (Assay Designs 900-091, Ann
Arbor, MI, USA) for detection of 8-iso-Prostaglandin F\textsubscript{2a} (8-iso-PGF\textsubscript{2a}). This is a competitive immunoassay which utilises a polyclonal antibody specific for 8-iso-PGF\textsubscript{2a}. The competitive nature of the assay means that the antibody will bind 8-iso-PGF\textsubscript{2a} present in the plasma sample, and also 8-iso-PGF\textsubscript{2a} conjugated with alkaline phosphatase which is available as part of the assay kit.

An initial hydrolysis step was carried out prior to conduction of the assay. Sodium hydroxide (NaOH) was added to the plasma samples in order to hydrolyse ester bonds binding 8-iso-PGF\textsubscript{2a} to lipoprotein or phospholipid molecules. This ensured that both free and bound 8-iso-PGF\textsubscript{2a} could be measured by the assay.

The wells of the assay plates are coated with rabbit immunoglobulinG (IgG). The plasma sample and the alkaline phosphatase conjugate, both containing 8-iso-PGF\textsubscript{2a}, were added to the wells along with anti-rabbit IgG. This anti-rabbit antibody is specific for, and binds to, the rabbit antibody coating the wells and has binding sites for 8-iso-PGF\textsubscript{2a}. Thus, during a 24 hour incubation period at 4 °C, the anti-rabbit antibody binds 8-iso-PGF\textsubscript{2a} and also binds to the antibody on the plate, thus capturing the antigen of interest. After the incubation period, the wells were washed to remove any excess reagent leaving the bound 8-iso-PGF\textsubscript{2a} attached to the wells. The greater the concentration of 8-iso-PGF\textsubscript{2a} in the plasma sample, the less of the conjugated 8-iso-PGF\textsubscript{2a} binds to the antibody, and therefore less alkaline phosphatase is retained in the well.

Following the removal of excess reagent, p-nitrophenyl phosphate (pNpp), a colourless substrate for the enzyme alkaline phosphatase, is added. This reaction generates a yellow colour due to formation of the yellow product p-nitrophenol. The intensity of colour is proportional to the amount of enzyme present. Thus, the intensity of colour is proportional
to the concentration of alkaline phosphatase in the well and inversely proportional to 8-iso-PGF$_{2\alpha}$ in the well.

The enzyme reaction is stopped after a brief incubation. In these studies, the optical density of each well was read immediately on a microplate reader (Spectra MR, Dynex Technologies, Worthing, UK) at a wavelength of 405 nm.

Standard solutions of 8-iso-PGF$_{2\alpha}$ at concentrations of 160 pg·ml$^{-1}$, 800 pg·ml$^{-1}$, 4000 pg·ml$^{-1}$, 20000 pg·ml$^{-1}$ and 100000 pg·ml$^{-1}$ were prepared and analysed to produce a standard curve from which the unknown concentrations of 8-iso-PGF$_{2\alpha}$ in the plasma samples could be determined. These calculations were done using 4 parameter curve fitting software ((Dynex Technologies Revelation 4.25, Worthing, UK) as recommended by the assay manufacturer. Concentrations were then corrected for dilution during the hydrolysis step.

All standards and samples were analysed in duplicate and the coefficient of variation was calculated as the ratio of standard deviation to mean of each pair. In cases where the coefficient of variation was high (> 10 %), the sample concentrations were examined to determine if there was justification for reporting the concentration of a single sample rather than the mean of the two samples.

2.3.6 Statistical analysis

All statistical analysis was carried out using SPSS version 17.0 statistical software. Details of specific statistical tests utilised are included in the relevant chapters. The mean and standard deviation have been used as the measures of central tendency and variability.
Chapter 3

Ethane and carbon monoxide responses to maximal dynamic exercise in human, equine and canine athletes
3.1 Introduction

3.1.1 Non-invasive assessment of oxidative stress

Non-invasive assessment of any physiological variable is generally preferable to invasive assessment from the perspectives of subject comfort and safety, especially if numerous repeated measurements are required. Oxidative stress can be assessed non-invasively via measurement of volatile hydrocarbon gases, specifically ethane and pentane, in the expired air.

Ethane (C$_2$H$_6$) and pentane (C$_5$H$_{12}$) are produced in the body as terminal reaction products of lipid peroxidation, that is, the oxidative degradation of polyunsaturated fatty acids (PUFAs). A description of this process can be found in section 1.1.4.1 (page 51). PUFAs are a major constituent of cell membranes and are those fatty acids which contain more than one double bond. Thus, PUFAs are liable to react with other molecules and, in the process, undergo structural change. They can be categorised according to the position of the first double bond; for example, in n-3 PUFAs, this is located at the third carbon atom from the methyl carbon end of the molecule. Ethane is derived from n-3 PUFAs such as alpha-linolenic acid. Pentane is produced from the peroxidation of n-6 PUFAs which include linoleic acid and arachidonic acid. N-3 and n-6 PUFAs are present in the body in an approximate 1:4 ratio (Lepage & Roy, 1986) and therefore, lipid peroxidation is likely to favour the production of pentane.

Both ethane (Leaf et al., 1997) and pentane (Dillard et al., 1978; Kanter et al., 1993; Leaf et al., 1997; Pincemail et al., 1990) have been utilised as markers of lipid peroxidation in healthy human subjects during and following exercise. The output of any substance in the expired air must reflect the actual production of the substance, its metabolism and
elimination by organs other than the lungs, and any washout of the substance from body stores. Ethane is generally considered to be the preferable marker, exhibiting lower intra-day variation than pentane (Knutson et al., 1999). This may be the case due to different rates of hepatic metabolism. Hydrocarbons are metabolised in the liver by mono-oxygenases and eliminated from the body via the kidneys as well as from the lungs (Kneepkens et al., 1994). The rate of hepatic metabolism has been shown to be lower in ethane (Wade & van Rij, 1985); therefore, any underestimation of true hydrocarbon production would be relatively more exaggerated in the case of pentane. In addition, both ethane and pentane are non-polar molecules and therefore have a low solubility in water. The solubility co-efficient of ethane in body fluids is 0.14 in comparison to that of pentane which is 0.42 (Dale et al., 2003). It has also been suggested that ethane has lower solubility in fat; lower molecular weight hydrocarbons are less lipophilic and, therefore, are less likely to be distributed within the adipose tissue (Kneepkens et al., 1994). Thus, washout of stored ethane from body tissues is likely to contribute less markedly to measured output values.

Ethane is rapidly washed out of the lungs. In one study (Dale et al., 2003), subjects inhaled gas containing a high ambient ethane concentration (19-29 ppm) for approximately 20 minutes. Subjects then breathed room air, and expired ethane was measured at numerous time points over a 210 minute period. The expired ethane concentration was found to drop to 5% of the initial inspired value within 1.5 minutes.

The washout of body tissues may take longer. A study by Morita and colleagues (1986) investigated pentane washout following a two hour period of breathing hydrocarbon-free air. A rapid decrease in expired pentane from 10.2 pmol·kg⁻¹·min⁻¹ to 1.6 pmol·kg⁻¹·min⁻¹ was reported in the first 30 minutes, followed by a further, much smaller decrease to 1.2 pmol·kg⁻¹·min⁻¹ after 60 minutes, with no further reduction in pentane output.
thereafter. As ethane is reportedly less soluble in body tissues than pentane, washout would be predicted to occur more quickly.

A recent study (von Basum et al., 2003) has considered the washout of ethane from body tissues following 5 minutes of breathing 1 ppm ethane. The results suggested that washout from the lungs was complete within approximately 20 seconds. This was followed by washout from two further body compartments, likely the blood and well-perfused tissue; washout from the blood was complete within approximately 1 minute, and from well-perfused tissue within approximately 12 minutes. This study was limited to some extent since only three subjects were included; however, it does suggest a rapid excretion of ethane from body tissues. The authors suggested that 5 minutes may not have been adequate for full equilibration with ethane, although results from Dale and colleagues (2003) suggest that equilibration with a high ethane concentration (16-29 ppm) is more rapid and takes place within 2 minutes. Thus, for ethane to be used as a marker of oxidative stress, only a brief period would seem to be required prior to initial sampling in order for a subject to equilibrate with the environmental ethane concentration.

3.1.1.1 Expired and ambient ethane concentrations

Ethane concentration tends to be higher in ambient air than in the expired air. Expired ethane concentration in healthy individuals at rest has been reported between 10.4 pmol·l$^{-1}$ (Knutson et al., 1999) and 50 pmol·l$^{-1}$ (Zarling & Clapper, 1987) when corrected for ambient ethane. Ethane is present in the atmosphere, from sources such as vehicle exhaust fumes and tobacco smoke, and the ambient concentration has been variously reported within the following ranges: 73-726 pmol·l$^{-1}$ (Dumelin et al., 1978); 68-224 pmol·l$^{-1}$ (Knutson et al., 1999); 70-220 pmol·l$^{-1}$ (Sexton & Westberg, 1984); and at a mean concentration (± standard deviation) of 800 ± 390 pmol·l$^{-1}$ (Zarling & Clapper, 1987). However, this will be variable depending on proximity to sources of ethane (Sexton &
Westberg, 1984) and some variability may be attributable to different measurement techniques.

Due to the similarity between ambient and expired ethane concentrations, it is necessary to correct for the presence of ethane in the inspired air in order to be able to assess accurately ethane production due to lipid peroxidation. A widespread method used to achieve this is to ask the subject to breathe hydrocarbon-free air (HCFA) for a period of time immediately preceding the collection of the expired air sample in order to flush ambient hydrocarbons from the lungs and airways. It has been suggested that a period of 4 minutes of breathing HCFA is sufficient to clear ambient ethane and pentane from the lungs and that a longer washout of up to 30 minutes does not result in a further reduction of expired ethane concentration (Knutson et al., 1999). However, while this technique may be suitable for single breath measurement of expired ethane, it is both impractical and costly for use in exercise studies in which repeated expired air measurements are frequently required. In these cases, ambient ethane concentration can be measured simultaneously alongside the measurement of expired ethane, with the ambient concentration simply being subtracted from the expired concentration to remove the imprecision associated with the inspiration of ethane. This technique has been reported to be reproducible (Cheng & Lee, 1999; Risby & Sehnert, 1999).

3.1.1.2 Limitations of hydrocarbon measurement

There are other potential limitations to the use of hydrocarbon measurement. Firstly, there are other possible sources of hydrocarbons in the body other than lipid peroxidation; these include protein oxidation and colonic bacterial metabolism. However, these are generally considered to be of limited importance and should not interfere with the interpretation of results (Kneepkens et al., 1994). The relative contribution of protein oxidation to the output of hydrocarbons has been shown to be small in vitro (Clemens et al., 1983). Some
researchers have considered that intestinal bacterial flora may produce hydrocarbons in significant quantity using polyunsaturated fatty acids in the diet as a substrate (Kneepkens et al., 1994). However, it was observed in premature human babies that ethane and pentane excretion increased during the first four to five days following delivery, with a sharp decrease in excretion of both gases thereafter (Kuivalainen et al., 1991; Pitkänen et al., 1990). It is known that it takes several days for intestinal flora to become established after birth (Stevenson et al., 1982), therefore, it is unlikely that bacterial flora are major producers of breath hydrocarbons.

Secondly, the formation of hydrocarbons in the body is dependent on the presence of transition metal ions such as Fe$^{2+}$ which are required for the decomposition of lipid peroxides. Therefore, it is important to control for the availability of these ions, otherwise, a measured increase in hydrocarbon production may reflect increased ion availability rather than an increased lipid peroxidation.

Finally, the use of hydrocarbon measurement may be unreliable in smokers as smoking significantly increases both ethane and pentane output. Expired ethane concentration at rest has been found to be significantly higher in smokers compared to non-smokers: 2.9 pmol·kg$^{-1}$·min$^{-1}$ in smokers compared to 1.1 pmol·kg$^{-1}$·min$^{-1}$ in non-smokers (Habib et al., 1995).

### 3.1.1.3 Novel technique for the assessment of ethane

The concentration of ethane in expired air can normally be measured in the parts per billion range (Skeldon et al., 2005), which is generally too low for detection by most measurement systems. Traditionally, expired ethane has been measured using gas chromatography (Knutson et al., 2000) which requires expired air samples to be pre-concentrated prior to measurement in order to accumulate a sufficiently high concentration
for detection. This technique is sometimes used in combination with mass spectrometry for definitive identification of ethane since interference by other species is possible. This can be a time-consuming procedure, taking from 9 minutes (Dale et al., 2003) to 15 minutes per sample (Knutson et al., 2000). Due to time constraints, these methods may be less practical than necessary for the assessment of numerous expired air samples collected during or following exercise.

A recently developed technique for quantifying expired ethane concentration utilising laser spectroscopy has been described (Gibson et al., 2002). A brief summary of this technique can be found in section 2.3.3 (page 112). Advantages of this novel technique include ease of use; the instrument can be controlled by a laptop computer interface without specialist knowledge. Sampling time is very rapid in comparison to traditional methods for ethane analysis in expired air; pre-concentration of the sample is not required and a single breath sample can be analysed in approximately 2 seconds (Skeldon et al., 2005). In addition, the technique has very high specificity for ethane without interference from other species (Skeldon et al., 2005).

To date, this technique has been utilised for the assessment of oxidative stress during transplantation surgery in animals, during radiation therapy in human patients and in cases of respiratory inflammation in horses (Skeldon et al., 2005). A similar technique has been used previously for investigating the profile of ethane washout after the inhalation of tobacco smoke (Dahnke et al., 2001), and for recording single exhalations of ethane in non-smokers (von Basum et al., 2003).

Thus, due to easy, rapid analysis, the laser spectroscopy technique is well suited to the non-invasive assessment of exercise-induced oxidative stress, however, has not been used previously for this purpose.
3.1.2 Previous exercise studies utilising ethane measurement

3.1.2.1 Previous studies in humans

Although exercise-induced oxidative stress has been measured in humans on numerous occasions, the majority of studies have utilised plasma markers of lipid peroxidation. Expired ethane has been assessed in association with exercise in only a small number of studies, all by the same group of authors (Leaf et al., 1997, 1999, 2004).

All three studies assessed lipid peroxidation via ethane output at three time points: at rest; at lactate threshold during incremental exercise to voluntary exhaustion; and five minutes into a period of post-exercise passive recovery. Ethane output was also measured at peak exercise in the 1997 study. A modified Bruce protocol was performed on a treadmill in the initial two studies (Leaf et al., 1997, 1999), whilst cycle ergometer exercise was utilised in the later study (Leaf et al., 2004). The subject group of interest was different in each study: healthy individuals (Leaf et al., 1997); patients with coronary artery disease (Leaf et al., 1999); and patients receiving chronic renal dialysis (Leaf et al., 2004).

Only the study utilising healthy subjects (Leaf et al., 1997) is suitable for comparison with resting ethane concentrations previously reported in healthy subjects. Ethane concentration at rest in this study was not reported, however it can be estimated. Ethane output at rest was approximately 85 pmol-min⁻¹; this was not reported numerically but presented only graphically. Mean ventilation at rest was 13.5 l-min⁻¹; thus, a resting ethane concentration of approximately 6.3 pmol-l⁻¹ can be estimated. This is similar to the lower end of the range previously observed (Knutson et al., 1999).

Incidentally, resting ethane output in coronary artery disease patients in the 1999 study was similar to that of the healthy individuals in the 1997 study. Ethane output was
approximately 75 pmol·min\(^{-1}\) in the training intervention group and approximately 45 pmol·min\(^{-1}\) in the control group. Again, data were presented graphically only.

In contrast, the data presented in the 2004 study were inconsistent. At rest ethane output for the control group subjects was reported as 250158 pmol·min\(^{-1}\); approximately 3000 times the value reported previously in healthy individuals (Leaf et al., 1997). These individuals were matched by age, gender, medication, smoking status and medical condition to the renal dialysis patients in the group of interest; thus, five of the seven control group subjects were hypertensive and two suffered from diabetes mellitus. Even considering that greater lipid peroxidation may be expected at rest in individuals with disease in comparison to healthy individuals (Cross et al., 1987), the authors made no mention of this striking difference in resting ethane output, especially since the resting values of the coronary artery disease patients in the earlier study (Leaf et al., 1999) were similar to those of healthy subjects (Leaf et al., 1997). Ethane was measured using the same technique in all three studies and ethane output was calculated in the same manner. Thus, confidence in these data is diminished.

The pattern of response of ethane output to incremental treadmill exercise to voluntary exhaustion in healthy subjects (Leaf et al., 1997) was as follows. Ethane output rose from a resting value of 85 pmol·min\(^{-1}\) to 660 pmol·min\(^{-1}\) at lactate threshold. A further rise in ethane output to 2400 pmol·min\(^{-1}\) was observed at peak exercise and was followed by a decline to 840 pmol·min\(^{-1}\) after five minutes of passive recovery. Data were presented graphically so all values are approximate.

A similar pattern was seen in coronary artery disease patients (Leaf et al., 1999). A three- to four-fold increase in ethane output was seen from rest to lactate threshold in both the training and control group. This was followed by a decline in ethane output measured in
recovery in the training group, although ethane output increased in recovery in the control group.

Only one study utilising pentane measurement has employed an incremental exercise protocol (Leaf et al., 1997). Pentane was measured alongside ethane as already described. The pattern of response to incremental exercise was the same as that for ethane, that is, an increase from rest to lactate threshold, a further increase to peak exercise followed by a decline measured five minutes into passive recovery.

Most studies examining incremental exercise to exhaustion have utilised plasma markers of lipid peroxidation. Some have reported a post-exercise increase whilst others have reported no significant change following exercise, as described previously (section 1.3.1.1, page 70). Most of these studies assessed lipid peroxidation only at rest and immediately following, or within 15 minutes of, the end of exercise (Ashton et al., 1998, 1999; Bailey et al., 2001; Kretzschmar et al., 1991; Leaf et al., 1997, 1999; Lovlin et al., 1987; Szcześniak et al., 1998). A minority of studies have reported data from two or three time points in recovery up to 24 hours following the end of exercise; however none reported a significant increase in lipid peroxidation from baseline at any time point (Hartmann et al., 1995; Niess et al., 1996; Quindry et al., 2003; Sen et al., 1994).

Three studies assessed lipid peroxidation at lactate threshold, at peak exercise and at multiple time points within the first 30 minutes of recovery (Jammes et al., 2004, 2005; Steinberg et al., 2006). The general pattern of response was a gradual increase in lipid peroxidation to a peak at 5 minutes (Jammes et al., 2004; Steinberg et al., 2006) or 10 minutes (Jammes et al., 2005) into recovery with a gradual reduction in response thereafter. Thus, as discussed previously (section 1.4.1, page 90), the pattern of response to incremental exercise to exhaustion remains unclear.
There is a paucity of consistent information in the literature regarding lipid peroxidation during and following incremental exercise in humans. The evidence available from the ethane studies is particularly limited since so few studies have been undertaken to date. In addition, data were collected at only a few time points and, furthermore, some of the results reported are unconvincing. The authors make no mention of what technique, if any, was used for the correction of expired ethane for ambient ethane concentration, which casts some doubt on the reliability of the results. Thus, there is justification for further investigation. In order to better characterise the ethane response to incremental exercise, it would be valuable to measure ethane output at a greater number of time points throughout exercise. Non-invasive assessment would increase the practicality of repeated sampling.

### 3.1.2.2 Previous studies in horses and dogs

The focus of exercise-induced oxidative stress studies in horses and dogs has tended towards the investigation of the potential effects of oxidative stress on performance. Exercise-induced oxidative stress has been reported previously in racehorses (Chiaradia et al., 1998; White et al., 2001), greyhounds (Marshall et al., 2002) and sled dogs (Baskin et al., 2000; Hinchcliff et al., 2000) following strenuous exercise. However, these studies employed plasma markers of lipid peroxidation. To date, ethane measurement has not been utilised in horses or dogs.

A non-invasive method of oxidative stress assessment may be preferable in veterinary research as blood collection has been shown to be related to physiological stress in animals (Balcombe et al., 2004). It has been suggested that breath samples may be suitable for diagnostic use in animals and can be collected with little distress to the animal (Wyse et al., 2004a). Expired air samples have been collected successfully from both horses (Murphy et al., 1998; Sutton et al., 2003) and dogs (Papasouliotis et al., 1995; Wyse et al., 2001), although not for the purpose of ethane analysis. Pentane output has been measured
previously in both horses (Wyse et al., 2004b) and dogs (Kim et al., 1996), although not in relation to exercise. However, pentane may not be the most reliable indicator of lipid peroxidation due to high hepatic pentane metabolism (Filser et al., 1983). Therefore, it would be of value to determine if non-invasive assessment of lipid peroxidation via ethane measurement is viable following exercise in these species.

3.1.3 Carbon monoxide

Carbon monoxide (CO) is produced in the body via the breakdown of haemoglobin. The haem portion of the haemoglobin molecule is catabolised to CO and biliverdin by the action of the enzyme haemoxygenase-1 (HO-1) (Tenhunen et al., 1968). Biliverdin is subsequently enzymatically converted to bilirubin.

HO-1 is a heat shock protein, that is, a protein whose expression is increased by elevated temperature. However, expression can also be increased by other stressors such as release of cytokines, ROS and nitric oxide during the inflammatory response. Therefore, it may also be referred to as a stress protein. The up-regulation of HO-1 may have a protective function in oxidative stress and inflammation (Horváth et al., 2001) since haem, which is catabolised by the enzyme, is known to have oxidant properties (Jeney et al., 2002) and the reaction product bilirubin is known to have antioxidant function (Stocker et al., 1987).

CO has been suggested as a non-invasive marker of HO-1 activity (Tenhunen et al., 1968) and oxidative stress in the lung (Horváth et al., 2001). Exhaled CO has been used as a marker of oxidative stress in chronic inflammatory diseases of the respiratory system such as asthma (Zayasu et al., 1997), chronic obstructive pulmonary disease (Montuschi et al., 2001) and cystic fibrosis (Paredi et al., 1999). CO is easy to measure in both healthy and patient populations and has been found to be reproducible within 5 % (Horváth et al., 1998).
Since HO-1 activation may be induced by oxidative stress, and oxidative stress can be a consequence of strenuous exercise, it is possible that HO-1 activation and, therefore, CO production may be increased as a result of strenuous exercise. However, few studies to date have investigated the effect of exercise on CO production or HO-1 induction. CO output increased following symptom-limited exercise in children with cystic fibrosis (Horváth et al., 1999). In healthy adults, HO-1 expression in lymphocytes has been shown to increase following a half-marathon run (Fehrenbach et al., 2003) and a treadmill run at 70 % \( \dot{V}O_2 \)max for 75 minutes (Thompson et al., 2005) but not following short duration exhaustive exercise or an acute bout of eccentric exercise (Fehrenbach et al., 2003). CO was not measured in the latter studies. Thus, it may be worthwhile to investigate the potential of CO as a marker of exercise-induced oxidative stress.

No studies, to date, have investigated the CO response to exercise in either horses or dogs. The measurement of CO may be of particular interest in the horse; exercise-induced pulmonary haemorrhage (EIPH) is a common condition in racehorses, characterised by bleeding in the lower respiratory tract due to stress failure of pulmonary capillaries during strenuous exercise (West et al., 1993). It has been suggested that 73-100 % of racehorses experience EIPH during high intensity exercise (McKane et al., 1993; Meyer et al., 1998). In contrast, this condition is rarely reported in human or canine subjects (King et al., 1990; Weiler-Ravel et al., 1995). The breakdown of haemoglobin subsequent to EIPH may lead to the formation of CO (Horváth et al., 1999). However, increased oxidative stress at maximal exercise may also lead to the increased formation of CO. The comparison of CO output in the horse with other species which do not generally suffer EIPH may indicate the efficacy of CO in the detection of EIPH in the horse.
3.1.4 Aims

The aims of this study were as follows. Firstly, to utilise a novel non-invasive technique for the assessment of post-exercise lipid peroxidation in three species: human, equine and canine. The use of non-invasive measurement is desirable from the perspective of subject compliance and comfort in all three species. Non-invasive assessment of EIOS has not been evaluated in horses or dogs previously. Traditional non-invasive measurement of hydrocarbon gases is time-consuming; however, a fast, efficient, accurate technique of ethane measurement has become available and has not been tested previously in any species during exercise.

The second objective of the study was to better characterise the oxidative stress response during and following incremental exercise to exhaustion by the collection of data at frequent intervals during exercise and during recovery. Previous studies have suggested the existence of a post-exercise oxidative stress response; however, there is very little information available regarding any response during exercise, and the response during recovery has not been clearly described.

Thirdly, it was intended to determine the utility of carbon monoxide as a marker of exercise-induced oxidative stress in all three species.
3.2 Methods

3.2.1 Human experiments

3.2.1.1 Subjects

Eight healthy, regularly active males volunteered to participate in this study. All subjects adhered to the exclusion criteria presented in section 2.1.1 (page 95). In addition, subjects in this study were required to be endurance trained, having exercised aerobically for at least 20 minutes, at least three times per week for at least eight weeks prior to participation. This was assessed by completion of a physical activity questionnaire (see Appendix A.5). At the time of the study, all subjects were currently in training for amateur competitive sport (athletics, cycling or football). Subjects were medically screened for abnormalities in resting electrocardiogram (ECG) and blood pressure prior to participation in order to ensure suitability for maximal aerobic exercise.

Ethical approval for the human element of this study was granted on 19th May 2004 by the ethics committee of the Institute of Biomedical and Life Sciences at the University of Glasgow, and all subjects provided written, informed consent as outlined previously (section 2.1.2, page 96). The relevant information sheet and consent form can be seen in Appendix A.1.

3.2.1.2 Test protocols

3.2.1.2.1 Familiarisation

General familiarisation procedures, as described in section 2.2.2 (page 97) were carried out at the start of the subject’s visit to the laboratory. Formal treadmill familiarisation, as
described below, took place immediately prior to testing and was utilised as a warm up period for the incremental test. The subject was also given the opportunity to practise mounting and dismounting the treadmill belt whilst it was in motion.

3.2.1.2.2 Incremental exercise test

Each subject underwent an incremental run to voluntary exhaustion on a motor driven treadmill (LE200CE, Erich Jaeger GmbH, Hoechberg, Germany). The protocol utilised required the subject to run at a constant speed throughout the test (MacDougal et al., 1990). This speed was determined during treadmill familiarisation prior to the start of the test, and was selected to elicit a steady state heart rate of 140-160 beats·min\(^{-1}\). Experience has shown that this typically produces a test duration of 8-17 minutes, which is considered to be optimal (Buchfuhrer et al., 1983). Test duration for subjects in the current study was 10-14 minutes.

The period of familiarisation consisted of walking on the treadmill until the subject felt steady and comfortable, followed by a gradual increase in speed to a running pace. The speed of the treadmill was then adjusted until a pace which elicited a steady state heart rate of 140-160 beats·min\(^{-1}\) was established. Treadmill speed was then reduced to a walking pace and the gradient was increased briefly to each possible test gradient (2-14 %). Following familiarisation, a rest period of 5-10 minutes preceded the start of the test during which time the subject was connected to the gas exchange equipment.

Treadmill speed during the test was kept constant at the pace which was shown to elicit a steady state heart rate of 140-160 beats·min\(^{-1}\) during the familiarisation period. This pace varied between subjects, and is reported in table 3.1. The treadmill gradient was 0 % during the first two minutes of the test and was increased by 2 % every two minutes thereafter until the subject signalled that he was no longer able to continue. At this point
the treadmill gradient was immediately reduced to 0 % and the speed was reduced to a walking pace of 4.5 km·h⁻¹. The subject completed a six minute period of active recovery at this pace, followed by a period of passive recovery, during which the subject rested in a chair, until 30 minutes had elapsed since the end of exercise. Figure 3.1 shows a schematic representation of the exercise test protocol.

### 3.2.1.3 Measurements

#### 3.2.1.3.1 Respired air measurements

Samples of expired air, each of one-minute duration, were collected for the purposes of expired ethane analysis and for determination of peak $\dot{V}O_2$. Collection equipment and procedures have been described in sections 2.3.1.1 and 2.3.2.2 (pages 100 and 107).

Two samples of expired air were collected at rest, prior to the start of exercise, in order to establish a baseline ethane concentration. The subject remained seated for a period of 10 minutes between the two resting samples at approximately 20 and 10 minutes prior to the start of exercise. During incremental exercise, the first sample was collected in the second minute of exercise (i.e. starting one minute after the onset of exercise), and then at two minute intervals until exhaustion. The timing of the exercise samples was designed to coincide with the second minute of each work rate as shown in figure 3.1. In cases where volitional exhaustion occurred during, or at the end of, a minute in which a sample was being collected for ethane analysis, this sample was also used for determination of peak $\dot{V}O_2$. Samples were also collected in alternate minutes once the subject’s heart rate reached 170 beats·min⁻¹ so that if the subject discontinued exercise during a minute in which no sample was being collected for ethane analysis, a sample would still be available for the determination of peak $\dot{V}O_2$. 
Exercise was continued until voluntary exhaustion; therefore, the duration of the exercise period was subject dependent with a range of 10-14 minutes. Expired air samples were of one minute duration.

Figure 3.1. Schematic representation of the incremental exercise test protocol and sampling points for ethane and carbon monoxide (CO).
Samples were collected in the second, fourth and sixth minutes of active recovery and at 20 and 30 minutes following the end of exercise, during which the subject was at rest. The timings of expired air samples for ethane analysis are illustrated in figure 3.1.

3.2.1.3.2 Ethane sampling procedures

Immediately following the test, a 5 litre sample of expired air was extracted from each Douglas bag into a Tedlar bag for later ethane analysis. All samples were analysed, following calibration of the spectrometer, within 24 hours of collection (Kneepkens et al. 1994).

A sample of ambient air was collected into a 5 litre capacity Tedlar bag, using a hand operated pump, in the middle of each of the above expired air sampling periods in order to correct expired air samples for the presence of atmospheric ethane.

3.2.1.3.3 Carbon monoxide sampling procedures

Carbon monoxide was measured using a portable electrochemical monitor (MicroLyser, Bedfont Scientific Ltd., Rochester, UK), sensitive to 1 ppm, which was calibrated prior to each test with a mixture of 50 ppm CO in air, according to the manufacturer’s instructions. The subject was asked to exhale fully and then to inspire to total lung volume, followed by a breath-hold for 15 s prior to blowing directly into the monitor via a T-piece and disposable mouthpiece (Jarvis et al., 1980). Ambient CO was measured immediately prior to each sample to correct for background concentration of the gas. Two samples were taken at baseline: 20 minutes and 10 minutes prior to the start of exercise; and further samples were taken 20 minutes and 30 minutes into recovery from exercise. A mean CO concentration was recorded for baseline and for recovery. CO measurements were made immediately following the collection of expired air for ethane analysis. The time points for CO sampling are indicated in figure 3.1.
3.2.1.3.4 Heart rate measurement

Heart rate was monitored throughout each test using a portable heart rate monitor (Polar Favor, Polar Electro Oy, Kempele, Finland). Peak heart rate was recorded as the highest heart rate measured during the test.

3.2.1.4 Analysis

3.2.1.4.1 Determination of peak oxygen uptake

Expired air was analysed for oxygen and carbon dioxide content and volume as described in section 2.3.1.1 (page 100).

Peak $\dot{V}O_2$ (l·min$^{-1}$) was calculated using the following equation (Wasserman et al., 2004):

$$\dot{V}O_2 \text{ (STPD)} = [\dot{V}I \text{ (STPD)} \times FIO_2] - [\dot{V}E \text{ (STPD)} \times FEO_2]$$  \[3.1\]

In equation 3.1, $\dot{V}I$ (STPD) and $\dot{V}E$ (STPD) are inspired and expired ventilation respectively, both measured in l·min$^{-1}$ and corrected to standard temperature and pressure, dry (STPD); $FIO_2$ is fractional inspired oxygen concentration; $FEO_2$ is fractional expired oxygen concentration; $FIO_2$ was assumed to be 0.2093 (Wasserman et al., 2004) and $FEO_2$ was measured.

Expired air was collected under ambient conditions, that is, at ambient temperature and pressure, and saturated with water vapour (ATPS). Expired minute ventilation under ambient conditions ($\dot{V}E$ [ATPS]) was calculated from the volume in litres of expired air collected divided by the duration of the collection in minutes. $\dot{V}E$ (ATPS) was then corrected to STPD as follows (Wasserman et al., 2004):
\[ \dot{V}_E \text{(BTPS)} = \dot{V}_E \text{(ATPS)} \times \left[ \frac{273 + 37}{273 + T} \right] \times \left[ \frac{\text{P}_B - \text{P}_{\text{H}_2\text{O}}}{\text{P}_B - 47} \right] \]  

[3.2]

and:

\[ \dot{V}_E \text{(STPD)} = \dot{V}_E \text{(BTPS)} \times \left[ \frac{273}{273 + 37} \right] \times \left[ \frac{\text{P}_B - 47}{760} \right] \]  

[3.3]

In equations 3.2 and 3.3, \( \dot{V}_E \) (BTPS) is expired minute ventilation (l·min\(^{-1}\)) at body temperature (37°C), ambient pressure and saturated with water vapour; T is the temperature of the expired gas in °C; \( \text{P}_B \) is barometric pressure in mmHg; \( \text{P}_{\text{H}_2\text{O}} \) is water vapour pressure at temperature T.

If volumes of inspired and expired nitrogen and other inert gases are assumed to be equal, \( \dot{V}_I \) (l·min\(^{-1}\)) can be calculated using the following equation:

\[ \dot{V}_I = \dot{V}_E \text{(STPD)} \times \left[ \frac{1 - \text{F}_{\text{EO}_2} - \text{F}_{\text{ECO}_2}}{1 - \text{F}_{\text{IO}_2} - \text{F}_{\text{ICO}_2}} \right] \]  

[3.4]

In equation 3.4, \( \text{F}_{\text{ICO}_2} \) is fractional inspired carbon dioxide concentration; \( \text{F}_{\text{ECO}_2} \) is fractional expired carbon dioxide concentration; \( \text{F}_{\text{ICO}_2} \) was assumed to be 0.0004 (Wasserman et al., 2004) and \( \text{F}_{\text{ECO}_2} \) was measured.

Thus, substituting equation [3.4] into equation [3.1]:

\[ \dot{V}_O_2 \text{ (STPD)} = \dot{V}_E \text{ (STPD)} \times \left[ \frac{\text{F}_{\text{IO}_2} \times (1 - \text{F}_{\text{EO}_2} - \text{F}_{\text{ECO}_2}) - \text{F}_{\text{EO}_2}}{(1 - \text{F}_{\text{IO}_2} - \text{F}_{\text{ICO}_2})} \right] \]  

[3.5]
This method of calculating $\dot{V}O_2$ has been found to be adequate both at rest and during exercise (Wagner et al., 1973; Wilmore & Costill, 1973).

3.2.1.4.2 Determination of peak respiratory exchange ratio

Carbon dioxide output ($\dot{V}CO_2$) was calculated as follows:

$$\dot{V}CO_2 \text{ (STPD)} = [\dot{V}E \text{ (STPD)} \times FECO_2] - [\dot{V}I \text{ (STPD)} \times FICO_2]$$  \hspace{1cm} [3.6]

In equation 3.6, $\dot{V}E$ (STPD) and $\dot{V}I$ (STPD) and are expired and inspired ventilation respectively, both measured in l·min$^{-1}$ and corrected to standard temperature and pressure, dry (STPD); $FECO_2$ is fractional expired carbon dioxide concentration; $FICO_2$ is fractional inspired carbon dioxide concentration; $FECO_2$ was measured and $FICO_2$ was assumed to be 0.0004 (Wasserman et al., 2004).

The respiratory exchange ratio (R) at peak exercise was calculated using the following equation:

$$R = \frac{\dot{V}CO_2 \text{ (STPD)}}{\dot{V}O_2 \text{ (STPD)}}$$  \hspace{1cm} [3.7]

3.2.1.4.3 Ethane analysis

Samples were analysed for ethane by ultra-sensitive laser spectroscopy as described previously (section 2.3.3, page 112). The spectrometer measured ethane concentration ([C$_2$H$_6$]) in parts per billion (ppb). This was converted into mol·l$^{-1}$ as follows, since 1 ppb $= 1 \times 10^{-9}$ l·l$^{-1}$ and, according to the ideal gas law, one mole of a gas occupies 22.4 l at standard temperature and pressure.
\[
[C_2H_6] (\text{mol} \cdot \text{l}^{-1}) = \frac{[C_2H_6] (\text{ppb}) \times 10^{-9}}{22.4}
\]  

[3.8]

Ethane concentration in expired air samples was corrected for any atmospheric ethane present in the inspired air as follows:

\[
\text{Corrected } [C_2H_6]_E = [C_2H_6]_E - [C_2H_6]_I
\]  

[3.9]

In equation 3.9, \([C_2H_6]_E\) is expired ethane concentration (mol·l⁻¹); \([C_2H_6]_I\) is inspired ethane concentration (mol·l⁻¹).

It is considered to be more accurate to express ethane as a rate standardised for body mass rather than as a concentration (Knutson et al., 1999), therefore, ethane output was calculated as follows:

\[
\dot{V}_C_2H_6 \text{ (pmol} \cdot \text{min}^{-1} \cdot \text{kg}^{-1}) = \left[ \frac{\text{Corrected } [C_2H_6]_E \times \dot{V}_E \text{ (STPD)}}{\text{body mass (kg)}} \times 10^{-12} \right]
\]  

[3.10]

In equation 3.10, \(\dot{V}_C_2H_6\) is ethane output (pmol·min⁻¹·kg⁻¹); \(\dot{V}_E\) (STPD) was calculated as shown above in equations 3.2 and 3.3.

3.2.2 Animal testing

The animal testing in this study was carried out by Dr Cathy Wyse of the Division of Companion Animal Science at the University of Glasgow Veterinary School in order to satisfy ethical considerations. All experimental procedures involving animals were approved by University of Glasgow Animal Ethics and Welfare Committee.
3.2.2.1 Subjects

Twelve racing greyhounds were recruited from dogs competing at a local stadium. The dogs were aged 1.5-3 years and had a body mass of 30-32 kg. Twelve racehorses, aged 2-10 years, were recruited from those training at a local yard. It was not possible to directly measure the body mass of the horses due to a lack of suitable equipment, therefore, body mass was estimated at 470 kg for analysis purposes. This value was previously reported for thoroughbred horses of a similar age, size and fitness (Leukeux & Art, 1994). All animals were in good health and free from medication at the time of testing.

3.2.2.2 Test protocols

The dogs performed maximal exercise during a competitive race on a sand racetrack over a distance of 480 m. The exercise test protocol for horses involved two bursts of gallop on a sand track over a distance of 603 m (3 furlongs). This was preceded by a warm-up at walk, trot then canter.

3.2.2.3 Measurements

Expired air samples of one-minute duration were collected prior to and following exercise. In dogs, the samples were collected immediately before and after the race; however, in horses the collection took place approximately 10 minutes before and after exercise due to the location of the stables, where the samples were collected, in relation to the track. Expired air was sampled using a plastic facemask which covered both mouth and nose of the animal. This was attached to a non-rebreathing valve (Hans Rudolph, Kansas City, USA) and samples were collected in a 5-litre Douglas bag (Cranlea, Birmingham, UK). This equipment can be seen in use in figure 3.2.
A sample of ambient air was drawn into a 5 litre capacity Tedlar bag in the middle of each expired air sampling period in order to correct for the presence of atmospheric ethane in expired air samples.

Each expired air sample was transferred from the Douglas bag into a Tedlar bag immediately following the end of each test for later ethane analysis. All samples were analysed, following calibration of the spectrometer, within 24 hours of collection (Kneepkens et al. 1994).

No equipment was available to measure volume of gas expired and therefore, the breathing frequency of each animal was measured by monitoring inspiratory movements of the chest wall during the one-minute collection period in order to estimate minute ventilation. Tidal
volumes for each animal were not measured and were assumed from previous literature as 5.6 l at rest and 6.5 l following exercise at gallop in thoroughbred horses of a similar age and fitness as the horses in the current study (Leukeux & Art, 1994). Similarly, in dogs tidal volume was estimated from previous mean data from trained greyhounds as 15 ml·kg\textsuperscript{-1} body mass at rest and 54 ml·kg\textsuperscript{-1} body mass one minute following 30 s of sprint treadmill exercise (Staaden, 1998).

Expired carbon monoxide was measured using the equipment described above for the human experiments, although the collection procedure was different. The monitor was attached to the outlet port of the non-rebreathing valve and CO concentration was recorded following a one-minute period during which the animal breathed through the system. A background reading was recorded prior to each sample in order to account for variations in ambient CO concentration. CO measurements were made immediately following the collection of expired air for ethane analysis, at rest and following exercise.

3.2.2.4 Ethane analysis

Samples were analysed for ethane as described previously (section 2.3.3, page 112). Expired ethane concentration was corrected for ambient ethane as described for humans using equations 3.8 and 3.9 (page 140).

Ethane output was then calculated as follows:

$$\dot{V}_{\text{C}_2\text{H}_6} (\text{pmol·kg}^{-1}·\text{min}^{-1}) = \frac{V_T \times Bf \times \text{Corrected} [\text{C}_2\text{H}_6]_E}{\text{estimated body mass (kg)} \times 10^{-12}}$$ \hspace{1cm} [3.11]

In equation 3.11, $\dot{V}_{\text{C}_2\text{H}_6}$ is ethane output (pmol·kg\textsuperscript{-1}·min\textsuperscript{-1}); $V_T$ is estimated tidal volume (l) as described in the text; $Bf$ is breathing frequency (breaths·min\textsuperscript{-1}).
3.2.3. Statistical analysis

Repeated measures analysis of variance was used to determine if there were any differences of statistical significance in ethane output between time points at which measurements were made. The assumption of sphericity was examined using Mauchly’s W test. In cases where this assumption was contravened, the Greenhouse-Geisser adjustment was applied. Where the analysis of variance indicated at least one significant difference, pairwise comparisons, with the application of a Bonferroni adjustment for multiple comparisons, revealed the location.

Paired t-tests were used to determine if there was any significant difference between the two resting measurements with respect to both ethane concentration and ethane output. In addition, Paired t-tests were used to discover any statistically significant differences in ethane output and carbon monoxide concentration between rest and peak exercise, in all species. Where data did not conform to a normal distribution, a Wilcoxon Signed Ranks test was utilised. Normality was tested using the Shapiro-Wilk test.

One-Way analysis of variance was carried out in order to determine if there were any statistically significant differences amongst all three species in ethane output at baseline and at post-exercise. Initially, a Levene test was used to determine the equality of variance between species. Where a significant difference was indicated, and variances were unequal between species, a Dunnett’s C post-hoc test was used to compare mean ethane output at the relevant time point for each species.
3.3 Results

3.3.1 Human data

3.3.1.1 Subject Characteristics

Subject characteristics are reported in table 3.1. A mean $\dot{V}O_2$ peak of 54.6 ml·kg$^{-1}$·min$^{-1}$ suggested that subjects had a high level of aerobic fitness. All subjects in the group fell above the 80th percentile of age-matched counterparts, with a classification of excellent or superior maximal aerobic power (American College of Sports Medicine, 2009).

Only one subject attained a true maximal effort in the incremental exercise test according to established criteria (Duncan et al., 1997) which are: a change in $\dot{V}O_2$ not exceeding 2.1 ml·kg$^{-1}$·min$^{-1}$ with an increase in work rate (i.e. a plateau in $\dot{V}O_2$); a blood lactate concentration of greater than 8 mmol·l$^{-1}$ post-exercise; a respiratory exchange ratio of greater than or equal to 1.15; and a peak heart rate of within 10 beats·min$^{-1}$ of age-predicted maximum heart rate, which is typically estimated as 220 minus age in years (Fox & Haskell, 1970; Fox et al., 1980). It was not possible to determine if a plateau in oxygen uptake had been attained since only one sample of expired air was collected, and blood samples were not obtained post-exercise for the determination of lactate concentration. Therefore, only the peak respiratory exchange ratio and peak heart rate were available as indicators of true effort. Only one subject achieved both criteria, however, the R value of five of the remaining subjects ranged from 1.02 to 1.13 suggesting at least a near maximal effort, with three of these subjects also exceeding predicted maximum heart rate. It has been noted that variability in R tends to be high (Howley et al., 1995). In any case, a true maximal effort was not crucial to the outcome of the study since peak $\dot{V}O_2$ was measured.
## Table 3.1. Subject characteristics for study one.

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<th>Subject</th>
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<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>( \dot{V}_{O_2} ) peak (l·min(^{-1}))</th>
<th>( \dot{V}_{O_2} ) peak (ml·kg(^{-1})·min(^{-1}))</th>
<th>HR peak (beats·min(^{-1}))</th>
<th>R peak</th>
<th>Treadmill speed (km·h(^{-1}))</th>
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<td>7</td>
<td>34</td>
<td>68.6</td>
<td>183.5</td>
<td>4.0</td>
<td>57.6</td>
<td>187</td>
<td>0.96</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>22</td>
<td>90.7</td>
<td>185.4</td>
<td>5.2</td>
<td>58.2</td>
<td>183</td>
<td>0.94</td>
<td>10.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>26 ± 4</td>
<td>76.0 ± 8.2</td>
<td>178.1 ± 6.9</td>
<td>4.2 ± 0.6</td>
<td>54.6 ± 2.5</td>
<td>191 ± 12</td>
<td>1.06 ± 0.08</td>
<td>11.1 ± 1.0</td>
</tr>
</tbody>
</table>

HR heart rate; R respiratory exchange ratio; SD standard deviation; \( \dot{V}_{O_2} \) oxygen uptake
simply as a descriptor of training status to ensure that subjects could be classed as endurance trained for a more meaningful comparison with the equine and canine athletes participating in the study.

### 3.3.1.2 Ambient ethane concentration

Ambient air was sampled for ethane analysis at the same time points as each expired air sample. Mean ambient ethane concentration for each test is shown in table 3.2. Mean ambient [C$_2$H$_6$] across all background samples was 185.3 ± 64.9 pmol·l$^{-1}$ (4.2 ± 1.5 ppb) with a range of 67.0 pmol·l$^{-1}$ to 325.9 pmol·l$^{-1}$ (1.5 ppb to 7.3 ppb). Ethane concentration is expressed as both ppb and pmol·l$^{-1}$ to assist comparison with previous data.

### 3.3.1.3 Ethane concentration and output at rest

The two resting expired air samples were corrected for ambient ethane and averaged to give a mean resting ethane concentration for each subject. These values were converted to ethane output and both sets of data are shown in table 3.3. Ethane output is expressed as both pmol·min$^{-1}$ and pmol·kg$^{-1}$·min$^{-1}$ to facilitate comparison with previous literature. Paired t-tests showed no significant difference between the two resting samples for ethane concentration ($t(4) = 0.334; p = 0.755$) or for ethane output ($t(4) = -0.131; p = 0.902$). Resting ethane concentration as a mean and standard deviation of all eight subjects was 72.7 ± 35.8 pmol·l$^{-1}$, and mean resting ethane output was 11.5 ± 9.1 pmol·kg$^{-1}$·min$^{-1}$ (872.1 ± 722.0 pmol·min$^{-1}$).
Table 3.2. Mean ambient ethane concentration during each incremental exercise test.

<table>
<thead>
<tr>
<th>Subject</th>
<th>$[C_2H_6]$ (ppb)</th>
<th>$[C_2H_6]$ (pmol·l$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5.2 ± 1.0</td>
<td>229.9 ± 45.3</td>
</tr>
<tr>
<td>2</td>
<td>3.5 ± 0.3</td>
<td>154.0 ± 12.4</td>
</tr>
<tr>
<td>3</td>
<td>3.2 ± 0.3</td>
<td>143.2 ± 14.6</td>
</tr>
<tr>
<td>4</td>
<td>2.7 ± 0.3</td>
<td>122.2 ± 13.1</td>
</tr>
<tr>
<td>5</td>
<td>3.6 ± 0.3</td>
<td>159.8 ± 11.5</td>
</tr>
<tr>
<td>6</td>
<td>6.2 ± 0.6</td>
<td>276.8 ± 26.1</td>
</tr>
<tr>
<td>7</td>
<td>2.8 ± 0.6</td>
<td>127.1 ± 26.6</td>
</tr>
<tr>
<td>8</td>
<td>6.0 ± 0.5</td>
<td>269.6 ± 22.2</td>
</tr>
</tbody>
</table>

All values are mean ± standard deviation.
Table 3.3. Mean resting ethane concentration and ethane output in each subject prior to an incremental exercise test.

<table>
<thead>
<tr>
<th>Subject</th>
<th>([C_2H_6]) (pmol·L(^{-1}))</th>
<th>(\dot{V}C_2H_6) (pmol·min(^{-1}))</th>
<th>(\dot{V}C_2H_6) (pmol·kg(^{-1})·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>71.9</td>
<td>1002.3</td>
<td>13.4</td>
</tr>
<tr>
<td>2</td>
<td>34.8</td>
<td>237.7</td>
<td>3.3</td>
</tr>
<tr>
<td>3</td>
<td>57.6</td>
<td>613.3</td>
<td>7.4</td>
</tr>
<tr>
<td>4</td>
<td>53.3</td>
<td>496.8</td>
<td>6.7</td>
</tr>
<tr>
<td>5</td>
<td>148.0</td>
<td>2524.3</td>
<td>31.8</td>
</tr>
<tr>
<td>6</td>
<td>83.5</td>
<td>535.1</td>
<td>8.3</td>
</tr>
<tr>
<td>7</td>
<td>89.5</td>
<td>1068.5</td>
<td>15.6</td>
</tr>
<tr>
<td>8</td>
<td>42.9</td>
<td>498.6</td>
<td>5.5</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>72.7 ± 35.8</td>
<td>872.1 ± 722.0</td>
<td>11.5 ± 9.1</td>
</tr>
</tbody>
</table>

SD standard deviation.

3.3.1.4 Ethane output during exercise and recovery

Figure 3.3 illustrates the pattern of response of ethane output to incremental treadmill exercise to volitional exhaustion in all eight subjects. All subjects showed a similar pattern during exercise: in general, ethane output rose markedly at the onset of exercise and continued to rise as work rate increased, reaching a peak at peak work rate. During recovery, ethane output tended to fall sharply at the end of exercise and then return to baseline gradually over a 30 minute period. It should be noted that the magnitude of rise in ethane output differed between subjects.
Figure 3.3. Ethane output prior to, during and following an incremental treadmill test to volitional exhaustion in eight subjects. Exercise duration was subject dependent and varied from 10 to 14 minutes. The exercise period was followed by 6 minutes of active recovery and 24 minutes of passive recovery. The dashed vertical lines indicate the onset of incremental exercise, passive recovery and active recovery respectively. The number in the top right hand corner of each panel refers to the subject number.
Figure 3.4 shows mean ethane output for five subjects at rest, during 14 minutes of incremental exercise to exhaustion, during 6 minutes of active recovery and a further 24 minutes of passive recovery. The data are presented in this way since only five of the eight subjects completed 14 minutes of incremental exercise prior to voluntary exhaustion. The three remaining subjects completed 8, 10 and 12 minutes of incremental exercise respectively. It can be seen from the standard deviations in figure 3.4 that the variability was high between individuals; thus, mean ethane output was comparable only between time points at which data were available for all subjects. Ethane output rose clearly at the first sampling point in exercise, and continued to rise steadily throughout the exercise period. A reduction in ethane output occurred immediately following the end of exercise, continuing to fall throughout the period of active recovery and returning close to a resting value in the passive recovery period.

Repeated measures analysis of variance was carried out to determine if there were any significant differences in ethane output between time points. The assumption of sphericity was contravened ($p = 0.00$), thus the Greenhouse-Geisser adjustment was applied ($\varepsilon = 0.101$). At least one significant difference was indicated between time points ($F(1.211, 2.423) = 24.224; p = 0.026$). Post-hoc pairwise comparisons, using the Bonferroni adjustment for multiple comparisons, showed that ethane output was significantly different between 6 minutes and 12 minutes of exercise ($p = 0.032$). Thus, although ethane output was observed to increase incrementally as work rate increased, and to decrease in a similar fashion during recovery, the differences between time points were not significant. The lack of significance may have been attributable to the small sample size and the large number of multiple comparisons; thus, a Paired t-test was undertaken to determine if there was any significant difference between ethane output at rest compared to the initial exercise sample, when analysed in isolation. The result of this analysis indicated that ethane output was significantly greater at 2 minutes into exercise than at rest ($t(4) = -5.819; p = 0.004$). It
was also of interest to determine at which point ethane output returned to resting levels following the onset of recovery. An isolated Paired t-test indicated that ethane output was clearly not significantly different from the resting response by 20 minutes into the recovery period ($t(3) = -0.326; p = 0.766$).

Figure 3.4. Mean ethane output during rest, incremental treadmill exercise to volitional exhaustion, and active and passive recovery in five subjects. Values are mean ± standard deviation of five subjects who completed 14 minutes of incremental exercise. The dashed vertical lines indicate the onset of incremental exercise, passive recovery and active recovery respectively. * indicates significant difference from value at 6 minutes into exercise ($p < 0.05$).
3.3.1.5 Carbon monoxide concentration

Figure 3.5 shows carbon monoxide concentration prior to and following the exercise test for each subject. Each data point is the mean of two measurements. There was a tendency for [CO] to decline or to remain stable over the course of the exercise. Figure 3.6 shows mean and standard deviation of [CO] at baseline (2.0 ± 0.5 ppm) and at post-exercise (1.6 ± 0.4 ppm). A Wilcoxon Signed Ranks test indicated that the small decrease in [CO] was not significant ($Z = -1.857; p = 0.063$).

Figure 3.5. Carbon monoxide concentration at baseline and following an incremental treadmill run to exhaustion in eight subjects.
3.3.2 Comparative data

3.3.2.1 Ethane output

Figure 3.7 shows responses at baseline and following maximal exercise for each individual subject in all three species. Data are reported from eight of the twelve racehorses. Two data sets were discarded due to possible erroneous estimation of the breathing frequency in two animals post-exercise, which resulted in unexpectedly low estimated minute ventilation. Breathing frequency post-exercise in these animals was 24 and 28 breaths.min\(^{-1}\), whereas the mean frequency for the other horses was 45 breaths.min\(^{-1}\). Breathing frequency at rest was similar in all horses. Since ethane output is a function of minute ventilation (see
section 3.2.1.4.3, page 139), the data were discarded as unreliable. The remaining two data sets were discarded due to the measurement of ambient ethane concentration as greater than expired air concentration in the resting samples. Similarly, two data sets from the canine athletes were discarded due to higher ambient than expired ethane at the post-exercise time point. Thus, data from ten greyhounds are reported.

Examination of figure 3.7 indicates that the variability in equine data was lower than in greyhounds or in humans; the horses responded in a similar fashion to the exercise stress, with the exception of a substantially greater post-exercise response in one horse. Indeed, when this animal’s exercise response was omitted from the data set, the mean and standard deviation of the equine data were markedly reduced from $23.4 \pm 19.5$ pmol·kg$^{-1}$·min$^{-1}$ to $16.6 \pm 2.9$ pmol·kg$^{-1}$·min$^{-1}$. 
Figure 3.7. Ethane output at baseline and post-exercise for all individual equine, canine and human subjects.
An inter-species comparison of mean ethane output at baseline and following maximal exercise is shown in figure 3.8. Mean ethane output at baseline, standardised for body weight, was low in all three species. Values for mean and standard deviation were as follows: 5.1 ± 2.7 pmol·kg\(^{-1}\)·min\(^{-1}\) in equine subjects; 18.1 ± 13.2 pmol·kg\(^{-1}\)·min\(^{-1}\) in canine subjects; and 11.5 ± 9.1 pmol·kg\(^{-1}\)·min\(^{-1}\) in human subjects. One-way analysis of variance indicated at least one significant difference was present in mean baseline ethane output amongst the three species (\(F(2, 23) = 3.927; p = 0.034\)). A Dunnett’s C post-hoc test confirmed that mean baseline ethane output was higher in canine subjects than in equine subjects (\(p < 0.05\)).

Mean ethane output increased significantly in all three species following maximal exercise, as indicated in figure 3.8 A Paired t-test showed that the increase in ethane output was significant in canine subjects (\(t(9) = -4.433; p = 0.002\)). Both equine and human data did not conform to a normal distribution and a Wilcoxon Signed Ranks test was undertaken in both cases. The increase in ethane output from baseline to post-exercise in horses was shown to be significant (\(Z = -2.521; p = 0.012\)). The data were analysed again with the outlying value described in section 3.3.2.1 (page 154) omitted. In this case the data conformed to a normal distribution and a Paired t-test was carried out; however, the increase in ethane output from baseline to post-exercise was still shown to be significant (\(t(6) = -14.986; p = 0.000\)). The increase in ethane output from baseline to maximal exercise was significant in the human subjects (\(Z = -2.521; p = 0.012\)).

The mean percentage increase in ethane output was greatest in canine athletes by a substantial margin (equine: 491 % increase; canine: 2317 % increase; human: 1902 % increase). Post-exercise values for mean and standard deviation were as follows: 23.4 ± 19.5 pmol·kg\(^{-1}\)·min\(^{-1}\) in equine subjects (16.6 ± 2.9 pmol·kg\(^{-1}\)·min\(^{-1}\) omitting outlying value); 346.8 ± 245.7 pmol·kg\(^{-1}\)·min\(^{-1}\) in canine subjects; and 178.8 ± 72.9 pmol·kg\(^{-1}\)·min\(^{-1}\)
in human subjects. One-way analysis of variance indicated at least one significant difference was present in mean post-exercise ethane output amongst the three species \((F(2, 23) = 9.233; p = 0.001)\). A Dunnett’s C post-hoc test confirmed that mean post-exercise ethane output in equine subjects was significantly different from both canine and human subjects \((p < 0.05)\); however, there was no difference between canine and human subjects.

<table>
<thead>
<tr>
<th>Species</th>
<th>n</th>
<th>Mean Post-exercise Ethane Output (pmol·kg(^{-1})·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equine</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Canine</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>Human</td>
<td>8</td>
<td>200</td>
</tr>
</tbody>
</table>

Figure 3.8. Inter-species comparison of ethane output at baseline and following maximal exercise. Values are mean ± standard deviation. Post-exercise samples were taken immediately following the cessation of exercise in human and canine subjects, and approximately 10 minutes following exercise in equine subjects. * denotes significant difference from baseline to post-exercise within each species; # denotes significant difference in baseline ethane output from all other species; † denotes significant difference in post-exercise ethane output from all other species.
3.3.2.2 Carbon monoxide concentration

Resting [CO] was measured at 1 ppm in three horses and three dogs; however CO concentration was below detectable limits in all animals post-exercise.
3.4 Discussion

3.4.1 Human data

3.4.1.1 Ambient ethane concentration

Ethane concentration in ambient and expired air samples was measured using a novel laser spectroscopy technique, as described previously (section 2.3.3, page 112; section 3.1.1.3, page 123). The range of ambient ethane concentrations recorded in the current study was 67.0 to 325.9 pmol·l\(^{-1}\) (1.5 ppb to 7.3 ppb), with a mean and standard deviation of 185.3 ± 64.9 pmol·l\(^{-1}\) (4.2 ± 1.5 ppb). These values compare favourably with previously reported ambient ethane concentrations of approximately 1.5 ppb, 5 ppb (Wyse et al., 2005a), and 2.3 ppb (Skeldon et al., 2005), using the laser spectroscopy technique.

Ambient ethane concentration in the current study also fell within the range of values of 68 to 726 pmol·l\(^{-1}\) reported previously by authors using gas chromatography as a measurement technique (Dumelin et al., 1978; Knutson et al., 1999; Sexton & Westberg, 1984). The similarity in ambient ethane concentration with previous studies gives some confidence in the comparability of techniques.

Ethane is present in the atmosphere from sources such as car exhaust emissions and cigarette smoke, and ethane concentration can often be higher in the ambient air than in the expired air (Knutson et al., 1999). For this reason, it is important to consider the ambient concentration in the testing environment in order to minimise the possibility of contaminating expired air samples with exogenous ethane. Subjects rested in the laboratory for at least 30 minutes prior to collection of the first expired air sample in order to allow sufficient time for equilibration with ambient ethane (see section 2.3.2.4.1, page 109).
Ambient ethane concentration was greater than expired ethane concentration in only one of the ninety samples collected from human subjects in the current study; ambient concentration was not notably greater than the previous or subsequent samples, rather, the expired air ethane concentration was spuriously low. It has been suggested that the technique of subtracting ambient from expired ethane concentration in order to correct for the ethane content of ambient air may be prone to error due to potentially rapid variation in ambient concentration (Knutson et al., 2000). However, in the current study, ambient ethane concentration remained fairly steady throughout each test, as can be seen from the magnitude of the standard deviations in table 3.2. Thus, the potential for contamination appeared to be minimal in this testing environment.

3.4.1.2 Ethane concentration and output at rest

Two expired air samples were collected at rest, and the resting value for ethane concentration and for ethane output was expressed as an average of the two measurements. There was no significant difference between the two resting samples for either variable, suggesting some reproducibility of measurement.

Mean expired ethane concentration at rest was $72.7 \pm 35.8$ pmol·l$^{-1}$. This was somewhat higher than values of 10.4 to 50 pmol·l$^{-1}$ reported in previous studies (Knutson et al., 1999; Zarling & Clapper, 1987). There was one spuriously high value in subject 5; however, even with this value omitted, the mean ethane concentration at rest of $61.9 \pm 20.5$ pmol·l$^{-1}$ was still higher than the previously reported range.

Mean resting ethane output of $872.1 \pm 722.0$ pmol·min$^{-1}$ was substantially higher than previously reported values of approximately 85 pmol·min$^{-1}$ in healthy individuals (Leaf et al., 1997), and approximately 45 pmol·min$^{-1}$ and approximately 75 pmol·min$^{-1}$ in two groups of patients with coronary artery disease (Leaf et al., 1999). One other study by the
same group of authors (Leaf et al., 2004) reported resting ethane output in a control group of individuals, all of whom had hypertension or diabetes mellitus, as 250158 pmol·min⁻¹. Examination of the raw data showed that this was the mean of only four individuals, one of whom had an extremely high resting ethane output of 1634000 pmol·min⁻¹. Omitting this outlying value and recalculating the mean still gave a very high mean resting ethane output of 39035 pmol·min⁻¹. As noted earlier (section 3.1.2.1, page 125) confidence in these data are low, since the authors did not comment on the striking difference in these values to those they had measured previously utilising the same techniques (Leaf et al., 1997, 1999). In addition, there was no mention in any of the three studies from Leaf and colleagues of correction for ambient ethane concentration, and the nomenclature within each paper lacked clarity leading to some confusion as to whether values reported were of ethane concentration or ethane output.

Expired ethane output at rest, from non-exercise studies which have utilised well-documented methodology, has varied from a mean of 0.3 to 3.0 pmol·kg⁻¹·min⁻¹ (Knutson et al., 1999). Again, the mean and standard deviation of 11.5 ± 9.1 pmol·kg⁻¹·min⁻¹, as recorded in the current study, was substantially higher.

Ethane output is a function of ethane concentration and minute ventilation. Examination of the raw data showed that minute ventilation at rest was higher than expected in subject 5, with a mean of 24.2 l·min⁻¹. Mean (and standard deviation) minute ventilation for all other subjects was 10.8 ± 2.8 l·min⁻¹. Subject 5 had no previous experience with breathing using a mouthpiece and nose clip, and despite extensive familiarisation prior to collection of the first resting sample, it appears that hyperventilation was present. Omitting the resting ethane output value for this subject gave a mean resting ethane output from the current study of 8.6 ± 4.4 pmol·kg⁻¹·min⁻¹. This value was still higher than expected in relation to previous literature.
It is unlikely that this discrepancy was due to the measurement technique, since ambient ethane concentration measured by laser spectroscopy was within the expected range. In addition, it has been reported that ethane concentration measured by the laser spectroscopy technique is accurate to within 10% of certified ethane standards (Skeldon et al., 2006). It may be that the differences noted are simply due to high inter-individual variability, which is a common feature of ethane measurement (Thekedar et al., 2009). The variation in resting ethane concentration and ethane output between individuals in the current study was notable, as can be seen from the large standard deviations in table 3.3, and high variability has been reported in previous studies (Kneepkens et al., 1999). This variability has been proposed to be influenced by factors such as antioxidant status and training status (Leaf et al., 1999). The current study is the first to measure ethane concentration and output at rest in endurance trained individuals. There is some evidence to suggest that the antioxidant enzyme status of skeletal muscle may be upregulated by endurance training (Gomez-Cabrera et al., 2008b; Powers & Lennon, 1999). However, if this was the case, trained individuals might be expected to show lower ethane concentration and output than untrained individuals, although studies have not equivocally shown reduced oxidative stress, measured by alternative markers, following a period of training (Vollaard et al., 2005). With the exception of ensuring that subjects were not currently taking any antioxidant supplementation, nutritional status was not controlled in the current study, since only one visit to the laboratory was required. Further investigation of the variability in resting ethane would be warranted.
3.4.1.3 Ethane output during exercise

Several studies have measured oxidative stress immediately following incremental exercise to exhaustion, and for up to 24 hours during recovery (Vollaard et al., 2005). However, it has been indicated that free radical production may decrease rapidly within 1-2 minutes following the cessation of muscle contraction (O’Neill et al., 1996) suggesting that it may be of value to measure oxidative stress during exercise as well.

Very few studies have investigated ethane output during dynamic exercise (Leaf et al., 1997, 1999, 2004), and those which have, have reported values only at peak exercise following incremental treadmill exercise to exhaustion, and at lactate threshold. The results of the current study confirmed previous findings that oxidative stress, as measured by ethane output, is significantly increased at peak exercise from the resting value. Mean ethane output at peak exercise in the current study was 13603.1 ± 5783.9 pmol·min⁻¹. This mean value was substantially higher than the 2400 pmol·min⁻¹ reported at peak treadmill exercise in healthy, untrained individuals by Leaf and colleagues (1997). This is unsurprising since resting ethane output was substantially higher in the current study. In addition, the subjects in the current study were trained individuals, and therefore, are likely to have exercised to a greater absolute work rate than untrained individuals. Thus, this study extends the finding of a significant rise in ethane output at peak exercise to a sample of trained individuals. A significant rise in lipid peroxidation at peak exercise has been reported previously in trained individuals using alternative markers (Vollaard et al., 2005).

No previous study has measured oxidative stress throughout a period of incremental exercise. In the current study, ethane output was measured at two minute intervals throughout the entire exercise period. The rise in ethane output above the resting value was shown to be not significant at any time point using Repeated measures analysis of
variance; however, an isolated Paired t-test suggested that ethane output was significantly increased as early as 2 minutes into the period of incremental exercise.

The pattern of response of ethane output during exercise has not been reported before. A trend for ethane output to increase as work rate increased was seen, although the increase in ethane output was not significant at each subsequent time point. This may have been due to the relatively small sample size, but it may have been that the increase in work rate at each time point was insufficient to trigger more than a small, insignificant increase in lipid peroxidation.

3.4.1.4 Ethane output during recovery

Ethane output was measured at 2 minutes, 4 minutes and 6 minutes of active recovery during which the subject walked comfortably on the treadmill. Further measurements were made at 20 minutes and 30 minutes into the recovery period during which the subject was seated comfortably in a chair. The ethane output response fell on termination of exercise and returned to a resting value by 20 minutes into the recovery period. Statistically it was unclear if the response could have reached a resting value earlier in the recovery period. A larger sample size could perhaps have delineated this more clearly.

Only one previous study has used ethane output as a means of assessment of lipid peroxidation following an incremental exercise test to exhaustion in healthy individuals (Leaf et al., 1997). Ethane output was highest at peak exercise and was still significantly elevated above the resting response at 5 minutes into the recovery period. It was stated that this was a “resting-recovery” period which would suggest that subjects did not undertake an active cool down from maximal exercise. This is a rather unconventional design, since a cool down period is advised to minimise risk of venous pooling following strenuous exercise (American College of Sports Medicine, 2009). However, this pattern of a
reduction in ethane output following peak exercise was similar to the current study. Other studies have utilised invasive markers of lipid peroxidation during the first 30 minutes of recovery from an incremental exercise test to exhaustion (Jammes et al., 2004, 2005; Steinberg et al., 2006; Vider et al., 2001b). One study made only one measurement during the recovery period, at 30 minutes following the end of exhaustive exercise, and the response had returned to resting level by this time (Vider et al., 2001b). Three studies by the same group (Jammes et al., 2004, 2005; Steinberg et al., 2006) reported that the peak lipid peroxidation response occurred 5 minutes (Jammes et al., 2004; Steinberg et al., 2006) or 10 minutes (Jammes et al., 2005) into the recovery period, rather than at peak exercise. It is possible that this could have been influenced by a delay in lipid peroxidation products entering the circulation from the working muscles, along with transit time to the site of blood collection. In all three studies, the response was still elevated above the resting response at 20 minutes (Steinberg et al., 2006) and 30 minutes (Jammes et al., 2004, 2005) into the recovery period. Thus, the current study provides support for some aspects of previous findings; however, overall, there is no agreement between studies with regard to the timing of the oxidative stress response.

3.4.2 Comparative data

3.4.2.1 Ethane output

The results demonstrated an increased oxidative stress following maximal exercise in all three species, as measured by expired ethane. This is the first time that oxidative stress has been measured in relation to exercise using this technique in any species, and the first time that oxidative stress has been measured non-invasively in horses and dogs. These data support the previous literature which has reported exercise-induced oxidative stress in all three species measured using plasma markers of lipid peroxidation (Chiaradia et al., 1998; Jammes et al., 2004, 2005; Marshall et al., 2002; Steinberg et al., 2006; White et al.,
A straightforward comparison between species cannot be made since exercise by the horses and dogs was undertaken in the field, and thus was not well quantified. In addition, the expired air measurements in the horses were collected 10 minutes following the end of exercise. Results from the current study, related to the recovery period in humans, have suggested that ethane output falls rapidly on cessation of exercise, and therefore, it is unsurprising that the post-exercise increase in ethane output was lowest in the equine athlete. This may also help to explain the lower variability seen between individual horses in comparison to that seen in the other species. Furthermore, since minute ventilation had to be estimated in both horses and dogs, errors in true ethane output in both species were expected.

3.4.2.2 Carbon monoxide concentration

Carbon monoxide concentration in the breath was measured at pre- and post-exercise in all three species. Carbon monoxide concentration at rest in human subjects was similar to that measured previously in healthy human subjects (Horváth et al., 1998, 1999; Paredi et al., 1999; Zayasu et al., 1997). A recent study measured resting [CO] in horses with respiratory inflammation and found similar values to those reported in the healthy equine subjects in the current study (Wyse et al., 2005a). Since both studies utilised the same measurement procedure, this suggests that carbon monoxide measurement may not be useful for the detection of respiratory inflammation in the horse.

The somewhat higher resting carbon monoxide concentration in the breath of the human subjects in the current study was likely due to the difference in measurement procedures. Human subjects were asked to perform a 15 second breath-holding manoeuvre prior to exhaling into the carbon monoxide monitor. This procedure is common in carbon monoxide measurement in humans (Horváth et al., 1999; Irving et al., 1988); however, is not possible in animals.
Carbon monoxide concentration tended to remain stable or decrease marginally from rest to post-exercise in human subjects; however, no significant difference was shown. There was also a suggestion of a reduction in [CO] in horses and dogs, as mean resting concentration was 1 ppm, and post-exercise concentration was below detectable limits in all animals. Carbon monoxide measurement in human and equine subjects did not take place immediately following exercise, but rather 20 minutes and 30 minutes after the end of maximal exercise in human subjects, and 10 minutes after the end of exercise in equine subjects; thus, it is possible that any increase in [CO] is transient and may have returned to resting values in these species by the time measurements were made. One previous study measured [CO] following exercise in children with cystic fibrosis and in healthy control subjects (Horváth et al., 1999). The authors reported a decrease in [CO] in both groups immediately following incremental cycle ergometer exercise to a symptom limited maximum, thus supporting the current findings. The expected rise in [CO] following maximal exercise did not occur. It may be that the exercise stimulus was not sufficient to promote upregulation of HO-1 and a consequent increase in CO production. An increase in HO-1 has been reported following a half-marathon performance (Fehrenbach et al., 2003) and a prolonged treadmill run (Thompson et al., 2005) in human subjects; however, short duration incremental treadmill exercise had no effect on HO-1 (Fehrenbach et al., 2003). It is possible that any increase in CO may have been localised to the working skeletal muscle and may not have been detectable at the lung at sufficient concentration at the time of measurement.

Carbon monoxide concentration may not be a suitable indicator of lipid peroxidation in any of the three species under investigation. However, it would be of interest to investigate carbon monoxide concentration in human subjects immediately following exercise to determine if there is any influence of time on the measurement.
3.4.3 Limitations and further work

The small sample size has already been alluded to as a limitation in this study, making it difficult to achieve statistical power, and obscuring clarity in cases where a statistical difference was not found. This was due, in part, to the varying duration of incremental exercise amongst individuals. This was unavoidable since exhaustive exercise tests naturally vary in duration amongst individuals of varying aerobic capacity.

The design of the exercise protocol in the current study was limited to some extent. It fulfilled the requirements of the study, in that a treadmill protocol was necessary in order to standardise the mode of exercise to running to make comparisons amongst species possible. However, it did not allow measurement of oxidative stress throughout the entire work rate range from rest to maximal exercise since the initial work rate on the treadmill corresponded to a heart rate of approximately 150 beats·min$^{-1}$. It is more difficult to standardise increments in work rate on the treadmill, in comparison to the cycle ergometer, due to individual differences in running style, and since the transition from walking to running must be taken into account (Wasserman et al., 2004). In order to eliminate this constraint, a further study was undertaken utilising a cycle ergometer protocol during which oxidative stress was measured throughout the entire work rate range from rest to exhaustion (see Chapter 5).

The results of the current study suggest that lipid peroxidation, as measured by ethane output, increases as work rate increases. However, it is not known to what extent the duration of exercise may also have an influence. Each successive work rate was performed for 2 minutes, immediately following the previous work rate; however it is unknown how much of the response to subsequent work rates may have been due to a cumulative effect of the exercise at preceding work rates. It was not possible to investigate this potential effect of exercise duration within the design of the current study, and it is planned to
address this issue in a later study by investigation of the time course of the oxidative stress response (see Chapter 5).

Further work could focus on the validation of oxidative stress as assessed by expired ethane measured by laser spectroscopy against more traditional plasma markers of oxidative stress. It has been suggested that, since there is no “gold standard” technique available for the assessment of oxidative stress, more than one plasma marker should be used in any investigation (Halliwell & Gutteridge, 1999). However, the use of ethane as a marker of oxidative stress has not yet been validated against any plasma marker in vivo (Kneepkens et al., 1994).

3.5 Conclusions

The results of this study demonstrate that oxidative stress can be assessed by expired ethane in exercising humans, horses and dogs using the novel technique of ultra-sensitive laser spectroscopy. The previous finding of an increased oxidative stress at peak exercise was confirmed in a trained population. The pattern of response during incremental treadmill exercise to volitional exhaustion was characterised for the first time. Oxidative stress was shown to increase as early as 2 minutes following the onset of exercise, and continued to increase as work rate increased during this mode of exercise until exhaustion. The response peaked at maximal exercise and fell rapidly on recovery, reaching resting values by 20 minutes into the recovery period. Carbon monoxide does not appear to be a viable marker of exercise-induced oxidative stress in any of the species under investigation.
Chapter 4

Pilot tests investigating real-time ethane measurement
4.1 Introduction

It has been suggested that the laser spectroscopy technique described by Gibson and colleagues (2002) may be utilised for the real-time measurement of ethane concentration in the expired air. These authors reported results from a test in which four subjects, two smokers and two non-smokers breathed directly into the laser spectrometer over a period of approximately 15 s and recorded the ethane concentration over that period of time. The recording from the smokers showed a sharp increase in ethane after an initial delay, presumably due to the time taken for the expired air to reach the measurement cell within the instrument. Dahnke and colleagues (2001) utilised a similar design to demonstrate the gradual reduction in expired ethane concentration in one subject after smoking a cigarette. Expired air samples were collected into gas-impermeable bags approximately every 30 minutes for 4 hours. Therefore, the time course of ethane exhalation could be determined, although the samples were collected and analysed following the experiment, and time intervals between samples were long. A later study by the same group (von Basum et al., 2003) reported data from three subjects who were asked to inhale and exhale directly into the spectrometer. A tracing of real-time ethane concentration was displayed, although subjects were asked to adhere to a pre-specified breathing pattern.

Real-time measurement of ethane has not been applied previously to exercise. An earlier study (Wyse et al., 2005b), reported in the preceding chapter, reported a gradual increase in ethane output throughout incremental exercise to exhaustion by collecting data at discrete time points throughout the exercise. Real-time measurement of ethane output would constitute a useful extension to this work by allowing an extremely high density of data collection and substantial improvement in characterisation of the time course of the oxidative stress response during exercise. Thus, it was proposed to undertake pilot tests to determine if real-time ethane spectroscopy could be developed for use during exercise. The
specific aims of these pilot tests were to develop measurement procedures and analysis techniques.

## 4.2 Zero calibration tests

### 4.2.1 Rationale

The spectrometer had been utilised previously for the measurement of ethane in single, discrete samples of expired air introduced to the inlet port of the spectrometer, but never for the continuous measurement of ethane in expired air over a prolonged duration. Thus, the initial two pilot tests were undertaken to determine if a useful output could be recorded from the spectrometer under its normal operating conditions which include a regular zero calibration using hydrocarbon-free nitrogen as described in section 2.3.3 (page 112).

### 4.2.2 Methods

#### 4.2.2.1 Subjects

Subjects were two young, healthy males. Both individuals had been regularly active for at least eight weeks prior to participation as determined by the physical activity questionnaire shown in Appendix A.5, and adhered to the exclusion criteria described in section 2.1.1 (page 95). Subjects were medically screened for abnormalities in resting electrocardiogram (ECG) and blood pressure prior to participation in order to ensure suitability for maximal aerobic exercise.
4.2.2.2 Test protocol

Each subject was familiarised with all equipment prior to the test as described in section 2.2.2 (page 97). The test protocol consisted of incremental exercise to voluntary exhaustion on a mechanically-braked cycle ergometer (Monark 828E, Vansbro, Sweden). The cycle ergometer was chosen as the mode of exercise since the location of testing was required to be the laboratory housing the laser spectrometer, thus the treadmill was not a viable option. Subjects rested whilst seated on the cycle ergometer prior to the start of exercise for 4 minutes, or until resting respiratory responses were stable and within the following ranges: 

\( \dot{V}E \) of 5-10 l·min\(^{-1}\), R of 0.7-0.9; and \( \text{PETCO}_2 \) of 37-43 mmHg. Subjects began by pedaling at a steady, comfortable cadence against an unloaded flywheel for 4 minutes. They were then asked to increase the cadence as quickly as possible to 60 rev·min\(^{-1}\) and were asked to maintain this cadence throughout the test. Work rate was incremented at 15 W·min\(^{-1}\) until the subject was no longer able to maintain the required cadence. At this point, the flywheel was rapidly unloaded and the subject was allowed to cool down at a slow and comfortable cadence for a recovery period of 6 minutes.

4.2.2.3 Measurements

4.2.2.3.1 Respired air measurements

Respired air measurements including ventilation (\( \dot{V}E \)), breathing frequency (Bf) and end-tidal oxygen (\( \text{PETO}_2 \)) and carbon dioxide (\( \text{PETCO}_2 \)) were made using a portable breath by breath metabolic cart (Jaeger Oxycon Mobile, Viasys Healthcare, Hoechberg, Germany).

A schematic representation of the breathing assembly used for real-time testing is shown in figure 4.1. The subject wore a nose-clip and breathed through a mouthpiece which was connected to the measuring sensor of the Oxycon Mobile. The measuring sensor housed a volume sensor which was connected by cable to a measuring unit. The lightweight
measuring unit was connected to a transmitter, both of which were fixed to a harness worn over the subject’s shoulders like a rucksack. The measuring unit contained oxygen and carbon dioxide sensors; this was connected to the measuring sensor at the subject’s mouth by a gas sample line. Data were transmitted from the transmitter to the base unit of the system by telemetry. The base unit was connected to a laptop computer (Hewlett-Packard zt3000, Berkshire, UK).

The measuring sensor was connected to a two-way non-rebreathing valve (Hans Rudolph 2700, Kansas City, USA) by a short piece of rubber tubing. The subject wore a head-support (Hans Rudolph 2726, Kansas City, USA) to bear the weight of the breathing assembly.

The metabolic cart was calibrated prior to each use according to the manufacturer’s instructions. This consisted of an automatic volume calibration in which flow rates of 0.2 and 2 l·s\(^{-1}\) were measured. The gas sensors were calibrated using a gas mixture of 5 % CO\(_2\), 16 % O\(_2\) with a balance of N\(_2\), and with ambient air. In addition, the delay time between mouth and gas sensors was determined as part of the calibration procedure.

4.2.2.3.2 Heart rate measurement

Heart rate was measured throughout each test using a portable heart rate monitor (Polar Favor, Polar Electro Oy, Kempele, Finland).
Figure 4.1. Schematic representation of breathing assembly for real-time measurement of ethane output. Components are not drawn to scale.

- Inspired air
- Rubber connector
- Measuring sensor
- Mouthpiece
- Measuring unit
- Transmitter
- Tubing for capture of expired air
- Two-way non-rebreathing valve
- Gas sample line
- Volume signal
- To laser spectrometer
- To base unit connected to laptop computer
4.2.2.3.3 Ethane sampling procedures

Expired air for sampling ethane was drawn continuously into the laser spectrometer from a sample line connected to the breathing valve as shown in figure 4.1. The spectrometer was initially set up to sample the expired air continuously with a check of the zero reading with a gas mixture containing less than 0.1 ppb of ethane at intervals of 78 seconds. The tubing for capture of expired air shown in figure 4.1 was not used in the first two pilot tests.

4.2.2.4 Analysis

4.2.2.4.1 Data editing

Prior to determination of peak oxygen uptake and estimation of the lactate threshold, ventilatory and pulmonary gas exchange variables were edited to remove any breaths which were uncharacteristic of the underlying physiological response. This can occur due to the subject voluntarily taking an atypical breath, or due to a mis-triggering by the computer software. For example, if the subject were to swallow or cough during a breath, the software may interpret this as two breaths rather than one. These instances were found by examining plots of Bf, PETO₂ and PETCO₂ versus time and any clearly atypical breaths were removed from the data set. Subsequently, \( \dot{V}_E \), \( \dot{V}_O_2 \) and \( \dot{V}_C O_2 \) were each plotted against time. The response to each phase of the test: rest, unloaded pedaling, incremental exercise and recovery, was fitted with a regression line and prediction bands were added. It has been established that the noise associated with breath by breath measurement of ventilatory variables follows a normal distribution (Lamarra et al., 1987), and, therefore, any data points lying outwith four standard deviations of the mean response were also removed from the data set since these were highly unlikely to be part of the underlying physiological response.
4.2.2.4.2 Determination of peak oxygen uptake

Oxygen uptake (\(\dot{V}O_2\)), carbon dioxide output (\(\dot{V}CO_2\)) and respiratory exchange ratio (R) were calculated according to equations 3.1 to 3.7 (pages 137-139). Peak oxygen uptake was recorded as the mean oxygen uptake over the final 10 s of incremental exercise.

4.2.2.4.3 Estimation of lactate threshold

Lactate threshold was estimated according to the V-slope method of Beaver and colleagues (1986) in association with other relevant ventilatory and pulmonary gas exchange variables (Whipp et al., 1986). This method is based upon the production of non-metabolic \(CO_2\) from the following reactions, which occur as lactic acid (HLa) is produced, dissociates into the lactate anion (La\(^{-}\)) and a proton (H\(^{+}\)), which is buffered by sodium bicarbonate in the muscle, or potassium bicarbonate in the blood.

\[
HLa \rightarrow La^{-} + H^{+} \quad [4.1]
\]

\[
H^{+} + HCO_{3}^{-} \rightarrow H_{2}CO_{3} \rightarrow CO_{2} + H_{2}O \quad [4.2]
\]

The result of this is that, as \(\dot{V}O_2\) continues to increase with increasing work rate during incremental exercise, the relationship between \(\dot{V}O_2\) and \(\dot{V}CO_2\) is altered such that \(\dot{V}CO_2\) begins to increase proportionately more than \(\dot{V}O_2\). The point at which this occurs, the estimated lactate threshold (\(\hat{\theta}\)) can be ascertained from a plot of \(\dot{V}CO_2\) versus \(\dot{V}O_2\).

The increased \(\dot{V}CO_2\) triggers a concomitant and proportional increase in \(\dot{V}E\), however this increase in \(\dot{V}E\) will be out of proportion to \(\dot{V}O_2\), such that the ventilatory equivalent for \(CO_2\) (\(\dot{V}E/\dot{V}CO_2\)) will remain stable immediately above the lactate threshold, whilst the ventilatory equivalent for \(O_2\) (\(\dot{V}E/\dot{V}O_2\)) will rise. Similarly, an increase would be expected...
in $\text{PETO}_2$ but not in $\text{PETCO}_2$ at the lactate threshold. The presence of these relationships can be used to check that the response under examination is, in fact, the lactate threshold, rather than a non-specific hyperventilation due to, for example, subject anxiety.

4.2.2.4.4 Determination of peak heart rate

Peak heart rate was determined as the mean heart rate recorded over the final 10 s of incremental exercise.

4.2.2.4.5 Ethane analysis

The output from the spectrometer consisted of ethane concentration measured in parts per billion at each sampling time point; sampling occurred at 1.1 second intervals. Figure 4.2 illustrates a typical example of the raw ethane concentration signal from the spectrometer, in which expired air was sampled throughout the testing period, with the exception of a 12 second period at intervals of 78 seconds during which the zero calibration of the spectrometer was checked. Data were edited for any unusual ethane responses, for example, at approximately 820 seconds in figure 4.2. Examination of the response pattern over a shorter time interval, as shown in figure 4.3, indicated the approximate stabilisation of the ethane concentration around 0 ppb during these calibration check periods. The mean zero reading at each calibration point was calculated by examining the plot constructed for each calibration period and calculating a mean of the points closely clustered about the nadir. Mean minute ventilation was calculated for each expired air sampling period and, subsequently, mean expired ethane output was calculated, according to equations 3.8 and 3.10 (page 140). This pattern of analysis was reproduced for pilot test two which shared the same sampling procedure.
Figure 4.2. Real-time raw ethane concentration signal from the spectrometer before and during incremental exercise to exhaustion in one male subject (pilot test one). Sampling pattern shows expired air sampling interspersed with verification of the zero reading of the spectrometer. The dashed vertical lines indicate the onset of unloaded pedaling and incremental exercise respectively.
Figure 4.3. Real-time raw ethane concentration signal during an incremental exercise test to exhaustion in one male subject (pilot test one). The data have been truncated to show a short time period of the response to emphasise the effect of zeroing of the spectrometer.

4.2.3 Results

4.2.3.1 Subject characteristics

Subject characteristics are displayed in table 4.1. Subjects were young, healthy males with a mean age of 24 ± 3 yr and a mean body mass of 73.5 ± 12.8 kg (mean ± standard deviation). Mean $\dot{V}_O_2$ peak, estimated lactate threshold and peak heart rate were recorded for one subject only since the test for the other subject had to be terminated prematurely due to failure of the battery in the portable metabolic cart. This could not be replaced in time to continue recording data prior to the end of the incremental phase of the test.
However, the attainment of a true maximum effort was not the primary aim of these tests and peak data have been included for the purposes of subject description only.

4.2.3.2 Zero calibration measurement

A typical raw ethane concentration signal is shown in figure 4.2, and the fall in ethane concentration to around 0 ppb at regular intervals can be seen clearly. Analysis of the raw data indicated that the mean response to zeroing of the spectrometer was -0.1 ± 0.2 ppb in pilot test one and 0.0 ± 0.3 ppb in pilot test two (mean ± standard deviation), suggesting that the zero calibration was adequate under these testing circumstances.

4.2.3.3 Ethane concentration response

Ethane concentration and ethane output in response to the exercise stimulus in both tests are reported, however, it must be noted that no measurement of ambient ethane concentration was undertaken in the initial two pilot tests, therefore the data cannot be compared to previous or future data. The data are reported as an indicator of the pattern of response, since in previous tests, ambient ethane has been shown to be quite consistent throughout the testing period as reported in section 3.3.1.2 (page 147).

Figure 4.4 shows post-analysis mean ethane concentration from pilot tests one and two. It can be seen that ethane concentration in the expired air varied little between the different phases of the test: rest; unloaded exercise; incremental exercise; and recovery. Pilot test one was terminated prior to attainment of voluntary exhaustion due to failure of the battery in the portable metabolic cart and, therefore, no data for the recovery phase were available. A similar pattern was seen in pilot test two.
4.2.3.4 Ethane output response

Figure 4.5 shows post-analysis ethane output from pilot tests one and two. There was evidence of a small rise in ethane output during rest and at unloaded pedalling, however, in general, the response remained low and fairly stable during both conditions. In incremental exercise, ethane output began to rise more evidently as work rate increased. In test two (figure 4.5; bottom panel) ethane output increased as work rate increased during incremental exercise, and began to rise more steeply at approximately 1000 s into the test. This reflected the pattern of change in ventilation following lactate threshold. The estimated lactate threshold of this subject was 1.9 l·min\(^{-1}\), which occurred at approximately 1000 s into the test. In pilot test one, a more rapid rise in ethane output was not seen, however, this test had to be terminated prematurely due to equipment failure. A glance at the two plots in figure 4.5 shows a difference in the scale of the rise in ethane output. In the first test, ethane output rose to approximately 30 pmol·kg\(^{-1}\)·min\(^{-1}\) at test termination, whilst the peak ethane output response in pilot test two was approximately 180 pmol·kg\(^{-1}\)·min\(^{-1}\). It is likely that the subject in pilot test one had not reached lactate threshold by the time the test was terminated. A further test utilising the same protocol and under the same conditions was conducted with the same subject 6 days following the test reported here and lactate threshold was estimated at 2.8 l·min\(^{-1}\). The \(\dot{V}O\_2\) at test termination of pilot test one was 2.8 l·min\(^{-1}\), therefore it is likely that the subject was just below or at lactate threshold at this time. Therefore, a sharp increase in ventilation and concomitant rise in ethane output were not expected by this stage of the test. Ethane output decreased very rapidly towards baseline in the recovery phase of pilot test two.
Table 4.1. Subject characteristics for real-time pilot tests.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Body mass (kg)</th>
<th>Height (cm)</th>
<th>( \dot{V}O_2 ) peak (l·min(^{-1}))</th>
<th>( \dot{V}O_2 ) peak (ml·kg(^{-1})·min(^{-1}))</th>
<th>( \hat{\theta} ) (l·min(^{-1}))</th>
<th>HR peak (beats·min(^{-1}))</th>
<th>R peak</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Zero calibration tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>82.5</td>
<td>187.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>64.4</td>
<td>171.5</td>
<td>2.6</td>
<td>40.4</td>
<td>1.9</td>
<td>195</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>24 ± 3</td>
<td>73.5 ± 12.8</td>
<td>179.3 ± 11.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Ventilatory challenge tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 *</td>
<td>38</td>
<td>49.0</td>
<td>157.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>28</td>
<td>82.3</td>
<td>187.0</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>33 ± 7</td>
<td>65.7 ± 23.5</td>
<td>172.0 ± 21.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Real-time measurement tests</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1</td>
<td>26</td>
<td>82.5</td>
<td>187.0</td>
<td>4.1</td>
<td>49.7</td>
<td>2.8</td>
<td>183</td>
<td>1.3</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>64.4</td>
<td>171.5</td>
<td>2.4</td>
<td>37.3</td>
<td>1.7</td>
<td>191</td>
<td>1.6</td>
</tr>
<tr>
<td>3</td>
<td>24</td>
<td>73.0</td>
<td>167.0</td>
<td>3.1</td>
<td>42.5</td>
<td>2.2</td>
<td>168</td>
<td>1.3</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>24 ± 1</td>
<td>68.7 ± 6.1</td>
<td>175.2 ± 10.5</td>
<td>3.2 ± 0.9</td>
<td>43.2 ± 6.2</td>
<td>2.2 ± 0.6</td>
<td>181 ± 12</td>
<td>1.4 ± 0.2</td>
</tr>
</tbody>
</table>

\( \hat{\theta} \) estimated lactate threshold; HR heart rate; R respiratory exchange ratio; SD standard deviation; \( \dot{V}O_2 \) oxygen uptake.

* data not available. Zero calibration test for subject 1 was terminated early due to equipment failure; Ventilatory challenge tests did not involve peak gas exchange or heart rate measurements. * All subjects were male with the exception of subject 1 in ventilatory challenge tests.
Figure 4.4. Mean ethane concentration prior to, during and following an incremental exercise test to exhaustion in two male subjects. 
Top panel shows pilot test one; bottom panel shows pilot test two. Pilot test one was terminated early due to equipment failure and shows no data for the recovery phase. The dashed vertical lines indicate the onset of unload pedalling, incremental exercise and recovery respectively.
Figure 4.5. Mean ethane output prior to, during and following an incremental exercise test to exhaustion in two male subjects.

Top panel shows pilot test one; bottom panel shows pilot test two. Pilot test one was terminated early due to equipment failure and shows no data for the recovery phase. The dashed vertical lines indicate the onset of unload pedalling, incremental exercise and recovery respectively.
4.2.4 Discussion

The ethane concentration varied little throughout the different phases of the exercise tests (figure 4.4). Since ethane output is a factor of ethane concentration and ventilation, it was unclear if the pattern of response of ethane output was simply a near duplicate of the ventilation response rather than an indication of the extent of lipid peroxidation. Further pilot tests were designed to investigate this issue, and are described in section 4.3 (page 188).

The ethane output response did not appear to be related to absolute work rate. The work incrementation rate was identical in both tests. The first pilot test was terminated at a work rate of 195 W at which point ethane output had risen to approximately 30 pmol·kg\(^{-1}\)·min\(^{-1}\). Ethane output at the same work rate in the second subject, which occurred at 20 min into pilot test two, was approximately 140 pmol·kg\(^{-1}\)·min\(^{-1}\), as shown in the bottom panel of figure 4.5. The second subject reached voluntary exhaustion at a work rate of 210 W.

The data suggested that a useful output could be recorded in real-time, and directly from the spectrometer, to begin to characterise the ethane output response during an extended period of exercise. However, ambient ethane concentration was not monitored during these pilot tests. This is necessary for the correction of expired ethane concentration for background ethane levels. The next step would be to determine if ambient ethane could be measured in real-time along with expired ethane. Pilot tests to investigate this issue are described in section 4.4 (page 197).
4.3 Ventilatory challenge tests

4.3.1 Rationale

Previous pilot tests (described in section 4.2, page 173) revealed little variation in ethane concentration with incremental exercise, whilst ethane output rose along with increased ventilation. The aim of the pilot tests described below was to investigate if the rise in ethane output was simply a manifestation of the ventilatory response to exercise, rather than an indication of exercise-induced oxidative stress. This was accomplished by forcing an increase in ventilation in circumstances which would not be expected to induce a significant oxidative stress.

4.3.2 Methods

4.3.2.1 Subjects

Two healthy individuals, one female and one male, volunteered to participate in these pilot tests.

4.3.2.2 Test protocol

Subjects were familiarised with personnel and equipment prior to undertaking the pilot test, as described in section 2.2.2 (page 97). During the test, the subject sat comfortably on a chair and was asked to breathe as normally as possible throughout the test. During pre-specified periods, as indicated in table 4.2, the subject breathed through a mouthpiece attached to a two-way non-rebreathing valve, and expired air was collected in Douglas bags, as described in section 2.3.1.1 (page 100). The mouthpiece was inserted, and nose-clip attached, two minutes prior to the start of each expired air collection period. A ventilatory challenge, in the form of additional dead space, was imposed at pre-specified
time points, again indicated in table 4.2, by adding a length of wide bore tubing to the inspired port of the breathing valve. The length of tubing was varied to alter the extent of the challenge. The female subject inspired through a tube with a volume of one litre, whereas the male subject inspired through tubes with volumes of both one litre and two litres.

Both subjects rested for a variable time prior to and following the first ventilatory challenge, and following the second ventilatory challenge. Timings for each test phase are indicated in table 4.2. An additional dead space of one litre was imposed on the female subject on two occasions during the test, interspersed by a rest period, whilst the male subject was challenged by an additional dead space of one litre on the first occasion, and by two litres on the second occasion.

4.3.2.3 Measurements

4.3.2.3.1 Respired air measurements
Expired air samples were of 2 minutes duration and were collected as indicated in table 4.2. In the female subject, two resting samples were collected prior to the first ventilatory challenge in order to establish a baseline response. Resting samples were also collected following each ventilatory challenge to check that ventilation had returned to rest following the challenge. Samples were collected during the final two minutes of each ventilatory challenge. A similar pattern was followed in the male subject although two samples were collected during each ventilatory challenge and no resting sample was collected between the two challenges to allow the subject some respite from the mouthpiece.
Table 4.2. Timing of expired air samples in relation to test phase for ventilatory challenge tests.

<table>
<thead>
<tr>
<th>Test Phase</th>
<th>Timing of phase (min)</th>
<th>Timing of expired air samples (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Subject 1 (female)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>0-14</td>
<td>2-4; 9-11</td>
</tr>
<tr>
<td>Ventilatory challenge (1 l)</td>
<td>14-19</td>
<td>17-19</td>
</tr>
<tr>
<td>Rest</td>
<td>19-28</td>
<td>24-26</td>
</tr>
<tr>
<td>Ventilatory challenge (1 l)</td>
<td>28-33</td>
<td>31-33</td>
</tr>
<tr>
<td>Rest</td>
<td>33-40</td>
<td>38-40</td>
</tr>
<tr>
<td><strong>Subject 2 (male)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>0-10</td>
<td>0-2; 7-9</td>
</tr>
<tr>
<td>Ventilatory challenge (1 l)</td>
<td>10-14.25</td>
<td>10-12; 12.25-14.25</td>
</tr>
<tr>
<td>Rest</td>
<td>14.25-17.25</td>
<td>No sample</td>
</tr>
<tr>
<td>Ventilatory challenge (2 l)</td>
<td>17.25-21.5</td>
<td>17.25-19.25; 19.5-21.5</td>
</tr>
<tr>
<td>Rest</td>
<td>21.5-34.5</td>
<td>25.5-27.5; 32.5-34.5</td>
</tr>
</tbody>
</table>

The duration of all expired air samples was 2 minutes.
4.3.2.3.2 Ethane sampling procedures

Expired air was sampled for the determination of ethane concentration as described in section 2.3.2.2 (page 107).

Ambient ethane concentration was monitored directly by the spectrometer continuously throughout the test. Ambient air was drawn into the sample cell continuously with the exception of 12 second periods at 78 second intervals during which the sample cell was flushed with hydrocarbon-free nitrogen in order to check the zero calibration of the spectrometer.

4.3.2.4 Analysis

4.3.2.4.1 Respired air analysis

Ventilation and \( \dot{V} \text{O}_2 \) were calculated by equations 3.3 and 3.5 respectively.

4.3.2.4.2 Ethane analysis

All expired air samples were analysed for ethane concentration within 30 minutes of the end of each experiment as described in section 3.2.1.4.3 (page 139). The ambient ethane signal from the spectrometer was plotted against time, as described in section 4.2.2.4.5 (page 179), for each of the time periods during which expired air was collected for later ethane analysis. Data points corresponding to the zeroing phase of the spectrometer were removed, and a mean of the remaining data was taken to represent mean ambient ethane during the expired air collection period. Expired air ethane concentration was corrected for ambient ethane as described in section 3.2.1.4.3 (page 139).
4.3.3 Results

4.3.3.1 Subject characteristics

Subject characteristics are shown in table 4.1.

4.3.3.2 Ventilation and ethane responses

Figures 4.6 and 4.7 show ventilation, ethane concentration and ethane output responses in the female and male subjects respectively. As expected, ventilation increased markedly during the periods of ventilatory challenge in both subjects and returned to baseline thereafter.

The ethane concentration response remained fairly stable in the female subject from baseline to the first ventilatory challenge. In the final three samples, ambient ethane concentration was greater than expired ethane concentration, therefore, expired ethane concentration was reported as $0 \text{ pmol} \cdot \text{L}^{-1}$. In the male subject (figure 4.7), expired ethane concentration fell during the first ventilatory challenge and fell further during the second, greater, challenge. It then rose again to baseline following the final challenge.

Ethane output, shown in the bottom panels of figures 4.6 and 4.7, rose during the first ventilatory challenge in the female subject. In the final three samples, calculation of ethane output was affected by the high ambient ethane as described previously. In the male subject, ethane output remained fairly stable from baseline through the first ventilatory challenge. Ethane output fell during the second challenge, despite the rise in ventilation, and increased again towards baseline during the following rest period.
Figure 4.6. Responses to a ventilatory challenge in one female subject (pilot test three). Top panel shows ventilation; middle panel shows ethane concentration; bottom panel shows ethane output. The first and second dashed vertical lines mark the onset and termination, respectively, of a one litre ventilatory challenge; the third and fourth dashed vertical lines mark the onset and termination, respectively, of a second one litre ventilatory challenge; the subject was at rest outwith these periods of challenge.
Figure 4.7. Responses to a ventilatory challenge in one male subject (pilot test four).
Top panel shows ventilation; middle panel shows ethane concentration; bottom panel shows ethane output. The first and second dashed vertical lines mark the onset and termination, respectively, of a one litre ventilatory challenge; the third and fourth dashed vertical lines mark the onset and termination, respectively, of a two litre ventilatory challenge; the subject was at rest outwith these periods of challenge.
4.3.3.3 Oxygen uptake response

Figure 4.8 shows the oxygen uptake response to the ventilatory challenge in the female subject (top panel) and in the male subject (bottom panel).

Figure 4.8. Oxygen uptake response to a ventilatory challenge in one female subject (top panel) and one male subject (bottom panel).

The first and second dashed vertical lines mark the onset and termination, respectively, of the first ventilatory challenge; the third and fourth dashed vertical lines mark the onset and termination, respectively, of the second ventilatory challenge; the subject was at rest outwith these periods of challenge. All challenges were of one litre, with the exception of the second challenge in the male subject which was of two litres.
Oxygen uptake was increased to a minor extent in both subjects during each of the ventilatory challenges, likely due to the increased work of breathing, and returned to near baseline following the challenges in which a sample was collected for analysis.

4.3.4 Discussion

The aim of the two preceding tests was to investigate if the rise in ethane output reported in previous tests was simply a representation of increased ventilation during exercise, rather than a marker of exercise-induced oxidative stress. To this end, subjects were presented with a ventilatory challenge in the form of inspiration through an additional dead space volume in order to increase ventilation in a situation unlikely to induce significant oxidative stress. Ethane production would not be expected to increase under these circumstances, and therefore, ethane output should not increase, unless simply an artifact of increased ventilation. In addition, the increase in ventilation was imposed without accompanying hyperventilation, allowing avoidance of a fall in arterial carbon dioxide pressure (PaCO$_2$) and potential decreased ventilatory drive in response to respiratory alkalosis.

The magnitude of the oxygen uptake response to the dead space challenge was minimal, and comparable to the increase seen in sustained isometric handgrip exercise (Alessio et al., 2000), however, the majority of the increase in this case was likely due to the increased oxygen cost of breathing (Coast & Krause, 1993). The ethane output responses suggested that no significant oxidative stress was induced by this minimal increase in oxygen uptake.
Ethane concentration tended to decline during the ventilatory challenges since any ethane expelled in the breath would be diluted by a greater volume of expired air due to the increase in ventilation.

In general, the pattern of ethane output did not mirror the pattern of ventilation. In fact, as ventilation rose, there was a tendency for ethane output to remain stable, or decline, in the case of the greater ventilatory challenge in the male subject. Thus, the rise in ethane output seen with incremental exercise does not appear to be simply a manifestation of the rise in ventilation.

4.4 Real-time measurements

4.4.1 Rationale

The initial pilot tests suggested that a useful real-time expired ethane signal could be recorded directly from the spectrometer whilst it was operating in its normal mode; that is, with regular zero calibration checks. However, ambient ethane measurement is also required in order to correct expired concentration for background ethane concentration. Therefore, it would be useful if the spectrometer could sample both expired air and ambient air during a period of exercise. This would remove the necessity for collection of independent ambient air samples in Tedlar bags throughout the exercise period. The purpose of the following pilot tests was to investigate the possibility of sampling both expired and ambient air for ethane concentration in real-time, and to develop a system for analysis of the output.
4.4.2 Methods

4.4.2.1 Subjects
Subjects were three regularly active males with a mean age of 24 ± 1 years and a mean body mass of 68.7 ± 6.1 kg (mean ± standard deviation). Activity level was assessed prior to participation using the physical activity questionnaire shown in Appendix A.5. Subjects were apparently healthy and were medically screened for abnormalities in resting electrocardiogram (ECG) and blood pressure prior to participation in order to ensure suitability for maximal aerobic exercise. Exclusion criteria were adhered to, as described in section 2.1.1 (page 95).

4.4.2.2 Test protocol
The test protocol was as described in section 4.2.2.2 (page 174). The intention was to impose the same gradually increasing work rate to voluntary exhaustion on each subject to assist comparison of responses between subjects. The incrementation rate of 15 W·min\(^{-1}\) was chosen to provide a maximum number of work rates for analysis of the oxidative stress response to the changing work rate.

4.4.2.3 Measurements
Respired air and heart rate measurements were made as described previously (sections 4.2.2.3.1 and 4.2.2.3.2, pages 174-175). Expired air for sampling ethane was drawn continuously into the laser spectrometer from a sample line connected to the breathing valve as shown in figure 4.1. However, the operating conditions of the spectrometer were altered so that there was no regular check of the zero calibration. Instead, the spectrometer was set up to sample the expired air for 12 s followed immediately by a 12 s period of sampling ambient air. This cycle was continued throughout each test. This was the case for all tests with the exception of pilot test six, in which the respective sampling periods were
extended to 15 s each. Ambient air sampling took place through a sample line separate from the breathing assembly.

The elephant tubing for capture of expired air was added to the expired port of the breathing valve in order to ensure that during the 12 s or 15 s sampling period, mixed expired air was sampled by the spectrometer. The sampling line drew gas into the spectrometer at a constant rate of $5 \text{ l} \cdot \text{min}^{-1}$. It had to be taken into consideration that mixed expired air would be drawn into the spectrometer from the expired port of the breathing valve for the duration of the expiration, however, during inspiration, the spectrometer would begin to draw gas from the ambient air. The elephant tube was used to create a ‘reservoir’ of mixed expired gas and was of sufficient volume to retain mixed expired air during the subsequent inspiration before it was refilled by the next expiration.

4.4.2.4 Analysis

Pulmonary and gas exchange variables were edited as described previously in section 4.2.2.4.1 (page 177). Determination of peak oxygen uptake, estimation of lactate threshold and determination of peak heart rate were carried out as described previously (sections 4.2.2.4.2, 4.2.2.4.3 and 4.2.2.4.4, pages 178-179).

4.4.2.4.1 Ethane analysis

Figure 4.9 shows the ethane concentration from pilot test six, in which expired air was sampled for a 15 s period, immediately followed by a 15 s period of sampling of ambient air. It can be seen clearly that ethane concentration shifts cyclically between expired air values of approximately 200 pmol·l$^{-1}$ and ambient air values of approximately 0 pmol·l$^{-1}$. 
Figure 4.9. Real-time raw ethane concentration signal from the spectrometer prior to, during and following incremental exercise to exhaustion in one male subject. Sampling pattern shows repetitive cycles of ambient air and expired air sampling. The dashed vertical lines mark the onset of unloaded pedaling, incremental exercise and recovery respectively.

If this pattern is examined over a shorter time interval, as shown in figure 4.10, it can be seen that ethane concentration rose quite rapidly to a plateau when expired air was sampled and fell rapidly to a baseline level when ambient air was sampled.
Figure 4.10. Real-time raw ethane concentration signal during an incremental exercise test to exhaustion in one male subject.

The data have been truncated to show a short time period of the response to emphasise the cyclical rise and fall in ethane concentration with successive sampling of expired air and ambient air.

It was necessary to subtract ambient ethane concentration from expired ethane concentration throughout the test in order to correct expired ethane concentration for the influence of ambient ethane. To this end, each sampling interval was analysed separately; a plot was constructed of each ambient sampling interval showing both the previous and subsequent expired air sampling period; an example of this is shown in figure 4.11. It was subjectively determined at which point ethane concentration started to fall from the average expired concentration towards ambient concentration, and at which point it again started to rise towards expired concentration again. All data points which lay between either plateau were discarded; an example is shown in figure 4.11. In addition, any points
which clearly lay outwith the underlying response, for example, as seen at approximately 1100 s in figure 4.9, were discarded.

Figure 4.11. Real-time ethane concentration signal from the spectrometer during an incremental exercise test to exhaustion in one male subject. The data have been truncated to show two cycles of expired air sampling and one cycle of ambient air sampling to demonstrate the data editing technique. Points indicated by arrows on the plot were removed from the data set.
Following this editing process, a mean concentration for each sampling period, both expired air and ambient air, was calculated. Mean ambient ethane concentration from the previous sampling period was subtracted from mean expired ethane concentration using equation 3.9 (page 140) in order to correct for background ethane. Mean minute ventilation was calculated for each expired air sampling period and ethane output was calculated according to equation 3.10 (page 140). This analysis procedure was followed for pilot tests three to six, which shared similar sampling characteristics.

4.4.3 Results

4.4.3.1 Subject characteristics

Subject characteristics are displayed in table 4.1. Subject 1 took part in two tests (pilot tests five and six). Work rate at voluntary exhaustion was 345 W, 375 W, 255 W and 210 W for pilot tests 5, 6, 7 and 8 respectively.

4.4.3.2 Ethane concentration response

Figure 4.12 shows the ethane concentration response throughout the different test phases for each test. It was not possible to display a mean response since the incremental phase of the test varied in duration between subjects. Ethane concentration remained fairly stable throughout each test.
Figure 4.12. Mean ethane concentration prior to, during and following an incremental exercise test to exhaustion.
Panel (a) shows pilot test five, panel (b) shows pilot test six, panel (c) shows pilot test seven and panel (d) shows pilot test eight. Pilot tests five and six show data from the same subject. The initial test phase is rest; the first dashed vertical line marks the onset of unloaded pedaling; the second dashed vertical line marks the onset of incremental exercise to exhaustion; the third dashed vertical line marks the onset of recovery.
### 4.4.3.3 Ethane output response

Figure 4.13 shows ethane output before, during and following incremental exercise to exhaustion.

![Ethane Output Graph](image)

Figure 4.13. Mean ethane output prior to, during and following an incremental exercise test to exhaustion.

Panel (a) shows pilot test five, panel (b) shows pilot test six, panel (c) shows pilot test seven and panel (d) shows pilot test eight. Pilot tests five and six show data from the same subject. The initial test phase is rest; the first dashed vertical line marks the onset of unloaded pedaling; the second dashed vertical line marks the onset of incremental exercise to exhaustion; the third dashed vertical line marks the onset of recovery.
In general, ethane output remained close to resting level during unloaded pedaling, rose gradually throughout the incremental phase of the test and declined rapidly at the onset of active recovery. The magnitude of the response was different between different subjects, and even between two tests with the same subject (figure 4.13, panels a and b), however, the pattern of response was similar in all four pilot tests.

4.4.4 Discussion

It was possible to measure ethane in both ambient air and expired air throughout the test by sampling each source in a cyclical fashion. An alternative method of determining ambient ethane would have been to collect ambient air samples in Tedlar bags during each expired air sampling interval for later analysis following the end of the exercise test. However, measuring ambient air directly by the spectrometer is more practical for two reasons. Firstly, a large number of expensive Tedlar bags would be required. In the four pilot tests described here, between sixty one and eighty two samples of ambient ethane were analysed by the spectrometer. This would have required a supply of this number of Tedlar bags, since the spectrometer cannot analyse independent samples whilst also directly sampling ethane from the expired air or from the environment. Thus, the use of Tedlar bags to collect ambient air samples would have been prohibitively expensive and would have required considerable time for later analysis of the samples. Secondly, the use of direct measurement allowed the measurement of both ambient and expired ethane by the same method, thus eliminating any errors caused by the use of separate techniques of sample collection.

The data analysis procedures required to generate real-time results were cumbersome and time-consuming. However, they did produce a response profile similar to that reported in the previous chapter (figure 3.3, page 150; figure 3.4, page 152) in which incremental exercise was performed on a treadmill, and expired air was collected in Douglas bags at
discrete time points throughout the test. This supports the validity of the real-time technique to some extent; however, further work should focus on the investigation of the ethane response by both methods within the same testing session in order to formally validate the real-time technique.

4.5 Discussion

The data suggested that the real-time measurement of ethane output may be a useful non-invasive tool for the assessment of lipid peroxidation during long periods of exercise. A potential drawback is the limited portability of the spectrometer. The spectrometer has been described as a portable device by the designers (Gibson et al., 2002), however, this is with regard to its use within the oil industry. The spectrometer can be transported to any location around the world for a period of exploration. However, for the current exercise studies, although theoretically the spectrometer could have been moved to the laboratory setting, affording the advantages of air conditioning, a wider range of potential equipment for work rate forcing, and a suitable environment for blood sampling, in reality, this was impractical. It would have been necessary to move the spectrometer to the exercise laboratory and for it to remain in situ throughout all exercise tests. However, these took place over a period of several weeks and with other users requiring the use of the spectrometer, this was unfeasible. For the future, a truly portable version of the spectrometer is currently under development with a view to transport for use within the clinical setting. The validation of this model with enhanced portability could be useful for the study of exercise-induced oxidative stress.
4.5.1 Further Work

A useful extension to this pilot work would be its continuation in a greater number of subjects using standardised procedures. Formal validation of the real-time technique could be accomplished by the assessment of the ethane response by both real-time and by Douglas bag within the same testing session. Unfortunately, it was not possible to carry out further tests within the constraints of the current series of studies. Following the completion of the tests reported here, the laser spectrometer required a new laser and was out of use for an extended period due to difficulty encountered in sourcing the correct specification for this bespoke piece of equipment.

The utility of real-time monitoring of ethane output could be harnessed in a more practical way if a computer programme could be developed to perform the time consuming calculations required. This could potentially benefit both the exercise scientist and the clinician.
Chapter 5

A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains
5.1 Introduction

5.1.1 Oxidative stress response to varying exercise intensity

It has been established that aerobic exercise can result in exercise-induced oxidative stress (Vollaard et al., 2005), however, the oxidative stress response to increasing exercise intensity has not been well characterised. It is difficult to draw conclusions regarding any relationship between exercise intensity and oxidative stress since previous studies have utilised varying exercise modes, testing protocols and means of assessment of oxidative stress. In addition, it has not been established if there is a threshold intensity below which EILOS will not occur.

Eleven previous studies have reported data relating oxidative stress to exercise intensity in human subjects performing aerobic exercise (Dillard et al., 1978; Jammes et al., 2004, 2005; Kanter et al., 1993; Leaf et al., 1997, 1999; Lovlin et al., 1987; Quindry et al., 2003; Sen et al., 1994; Steinberg et al., 2006; Watson et al., 2005), although this was the main aim of only four of these studies (Dillard et al., 1978; Leaf et al., 1997; Quindry et al., 2003; Sen et al., 1994). The methodology of each study is summarised in tables 5.1, 5.2 and 5.3. Exercise test protocols were varied, but generally consisted of one of the following: the performance of one or more sub-maximal exercise intensities within the same test session with oxidative stress assessed following each work rate (table 5.1); incremental exercise to exhaustion with assessment of oxidative stress at, or close to, estimated lactate threshold and at peak exercise (table 5.2); and the assessment of oxidative stress following separate trials of constant load exercise (table 5.3). All studies assessed the extent of lipid peroxidation as a marker of oxidative stress; however, different techniques were used making direct comparison between studies difficult.
Table 5.1. Summary of methodology of previous studies relating oxidative stress to various sub-maximal exercise intensities.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Exercise mode</th>
<th>Test Protocol</th>
<th>Oxidative stress markers</th>
<th>Sampling points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dillard et al. (1978)</td>
<td>CE</td>
<td>20 min at 25% ( \dot{V}O_2 ) max, 20 min at 50% ( \dot{V}O_2 ) max, 20 min at 75% ( \dot{V}O_2 ) max, 20 min rest</td>
<td>Pentane</td>
<td>Rest; after each WR; after 20 min recovery</td>
</tr>
<tr>
<td>Lovlin et al. (1987)</td>
<td>CE</td>
<td>5 min at 40% ( \dot{V}O_2 ) max, 5 min rest, 5 min at 70% ( \dot{V}O_2 ) max, 5 min rest, 30 W·min(^{-1}) to exhaustion</td>
<td>MDA (TBARS)</td>
<td>Rest; 2.5 min after each submaximal WR; after exhaustion</td>
</tr>
<tr>
<td>Kanter et al. (1993)</td>
<td>TM</td>
<td>30 min at 60% ( \dot{V}O_2 ) max, 5 min with intensity increase to 90% ( \dot{V}O_2 ) max within first 2.5 min</td>
<td>Pentane, MDA (TBARS)</td>
<td>Rest; 15 s after 60% ( \dot{V}O_2 ) max WR; after 90% ( \dot{V}O_2 ) max WR</td>
</tr>
<tr>
<td>Watson et al. (2005)</td>
<td>TM</td>
<td>30 min at 60% ( \dot{V}O_2 ) max, 2 min at 10 km·h(^{-1}) at 0%, 2 km·h(^{-1}) increase every 2 min until subject-selected speed, 2%·min(^{-1}) to exhaustion</td>
<td>F(_2)-isoprostanes</td>
<td>Rest; after 60% ( \dot{V}O_2 ) max WR; immediately &amp; 1 h post-exercise</td>
</tr>
</tbody>
</table>

CE cycle ergometer; TM treadmill; MDA malondialdehyde; TBARS thiobarbituric acid reactive substances; WR work rate
Table 5.2. Summary of methodology of previous studies relating oxidative stress to incremental exercise to exhaustion.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Exercise mode</th>
<th>Test Protocol</th>
<th>Oxidative stress markers</th>
<th>Sampling points</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leaf et al. (1997)</td>
<td>TM</td>
<td>Modified Bruce protocol, 5 min rest</td>
<td>Ethane, Pentane, MDA</td>
<td>Rest, at ( \hat{\theta} ), at peak exercise, 5 min into recovery (rest and recovery samples only for MDA)</td>
</tr>
<tr>
<td>Leaf et al. (1999)</td>
<td>TM</td>
<td>Modified Bruce protocol, 5 min rest</td>
<td>Ethane, Pentane, MDA</td>
<td>Rest, at ( \hat{\theta} ), 5 min into recovery (rest and recovery samples only for MDA)</td>
</tr>
<tr>
<td>Jammes et al. (2004)</td>
<td>CE</td>
<td>2 min at 0 W, 20 W·min(^{-1}) to exhaustion, 5 min unloaded, 25 min rest</td>
<td>TBARS</td>
<td>Rest, at ( \hat{\theta} ), at peak exercise, during recovery at 5, 10, 20 and 30 min</td>
</tr>
<tr>
<td>Jammes et al. (2005)</td>
<td>CE</td>
<td>2 min at 0 W, 20 W·min(^{-1}) to exhaustion, 5 min unloaded, 25 min rest</td>
<td>TBARS</td>
<td>Rest, at ( \hat{\theta} ), at peak exercise, during recovery at 5, 10, 20 and 30 min</td>
</tr>
<tr>
<td>Steinberg et al. (2006)</td>
<td>CE</td>
<td>2 min at 0 W, 20 W·min(^{-1}) to exhaustion, 5 min unloaded, 25 min rest</td>
<td>TBARS</td>
<td>Rest, at ( \hat{\theta} ), at peak exercise, during recovery at 5 and 20 min</td>
</tr>
</tbody>
</table>

CE cycle ergometer; TM treadmill; MDA malondialdehyde; TBARS thiobarbituric acid reactive substances; \( \hat{\theta} \) estimated lactate threshold
Table 5.3. Summary of methodology of previous studies relating oxidative stress to constant load exercise.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Exercise mode</th>
<th>Test Protocol</th>
<th>Oxidative stress markers</th>
<th>Sampling points</th>
</tr>
</thead>
</table>
| Sen et al. (1994) | CE            | 20 W·min\(^{-1}\) to exhaustion  
30 min at aerobic threshold  
30 min at anaerobic threshold | TBARS                    | Rest; 2 min & 24 h post-exercise |
| Quindry et al. (2003) | TM           | Incremental test using Broeder protocol  
45 min at 10% > \(\hat{\theta}_L\)  
45 min at 10% < \(\hat{\theta}_L\) | MDA, Neutrophil count  | Rest; immediately, 1 h & 2 h post-exercise |

CE cycle ergometer; TM treadmill; MDA malondialdehyde; TBARS thiobarbituric acid reactive substances; \(\hat{\theta}_L\) estimated lactate threshold
The first group of studies (table 5.1) assessed oxidative stress following various submaximal exercise intensities. Dillard and colleagues (1978) were the first authors to investigate the effect of exercise intensity on pentane production in humans. Six subjects performed 20 minutes of exercise at an intensity of 25% \( \dot{V}O_2 \) max, followed immediately by 20 minutes at 50% \( \dot{V}O_2 \) max and finally 20 minutes at 75% \( \dot{V}O_2 \) max. The authors suggested that pentane production increased during exercise, with an average 1.8-fold increase in pentane production at 75% \( \dot{V}O_2 \) max compared to rest, however, means and standard deviations were not reported for each exercise intensity. Individual plots of pentane production versus time, which are reproduced in figure 5.1, did not suggest a clear relationship between exercise intensity and pentane production due to inter-individual variability in the pattern of response. It was also shown, within the same series of experiments, that pentane production appeared to increase compared to rest following 20 minutes of exercise at an intensity of 50% \( \dot{V}O_2 \) max, however, this was investigated in only four subjects and was not verified statistically. However, this observation was suggestive of increased lipid peroxidation at an exercise intensity as low as 50% \( \dot{V}O_2 \) max.

Lovlin and colleagues (1987) investigated 5-minute periods of exercise at two submaximal intensities and observed a trend towards increased serum MDA with increased exercise intensity, i.e. MDA increased from 40% \( \dot{V}O_2 \) max to 70% \( \dot{V}O_2 \) max to peak exercise. However, MDA was actually significantly reduced at 40% \( \dot{V}O_2 \) max in comparison to resting levels, and only became significantly higher than baseline at peak exercise at which there was a 26% increase from baseline. The authors concluded that short periods of submaximal exercise, below 70% \( \dot{V}O_2 \) max, may inhibit lipid peroxidation. Baseline MDA was comparable to other studies which assessed oxidative stress by TBARS (Jammes et al., 2004, 2005; Steinberg et al., 2006) and therefore this finding was unlikely to be due to a spuriously high resting level. The duration of exercise at the two submaximal intensities
was very low in comparison to other studies which have investigated a minimum period of 20 minutes at each intensity. There are currently no data to describe the effect of exercise duration on oxidative stress.

Kanter and colleagues (1993) reported a significant increase in both pentane production and MDA from rest to 60% $\dot{V}O_2$ max, with a further rise to 90% $\dot{V}O_2$ max thus indicating a similar trend with increased exercise intensity.

More recently, Watson et al. (2005) observed a non-significant fall in F$_2$-isoprostanes following a 30 minute treadmill run at 60% $\dot{V}O_2$ max with a further significant fall at peak exercise. In contrast, subjects consuming a reduced antioxidant diet showed some increase in oxidative stress at 60% $\dot{V}O_2$ max which was sustained at exhaustion. In this group, the increase in F$_2$-isoprostanes did not become significant until recovery. No other studies have utilised F$_2$-isoprostanes as a marker of oxidative stress related to exercise intensity and therefore, there is no direct comparison available.
Figure 5.1. Pentane production versus exercise intensity in six individuals. Subjects exercised for 20 minutes at each intensity. A post-exercise value was recorded following 20 minutes of rest. (Modified from Dillard et al., 1978).
The second group of studies (table 5.2) assessed oxidative stress close to lactate threshold and at peak exercise during incremental exercise to exhaustion. The investigation of the effect of exercise intensity on lipid peroxidation was the main aim of a study by Leaf and colleagues (1997). Lipid peroxidation was assessed, by measurement of expired ethane and pentane, at rest, during the minute following attainment of the lactate threshold, at peak exercise and 5 minutes following the end of exercise. Results showed a rise in both ethane and pentane production from rest to the lactate threshold, although this was significant only for ethane. A further significant rise was observed in both markers to peak exercise followed by a decline during recovery. The same authors (1999) employed a similar protocol in patients with coronary artery disease and found an increase in ethane and pentane at lactate threshold in comparison to resting values, but little change in MDA between measurements at rest and post-exercise.

Jammes and colleagues (2004) also assessed oxidative stress at lactate threshold and peak exercise but found no significant increase in TBARS with exercise although there was a tendency for increase alongside exercise intensity. A significant increase in lipid peroxidation was only found during the recovery period. The same authors (Jammes et al., 2005) repeated this testing protocol in a comparison of control subjects versus patients with chronic fatigue syndrome. These patients displayed a significant increase in TBARS at lactate threshold, with a further increase at peak exercise. However, the pattern of response in the control subjects was entirely different: there was a non-significant reduction in TBARS at lactate threshold followed by a rise at peak exercise; however the value at peak exercise was not significantly different from baseline. Steinberg and colleagues (2006) again utilised the same protocol in healthy subjects and found no increase in TBARS at lactate threshold but a significant rise at peak exercise.
Therefore, although it appears that there is a tendency for lipid peroxidation to increase above baseline at lactate threshold and to rise further at peak exercise, this is inconclusive due to the relatively few data available and the inconsistency of findings.

The final group of studies (table 5.3) related oxidative stress to constant load exercise performed in separate trials. In one study (Sen et al., 1994), subjects completed an incremental test to exhaustion in order to determine aerobic and anaerobic threshold according to the method of Skinner & McLellan (1980). Thereafter, subjects completed 30 minutes of exercise at each work rate on separate days. Oxidative stress was assessed immediately post-exercise and was found to increase significantly following both work rates and increased more following the higher intensity test. Interestingly, oxidative stress increased following exhaustion in the incremental test, however it was a smaller increase than in either of the constant load tests and did not reach statistical significance suggesting that exercise duration may perhaps have been a factor.

A similar study (Quindry et al., 2003) assessed oxidative stress following maximal incremental exercise and constant load exercise below and above the lactate threshold. There was a significant positive relationship between neutrophil count, suggestive of a subsequent oxidative stress response, and post-exercise blood lactate concentration, although markers of lipid peroxidation were not elevated at any work rate. Oxidative stress was indicated following maximal exercise only, by a reduction in ascorbic acid, with no change in uric acid status.

Thus, the majority of the studies available have reported a trend for increased lipid peroxidation with increasing exercise intensity; however, all prior studies have been limited to some extent by a paucity of data points. Sampling has been performed only at, or close to lactate threshold, at one to three submaximal intensities, and at peak exercise. Few
studies have assessed oxidative stress during, rather than following, exercise, and of those that have, only one time point (close to lactate threshold) has been investigated. Most authors have compared baseline levels to those immediately following exercise and during recovery. In general, there is a lack of consistency in findings.

5.1.2 Exercise intensity domains

Exercise may have a different impact on the oxidative stress response if performed below in comparison to above the lactate threshold. Lactic acid is produced as a product of anaerobic glycolysis which takes place in the cytoplasm of the muscle cell. The lactic acid dissociates rapidly as follows:

\[ \text{HLa} \rightarrow \text{La}^- + \text{H}^+ \]  

[5.1]

In order to prevent a change in the pH of the blood, \( \text{H}^+ \) is buffered by \( \text{HCO}_3^- \) as follows:

\[ \text{H}^+ + \text{HCO}_3^- \rightarrow \text{H}_2\text{CO}_3 \rightarrow \text{CO}_2 + \text{H}_2\text{O} \]  

[5.2]

At work rates above the lactate threshold, this buffering is insufficient to prevent a drop in pH, and thus a metabolic acidosis.

Lactate has been shown to have antioxidant properties \textit{in vitro} (Groussard \textit{et al.}, 2000); however, it has been reported that the condition of acidosis, present after the lactate threshold, creates a pro-oxidant environment (Rehncrona \textit{et al.}, 1989; Siesjö \textit{et al.}, 1985). In relation to exercise, Lovlin and colleagues (1987) observed a positive correlation between MDA and blood lactate concentration during 5 minute periods of cycle ergometer exercise. In addition, a positive correlation between neutrophil count, suggestive of a
subsequent oxidative stress response, and post-exercise blood lactate concentration has been observed (Quindry et al., 2003). Acidosis has also been linked to oxidative stress in the circumstance of breath-holding (Joulia et al., 2002). One postulated mechanism for these effects is the conversion of the weaker superoxide radical into the more highly reactive hydroxyl radical by lactic acid (Clarkson & Thompson, 2000). Thus it might be expected that exercise at a work rate above the lactate threshold, during which metabolic acidosis is present, may result in a greater oxidative stress response.

Exercise intensity is often characterised with reference to maximum oxygen uptake, i.e. as \%\hat{\text{VO}}_2\text{ max}, and most studies which have assessed oxidative stress at various submaximal intensities have reported work rate in this way. However, this system is not ideal as it does not take into account inter-individual differences in the ability to sustain exercise. This ability can be characterised by the lactate threshold (\(\theta\text{L}\)), i.e. the onset of metabolic acidosis, and by the critical power, i.e. the highest work rate during a constant load test which can be achieved without a persistent increase in \(\hat{\text{VO}}_2\) and arterial lactate concentration (\([\text{La}^-]\text{a}\)).

In healthy individuals, \(\theta\text{L}\) occurs at approximately 50-60 \% \(\hat{\text{VO}}_2\text{ max}, however, can be much higher in individuals with good aerobic fitness (Wasserman et al., 2004). Therefore, one individual exercising at a specified intensity, e.g. 60\% \(\hat{\text{VO}}_2\text{ max, may be exercising at a sub-}\theta\text{L intensity and another may be exercising at a supra-}\theta\text{L intensity with consequent differences in ventilatory and pulmonary gas exchange variables and the extent of metabolic acidosis. On consideration of the above, three intensity domains have been described (Wasserman et al., 2004). These are moderate, heavy and very heavy, where moderate exercise describes work rates below \(\theta\text{L}\) at which there is no sustained increase in \([\text{La}^-]\text{a,} \hat{\text{VO}}_2\), \(\hat{\text{CO}}_2\) and \(\hat{\text{VE}}\) reach steady state. Heavy exercise encompasses the range of
work rates above $\theta_c$ and up to and including critical power at which $[\text{La}^-]_a$ is elevated to a steady state, and a steady state of $\dot{V}O_2$ can be achieved. Very heavy exercise occurs at higher work rates at which $[\text{La}^-]_a$ increases continually throughout the duration of exercise and steady states of $\dot{V}E$ and $\dot{V}O_2$ cannot be achieved. Thus, the lactate threshold occurs at the highest work rate at which exercise can be maintained without a sustained increase in blood lactate concentration (Wasserman et al., 1973).

5.1.3 Aims

Even though some previous studies have assessed oxidative stress at the lactate threshold, investigation of oxidative stress at a sub-lactate threshold work rate has been undertaken in only one study (Quindry et al., 2003). In addition, one group of authors assessed oxidative stress at a low relative exercise intensity of 40% $\dot{V}O_2$ max (Lovlin et al., 1987). It is likely that this was a sub-lactate threshold work rate since subjects were Physical Education students with a high aerobic capacity; however, since lactate threshold was not measured, this remains uncertain. Neither study reported an oxidative stress response. Quindry and colleagues (2003) suggested that the lack of response following both sub- and supra-lactate threshold work rates may have been due to a lack of sensitivity of measurement technique.

Thus, the aims of this study were to better characterise the oxidative stress response to incremental exercise by measurement throughout the entire voluntary work rate range; to examine the magnitude and time course of the oxidative stress response during steady state exercise both above and below the lactate threshold; and to determine if this response is affected by the duration of steady state exercise. It was also intended to compare the assessment of oxidative stress by both the novel ethane technique and by a traditional plasma marker.
5.2 Methods

5.2.1 Subjects

Subjects in this study were six healthy, active males, each of whom was eligible to participate according to the exclusion criteria described in section 2.1.1 (page 95). In addition, subjects were medically screened for abnormalities in resting ECG and blood pressure prior to participation since this study required maximal aerobic exercise. Each potential subject was also required to complete a physical activity questionnaire to ascertain the extent of participation in regular exercise.

Ethical approval for the study was granted on 4th November 2005, and all subjects provided written, informed consent as described in section 2.1.2 (page 96) (see Appendix A.2 for information sheet and consent form).

5.2.2 Test protocols

Subjects were asked to visit the laboratory on four separate occasions and each visit took place at the same time of day whenever possible. There was a rest period of at least 48 hours between each visit. The first visit consisted of a familiarisation session. At the second visit, the subject performed an incremental exercise test on a cycle ergometer for the purpose of non-invasive estimation of the lactate threshold ($\hat{\theta}_L$) and measurement of $\dot{V}O_2$ peak ($\mu\dot{V}O_2$). The final two visits each involved a constant load test on the cycle ergometer, one at a sub-$\hat{\theta}_L$ work rate and one at a supra-$\hat{\theta}_L$ work rate.

5.2.2.1 Familiarisation

The familiarisation visit to the laboratory included the general procedures described in section 2.2.2 (page 97). In addition to this, the subject was familiarised with the cycle
ergometer. This involved correct seat adjustment so that the subject’s leg was almost completely extended with the pedal at the lowest point, and adjustment of the handgrips to a comfortable position. These settings were recorded and replicated for each subsequent visit.

5.2.2.2 Ramp test

The subject performed a ramp test to voluntary exhaustion on an electromagnetically-braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). With this style of ergometer, an electromagnetic field produces a resistance to pedalling which varies with pedalling cadence; in this way, a specific work rate can be maintained despite variations in cadence. Work rate was controlled by a computerised workload programmer (Lode BV, Groningen, The Netherlands). This allowed work rate to be incremented in small steps of 1 W every 4 seconds, allowing a near continuous increase in work rate to create a ramp profile, that is, one in which the work rate is increased continuously at a constant rate (Wasserman et al., 2004).

In cycle ergometer testing, the incrementation rate can be selected according to the subject’s predicted $\mu \dot{V}O_2$ so that exercise duration can be optimised (Buchfuhrer et al., 1983). If test duration is too long, the subject may terminate the test due to boredom or seat discomfort rather than cardiorespiratory limitation. However, test duration was not the most important factor determining rate of incrementation in the current study since the primary aim was not to establish $\mu \dot{V}O_2$. In fact, the aim was to investigate the oxidative stress response to exercise work rates from rest to maximal exercise. In order to compare inter-individual responses, work rate incrementation was standardised between subjects and was selected so that all subjects could perform the same protocol.
Exercise was preceded by at least 2 minutes of rest whilst seated on the ergometer; this allowed the subject time to become accustomed to the respiratory measuring apparatus. Exercise commenced only once it was clear that the subject’s resting responses were stable and that $\dot{V}_E$ was in the range of 5-10 l·min$^{-1}$, RER was between 0.7 and 0.9 and $P_{ETCO_2}$ was 37-43 mmHg.

Exercise testing on a cycle ergometer commonly includes a period of two to three minutes of “unloaded” pedalling before the incremental phase of the test begins. During this time the subject is pedalling against zero resistance, however, about 10-15 W of work is done in moving the legs and maintaining the momentum of the flywheel. In this study, the ramp phase of the test was started immediately from rest in order to prevent any potential effect of this initial work done on oxidative stress. The incrementation rate of 15 W·min$^{-1}$ was implemented as 1 W·4s$^{-1}$ for all subjects which led to ramp phase durations of 14-24 minutes. Thus, work rate at voluntary exhaustion ranged from 210 W to 360 W. The subject was encouraged to maintain a steady, comfortable cadence of at least 50 rev·min$^{-1}$ throughout the test, and to continue until exhaustion. The mass of the legs exerts an influence on the oxygen cost of cycling, and therefore, a constant cadence would ensure a constant influence at all work rates. The ramp was terminated when the subject was unable to maintain a cadence of at least 50 rev·min$^{-1}$ or if the subject wished to voluntarily end the test for any other reason. At this point the work rate was reduced to 20 W and the subject completed a 6 minute period of active recovery.

5.2.2.3 Constant load tests

Each subject performed two constant load tests: one at a sub-$\dot{\theta}_L$ work rate and one at a supra-$\dot{\theta}_L$ work rate. Each test was preceded by at least 2 minutes of rest whilst the subject was seated on the ergometer in order to ensure stable resting responses, as in the ramp test. The subject then pedalled at a minimum cadence of 50 rev·min$^{-1}$ for 20 minutes, or until
fatigue, at a pre-specified work rate as described below (section 5.2.2.3.1, page 225), followed by a 6 minute period of recovery at 20 W.

5.2.2.3.1 Determination of work rates

The work rates used for the constant load tests were calculated according to the non-invasive lactate threshold estimated from the ramp test. The $\dot{V}O_2$ at $\hat{\theta}_L$ was estimated according to the V-slope method of Beaver and colleagues (1986) as described in section 4.2.2.4.3 (page 178). The work rate at $\hat{\theta}_L$ was then ascertained from a plot of $\dot{V}O_2$ versus work rate. The work rate at $\hat{\theta}_L$ determined from a ramp test is not equivalent to the steady state work rate at the same $\dot{V}O_2$ (Whipp et al., 1981). The relationship between $\dot{V}O_2$ and work rate is linear for steady state exercise. During rapid incremental exercise (i.e. a ramp) the slope of the $\dot{V}O_2$ response to increasing work rate is identical to that for steady state exercise; however, there is an initial lag period before $\dot{V}O_2$ begins to increase. The lag time is equal to the mean response time ($\tau'$), which is the sum of the phase I delay plus the phase II time constant ($\tau$) (Whipp et al., 1981). The time constant for $\dot{V}O_2$ below the lactate threshold is 35-45 s in healthy individuals (Wasserman et al., 2004), and a mean response time of 60 s can be used as a practical approximation. Thus, in order to calculate the work rate at lactate threshold for steady state exercise in the current study, in which the ramp incrementation rate was 15 W·min$^{-1}$, 15 W was subtracted from the work rate at lactate threshold as measured in the ramp test.
The following calculations were used to determine work rates (WR) for the constant load tests:

\[
\text{Sub-} \hat{\theta}_L \text{ WR} = 90\% (\hat{\theta}_L - 15 \text{ W}) \quad [5.3]
\]

\[
\text{Supra-} \hat{\theta}_L \text{ WR} = 40\% (\text{Peak WR} - [\hat{\theta}_L - 15 \text{ W}]) + (\hat{\theta}_L - 15 \text{ W}) \quad [5.4]
\]

The calculation for the supra-lactate threshold work rate was chosen to ensure that the work rate was above lactate threshold, but not above critical power, and thus, in the heavy rather than the very heavy domain.

5.2.3 Measurements

5.2.3.1 Respired air measurements

Ventilatory and pulmonary gas exchange variables were measured continuously throughout each test using mass spectrometry (QP9000, Morgan Medical, Kent, UK) as previously described (sections 2.3.1.2, 2.3.1.2.1 and 2.3.1.2.2, pages 102-105).

5.2.3.2 Blood sampling procedures

Blood was sampled from an indwelling catheter inserted into an antecubital vein as described in section 2.3.4 (page 114). All samples were approximately 6 ml in volume. Two baseline samples, separated by a 10 minute period, were collected prior to the start of exercise whilst the subject was seated in a comfortable chair. The test protocol commenced approximately 10 minutes following the second baseline sample. During the intervening period, ECG electrodes were applied, the subject was made comfortable on the cycle
ergometer, respiratory gas exchange apparatus was attached, and the subject was allowed two to three minutes to become comfortable with breathing through the mouthpiece.

During the ramp phase of the protocol, blood samples were drawn at 2 minute intervals, starting 2 minutes after the onset of exercise. An additional sample at peak exercise was attempted in circumstances in which the previous sample had been drawn at least 30 s previously. Blood samples were drawn at 2, 4 and 6 minutes into the active recovery period. The time points for blood sampling in the constant load tests are illustrated in figure 5.2.

5.2.3.3 Heart rate

Heart rate was monitored continuously as the R-R interval using a six-lead ECG (Q710, Quinton, Kent, UK) and disposable electrodes (Blue Sensor R-00-S, Ambu Ltd., Cambridgeshire, UK). This was done for two reasons: firstly, in order to measure the subject’s maximal heart rate; and secondly, to monitor the pattern of response during exercise and recovery to provide warning of potential subject difficulty in the event of a significant deviation from the expected response.

5.2.3.4 Arterial oxygen saturation

Arterial oxygen saturation (SaO₂) was monitored continuously throughout each experiment using a near-infra-red pulse oximeter (Satlite trans, Datex Engstron, Helsinki, Finland) placed on the ring finger of the left hand. Any test in which SpO₂ fell by 4 % was terminated immediately (American Association for Respiratory Care, 2001). This was done in order to ensure that an adequate oxygen supply to the subject’s tissues was maintained.
Figure 5.2. Schematic representation of the constant load test protocol and blood sampling points.
5.2.4 Analysis

5.2.4.1 Determination of peak oxygen uptake
Oxygen uptake, carbon dioxide output and respiratory exchange ratio were calculated on a breath by breath basis according to equations 3.1 to 3.7 (pages 137-139). Peak oxygen uptake was recorded as the mean oxygen uptake over the final 10 s of incremental exercise.

5.2.4.2 Estimation of lactate threshold
Lactate threshold was estimated using the V-slope method (Beaver et al., 1986) as described in section 4.2.2.4.3 (page 178).

5.2.4.3 Determination of peak heart rate
Peak heart rate was determined as the mean heart rate recorded over the final 10 s of incremental exercise.

5.2.4.4 Blood analysis
Blood samples were centrifuged at 3000 g at 4 °C for 15 minutes. Immediately thereafter, samples were stored on dry ice whilst the plasma was divided into aliquots of approximately 500 μl and stored at -80 °C until further analysis.

5.2.4.4.1 MDA assay
Preliminary analysis was carried out to determine plasma MDA concentration in samples from the first two tests (Bioxytech MDA-586, OxisResearch, Oregon, USA). MDA has been criticised as a sole indicator of lipid peroxidation (Janero, 1990), however, it was more cost effective to use this marker as a preliminary indicator of the pattern of response.
5.2.4.4.2 Isoprostane assay

Plasma isoprostane concentration was measured using the Direct 8-iso-Prostaglandin F$_{2\alpha}$ enzyme immunoassay kit (Assay Designs 900-091, Ann Arbor, MI, USA), as described in section 2.3.5 (page 115).
5.3 Results

5.3.1 Subjects

Subject characteristics are displayed in table 5.4. All subjects were involved in regular exercise participation, and the mean \( \dot{V}O_2 \) peak response was 46.3 ml·kg\(^{-1}\)·min\(^{-1}\), suggesting a good level of cardiorespiratory fitness (American College of Sports Medicine, 2009). However, subjects’ fitness was heterogeneous based on the American College of Sports Medicine classification system (American College of Sports Medicine, 2009); subjects ranged from excellent to poor maximal aerobic power. In relation to the criteria put forward for the indication of a true maximal effort (Duncan et al., 1997), a plateau in the \( \dot{V}O_2 \) response was not evident for any subject, according to the procedure of Poole and colleagues (Poole et al., 2008). Four subjects reached a peak heart rate which was within 10 beats·min\(^{-1}\) of age-predicted maximum heart rate, whereas peak heart rate in the other two subjects was well below age-predicted maximum. Respiratory exchange ratio was less than 1.15 for all subjects. Thus, it is likely that most subjects did not reach a true maximal effort; however, this was not critical for achievement of the proposed outcomes of the study.
Table 5.4. Subject characteristics for study two.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>( \dot{\theta}_L ) (l·min(^{-1}))</th>
<th>( \dot{V}_O_2 ) peak (l·min(^{-1}))</th>
<th>( \dot{V}_O_2 ) peak (ml·kg(^{-1})·min(^{-1}))</th>
<th>HR peak (beats·min(^{-1}))</th>
<th>R peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>27</td>
<td>82.8</td>
<td>187.0</td>
<td>3.2</td>
<td>4.5</td>
<td>54.4</td>
<td>183</td>
<td>0.95</td>
</tr>
<tr>
<td>2</td>
<td>23</td>
<td>61.5</td>
<td>171.0</td>
<td>1.8</td>
<td>2.7</td>
<td>43.9</td>
<td>195</td>
<td>1.02</td>
</tr>
<tr>
<td>3</td>
<td>40</td>
<td>99.6</td>
<td>187.0</td>
<td>2.3</td>
<td>4.2</td>
<td>42.2</td>
<td>151</td>
<td>0.94</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>80.2</td>
<td>171.0</td>
<td>1.8</td>
<td>3.3</td>
<td>41.2</td>
<td>193</td>
<td>1.00</td>
</tr>
<tr>
<td>5</td>
<td>28</td>
<td>74.5</td>
<td>175.5</td>
<td>1.6</td>
<td>3.5</td>
<td>47.0</td>
<td>184</td>
<td>1.00</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>66.9</td>
<td>165.9</td>
<td>1.7</td>
<td>3.3</td>
<td>49.4</td>
<td>170</td>
<td>0.95</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>28 ± 6</td>
<td>77.6 ± 13.4</td>
<td>176.2 ± 8.9</td>
<td>2.1 ± 0.6</td>
<td>3.6 ± 0.7</td>
<td>46.3 ± 5.0</td>
<td>179 ± 16</td>
<td>0.98 ± 0.03</td>
</tr>
</tbody>
</table>

HR heart rate; R respiratory exchange ratio; SD standard deviation; \( \dot{V}_O_2 \) oxygen uptake; \( \dot{\theta}_L \) estimated lactate threshold
Table 5.5 contains details of peak work rate generated during maximal incremental exercise; work rate at the estimated lactate threshold; and work rates used for sub- and supra-lactate threshold constant load exercise, calculated according to equations 5.1 and 5.2 (page 219).

Table 5.5. Work rates measured at peak exercise and estimated lactate threshold, and calculated for sub- and supra-lactate threshold constant load exercise.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Peak</th>
<th>$\hat{\theta}_L^{\text{RAMP}}$</th>
<th>$\hat{\theta}_L^{\text{STEDY STATE}}$</th>
<th>Sub-$\hat{\theta}_L$</th>
<th>Supra-$\hat{\theta}_L$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>365</td>
<td>233</td>
<td>218</td>
<td>196</td>
<td>277</td>
</tr>
<tr>
<td>2</td>
<td>204</td>
<td>97</td>
<td>82</td>
<td>74</td>
<td>131</td>
</tr>
<tr>
<td>3</td>
<td>321</td>
<td>152</td>
<td>137</td>
<td>123</td>
<td>211</td>
</tr>
<tr>
<td>4</td>
<td>253</td>
<td>114</td>
<td>99</td>
<td>89</td>
<td>161</td>
</tr>
<tr>
<td>5</td>
<td>255</td>
<td>98</td>
<td>83</td>
<td>75</td>
<td>152</td>
</tr>
<tr>
<td>6</td>
<td>235</td>
<td>102</td>
<td>87</td>
<td>78</td>
<td>146</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>272 ± 59</td>
<td>133 ± 53</td>
<td>118 ± 53</td>
<td>106 ± 48</td>
<td>180 ± 55</td>
</tr>
</tbody>
</table>

SD standard deviation; $\hat{\theta}_L$ estimated lactate threshold
5.3.2 Oxygen uptake responses to constant load exercise

The steady state oxygen uptake response for each constant load test is displayed in table 5.6. The value reported for each test is the mean response over the final minute of the constant load phase. It can be seen that all sub-\( \hat{\theta}_L \) work rates did provoke a sub-lactate threshold \( \dot{V}O_2 \) response. The range was 88-96 % of the oxygen uptake response measured at the lactate threshold. All supra-\( \hat{\theta}_L \) work rates produced a supra-lactate threshold \( \dot{V}O_2 \) response. The supra-\( \hat{\theta}_L \) constant load test for subject 5 could not be carried out due to limitations imposed on equipment usage which were outwith the control of the author.

Table 5.6. Estimated lactate threshold and oxygen uptake responses to sub- and supra-lactate threshold constant load tests.

<table>
<thead>
<tr>
<th>Subject</th>
<th>( \hat{\theta}_L ) (l·min(^{-1}))</th>
<th>Sub-( \hat{\theta}_L ) (l·min(^{-1}))</th>
<th>Supra-( \hat{\theta}_L ) (l·min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3.2</td>
<td>2.8</td>
<td>3.7</td>
</tr>
<tr>
<td>2</td>
<td>1.8</td>
<td>1.7</td>
<td>2.5</td>
</tr>
<tr>
<td>3</td>
<td>2.3</td>
<td>2.2</td>
<td>3.4</td>
</tr>
<tr>
<td>4</td>
<td>1.8</td>
<td>1.6</td>
<td>2.6</td>
</tr>
<tr>
<td>5</td>
<td>1.6</td>
<td>1.5</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>1.7</td>
<td>1.6</td>
<td>2.6</td>
</tr>
</tbody>
</table>

\( \hat{\theta}_L \), estimated lactate threshold
5.3.3 Oxidative stress response to incremental exercise

Blood samples were collected at 2 minute intervals during incremental exercise and during 6 minutes of active recovery on the cycle ergometer. A blood sample was collected at peak exercise in three of the six subjects, and in these cases was collected within 16 seconds following the end of the ramp phase of exercise. A blood sample was attempted at peak exercise in two of the remaining subjects since the previous sample had been collected at least one minute prior to the end of the ramp in both subjects. However, it was not possible to withdraw a sample in either case due to a kink in the cannula in one subject and poor blood flow in the other. A peak blood sample was not attempted in the remaining subject since only 20 s had passed since collection of the previous sample.

Preliminary analysis of samples from the first two subjects was undertaken to determine if an oxidative stress response was apparent, and to justify continuation of the study. MDA concentration, a marker of lipid peroxidation, from the first two incremental tests can be seen in figure 5.3. The data from subject 1, shown in the upper panel, was as expected: an incremental rise in MDA concentration as work rate increased incrementally. It can be seen that the MDA concentration was starting to fall during the 6 minute period of active recovery, but was still well above the resting value in the final sample collected. In contrast, data from the second subject did not follow this pattern. The resting value was greater than that seen in subject 1, and the concentration remained steady at this value for 10 minutes into the ramp exercise period, at which point it fell close to the resting value seen in subject 1, and remained steady at this value for the remainder of the exercise test. This pattern of response does not suggest a physiological explanation; the ramp phase was 14 minutes in duration for this subject, and an abrupt fall in lipid peroxidation at this time point was unexpected, perhaps suggesting an issue with the assay.
Figure 5.3. MDA response to incremental cycle ergometer exercise to volitional exhaustion in two male subjects.
The dashed vertical lines indicate the onset of exercise and active recovery respectively. The upper panel shows the response of subject 1; the lower panel shows the response of subject 2.
Figure 5.4 shows the F₂-isoprostane response to incremental exercise in all six subjects. The most salient feature is the abundance of missing data. Table 5.7 presents the data shown in these plots in numerical form and includes a key indicating the reason for each missing data point. Some samples could not be collected for technical reasons, for example, poor blood flow; however, the majority of data points were missing due to the measured concentration falling outside the range of the assay. Of the data available, no obvious pattern of response was present, thus no statistical analysis was performed.

The pattern of response for subject 1 is noteworthy. The blood samples collected were analysed for both MDA concentration (as displayed in figure 5.3) and F₂-isoprostane concentration. At first glance, the pattern of response between the two markers of lipid peroxidation appears very different. However, if considered in isolation, samples at 2 min, 6 min and 18 min in the F₂-isoprostane concentration plot for this subject did increase incrementally in a similar manner to that seen in the [MDA] plot.
Figure 5.4. \(F_2\)-isoprostane concentration prior to, during and following incremental cycle ergometer exercise to volitional exhaustion in six male subjects. Incremental exercise duration was subject dependent and varied from 14 to 24 minutes. The exercise period was followed by 6 minutes of active recovery. The dashed vertical lines indicate the onset of incremental exercise and active recovery respectively. The number in the top right hand corner of each panel refers to the subject number.
Table 5.7. F₂-isoprostane response prior to, during and following incremental cycle ergometer exercise to volitional exhaustion.

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
<td>203.2</td>
<td>HI</td>
<td>LO</td>
<td>91.7</td>
<td>174.3</td>
<td>235.6</td>
</tr>
<tr>
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<td>591.1</td>
<td>-</td>
</tr>
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<td>HI</td>
<td>84.9</td>
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</tr>
<tr>
<td>6</td>
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<td>LO</td>
<td>60.8</td>
<td>HI</td>
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</tr>
<tr>
<td>8</td>
<td>LO</td>
<td>LO</td>
<td>-</td>
<td>HI</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
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<td>LO</td>
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</tr>
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<td>104.5</td>
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</tr>
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<td>81.6</td>
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</tr>
<tr>
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<td>LO</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
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<td></td>
</tr>
<tr>
<td>22</td>
<td>64.5</td>
<td>LO</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>24</td>
<td>142.7</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>R2</td>
<td>HI</td>
<td>HI</td>
<td>LO</td>
<td>HI</td>
<td>HI</td>
<td>138.5</td>
</tr>
<tr>
<td>R4</td>
<td>-</td>
<td>HI</td>
<td>87.6</td>
<td>401.0</td>
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<td>89.8</td>
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<tr>
<td>R6</td>
<td>HI</td>
<td>LO</td>
<td>HI</td>
<td>113.0</td>
<td>-</td>
<td>100.5</td>
</tr>
</tbody>
</table>

Value at rest is mean of two samples; R2 to R6 are samples taken at 2 to 6 min into recovery; - sample not collected for technical reasons; HI measured concentration was above the upper limit of the assay; LO measured concentration was below the lower limit of the assay; a blank cell indicates that the subject was not exercising at this time point.
5.3.4 Oxidative stress response to constant load exercise

The data from preliminary analysis of the blood samples collected from the constant load tests of the first two subjects are shown in figure 5.5. The response to the sub-lactate threshold constant load test in subject 1 (upper left panel) was somewhat as expected, with a rise in [MDA] close to the onset of exercise, followed by a fairly steady response over the first 10 minutes of the exercise period. However, the response then fell close to the resting value for the latter half of the exercise period, and fell below the resting value during the active recovery period. There was no apparent response in the supra-lactate threshold constant load test for subject 1 until the active recovery period, at which point there was a noticeable increase in [MDA]. There was no clear response for subject 2 in either test.

The resting [MDA] response was not consistent within each subject. For subject 1, the resting response in the incremental test was 1.9 µmol, whilst it was 5.9 µmol and 5.2 µmol in the sub- and supra-lactate threshold constant load tests respectively. In subject 2, the resting value was 5.7 µmol in the incremental test, but was lower at 2.3 µmol and 2.6 µmol in the constant load tests. The observed range in [MDA] through all tests was 1.8 µmol to 8.6 µmol.

Figures 5.6 and 5.7 show the F₂-isoprostane responses to sub- and supra-lactate threshold constant load exercise respectively for each individual. As mentioned previously, the supra- constant load test for subject 5 was not carried out due to external constraints imposed on equipment usage. Similar to the incremental exercise data shown in figure 5.4, it can be seen that there are several missing data points. Tables 5.8 and 5.9 show the sub- and supra-lactate threshold constant load data in numerical form, with a key explaining the reason for each missing data point. Again, this was mainly due to the measured
concentrations falling outwith the range of the assay. The lack of any ostensible pattern of
response precluded any statistical analysis.

Resting F₂-isoprostane values measured in all three exercise tests ranged from 80.6 pg·ml⁻¹
to 235.6 pg·ml⁻¹, as shown in tables 5.7, 5.8 and 5.9, omitting the spuriously high value of
692.2 pg·ml⁻¹ for subject 1 measured prior to the supra-lactate threshold constant load test.
More than one resting value was available for four subjects. Values were consistent
between tests for only two of these subjects (subject 3: mean ± SD = 83.2 ± 3.6; subject 5:
mean ± SD = 169.9 ± 6.3). The overall range of values through all tests was 11.5 pg·ml⁻¹ to
2345.7 pg·ml⁻¹.
Figure 5.5. MDA response to constant load cycle ergometer exercise at sub- and supra-lactate threshold work rates in two male subjects.

The dashed vertical lines indicate the onset of exercise and active recovery respectively. The upper panels show sub-lactate threshold responses; the lower panels show supra-lactate threshold responses. The left hand panels show the responses of subject 1; the right hand panels show the responses of subject 2.
Figure 5.6. \( F_2 \)-isoprostane concentration prior to, during and following constant load cycle ergometer exercise at a sub-lactate threshold work rate in six male subjects. The steady state exercise period was followed by 6 minutes of active recovery. The dashed vertical lines indicate the onset of steady state exercise and active recovery respectively. The number in the top right hand corner of each panel refers to the subject number.
Table 5.8. F₂-isoprostane response prior to, during and following constant load cycle ergometer exercise at a sub-lactate threshold work rate.

<table>
<thead>
<tr>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rest</td>
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<td>LO</td>
<td>80.6</td>
<td>104.1</td>
<td>165.4</td>
<td>97.0</td>
</tr>
<tr>
<td>2</td>
<td>HI</td>
<td>LO</td>
<td>90.8</td>
<td>233.9</td>
<td>HI</td>
<td>292.1</td>
</tr>
<tr>
<td>4</td>
<td>1346.4</td>
<td>HI</td>
<td>114.7</td>
<td>176.0</td>
<td>110.4</td>
<td>115.3</td>
</tr>
<tr>
<td>6</td>
<td>47.7</td>
<td>LO</td>
<td>161.9</td>
<td>70.8</td>
<td>HI</td>
<td>42.8</td>
</tr>
<tr>
<td>8</td>
<td>LO</td>
<td>1778.1</td>
<td>HI</td>
<td>123.5</td>
<td>-</td>
<td>74.8</td>
</tr>
<tr>
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<td>279.4</td>
<td>HI</td>
</tr>
<tr>
<td>15</td>
<td>39.2</td>
<td>LO</td>
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<td>HI</td>
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</tr>
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<td>53.7</td>
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</tr>
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</tr>
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<td>HI</td>
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<td>-</td>
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</tr>
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<td>R6</td>
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<td>LO</td>
<td>81.2</td>
<td>HI</td>
<td>182.9</td>
<td>68.4</td>
</tr>
</tbody>
</table>

Value at rest is mean of two samples; R2 to R6 samples taken at 2 to 6 min into recovery; - sample not collected for technical reasons; HI measured concentration was above the upper limit of the assay; LO measured concentration was below the lower limit of the assay.
Figure 5.7. F₂-isoprostane concentration prior to, during and following constant load cycle ergometer exercise at a supra-lactate threshold work rate in five male subjects. The steady state exercise period was followed by 6 minutes of active recovery. The dashed vertical lines indicate the onset of steady state exercise and active recovery respectively. The number in the top right hand corner of each panel refers to the subject number.
Table 5.9. F$_2$-isoprostane response prior to, during and following constant load cycle ergometer exercise at a supra-lactate threshold work rate in six male subjects.

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Subject</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rest</td>
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<td>LO</td>
<td>85.7</td>
<td>214.4</td>
<td>-</td>
<td>217.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>26.4</td>
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<td>HI</td>
<td>-</td>
<td>99.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>HI</td>
<td>LO</td>
<td>188.4</td>
<td>HI</td>
<td>-</td>
<td>75.8</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>HI</td>
<td>2345.7</td>
<td>151.8</td>
<td>HI</td>
<td>-</td>
<td>89.3</td>
<td></td>
</tr>
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<td>-</td>
<td>115.9</td>
<td>188.1</td>
<td>-</td>
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</tr>
<tr>
<td>10</td>
<td>388.5</td>
<td>-</td>
<td>310.3</td>
<td>87.1</td>
<td>-</td>
<td>HI</td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>HI</td>
<td>-</td>
<td>-</td>
<td>68.7</td>
<td>-</td>
<td>HI</td>
<td></td>
</tr>
<tr>
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<td>516.0</td>
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<td>HI</td>
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<tr>
<td>R2</td>
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<td>430.2</td>
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</tr>
<tr>
<td>R4</td>
<td>-</td>
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<td>HI</td>
<td>HI</td>
<td>-</td>
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<td></td>
</tr>
<tr>
<td>R6</td>
<td>HI</td>
<td>LO</td>
<td>111.2</td>
<td>HI</td>
<td>-</td>
<td>64.6</td>
<td></td>
</tr>
</tbody>
</table>

Value at rest is mean of two samples; R2 to R6 samples taken at 2 to 6 min into recovery; - sample not collected for technical reasons; HI measured concentration was above the upper limit of the assay; LO measured concentration was below the lower limit of the assay.
5.4 Discussion

Data reported in Chapter 3 indicated a linear relationship between work rate and oxidative stress during incremental treadmill exercise to exhaustion. However, this data captured only the work rate range between that which elicited a heart rate response of approximately 150 beats·min\(^{-1}\) and exhaustion. In those subjects, this heart rate response equated to approximately 78 % of maximum heart rate. It was hoped to extend this investigation in the current chapter to the entire work rate range from rest to exhaustion. It was of interest to determine if the relationship between work rate and oxidative stress was similar at the lower end of the work rate range, and indeed, if an oxidative stress response was present at all at the lower work rates. The concept of a threshold intensity for an oxidative stress response remains an intriguing question yet to be answered. In addition, it was of interest to observe if the relationship between work rate and oxidative stress remained the same at work rates above the lactate threshold, or whether the presence of a metabolic acidosis may have magnified the response. Lactate threshold was not measured in the study reported in Chapter 3, and therefore it was not possible to identify any change in relationship at this point.

5.4.1 Preliminary MDA analysis

The preliminary MDA analysis carried out on blood samples collected from the first subject indicated a positive progressive relationship between work rate and oxidative stress very clearly. However, the samples analysed from subject 2 were not consistent with this finding. Some confidence in the pattern of response observed in subject 1 can be taken from the similarity in response to that reported in Chapter 3.

The resting response of 1.9 \(\mu\text{mol}\) in subject 1 was similar to the majority of previous reports in the literature; however, a few studies have reported higher resting [MDA],
similar to that seen in subject 2 (Vollaard et al., 2005). The difference between the two subjects may simply highlight individual variation; however, it has been suggested that differences in technique between MDA assays have not always been clearly reported in the literature and may lead to wide variations in resting concentration (Vollaard et al., 2005). The peak response in subject 1 was 7.0 μmol. This was higher than that reported in many studies utilising a maximal incremental cycle ergometer exercise protocol (Ashton et al., 1998, 1999; Jammes et al., 2004; Lovlin et al., 1987; Sen et al., 1994; Steinberg et al., 2006; Viinikka et al., 1984), yet the progressive nature of the response seen in the current study is convincing.

The lack of consistency in the pattern of response between these two subjects was likely due to a technical issue with the MDA assay which did not become apparent until at least after the samples from the first subject’s incremental test had been analysed. The samples were processed in the following order: all samples from subject 1 before samples from subject 2; and for each subject samples from the incremental test first, followed by samples from the sub-lactate threshold test, and finally, samples from the supra-lactate threshold test. The responses observed for the first six samples of the sub-lactate threshold test in subject 1 were mostly plausible, but the rest of the data from the constant load tests were less convincing. It is probable that experimenter error was the problem in light of the time dependent pattern of appearance of less credible data.

5.4.2 F₂-isoprostane analysis

Normal plasma concentration of F₂-isoprostanes has been reported as 35 ± 6 pg.ml⁻¹ (Morrow & Roberts, 1997). Several previous studies have measured F₂-isoprostane concentration prior to and following exercise. Reported values at rest have ranged from 28 ± 2 pg.ml⁻¹ (Mastaloudis et al., 2004b) to 75 ± 7 pg.ml⁻¹ (Mastaloudis et al., 2001); however, most values have been at the lower end of this range: 34 ± 3 pg.ml⁻¹ (Steensberg...
et al., 2002), 35.0 ± 4.7 pg·ml\(^{-1}\) (Waring et al., 2003) and 44.8 ± 2.8 pg·ml\(^{-1}\) (Nieman et al., 2002). One study reported a substantially higher resting concentration of approximately 2000 pg·ml\(^{-1}\) (Sacheck et al., 2003).

In the current study, resting F\(_2\)-isoprostane concentration was available for four subjects in each of the three testing sessions, and mean concentration was 176.2 ± 61.6 pg·ml\(^{-1}\) for the incremental test, 111.8 ± 37.1 pg·ml\(^{-1}\) for the sub-lactate threshold constant load test and 302.5 ± 267.0 pg·ml\(^{-1}\) for the supra-lactate threshold constant load test. The range of values spanned from 80.6 pg·ml\(^{-1}\) to 692.2 pg·ml\(^{-1}\) over all tests and all subjects. In some subjects for which there was more than one resting value available, values were fairly similar. For example, in subject 3, the two resting values available were 80.6 pg·ml\(^{-1}\) and 85.7 pg·ml\(^{-1}\). Similarly, in subject 5, resting values were 174.3 pg·ml\(^{-1}\) and 165.4 pg·ml\(^{-1}\). However, in other subjects, resting values were quite different. For example, in subject 4 resting values were measured as 91.7 pg·ml\(^{-1}\), 104.1 pg·ml\(^{-1}\) and 214.4 pg·ml\(^{-1}\); and for subject 1, 203.2 pg·ml\(^{-1}\) and 692.2 pg·ml\(^{-1}\) were measured. Inter-individual variation in resting values was not high in previous studies, and thus high intra-individual variation would not be expected. The large differences between these values compared to those previously published for resting F\(_2\)-isoprostane concentration, coupled with the low reproducibility of measurement within individual subjects, casts doubt upon the overall reliability of these data.

Previously published post-exercise F\(_2\)-isoprostanes concentrations have fallen in the range of 41-131 pg·ml\(^{-1}\) following exercise as diverse as ultramarathon running (Mastaloudis et al., 2001, 2004b; Nieman et al., 2002), 20 minutes of cycle ergometer exercise at a work rate of 80 W (Waring et al., 2003), a 2.5 hour treadmill run at 75 % \(\dot{V}O_2\) max (Steensberg et al., 2002) and a 3 hour treadmill run at 70 % \(\dot{V}O_2\) max (McAnulty et al., 2003). Responses during or following exercise in the current study ranged from 11.5 pg·ml\(^{-1}\) to
2345.7 pg·ml\(^{-1}\). Although many values in the current study were within the expected range, again, the lack of a clear pattern in response reduces the dependability of the data.

Unfortunately, the large number of missing data points, alongside the lack of any clear pattern of response in the available data, and lack of confidence regarding the reliability of the data precluded any meaningful analysis and interpretation of the data collected.

The concentration of 45 % of all samples was found to be outwith the range of the assay. Of all out of range samples, 64 % were above the upper range, and in 36 % the concentration was too low to be measured. This upper range is reported by the manufacturer as 100000 pg·ml\(^{-1}\) (Assay Designs 900-091, Ann Arbor, MI, USA), thus, there was no prior indication that this means of assessment may be unsuitable for the experimental conditions in the current study. The apparent random occurrence of the out of range samples, both below and above the manufacturer’s indicated range suggests an inconsistent error, and thus, likely not due to experimenter error. Autoxidation of arachidonic acid in plasma (Morrow & Roberts, 1997) may occur in biological samples containing lipid, and this may artificially elevate the F\(_2\)-isoprostane concentration in the sample. However, Morrow and Roberts (1997) suggested that autoxidation was not seen to occur in samples snap frozen in liquid nitrogen and stored at -70 °C for 6 months. Samples in the current study were frozen immediately upon collection and stored at -80 °C; however, the storage period prior to analysis was 20 months in the current study. This delay in analysis was unavoidable due to severe adverse circumstances. It is possible that during the storage time, some autoxidation occurred in some of the stored samples, which could explain the unexpectedly high values measured here. However, it is unclear why some samples should have been affected in this way, and not others. Some concerns have been put forward regarding the reliability of ELISA measurement of isoprostanes (Roberts & Morrow, 2000); however, unfortunately it was not possible to use HPLC in the current
study due to lack of access to equipment. The analysis issues experienced in the current study underline the potential value of non-invasive assessment of oxidative stress.

5.4.3 Study design

The use of a standard incrementation rate for all subjects brought about a test duration which was longer than optimal for assessment of peak \( \dot{V}O_2 \) in some subjects (Buchfuhrer et al., 1983). However, this was not perceived to be a limitation in design since this variable was measured simply as an aid to subject characterisation and for the estimation of a suitable supra-lactate threshold work rate. It was more important that all subjects utilised the same incrementation rate so that any changes in oxidative stress were comparable. This meant relatively long duration tests for a minority of subjects, however, allowed a greater density of blood sampling. It is well established that peak \( \dot{V}O_2 \) is higher in most subjects when measured using a treadmill in comparison to a cycle ergometer due to the use of a larger working musculature (Buchfuhrer et al., 1983; Porszasz et al., 2003). Wasserman and colleagues (2004) indicated that peak \( \dot{V}O_2 \) may be 5-11 % higher when utilising a treadmill protocol in comparison to cycle ergometry, although Porszasz and colleagues (2003) reported a difference of 23 % between modes. However, this was not a limitation in the present study since a key aim was to investigate the oxidative stress response to incremental exercise throughout the entire work rate range from rest to maximal exercise, rather than to accurately measure a maximal value. A further advantage of using the cycle ergometer in this study was the relative ease of obtaining blood samples due to minimal movement in the upper body during exercise.

5.4.4 Limitations and further work

The major limitation in this study was clearly the problems encountered with the blood assays as discussed in sections 5.4.1 and 5.4.2 (pages 247 and 248). In the case of the
F₂-isoprostane analysis, it is likely that the extended period of storage had a detrimental impact on the integrity of the samples.

It would be useful to replicate the experiments undertaken within this study, but to use the non-invasive measurement technique which has been used with success in other studies reported here. In addition, more than one plasma marker could be utilised, if financially viable, for confirmation of results as advised by Halliwell & Gutteridge (1999). The verification of the laser spectroscopy technique for the measurement of ethane output remains to be validated against traditional plasma markers of oxidative stress and this is one of the objectives for the work described in Chapter 7.

### 5.5 Conclusions

Preliminary analysis suggested that the oxidative stress response may increase progressively alongside work rate throughout the entire range from rest to exhaustion. However, this observation is far from conclusive as it is based on data from a single subject only.
Chapter 6

Pilot tests investigating the potential for non-invasive assessment of oxidative stress during and following isometric exercise
6.1 Introduction

Very few studies have investigated the effect of isometric exercise on oxidative stress (Bloomer & Goldfarb, 2004). Three previous studies (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) reported increased oxidative stress following static isometric handgrip exercise at 50 % or 60 % of maximal voluntary contraction (MVC). However, one previous study (Sahlin et al., 1992) suggested the existence of a threshold intensity below which a response was not stimulated, although this study utilised intermittent isometric contractions of the knee extensors. Further investigation of the oxidative stress response to sustained isometric contraction at a range of intensities was planned within the current series of studies. Thus, pilot tests were undertaken in order to determine firstly, whether the previously reported oxidative stress response could be replicated prior to commencing a full-scale study; secondly, to determine if oxidative stress could be assessed non-invasively in relation to this mode of exercise since this has not been investigated previously; and thirdly, to adjust the methodological design.

6.2 Methods

6.2.1 Subjects

Two subjects participated in these pilot tests; one healthy male (age 28 yr; body mass 82.3 kg) and one healthy female (age 38 yr; body mass 49.0 kg). Both were eligible according to the exclusion criteria set out in section 2.1.1 (page 95). No further inclusion or exclusion criteria were applied.

6.2.2 Test protocols

Subjects performed isometric contractions of the flexor digitorum muscles using a handgrip dynamometer. The handgrip dynamometer is a customised device incorporating a
strain gauge attached to a horizontal handle which the subject can grip to produce force. The strain gauge was connected to data acquisition software (Spike2 v.5.03, Cambridge Electronic Design Ltd., Cambridge, UK), running on a laptop computer (Tecra A4, Toshiba Information Systems (UK) Ltd., Surrey, UK), via a data capture unit (Micro 1401 mk II, Cambridge Electronic Design Ltd., Cambridge, UK) and amplifier (IBLS Mechanical Workshop, University of Glasgow, Glasgow, UK). The dynamometer was calibrated with known weights prior to use in order to quantify the forces recorded. Figure 6.1 illustrates a typical calibration plot which shows the response of the strain gauge to be linear ($R^2 = 0.99$) throughout the range calibrated. It was possible to calibrate the dynamometer only throughout the range from 0-28 kg due to lack of availability of additional weights.

![Figure 6.1. A typical calibration plot for the handgrip dynamometer.](image)
A diagrammatic representation of the experimental set-up is shown in figure 6.2. The distance between the wooden handle attached to the strain gauge and the foam grip could be adjusted to take account of variations in hand size between individuals and this distance was kept constant for each individual between trials.

Figure 6.2. Experimental set-up for the recording of force during isometric contraction of the flexor digitorum muscles.
6.2.2.1 Determination of maximal voluntary contraction

Subjects visited the laboratory on three separate occasions and all visits for each individual took place at the same time of day whenever possible since maximal isometric force production has been reported to vary with time of day (Martin et al., 1999). At the first visit, subjects were introduced to personnel and were familiarised with all equipment and procedures to be used, as described in section 2.2.2 (page 97). This included instruction on the correct use of the handgrip dynamometer; the subject was seated and was asked to adjust the height of the seat so that he could grip the dynamometer with the arm relaxed and hanging perpendicular to the handle of the dynamometer. The grip size was adjusted for comfort as described above. Subjects performed both trials with the dominant hand.

The subject was asked to perform a maximal voluntary contraction by grasping the wooden handle and foam grip together as hard as possible for 5 seconds. This was followed by a 5 minute rest period. The subject then repeated the MVC manoeuvre twice more, with a 5 minute rest period between each trial. The percentage difference between the highest MVC attempt and each other attempt was calculated. If the difference between any trial and the highest attempt was less than 5%, MVC was recorded as the highest of the three trials. If the difference between any trial and the highest attempt was greater than 5%, the subject was asked to perform a further two trials, and the overall MVC was recorded as the highest of all five trials. Force in kg was calculated according to the calibration data and then converted to newtons (N) by multiplying by 9.8, the known acceleration due to gravity, as indicated in equation 6.1.

\[
1 \text{ N} = 1 \text{ kg} \cdot \text{m} \over \text{s}^2
\]  

[6.1]
Healthy subjects typically exhibit peak force early in a maximal voluntary contraction followed by a gradual decline in force. Good reliability of measurement has been reported after 5 seconds of a 6 second sustained maximal grip strength contraction (Kamimura & Ikuta, 2001). Studies have indicated that three to five repetitions are adequate to determine MVC (Wilson & Murphy, 1996). MVC has been measured in a similar manner in previous studies in this area (Alessio et al., 2000; Dousset et al., 2002).

6.2.2.2 Sustained contraction trials

At the second and third visits respectively, the subject was asked to sustain a voluntary isometric muscle contraction at 40% and 60% of the previously determined MVC. During each trial, the subject sustained the specified contraction intensity until fatigue. The subject was able to view the computer monitor at all times during the contraction for feedback on performance. Reference lines were drawn across the screen at the required intensity level and at ± 2% of this level to provide visual cues to assist the subject in maintaining the contraction at the correct intensity. If the force fell more than 2% below the specified level at any point during the trial, the subject was given verbal feedback to encourage him or her to regain the correct intensity. If the subject was unable to regain the set level immediately, the trial was terminated. On cessation of the contraction, the subject rested comfortably in a chair for 30 minutes in all tests with the exception of the 60% MVC sustained contraction trial in the female subject who rested for 65 minutes.

6.2.3 Measurements

6.2.3.1 Respired air measurements

Samples of expired air of two minutes duration were collected in Douglas bags at the following time points: two baseline samples, separated by a period of ten minutes, were taken at rest prior to exercise; a sample was taken immediately following cessation of the
contraction; a recovery sample was collected five minutes after the cessation of exercise and then at 5 minute intervals up to 30 minutes following the end of exercise in all tests, with the exception of the 60 % MVC sustained contraction trial in the female subject in which samples were collected at 5 minute intervals for 65 minutes following the end of the contraction. Expired air collection procedures are detailed in section 2.3.1.1 (page 100). The subject was linked to the gas collection equipment one minute prior to the start of each sample in order to provide a short familiarisation to minimise the likelihood of hyperventilation during the sample, and the equipment was removed at the end of each sample for subject comfort. There was one exception to this procedure; there was no familiarisation period before the sample immediately following exercise so that the gas collection equipment did not interfere with the exercise. The mouthpiece and nose-clip were introduced as soon as possible after the end of exercise and the expired air sample was started immediately thereafter.

6.2.3.2 Ethane sampling procedures and analysis

Expired and ambient ethane samples were collected are described in section 2.3.2.2 (page 107). All samples were analysed for ethane content as described in section 2.3.3 (page 112).
6.3 Results

6.3.1 Subject characteristics

Subject characteristics are displayed in table 6.1.

Table 6.1. Subject characteristics for isometric pilot tests.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>MVC (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>male</td>
<td>28</td>
<td>82.3</td>
<td>187.0</td>
<td>519.4</td>
</tr>
<tr>
<td>2</td>
<td>female</td>
<td>38</td>
<td>49.0</td>
<td>157.0</td>
<td>303.8</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td></td>
<td>33 ± 7</td>
<td>65.7 ± 23.5</td>
<td>172.0 ± 21.2</td>
<td>411.6 ± 152.5</td>
</tr>
</tbody>
</table>

MVC maximal voluntary contraction; SD standard deviation

The duration of the sustained contraction at 40 % MVC was 129 s for subject 1 and 161 s for subject 2. Shorter contraction times were recorded at the higher contraction intensity of 60 % MVC: 58 s for subject 1 and 52 s for subject 2. Both subjects closely matched the target intensity throughout each trial, sustaining 97-105 % of target force.

6.3.2 Ethane output response

Figures 6.3 and 6.4 show the ethane output response following a sustained isometric contraction at an intensity of 40 % MVC in two subjects. Figures 6.5 and 6.6 show ethane output in response to a sustained contraction at 60% MVC in two subjects.
Mean ethane output at rest across the four pilot tests was $7.5 \pm 1.6 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$. This is somewhat lower than the baseline ethane output of $12.0 \pm 9.4 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ reported in the previous study. However, with the omission of the unusually high baseline value of one subject bringing the mean response in the previous study to $9.2 \pm 5.4 \text{ pmol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$, ethane output was more comparable between studies.

There appeared to be a tendency for ethane output to be elevated substantially above baseline immediately upon recovery from the sustained contraction at both intensities in both subjects indicated by a mean increase in ethane output above baseline of 174 % in the 40 % MVC trials and of 249 % in the 60 % MVC trials.

The pattern of response throughout the remainder of the recovery period suggested a reduction in ethane output towards baseline output by 5 minutes into the recovery period, generally followed by stabilisation of the response. In some tests there did seem to be a less evident rise in ethane output at 10 minutes into recovery, however, this pattern was equivocal.

The recovery period was extended to 65 minutes in the 60 % MVC trial in subject 2 as shown in figure 6.6. This was done in order to determine the potential value of utilising a longer recovery period in the main study described in chapter 7. The ethane output response may have been somewhat elevated above baseline between 35 and 55 minutes into recovery; however, there was little variation in the response over time until a reduction towards baseline at 65 minutes into recovery.
Figure 6.3. Ethane output at rest and during passive recovery from a sustained isometric contraction at 40 % maximal voluntary contraction in one male subject.

B baseline sample; R0 immediate post-exercise sample; R5 to R30 samples collected at 5 to 30 min into recovery.
Figure 6.4. Ethane output at rest and during passive recovery from a sustained isometric contraction at 40 % maximal voluntary contraction in one female subject.

B baseline sample; R0 immediate post-exercise sample; R5 to R30 samples collected at 5 to 30 min into recovery.
Figure 6.5. Ethane output at rest and during passive recovery from a sustained isometric contraction at 60 % maximal voluntary contraction in one male subject.

B baseline sample; R0 immediate post-exercise sample; R5 to R30 samples collected at 5 to 30 min into recovery.
Figure 6.6. Ethane output at rest and during passive recovery from a sustained isometric contraction at 60 % maximal voluntary contraction in one female subject.

B baseline sample; R0 immediate post-exercise sample; R5 to R30 samples collected at 5 to 30 min into recovery.
6.4 Discussion

The first aim of these pilot tests was to determine whether or not a previously reported oxidative stress response to sustained, static isometric exercise (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) could be replicated prior to commencing the main study. The current data indicated that oxidative stress, as assessed by expired ethane, increased in response to a sustained isometric contraction. The peak response occurred immediately following the end of exercise, and thereafter returned towards baseline values, although there may have been some elevation above baseline later in the recovery period.

Ethane output in recovery could not be compared to previous studies since this is the first known study to utilise a non-invasive measurement technique with this mode of exercise. However, the pattern of response was somewhat similar to that reported previously (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) with an elevation in oxidative stress following isometric handgrip exercise; however the timing of the peak response was unclear. Two previous studies suggested the peak response occurred 5 minutes into the recovery period (Steinberg et al., 2004; Steinberg et al., 2006), slightly later than in the current tests, and one previous study (Dousset et al., 2002) indicated a peak response at 20 minutes into recovery, although in this latter study only one measurement was made during the recovery period.

An elevation in ethane output later in recovery may be expected since ethane produced by the muscle during exercise may naturally be trapped to some extent in the muscle during the contraction due to elevated intramuscular pressure (Sejersted et al., 1984) with subsequent, at least partial, venous occlusion. Thus, at the onset of recovery, as blood flow is restored to the exercising muscle, ethane may be able to flow through the systemic circulation with subsequent increased output at the lung. However, it is possible that the small volume of ethane liberated by the muscle during exercise may have cleared to the
lung within the 2 minute period of the expired air sample. Thus, further investigation of the pattern of response would be valuable.

With reference to the second aim of these tests, to assess the measurement technique for this mode of exercise, it can be concluded that an oxidative stress response to isometric exercise can be shown using a non-invasive measurement technique and further investigation would be warranted. If this result could be replicated in further subjects, the findings of previous studies (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) could be confirmed and extended. The findings of these studies are reviewed in section 7.1.3.1 (page 271).

The final aim of these pilot tests was to adjust the methodological design. To this end, one test was conducted with an extended recovery period to determine if this would have potential utility for the main study. Although there may have been some elevation in ethane output above baseline after the 30 minute recovery period, the response was not sufficiently prominent to recommend an extended recovery period in the main study, especially since time constraints for testing became a consideration (as described in section 7.2.2, page 278).
Chapter 7

A comparison of invasive and non-invasive means of assessment of oxidative stress during recovery from isometric exercise
7.1 Introduction

7.1.1 Isometric exercise

An isometric muscle contraction is defined as one during which there is no change in muscle length (Faulkner, 1995). This type of contraction is common in sporting activities such as rock climbing, gymnastics and horse riding, and is a frequent occurrence even in the sedentary population, being utilised in everyday activities, such as carrying shopping bags.

In a sustained isometric contraction, intramuscular pressure increases in relation to the intensity of the contraction (Sejersted et al., 1984). Consequently, blood flow to the exercising muscle may be hindered by occlusion of supply vessels and it has been suggested that blood flow may be impaired by a contraction intensity as low as 15 % of maximal voluntary contraction (Sjøgaard et al., 1988). Simultaneous obstruction of outflow vessels may encourage the accumulation of metabolites such as K⁺, which have been associated with the onset of fatigue (Sjøgaard et al., 1988).

Isometric exercise can be performed in a static manner, in which the contraction is sustained as described above, or can be performed in an intermittent fashion, in which contraction and relaxation follow a cyclical pattern, for example, contraction for ten seconds followed by relaxation for ten seconds. If the contraction is static, and is performed at an intensity above that which impairs blood flow, blood flow to the exercising muscle will be reduced or interrupted for the entire duration of the contraction. If the contractions are of an intermittent nature, blood flow will decrease and increase in a cyclical fashion in tandem with contraction and relaxation of the muscle (Sjøgaard et al., 1988). It has been reported that transient reactive hyperaemia occurs during recovery from isometric contraction (Sjøgaard et al., 1988).
7.1.2 Ischaemia-reperfusion injury

Ischaemia is defined as an inadequate blood supply; hypoxia is a deficiency of $O_2$ (Martin, 2007). Different tissues have varying resistance to ischaemic or hypoxic conditions, for example, skeletal muscle is fairly resistant to hypoxia, however, any cell, with the exception of erythrocytes, will become injured if exposed to ischaemic conditions for a sufficient period of time.

A period of ischaemia during isometric exercise followed by restoration of blood flow shares some characteristics with ischaemia-reperfusion injury, which can be a serious complication in cases of organ transplantation, myocardial infarction or stroke (Dröge, 2002). Ischaemia-reperfusion injury is tissue damage caused by the return of blood flow to ischaemic tissue, and this damage has been attributed, at least in part, to the production of free radicals once blood flow has been re-established at the ischaemic site (Babior, 2000). Several sources for this free radical production have been postulated including: neutrophil activation by the release of inflammatory cytokines in response to ischaemia (Liao et al., 1991); and production of $O_2^\cdot$ via the conversion of xanthine hydrogenase to xanthine oxidase (McCord, 1985). Both putative mechanisms were described in Chapter 1 (section 1.3.3.3, page 86; section 1.3.3.4, page 87). These mechanisms may be responsible, to some extent, for the oxidative stress response associated with recovery from isometric exercise. As described previously, it is unlikely that increased oxygen consumption with concomitant increased mitochondrial production of reactive species contributes significantly to oxidative stress in isometric exercise since oxygen uptake does not increase substantially during this mode of exercise (Alessio et al., 2000).
7.1.3 Evidence of oxidative stress in isometric exercise

7.1.3.1 Static isometric exercise

There is little information in the literature regarding the effect of static isometric exercise on oxidative stress. One research group has studied the oxidative stress response to static isometric exercise in three separate investigations (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006); the methodologies and main findings of which are summarised in table 7.1. In each study, subjects were asked to sustain a static isometric handgrip contraction at a specified intensity until fatigue. Contraction intensity was 60% of maximal voluntary contraction (MVC) in the first study (Dousset et al., 2002) and 50% MVC in the latter two studies (Steinberg et al., 2004; Steinberg et al., 2006), both of which would be expected to induce ischaemia to some extent. Oxidative stress was assessed at baseline, immediately following exercise and during up to 20 minutes of recovery. All three studies reported a significant rise in oxidative stress immediately following cessation of exercise. This was measured by a rise in plasma TBARS in all three studies and by a decrease in plasma RAA (Dousset et al., 2002; Steinberg et al., 2006) or erythrocyte GSH (Steinberg et al., 2004; Steinberg et al., 2006).

The pattern of recovery was slightly different between studies. In two studies (Steinberg et al., 2004; Steinberg et al., 2006), oxidative stress was reported to reach a peak, as reflected by all assessment methods, at 5 minutes of recovery with a return to baseline at 20 minutes of recovery in one study (Steinberg et al., 2006), whilst measures at this time point were not reported in the other study (Steinberg et al., 2004). The third study (Dousset et al., 2002) measured oxidative stress immediately following exercise and at 20 minutes of recovery and produced some conflicting data. Plasma RAA had returned to baseline by 20 minutes of recovery similar to the later study (Steinberg et al., 2006), however plasma TBARS indicated that peak oxidative stress occurred at 20 minutes of recovery. Thus, it
appears clear that static isometric exercise at the intensities studied leads to increased oxidative stress during recovery; however, the time course of the increase is not well established.

7.1.3.2 Intermittent isometric exercise

The oxidative stress response to isometric exercise has also been studied using intermittent protocols. For example, Sahlin and colleagues (1992) investigated isometric knee extension exercise performed at an intensity of 30 % MVC. Each contraction was sustained for 10 s followed by 10 s relaxation for a total period of 80 minutes, or until exhaustion. Blood was sampled every 20 minutes during exercise and was analysed for MDA and total glutathione (TGSH). Muscle biopsies at baseline, 20 minutes and at fatigue were analysed for TGSH. Results indicated no significant increase in oxidative stress as measured by plasma MDA or muscle TGSH, however an increase in plasma TGSH was reported at all time points, although this was statistically significant only up to 60 minutes; the data at 80 minutes were not included in the analysis since some subjects fatigued before this time.

It was suggested that the intensity of contraction may have been too low to elicit change in some of the measured variables. A threshold for oxidative stress has been indicated for aerobic exercise (Leaf et al., 1997; Lovlin et al., 1987), however, this has not been explored for isometric exercise. A salient feature of the study design was the measurement of oxidative stress during, rather than following, a period of isometric exercise. All other studies cited in the literature have assessed oxidative stress only during recovery from isometric exercise.
Table 7.1. A summary of the methodology and main findings of previous studies of oxidative stress associated with static isometric handgrip exercise.

<table>
<thead>
<tr>
<th>Author(s)</th>
<th>Exercise intensity (% MVC)</th>
<th>Duration of exercise (s) (mean ± SE)</th>
<th>Means of assessment of oxidative stress</th>
<th>Time points of measurement</th>
<th>Main findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dousset et al. (2002)</td>
<td>60</td>
<td>42 ± 5</td>
<td>plasma TBARS; plasma RAA</td>
<td>Baseline; R0; R20</td>
<td>Significant increase in oxidative stress at R0; peak at R20 as assessed by TBARS; return to baseline by R20 as assessed by RAA.</td>
</tr>
<tr>
<td>Steinberg et al. (2004)</td>
<td>50</td>
<td>95 ± 12</td>
<td>plasma TBARS; erythrocyte GSH</td>
<td>Baseline; R0; R5; R10; R20</td>
<td>Significant increase in oxidative stress at R0; peak at R5; not reported if baseline reached by R20.</td>
</tr>
<tr>
<td>Steinberg et al. (2006)</td>
<td>50</td>
<td>100 ± 13</td>
<td>plasma TBARS; plasma RAA; erythrocyte GSH</td>
<td>Baseline; R0; R5; R20</td>
<td>Significant increase in oxidative stress at R0; peak at R5; return to baseline by R20.</td>
</tr>
</tbody>
</table>

GSH reduced glutathione; MVC maximal voluntary contraction; RAA reduced ascorbic acid; SE standard error; TBARS thiobarbituric acid reactive substances.

For time points of measurement, R denotes the recovery phase of the experiment and the subsequent number denotes the time in minutes into recovery.
Two previous studies have involved intermittent handgrip exercise. Alessio and colleagues (2000) investigated the oxidative stress response to isometric handgrip exercise at 50 % MVC using a 45 s contraction and 45 s relaxation cycle to fatigue. Markers of oxidative stress, including MDA, protein carbonyls and lipid hydroperoxides, were measured pre-exercise, immediately post-exercise and one hour following the cessation of exercise. There was a significant increase in lipid hydroperoxides on cessation of exercise, the concentration remaining high for up to one hour after exercise. Other markers did not change from pre-exercise values. The design of this study was unusual from the point of view that subjects were allowed to change hands during the test protocol if fatigue and discomfort warranted it. The duration of the trial was determined by a previous maximal treadmill run, and once this time had elapsed the subject was asked to sustain the final contraction until exhaustion which typically occurred a few seconds later. The duration of exercise was not standardised between individuals. The effect of exercise duration on oxidative stress is currently unknown; however, it seems intuitively likely that a greater response may be linked to a longer duration of exercise. Until there is evidence to the contrary it would be wise to control for this potential confounding factor.

Steinberg and colleagues (2002) measured TBARS, plasma RAA and erythrocyte GSH at rest, and then at 0 min, 5 min, 10 min, 20 min and 30 min during recovery from a 3 minute period of intermittent isometric handgrip exercise utilising a 1 s contraction and 1 s relaxation cycle which was deemed to be exhaustive. Intensity of exercise was approximately 20% MVC. Results showed a significant rise in TBARS immediately after exercise (greatest change at 5 min post-exercise) and a significant fall in GSH (greatest change at 20 min post-exercise) indicating increased oxidative stress. The results of this study make the idea of a threshold concept for oxidative stress in isometric exercise less clear. These authors (Steinberg et al., 2002) reported a significant rise in oxidative stress following intermittent isometric exercise at an intensity of approximately 20 % MVC,
whilst Sahlin and colleagues (1992) indicated the evidence for an oxidative stress response was less clear following similar exercise at 30% MVC. Clearly the two studies are not directly comparable due to differing methodology and further work would be required to explore this issue.

7.1.4 Limitations in the literature

One limitation of the few previous studies of isometric exercise and oxidative stress is that a narrow range of contraction intensities has been studied. This has necessarily precluded the investigation of any effect of contraction intensity on the oxidative stress response. In addition, differing exercise protocols and modes of assessment of oxidative stress have been utilised, creating a challenge to establish a clear relationship between this exercise mode and oxidative stress. No previous study has had the primary aim of characterising the time course of the oxidative stress response to isometric exercise; rather static isometric contractions have been used as a tool to investigate other issues, for example, the effect of acute hypoxaemia on the oxidative stress response, and the reliability of oxidative stress assessment by various blood markers. Thus, information regarding the time course of the response in recovery is ambiguous.

7.1.5 Assessment of oxidative stress

Exercise-induced oxidative stress has been assessed typically using whole blood or plasma markers, and solely assessed in this way for isometric exercise. The use of non-invasive assessment, utilising the measurement of ethane gas liberated in the process of lipid peroxidation, has been employed successfully during and following aerobic exercise (Wyse et al., 2005b), and this is appealing from the point of view of subject comfort and safety aspects related to blood sampling and handling. However, ethane output has not yet been validated against traditional blood markers of lipid peroxidation. The successful
correlation of ethane output with commonly used blood markers would support a case for the use of non-invasive assessment of oxidative stress in isometric exercise, resulting in an improved capacity to perform time course analysis of the response in future studies through real-time assessment of ethane.

The site of measurement of oxidative stress markers may be significant. End products of lipid peroxidation enter the circulation and can be measured in the plasma distant from the site of production and in the expired air. A measurement of oxidative stress markers at the venous outflow of an exercising muscle may give the most accurate assessment of the exercise-induced response in terms of both the size of response and the timing; however, this is not always possible due to the impracticality of access to some measurement sites. Blood sampled remotely may give a good indication of the response, as may ethane measured at the lung.

7.1.6 Aims

There have been numerous reports of an increase in exercise-induced oxidative stress following aerobic exercise (Vollaard et al., 2005); however, few studies have investigated the oxidative stress response to isometric exercise (Bloomer & Goldfarb, 2004). A limitation of the few previous studies is that differing exercise protocols and modes of assessment of oxidative stress have been utilised, creating a challenge to establish a relationship between exercise intensity and oxidative stress.

The first aim of the current study was to better characterise the oxidative stress response to isometric exercise using a within subject design to overcome the previous limitation of diverse methodologies; specifically to investigate the effect of contraction intensity; to look for the presence of a threshold intensity for response; and to clarify the time course of the response in greater detail by assessing oxidative stress at more regular intervals and for
a longer period of time during recovery from exercise than in previous studies (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006).

A further aim of this study was to investigate the relationship between oxidative stress measured in the plasma at the exercising muscle with that measured in the plasma at a remote site to determine the effect of measurement site.

Furthermore, oxidative stress has not been measured previously by non-invasive means in isometric exercise. Therefore, the final aim of this study was to allow comparison of invasive with non-invasive response to investigate the validity of non-invasive measurement of oxidative stress in this mode of exercise.
7.2 Methods

7.2.1 Subjects

Six healthy males, aged between 24 and 29 years, volunteered for this study. All met the exclusion criteria described in section 2.1.1 (page 95). No further inclusion or exclusion criteria were applied.

Ethical approval for this study was granted on 24th May 2005, and all volunteers gave written, informed consent prior to participation. The relevant information sheet and consent form are shown in Appendix A.3.

7.2.2 Test protocols

Subjects participated in two tests on separate occasions: firstly, determination of maximal voluntary contraction of the flexor digitorum muscles using a handgrip dynamometer; and secondly, a sustained contraction trial at 60 % of the previously determined MVC. Both tests took place at the same time of day to control for variations in maximal force with time of day (Martin et al., 1999).

The original intention was to test several intensities of contraction in each subject, specifically 20 %, 40 %, 60 % and 80 % MVC; however, unfortunately this was not possible due to constraints on laboratory availability. It was essential to carry out this testing in a low ethane environment in order to avoid unnecessary contamination of samples. All available laboratories had been tested for ambient ethane concentration prior to the start of this study and all others had prohibitively high concentrations ranging from 625-17857 pmol·l$^{-1}$ (14 to 400 ppb). The start of testing was substantially delayed due to malfunction of the laser spectrometer and the need to source bespoke replacement parts.
When testing was finally scheduled, it was advised that the laboratory to be used for this study was due for refurbishment and surrender to another faculty of the university. There was no other laboratory space available which was suitable for both ethane determination, requiring a low ambient ethane concentration, and for blood sampling. Therefore, it was necessary to amend the design of the study to include just one contraction intensity due to time constraints.

The intensity for the sustained contraction trials was chosen as 60 % MVC since previous studies have shown a significant oxidative stress response at 50 % and 60 % MVC (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) allowing for some comparison with previous work. The higher intensity was chosen since successful pilot work had been carried out using this intensity.

Test protocols and equipment are described in detail in sections 6.2.2, 6.2.2.1 and 6.2.2.2 (pages 254-258). One exception to the previously described sustained contraction protocol was that all subjects rested comfortably for 30 minutes following the cessation of contraction.

### 7.2.3 Measurements

A schematic representation of the test protocol showing expired air, ambient air and blood sampling points, is shown in figure 7.1.

#### 7.2.3.1 Respired air measurements

Expired air collection procedures are described in section 6.2.3.1 (page 258). Recovery samples were collected at 5 minute intervals for a period of 30 minutes following the cessation of contraction.
7.2.3.2 Ethane sampling procedures

Expired and ambient ethane sampling procedures are described in section 2.3.2.2 (page 107).

7.2.3.3 Blood sampling procedures

Blood was sampled from an indwelling catheter placed in an antecubital vein, in both the exercising and non-exercising arms, at the same time points as the start of the expired air samples. Details of the blood sampling procedure are described in section 2.3.4 (page 114). Blood samples were drawn from the non-exercising arm in order to facilitate correlation of whole body invasive and non-invasive markers of oxidative stress. The exercising arm provided a marker of regional changes for comparison with the whole body response measured at the lung and in the contra-lateral control arm.

7.2.4 Analysis

7.2.4.1 Ethane analysis

Expired air and ambient air samples were analysed for ethane content as described in section 2.3.3 (page 112).

7.2.4.2 Blood analysis

Following each test, blood samples were centrifuged at 3000 g at 4 °C for 15 minutes. Immediately thereafter, samples were stored on dry ice whilst the plasma was divided into aliquots of approximately 500 μl, and were then stored at -80 °C until further analysis.
Blood samples were analysed using a commercial ELISA kit (Assay Designs 900-091, Ann Arbor, MI, USA) for the presence of 8-iso-Prostaglandin F$_{2\alpha}$, as described in section 2.3.5.2 (page 115).

### 7.2.4.3 Statistical analysis

Repeated measures analysis of variance was used to reveal any statistically significant differences in oxygen uptake and in ethane output between measurement points during rest, exercise and recovery. The assumption of sphericity was examined using Mauchly’s W test. In cases where this assumption was contravened, the greenhouse-Geisser adjustment was applied. Where the analysis of variance indicated at least one significant difference, pairwise comparisons, with the application of a Bonferroni adjustment for multiple comparisons, revealed the location.

In cases where data conformed to a normal distribution, a Paired t-test was used to determine if there was any statistically significant difference between the two resting measurements of ethane concentration and ethane output. Where the data did not conform to a normal distribution, a Wilcoxon Signed Ranks test was utilised. Normality was tested using the Shapiro-Wilk test.

The relationships between oxygen uptake and ethane output; F$_2$-isoprostane concentration from the exercised versus the non-exercised arm; and ethane output versus F$_2$-isoprostane concentration from the non-exercised arm were each examined using correlation. A Spearman’s Rho test was utilised where data did not conform to a normal distribution, which was tested using the Shapiro-Wilk test.
Figure 7.1. Schematic representation of the sustained contraction trial protocol.

Exercise was continued until exhaustion. The duration of the exercise period was variable from 53-85 s. The reduction in force due to fatigue is not shown. Expired air samples were of 2 min duration. Blood samples were collected at the start of each expired air sample.
7.3 Results

7.3.1 Subjects

Subjects were healthy, male volunteers, all of whom were regularly active in football, rugby or cycling. Subject characteristics are shown in table 7.2.

Table 7.2. Subject characteristics for study three.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Age (yr)</th>
<th>Weight (kg)</th>
<th>Height (cm)</th>
<th>MVC (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>28</td>
<td>82.3</td>
<td>187.0</td>
<td>519</td>
</tr>
<tr>
<td>2</td>
<td>29</td>
<td>77.0</td>
<td>175.5</td>
<td>428</td>
</tr>
<tr>
<td>3</td>
<td>25</td>
<td>83.2</td>
<td>171.0</td>
<td>518</td>
</tr>
<tr>
<td>4</td>
<td>26</td>
<td>68.2</td>
<td>167.0</td>
<td>409</td>
</tr>
<tr>
<td>5</td>
<td>24</td>
<td>64.8</td>
<td>171.5</td>
<td>397</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>96.2</td>
<td>185.4</td>
<td>517</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>26 ± 2</td>
<td>78.6 ± 11.4</td>
<td>176.2 ± 8.2</td>
<td>465 ± 59</td>
</tr>
</tbody>
</table>

MVC maximal voluntary contraction; SD standard deviation

7.3.2 Maximal voluntary contraction

Each subject was asked to produce at least three maximal handgrip efforts in order to determine maximal voluntary contraction force. If the difference in force between any attempt and the highest force measured was less than 5 %, then the highest force from the three attempts was recorded as the subject’s MVC. If the difference in force was greater than 5 % between any attempt, the subject was asked to perform a further two attempts and the highest force of all five attempts was recorded as the subject’s MVC. This was the case
for only one subject as seen in table 7.3, which shows force produced at each attempt at maximal voluntary contraction for all subjects. Maximal voluntary contraction force, as a mean and standard deviation of all six subjects, was 465 ± 59 N, as shown in table 7.2.

<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
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<td>1</td>
<td>466</td>
<td>427</td>
<td>519</td>
<td>451</td>
<td>498</td>
<td>519</td>
</tr>
<tr>
<td>2</td>
<td>405</td>
<td>428</td>
<td>425</td>
<td>-</td>
<td>-</td>
<td>428</td>
</tr>
<tr>
<td>3</td>
<td>518</td>
<td>515</td>
<td>503</td>
<td>-</td>
<td>-</td>
<td>518</td>
</tr>
<tr>
<td>4</td>
<td>409</td>
<td>407</td>
<td>399</td>
<td>-</td>
<td>-</td>
<td>409</td>
</tr>
<tr>
<td>5</td>
<td>346</td>
<td>381</td>
<td>397</td>
<td>-</td>
<td>-</td>
<td>397</td>
</tr>
<tr>
<td>6</td>
<td>517</td>
<td>489</td>
<td>483</td>
<td>-</td>
<td>-</td>
<td>517</td>
</tr>
</tbody>
</table>

- not measured

Figure 7.2 shows a typical plot of the output from the data acquisition unit during a maximal voluntary contraction trial. The tracing shows the first, and highest, attempt at producing a maximal voluntary effort in subject 4. Maximum force was reached within 5 s of the onset of contraction in all subjects. The signal from the data acquisition unit was later converted from a voltage to a force measured in newtons (N) as described in section 6.2.2.1 (page 257).
Figure 7.2. Typical force output during a maximal voluntary contraction trial in one male subject.

7.3.3 Sustained contraction trial

Each subject participated in one sustained contraction trial. This involved maintaining a force equivalent to 60 ± 2 % MVC until exhaustion. Contraction time was measured from the point at which force first became equal to 60 % MVC minus 2 %, to the time point at which force fell below this level and could not be recovered immediately. Figure 7.3 shows
a typical recording of a sustained contraction trial in one subject and the dashed vertical lines indicate the start and end of the contraction. As can be seen from the figure, the subject was given visual feedback to guide force production throughout the trial by means of horizontal lines drawn at the target force and at 2 % above and below this value. In all subjects, force increased to the target force rapidly, within 5 s of the start of the contraction, and was sustained within 2 % of this value throughout the trial as described. Table 7.4 shows the mean force sustained during the contraction for each subject as an absolute force, and also as a percentage of the target force (60 % MVC). All subjects closely matched the target force, although the mean force sustained was lower than expected in some cases. This was due to the effect of large transient deviations below the target force where an immediate recovery was made and the subject was allowed to continue the contraction. Mean contraction time was 70.0 ± 14.1 s as shown in table 7.4.

Table 7.4. Sustained isometric handgrip contraction at 60 % maximal voluntary contraction.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Contraction time (s)</th>
<th>Mean Force sustained (N)</th>
<th>Target Force (N)</th>
<th>Mean Force sustained (% Target Force)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>52.9</td>
<td>301</td>
<td>311</td>
<td>97</td>
</tr>
<tr>
<td>2</td>
<td>69.4</td>
<td>251</td>
<td>257</td>
<td>98</td>
</tr>
<tr>
<td>3</td>
<td>53.9</td>
<td>303</td>
<td>311</td>
<td>98</td>
</tr>
<tr>
<td>4</td>
<td>85.2</td>
<td>239</td>
<td>245</td>
<td>98</td>
</tr>
<tr>
<td>5</td>
<td>83.1</td>
<td>233</td>
<td>238</td>
<td>98</td>
</tr>
<tr>
<td>6</td>
<td>75.7</td>
<td>298</td>
<td>310</td>
<td>96</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>70.0 ± 14.1</td>
<td>271 ± 33</td>
<td>279 ± 36</td>
<td>97 ± 1</td>
</tr>
</tbody>
</table>

MVC maximal voluntary contraction; SD standard deviation
Figure 7.3. Typical force output during a sustained contraction trial at 60 % maximal voluntary contraction in one male subject.

Horizontal lines at 0.365 V, 0.358 V and 0.351 V correspond to 62 % MVC, 60 % MVC and 58 % MVC respectively. Vertical lines indicate the start and end of contraction.
7.3.4 Oxygen uptake response

The oxygen uptake response to sustained isometric handgrip exercise and recovery is shown in figure 7.4. The figure shows the mean response of five of the six subjects. The first three expired air samples of subject 3 were discarded due to a temporary malfunction of the dry gas meter, and therefore, no measurement of oxygen uptake could be made. Thus, this subject has been omitted from the mean oxygen uptake response data. Oxygen uptake was seen to rise above baseline in the sample collected immediately upon cessation of exercise. Oxygen uptake then returned towards baseline within 5 minutes of passive recovery and remained stable for the remainder of the recovery period. Repeated measures analysis of variance was performed and the Greenhouse-Geisser adjustment was applied ($\varepsilon = 0.178$) since the assumption of sphericity was violated ($p = 0.000$). This analysis revealed borderline significance ($F(1.248, 4.991) = 6.542; p = 0.047$); however, pairwise comparisons indicated no significant differences between time points.
Figure 7.4. Mean oxygen uptake response of five subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60% maximal voluntary contraction. Data points show mean and standard deviation. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
7.3.5 Ethane response

7.3.5.1 Ambient ethane concentration

Ambient ethane concentration was measured at each expired air sampling point and mean values for each test are shown in table 7.5. Ambient ethane concentration ranged from 53.6 pmol·l⁻¹ to 281.3 pmol·l⁻¹ (1.2 ppb to 6.3 ppb) across all samples, with a mean and standard deviation of 115.2 ± 58.6 pmol·l⁻¹ (2.6 ± 1.3 ppb).

Table 7.5. Mean ambient ethane concentration during each sustained contraction trial.

<table>
<thead>
<tr>
<th>Subject</th>
<th>[C₂H₆] (ppb)</th>
<th>[C₂H₆] (pmol·l⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.3 ± 0.0</td>
<td>58.0 ± 2.2</td>
</tr>
<tr>
<td>2</td>
<td>2.1 ± 0.1</td>
<td>93.3 ± 4.1</td>
</tr>
<tr>
<td>3</td>
<td>2.1 ± 0.2</td>
<td>92.3 ± 7.7</td>
</tr>
<tr>
<td>4</td>
<td>5.1 ± 0.7</td>
<td>228.2 ± 32.8</td>
</tr>
<tr>
<td>5</td>
<td>3.2 ± 0.1</td>
<td>141.9 ± 6.6</td>
</tr>
<tr>
<td>6</td>
<td>1.7 ± 0.1</td>
<td>77.9 ± 2.4</td>
</tr>
</tbody>
</table>

All values are mean ± standard deviation.
7.3.5.2 **Ethane concentration and ethane output at rest**

Resting ethane concentration and ethane output were each determined as a mean of two baseline samples collected 10 minutes apart prior to the start of exercise, and were calculated using equations 3.8 to 3.10 (page 140). Values for each subject are displayed in table 7.6. The mean and standard deviations shown were inclusive of all six subjects for ethane concentration, but inclusive of only five subjects for ethane output. Ethane output could not be calculated for subject 3 due to the missing expired air volume data for the baseline samples, as explained in section 7.3.4 (page 288). The relatively high standard deviations underline the inter-individual variability in resting ethane. There was no significant difference between the two resting samples for ethane concentration ($Z = -0.954; p = 0.340$), or ethane output ($Z = -0.184; p = 0.854$), shown by a Wilcoxon Signed-Ranks test in both cases.

<table>
<thead>
<tr>
<th>Subject</th>
<th>$[C_2H_6]$ (pmol·l$^{-1}$)</th>
<th>$\dot{V}C_2H_6$ (pmol·min$^{-1}$)</th>
<th>$\dot{V}C_2H_6$ (pmol·kg$^{-1}$·min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>11.2</td>
<td>77.1</td>
<td>0.9</td>
</tr>
<tr>
<td>2</td>
<td>29.0</td>
<td>185.5</td>
<td>2.4</td>
</tr>
<tr>
<td>3</td>
<td>15.6</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>4</td>
<td>26.8</td>
<td>87.1</td>
<td>1.3</td>
</tr>
<tr>
<td>5</td>
<td>11.2</td>
<td>72.4</td>
<td>1.1</td>
</tr>
<tr>
<td>6</td>
<td>6.7</td>
<td>74.2</td>
<td>0.8</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>16.7 ± 9.1</td>
<td>99.3 ± 48.5</td>
<td>1.3 ± 0.6</td>
</tr>
</tbody>
</table>

NA not available; SD standard deviation.
7.3.5.3 Ethane output post-exercise

Ethane output was measured at rest, immediately post-exercise and at 5 minute intervals during a 30 minute period of passive recovery. Figure 7.5 shows the ethane output response in six individuals.

Ethane output could not be calculated for the first three expired air samples in subject 3 due to the lack of expired air volume measurement, as described in section 7.3.4 (page 288). Ethane output fell to 0 pmol·kg⁻¹·min⁻¹ in several samples in subjects 1, 4 and 5. Examination of the raw data indicated that expired ethane concentration was equal to ambient ethane concentration at these sampling points. This was due to a reduction in expired ethane concentration in each case since ambient ethane concentration was stable throughout each test. However, these samples were omitted from the data set since there was uncertainty regarding the true ethane output value. It is possible that ethane production was present, but masked by ambient ethane.

The temporal pattern of response was variable amongst subjects. The peak oxidative stress response tended to occur immediately following exercise or within 5 minutes of recovery. In subjects 1 and 2 the pattern of response was a rise in oxidative stress in the first 5 minutes of recovery from exercise and then a decline back to baseline by the end of the 30 minute recovery period. The lack of data for the first three time points precluded analysis of the pattern of response in subject 3. In subject 4 the response was elevated slightly from baseline by 5 minutes into the recovery period and fell at the next time point, however, rather than declining to baseline as in subjects 1 and 2, ethane output then rose throughout the remainder of the recovery period. In subject 5 the lack of values at 0 and 5 minutes into recovery concealed the timing of the peak response; however a decline in response to the end of the recovery period was noted. In subject 6 there was little deviation from the baseline response throughout the recovery period.
The magnitude of the peak ethane output response was similar in two subjects (subjects 1 and 6) with a range of 1.1 to 1.4 pmol·kg\(^{-1}\)·min\(^{-1}\). The peak response in subject 2 was 3.6 pmol·kg\(^{-1}\)·min\(^{-1}\); however, examination of the plots in figure 7.5 showed that this subject also had a higher baseline response than all other subjects. Since the responses immediately or 5 minutes post-exercise were missing in the other subjects, the peak response may have been missed.

The mean ethane output response of five subjects is shown in figure 7.6. The mean response was similar throughout the entire period of measurement and the large standard deviations indicated a high inter-individual variability. The peak response occurred immediately post-exercise and remained close to this value after 5 minutes of recovery. Repeated measures analysis of variance was performed and the Greenhouse-Geisser adjustment was applied (\(\varepsilon = 0.143\)) since the assumption of sphericity was violated (\(p = 0.000\)). This analysis indicated no significant difference in ethane output between any time points (\(F(1.000, 1.000) = 2.486; p = 0.360\)).

Figure 7.7 suggests no obvious correspondence between the ethane output response and the oxygen uptake response to isometric exercise. A Spearman’s Rho test indicated no significant correlation (\(\rho = -0.260; p = 0.115\)).
Figure 7.5. Ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in six male subjects. The number in the top right hand corner of each panel refers to the subject number. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
Figure 7.6. Mean ethane output response of five male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

Data points show mean and standard deviation. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
7.3.6 F₂-isoprostane response

Blood samples were collected at the same time points as the expired air measurements: at baseline, immediately post-exercise and at 5 minute intervals throughout a 30 minute passive recovery period. Samples were collected from both the exercised arm and the non-exercised arm, as shown in table 7.7 and in figure 7.8, and were analysed for the presence of F₂-isoprostanes, a marker of lipid peroxidation.
Data from the non-exercised arm are missing from subject 5, and data from the exercised arm are missing from subject 6. In both cases, this was due to inability to cannulate the subject within several attempts. Several further samples were not collected due to difficulty withdrawing blood at the sampling time. Other values, marked as ‘HI’ in table 7.7, were omitted from the data set since analysis indicated that the measured F$_2$-isoprostane concentration was above the upper limit of the assay, thus the true value was obscured.

Figure 7.8 shows a direct comparison between the exercised arm and the non-exercised arm. From this, and the mean data shown in figure 7.9, it can be seen that the pattern of response differed between the exercised and non-exercised arms. There was a clear peak response immediately post-exercise in the exercised arm; however, this may be somewhat misleading since this value was the response from only one subject, since responses from the other subjects were missing as already described. The response did not vary much from baseline at the other time points throughout recovery. In the non-exercised arm, there was a peak five minutes in to the recovery period and then again 30 minutes into recovery. The response rose above baseline immediately after exercise and continued to rise in the first five minutes of recovery. Thereafter it recovered towards baseline until the second peak at the end of the measured recovery period. The missing data precluded analysis of variance to investigate any differences between time points for either the exercised or the non-exercised arm. The baseline and peak responses were similar in magnitude in both arms. The standard deviations in figure 7.9 tended to be lower in the exercised arm. There are two instances where no standard deviation is shown; these values show the response of only one subject. Figure 7.10 illustrates no clear relationship between F$_2$-isoprostane concentration in the exercised versus the non-exercised arm, and there was no significant correlation between the two variables in the four subjects for whom data were available from both arms ($\rho = 0.063; p = 0.846$).
Table 7.7. $F_2$-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60% maximal voluntary contraction.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time Point</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$EX$</td>
<td>$N-EX$</td>
<td>$EX$</td>
<td>$N-EX$</td>
<td>$EX$</td>
<td>$N-EX$</td>
<td>$EX$</td>
</tr>
<tr>
<td>B</td>
<td>399.8</td>
<td>222.3</td>
<td>566.5</td>
<td>HI</td>
<td>156.2</td>
<td>255.1</td>
<td>199.1</td>
</tr>
<tr>
<td>R0</td>
<td>HI</td>
<td>475.2</td>
<td>946.9</td>
<td>HI</td>
<td>HI</td>
<td>-</td>
<td>HI</td>
</tr>
<tr>
<td>R5</td>
<td>228.4</td>
<td>733.8</td>
<td>-</td>
<td>1044.2</td>
<td>HI</td>
<td>HI</td>
<td>-</td>
</tr>
<tr>
<td>R10</td>
<td>237.1</td>
<td>246.1</td>
<td>HI</td>
<td>937.5</td>
<td>326.6</td>
<td>322.0</td>
<td>-</td>
</tr>
<tr>
<td>R15</td>
<td>188.2</td>
<td>HI</td>
<td>364.3</td>
<td>586.6</td>
<td>224.0</td>
<td>275.3</td>
<td>HI</td>
</tr>
<tr>
<td>R20</td>
<td>236.3</td>
<td>328.7</td>
<td>598.3</td>
<td>HI</td>
<td>252.0</td>
<td>HI</td>
<td>267.3</td>
</tr>
<tr>
<td>R25</td>
<td>221.4</td>
<td>HI</td>
<td>HI</td>
<td>-</td>
<td>256.8</td>
<td>HI</td>
<td>HI</td>
</tr>
<tr>
<td>R30</td>
<td>243.5</td>
<td>661.8</td>
<td>HI</td>
<td>-</td>
<td>285.8</td>
<td>907.4</td>
<td>HI</td>
</tr>
</tbody>
</table>

EX exercised arm; N-EX non-exercised arm; B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery; - sample not collected; HI measured concentration was above the upper limit of the assay.
Figure 7.8. F₂-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in the exercised and non-exercised arm in six male subjects.

The number in the top right hand corner of each panel refers to the subject number. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery. ■ exercised arm; □ non-exercised arm.
Figure 7.9. Mean $F_2$-isoprostane response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in the exercised arm (EX; upper panel) and non-exercised arm (N-EX; lower panel).

Data points show mean and standard deviation. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.

- ■ exercised arm; □ non-exercised arm.
Figure 7.10. Relationship between $F_2$-isoprostane concentration in the exercised arm versus the non-exercised arm in four male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.
7.3.7 Relationship between expired air and blood markers

Figure 7.11 shows a comparison of the F$_2$-isoprostane response in the non-exercised arm with the ethane output response in five subjects. Both markers give an indication of the systemic oxidative stress response. Figure 7.12 displays mean values of F$_2$-isoprostanes in the non-exercised arm alongside mean values of ethane output. These data were displayed previously in figure 7.9 and figure 7.6 respectively.

There was no obvious relationship seen in either the individual plots or in the plot of mean responses (figures 7.11 and 7.12 respectively) between invasive and non-invasive measures of lipid peroxidation. Peak responses occurred 5 minutes and 30 minutes into the recovery period in the blood as seen in figure 7.12. There was no clear peak response in the ethane output data: the highest values were recorded immediately post-exercise and at 5 minutes into the recovery period; however, these values were not significantly different from the baseline response as described in section 7.3.5.2 (page 291). Figure 7.13 indicates no clear relationship between ethane output and F$_2$-isoprostane concentration in the non-exercised arm. Statistical analysis confirmed no significant correlation between the two variables ($\rho = 0.270; p = 0.278$).
Figure 7.11, Panel 1. $F_2$-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in two male subjects. The number in the top right hand corner of the pair of panels refers to the subject number. For each subject, the upper panel shows the $F_2$-isoprostane response and the lower panel shows the ethane output response. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
Figure 7.11, Panel 2. $F_2$-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction in two male subjects.

The number in the top right hand corner of the pair of panels refers to the subject number. For each subject, the upper panel shows the $F_2$-isoprostane response and the lower panel shows the ethane output response. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
Figure 7.11, Panel 3. \( \text{F}_2 \)-isoprostane response and ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60% maximal voluntary contraction in one male subject.

The number in the top right hand corner of the pair of panels refers to the subject number. The upper panel shows the \( \text{F}_2 \)-isoprostane response and the lower panel shows the ethane output response. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
Figure 7.12. Mean $F_2$-isoprostane response in the non-exercised arm and mean ethane output response prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.

The upper panel shows the $F_2$-isoprostane response and the lower panel shows the ethane output response. B baseline sample: mean of two samples at rest; R0 immediate post-exercise sample; R5 to R30 samples taken at 5 to 30 min into recovery.
Figure 7.13. Relationship between mean ethane output and F$_2$-isoprostane concentration in the non-exercised arm in six male subjects prior to and following sustained isometric handgrip exercise to exhaustion at 60 % maximal voluntary contraction.
7.4 Discussion

7.4.1 Maximal voluntary contraction

Mean maximal voluntary contraction was 465 ± 59 N. This was greater than two of the three studies which have previously investigated the effect of isometric handgrip exercise on oxidative stress. Steinberg and colleagues reported mean maximal voluntary contractions of 367 N (Steinberg et al., 2004) and 333 N (Steinberg et al., 2006). However, it should be noted that the subjects in both studies were substantially older than subjects in the present study with a mean age of 59 yr and 44 yr respectively. Unfortunately, the remaining study (Dousset et al., 2002) which utilised a cohort of male subjects with a similar mean age (30 ± 3 yr) did not report absolute MVC.

7.4.2 Sustained contraction duration

The mean duration of the sustained contraction at a force equivalent to 60 % MVC was 70.0 ± 14.1 s as shown in table 7.4. This was substantially longer than the contraction duration of 42 ± 5 s reported by the only previous study to utilise this contraction intensity (Dousset et al., 2002). However, subjects in the previous study were reported to rest for only 10 minutes between the initial MVC trial and the sustained contraction trial, thus perhaps there was some residual fatigue. There was a minimum of 72 h between the MVC trial and the sustained contraction trial in the current study, with the exception of the tests for one subject whose sustained contraction trial took place 80 minutes following the MVC trial due to constraints on the availability of the subject. However, this subject had a contraction duration above the mean response (subject 6, table 7.4).
7.4.3 Oxygen uptake response

The oxygen uptake response was not measured in previous studies which utilised a similar isometric exercise protocol to the current study (Dousset et al, 2002; Steinberg et al., 2004, 2006); however, Alessio and colleagues (2000) did measure oxygen uptake during an intermittent handgrip exercise protocol. They reported a two-fold increase in \( \dot{V}O_2 \) from a baseline response of approximately 3-4 ml·kg\(^{-1}\)·min\(^{-1}\). The current study showed a similar response with a mean baseline \( \dot{V}O_2 \) of 3.5 ml·kg\(^{-1}\)·min\(^{-1}\) rising to a peak of 6.0 ml·kg\(^{-1}\)·min\(^{-1}\) immediately following exercise.

One postulated mechanism of exercise-induced oxidative stress is an increase in mitochondrial production of oxidant species as oxygen uptake increases during exercise, as described in section 1.3.3.1 (page 83). It has been suggested that this may not be a key mechanism in the oxidative stress response to isometric exercise since a greater oxidative stress response was found following isometric exercise in comparison to aerobic exercise over the same time period, despite a substantially lower oxygen uptake response in isometric exercise (Alessio et al., 2000). In the current study, the ethane output response pattern did not correlate well with the oxygen uptake response: the peak in ethane output did not coincide with the peak in oxygen uptake immediately following exercise, and there was no significant correlation between the two variables; thus supporting the premise that additional mechanisms of oxidant production may be operating in this mode of exercise.
7.4.4 Ethane response

7.4.4.1 Ambient ethane concentration

Ambient ethane concentration, as a mean of all time points measured in all subjects, was 115.2 ± 58.6 pmol·l$^{-1}$. This was within the range of 68 to 800 pmol·l$^{-1}$ reported in previous studies (Dumelin et al., 1978; Knutson et al., 1999; Sexton & Westberg, 1984; Zarling & Clapper, 1987), and was fairly similar to the mean ambient ethane concentration of 185.3 ± 64.9 pmol·l$^{-1}$ reported in our previous study (Wyse et al., 2005b), as described in chapter three. Statistical analysis confirmed no significant difference in mean ambient concentration between these two studies (Independent samples t-test; $t(12) = 2.064$, $p = 0.061$). A low ambient ethane concentration is desirable to minimise the possibility of contamination of expired air samples.

A high ambient ethane concentration should not affect the ability to detect ethane production, as long as the subject has been breathing the ambient air in the testing environment for a sufficient period for equilibration to occur (see section 2.3.2.4.1, page 109). This was the case for all experiments in this study. Ethane production was calculated as 0 pmol·kg$^{-1}$·min$^{-1}$ for several samples in this study since ambient ethane concentration and expired ethane concentration were equivalent. In each case, this was due to a reduction in expired ethane concentration, rather than a rise in ambient ethane concentration. This is perhaps a limitation with the use of the laser spectroscopy technology; however, it does not seem to occur with high frequency. This issue occurred in 1 % of the samples collected in our previous study described in chapter 3, and in 13 % of samples collected in the current study.
7.4.4.2 Ethane concentration and ethane output at rest

Ethane concentration and ethane output were both expressed as a mean of two samples of expired air collected at rest, prior to the start of exercise. No significant difference was found between the two resting measurements of either variable, thus strengthening the confidence in reproducibility of the measurement technique as reported in chapter three (section 3.4.1.2, page 161).

A mean ethane concentration at rest of $16.7 \pm 9.1$ pmol·l$^{-1}$ fell within the range of 10.4 to 50 pmol·l$^{-1}$ previously reported in the literature (Knutson et al., 1999; Zarling & Clapper), and was somewhat lower than the value of 72.7 pmol·l$^{-1}$ reported in our previous study described in chapter three (Wyse et al., 2005b). The same procedures were used for the collection and analysis of expired air for the presence of ethane in both studies; however, statistical analysis confirmed a significant difference in mean ethane concentration at rest between each study (Independent samples t-test; $t(12) = 3.698; p = 0.003$).

Similarly, the mean ethane output at rest of $99.3 \pm 48.5$ pmol·min$^{-1}$ in the current study was comparable to a previously reported values of approximately 85 pmol·min$^{-1}$ in healthy individuals (Leaf et al., 1997). However, again mean ethane output in the current study was substantially lower in comparison to the value of 872.1 pmol·min$^{-1}$ reported in our previous study (Wyse et al., 2005b), described in chapter three. Statistical analysis confirmed a significant difference in this variable between the two studies (Independent samples t-test; $t(11) = 2.351; p = 0.038$). When mean ethane output was standardised for body mass, the value in the current study fell within the range previously reported (Knutson et al., 1999). Both studies utilised a similar cohort of healthy, trained male subjects and the same testing environment. Thus, the reason for this discrepancy in the mean values between studies is unclear. Procedures for the measurement and analysis of ethane did not differ between studies, and the consistency of ambient ethane concentration
suggests no technical inconsistencies with equipment or experimenter error. It is possible that the difference between studies simply underlines a high inter-individual variability in ethane concentration at rest, which has been indicated as a common feature of ethane measurement (Thekedar et al., 2009), and is supported by the wide range of values reported in the literature (Knutson et al., 1999; Zarling & Clapper). This may not just be variability between different individuals, but potentially variability within individuals at different times. Three subjects participated in both studies described here, separated by a period of 2 years, and in two of these individuals resting ethane concentration was substantially higher in the first study. Factors such as training status or antioxidant status, not measured in either study, may impact on resting oxidative stress (Leaf et al., 1999), although the literature is far from conclusive. As indicated in chapter three, the variability in expired ethane requires further investigation.

### 7.4.4.3 Ethane output post-exercise

Previous data has indicated a significant rise in oxidative stress following static isometric handgrip exercise at 50-60 % MVC, with the peak response occurring at around 5 minutes into recovery (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006). However, due to some conflicting data in this small number of studies, one aim of the present study was to repeat these measurements over an extended 30 minute recovery period and to sample at 5-minute intervals throughout. The increase in ethane output following sustained isometric handgrip exercise to exhaustion in the current study was not significant, although the mean response suggested a rise in ethane output immediately after, to 5 minutes after the end of exercise. All studies used a similar exercise protocol; however, previous studies utilised plasma markers of lipid peroxidation, whereas the current study is the first to have used ethane output as a marker of lipid peroxidation in relation to isometric exercise. Subjects in the current study exercised at the same or a higher relative intensity in comparison to those in previous studies. It is still unknown if
there is a threshold exercise intensity below which an oxidative stress response will not manifest. Subjects in the current study may have been better trained than subjects in previous studies, who were described simply as healthy individuals. It has been suggested that a period of training may reduce the oxidative stress response to a specified exercise stimulus (Miyazaki et al., 2001), and therefore, it is possible that subjects in the current study may not have reached a potential threshold for developing a significant oxidative stress response. However, it is possible that a larger sample size may have produced significance at the contraction intensity utilised. It is unlikely that the lack of a significant oxidative stress response was due to a limited exercise duration. Mean contraction time was 70 s at 60 % MVC, in comparison to previous isometric studies which reported contraction times of 42 s at 60 % MVC (Dousset et al., 2002), 95 s at 50% MVC (Steinberg et al., 2004), and 100 s at 50% MVC (Steinberg et al., 2006).

The pattern of response as measured by ethane output in the current study was similar to that reported in previous studies in which an immediate increase following the end of sustained isometric contraction, shown in three studies (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006), was followed by a further increase to a peak response at 5 minutes into the recovery period, shown in the two studies in which oxidative stress was measured at this time point (Steinberg et al., 2004, 2006). One study showed that the peak response did not occur until 20 minutes into the recovery period (Dousset et al., 2002), although it was only measured at this time point and immediately after exercise. Thus, the results of the current study show partial agreement with previous literature in terms of the timing of the peak oxidative stress response following this mode of exercise. The 

\[ \text{F₂-isoprostane} \] response measured in the non-exercised arm in the current study suggested a peak response later in the recovery period, and perhaps this is evidence of a later response to the exercise stress, potentially due to a different mechanism. Further study would be required to investigate this.
In the current study, the ethane output response was observed to have returned close to the baseline value by 10 minutes into the recovery period, although there is some lack of clarity due to the lack of statistical significance. The previous studies assessed oxidative stress as far as 20 minutes into the recovery period; however, results were conflicting. Dousset and colleagues (2002) reported a peak response after 20 minutes of recovery, whilst Steinberg and colleagues (2006) reported that the ethane output response had returned to baseline following this time period. The third study (Steinberg et al., 2004) did not report at which time point the response had returned to baseline. Thus, again, there is partial agreement with some of the previous literature in terms of the temporal pattern of the ethane output response during the recovery period. The tendency for a quicker return to baseline here may have been influenced by the measurement technique. The previous studies cited utilised plasma markers of lipid peroxidation, and it is possible that ethane clears more quickly from the lungs than from the circulation, leading to a quicker return to baseline with ethane output as a marker.

Information about the pattern of oxidative stress response following isometric exercise may help to elucidate the mechanisms involved in the response. An immediate response would suggest an immediate mechanism, for example, mitochondrial production of reactive species; whereas a delayed peak response may indicate an alternative mechanism, for example, generation of free radicals by neutrophils (Hessel et al., 2000; Ji, 1999). The finding of two peak responses in the recovery period may indicate the involvement of more than one mechanism in this exercise mode. It must be emphasised that the aim of the current study was not to investigate the mechanistic basis of the oxidative stress response, but simply to delineate the timing of the response.

Interestingly, the magnitude of the peak response in the current study (1.9 ± 1.5 pmol·kg⁻¹·min⁻¹) was substantially lower than that reported following maximal
aerobic exercise (142.1 ± 27.5 pmol·kg\(^{-1}\)·min\(^{-1}\)), as reported in chapter 3. This may be expected since the activated muscle mass is lower in isometric exercise, and several mechanisms of production of reactive species are related to the process of muscle contraction (Vollaard et al., 2005). This finding contradicts that reported previously by Alessio and colleagues (2000) who measured lipid peroxidation as assessed by TBARS and lipid hydroperoxides in the same cohort of individuals following both maximal aerobic and isometric exercise. These authors found the increase in lipid peroxidation to be greater following isometric exercise when assessed by lipid hydroperoxides, with no significant change in TBARS in either condition. They concluded that different mechanisms of oxidative stress were likely in the different exercise modes, and that the mechanism of oxidative stress in isometric exercise is not primarily due to production of free radicals in the electron transport chain. Our results support this hypothesis since there was no clear relationship between oxygen uptake and lipid peroxidation measured by ethane output, as previously discussed (section 7.4.3, page 309).

7.4.5 F\(_2\)-isoprostane response

F\(_2\)-isoprostanes were measured in blood collected from both the exercised arm and from the non-exercised arm. The magnitude of the peak response was similar in both arms; however, the pattern of response differed. In the exercised arm, there was a clear peak response immediately following the cessation of exercise; however, this was based on only one observation since measurement at this time point was missing in all other subjects. In contrast, in the non-exercised arm the highest responses occurred 5 minutes into the recovery period, and then again 30 minutes into the recovery period. The earlier peak response in the exercised arm may have been influenced by the proximity of the sampling site to the site of production of reactive species in the exercising muscle. The presence of a later peak in the non-exercised arm may indicate the presence of an alternative mechanism of oxidative stress, as discussed in the previous section (section 7.4.4.3, page 312);
however, it is unclear why this response was not also present in the exercised arm. The high inter-individual variability, and the fact that samples were missing at some time points in some individuals may have obscured the underlying response. The lack of correlation between the sampling sites may have been due to the paucity of data; however may have been a function of the transit time required for markers of oxidative stress to arrive at the sampling site.

The \( \text{F}_2 \)-isoprostanes assay was not altogether successful in the current study, with the concentration of 33 % of all samples falling outwith the upper range of the assay, thus limiting the amount of useable data. The mean \( \text{F}_2 \)-isoprostane concentration at rest was \( 303.8 \pm 174.9 \) pg·ml\(^{-1}\) in the exercised arm and \( 259.0 \pm 42.5 \) pg·ml\(^{-1}\) in the non-exercised arm. Both values are substantially higher than those previously reported in the literature which fall within a range of 28-75 pg·ml\(^{-1}\) (Mastaloudis et al., 2001; Mastaloudis et al., 2004b; Nieman et al., 2002; Steensberg et al., 2002; Waring et al., 2003), although one study did report a resting concentration of approximately 2000 pg·ml\(^{-1}\) (Sachek et al., 2003). Previous studies investigating oxidative stress in aerobic exercise which have utilised \( \text{F}_2 \)-isoprostanes as a marker of lipid peroxidation have reported concentrations in the range of 41-131 pg·ml\(^{-1}\) at post-exercise or recovery (Mastaloudis et al., 2001, 2004b; McAnulty et al., 2003; Nieman et al., 2002; Steensberg et al., 2002; Waring et al., 2003). Only one study reported a relatively high post-exercise concentration of 3000-4000 pg·ml\(^{-1}\) following downhill running (Sacheck et al., 2003). One previous study reported a post exercise \( \text{F}_2 \)-isoprostane concentration of approximately 35 pg·ml\(^{-1}\) following 2 hours of resistance exercise (McAnulty et al., 2005b). No previous studies to date have utilised \( \text{F}_2 \)-isoprostanes as a marker in relation to isometric exercise. Thus, values in the present study are generally not comparable to previous literature.
Blood samples from the study reported in Chapter 5 and from this study were analysed at the same time, thus, the analysis issues regarding potential autoxidation of samples with subsequent elevation of \( F_2 \)-isoprostanes concentrations, discussed in section 5.4.2 (page 248), are also pertinent here. The storage period of samples from the current study was 17 months, and again the delay before analysis was unforeseen and unavoidable. The issues with analysis of blood samples emphasise the potential utility of non-invasive assessment of oxidative stress.

7.4.6 Relationship between expired air and blood markers

No significant relationship was observed between the ethane output response and the \( F_2 \)-isoprostane response in the non-exercised arm, both of which gave an indication of the systemic oxidative stress response. Again this was likely due to the small subject number, the high percentage of missing invasively measured data, and to the high inter-individual variability in response. Therefore, validation of ethane output against blood markers of lipid peroxidation is still required.

7.4.7 Limitations and further work

One of the original aims of the current study was to better characterise the oxidative stress response to isometric exercise, specifically by investigating the effect of contraction intensity, by looking for the presence of a threshold response and by clarifying the time course of the response. Unfortunately, it was not possible to achieve the first two objectives due to the unexpected withdrawal of a suitable testing environment, as described previously (section 7.2.2, page 278). Thus, these remain a valuable focus of future work.

Despite the above limitation, it was still possible to undertake a novel investigation into the suitability of non-invasive assessment of oxidative stress in relation to isometric exercise.
However, the ability to form clear conclusions regarding the time course of the oxidative stress response was limited by low subject number due to unavoidable early termination of data collection, as described (section 7.2.2, page 278). The lack of data at some time points was a substantial limitation to the interpretation of the findings.

The low subject number also hindered the analysis of data with regard to the validation of non-invasive assessment of lipid peroxidation against traditional blood markers. It would also have been useful to compare the ethane output response against more than one blood marker, as recommended by Halliwell & Gutteridge (1999), however economic constraints were prohibitive. F\textsubscript{2}-isoprostanes were chosen as a single blood marker of lipid peroxidation since they have been used increasingly within exercise-induced oxidative stress studies in recent years, and have been suggested as being well suited to this purpose (Vollaard et al., 2005), and have been reported as one of the best markers of lipid peroxidation (Dalle-Donne et al., 2006; Milne et al., 2005). Their use also allowed avoidance of the methodological issues associated with the use of MDA, as described earlier (section 1.2.2.3.1, page 64).

Rodriguez and colleagues (2003) reported an increase in oxidative stress related to intermittent isometric handgrip exercise during which a cuff was inflated around the upper arm to ensure ischaemia during exercise. It has previously been shown that an oxidative stress response is brought about through isometric exercise alone (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006) and through ischaemia alone (Cordis et al., 1993; Grissoto et al., 2000). It would be of interest to determine how much of the oxidative stress response to isometric exercise may be due to ischaemia rather than exercise \textit{per se}. In order to investigate this, oxidative stress could be measured following the conditions of ischaemia alone with no exercise, an isometric exercise protocol, and a repeat of the exercise protocol performed under conditions of total ischaemia.
7.5 Conclusions

The current study supported the previous finding of a peak oxidative stress response following isometric exercise within the first 5 minutes of the recovery period; however, further details of the time course of the oxidative stress response to isometric exercise sustained to exhaustion remain to be clarified. Oxidative stress was assessed by ethane output for the first time in relation to isometric exercise and was found to be a viable technique; however, its use remains to be validated against more traditional plasma markers, and both intra- and inter-individual variability in ethane output require further investigation. The potential value of non-invasive assessment was underlined by F₂-isoprostane analysis issues. Oxidative stress mechanisms additional to mitochondrial oxidant production may operate in isometric exercise.
Chapter 8

General Discussion
8.1 Experimental findings

The common themes linking the studies presented here are the time course of the oxidative stress response to exercise, and the means of assessment of oxidative stress during and following exercise. These investigations have initiated some thought-provoking findings.

Oxidative stress was assessed in the first study (Chapter 3) via ethane output measured by laser spectroscopy. This was a novel technique, and the focus of the investigation was to determine if the technique was viable for use during exercise in humans, horses and dogs. It had previously been utilised in a life sciences environment, specifically in lung cancer patients (Skeldon et al., 2006), and in horses with respiratory inflammation (Skeldon et al., 2005); however, had never been utilised in an exercise environment in any species. It was known from previous literature that an oxidative stress response was expected following maximal exercise (Vollaard et al., 2005); however, this response had been measured rarely by non-invasive means (Leaf et al., 1997, 1999, 2004). In addition, no previous study had measured oxidative stress during incremental exercise over several time points. Results indicated that measurement of ethane output by laser spectroscopy was a practicable technique for measuring oxidative stress during treadmill exercise, and a rise in oxidative stress at peak exercise measured by non-invasive means was shown for the first time in a trained population. A progressive relationship between work rate and ethane output was shown throughout incremental treadmill exercise to exhaustion, with an increase in oxidative stress apparent within 2 minutes of the onset of exercise, and a return to resting values by 20 minutes into the recovery period. The magnitude of the response at rest and following maximal exercise did not closely match that reported in previous studies (Knutson et al., 1999; Leaf et al., 1997, 1999, 2004; Zarling & Clapper, 1987), warranting further investigation. Limitations in study design were such that the exercise protocol did not allow assessment of oxidative stress across the entire work rate range from rest to exhaustion, and it was not possible to investigate the potential confounding factor of
exercise duration. In addition, it was not possible to compare oxidative stress measured by the new technique with that measured by an alternative technique (Halliwell & Gutteridge, 1999). It was intended that the limitations of the first study should be addressed in the second study (Chapter 5).

In the meantime, pilot work was undertaken to investigate and develop the potential of real-time monitoring of ethane output (Chapter 4). A similar technique had been reported previously (Dahnke et al., 2001; von Basum et al., 2003), but had not been applied in an exercise setting. This technique would permit an extremely high density of data collection, allowing a more detailed characterisation of the time course of the oxidative stress response to exercise, and could be particularly useful during long periods of exercise. Although the technique was time-consuming in terms of the lengthy data analysis required, it showed promise. The response profile during incremental treadmill exercise was similar to that reported in the previous chapter, thus validating the real-time technique to some extent.

In the second study (Chapter 5), it was intended to assess oxidative stress both by expired ethane and by blood marker. The original ethical application reflected this aim; however, this was unachievable due to long term failure of the laser spectrometer. It became apparent that a new bespoke laser was required for the instrument and there was substantial difficulty in sourcing this. Due to time constraints, the decision was made to proceed with the second study despite the loss of capability for ethane measurement, and to measure oxidative stress using only a blood marker. This did not impact too greatly on the main aims of this study which were to determine if the relationship between oxidative stress and work rate was linear throughout the entire work rate range from rest to maximal exercise; to determine the magnitude and time course of any increase in oxidative stress at both sub- and supra-lactate threshold work rates; and to establish if oxidative stress varied as a
function of exercise duration as well as work rate. These objectives could be met utilising only a blood marker of oxidative stress. Preliminary analysis suggested that oxidative stress may increase throughout the entire work rate range; however, unfortunately, due to technical problems with the analysis of the blood samples, it was not possible to reach any concrete conclusions regarding the aims of this study.

It was not until the third study (Chapter 7) that the opportunity arose to compare laser spectroscopy, as a method of oxidative stress measurement, with blood markers, although funding constraints limited the assessment to just one blood marker. This study utilised isometric exercise as a means to initiate an oxidative stress response. It was already known that isometric exercise at 50 or 60 % MVC could produce a significant increase in oxidative stress during the early recovery period (Dousset et al., 2002; Steinberg et al., 2004; Steinberg et al., 2006), and the original plan for this study was to extend this finding by investigating oxidative stress at a range of isometric intensities. This was intended to provide some insight into the existence, or not, of a dose-response relationship between exercise intensity and oxidative stress, and to determine a potential threshold for response, as has been suggested for aerobic exercise ((Leaf et al., 1997; Lovlin et al., 1987). However, time constraints allowed the investigation of a single exercise intensity only: 60 % maximal voluntary contraction was chosen following successful pilot work which utilised non-invasive measurement of oxidative stress for the first time in isometric exercise (as described in Chapter 6). Despite this, it was still possible to investigate the time course of the response in more detail during recovery from isometric exercise. Again problems with blood analysis were encountered, and thus it was not possible to compare ethane output against traditional plasma markers; however, results corroborated the previous finding of a peak oxidative stress response occurring early in the recovery period and indicated that ethane output was a viable technique for use in relation to isometric exercise.
8.2 Implications of findings

Although some advances in the characterisation of the oxidative stress response have been made, in both aerobic and isometric exercise, much remains unclear. The three main studies reported here investigated the time course of the oxidative stress response to varying modes of exercise: incremental treadmill and cycle ergometer exercise to voluntary exhaustion; constant load cycle ergometer exercise both above and below the lactate threshold; and isometric handgrip exercise sustained to exhaustion. Unfortunately due to limitations imposed during all three studies, the time course remains to be clarified for each mode of exercise. However, the importance of the pattern of response over time may be significant since it could have a bearing on adaptive responses to training.

8.2.1 Adaptations to training

Initial literature on oxidative stress focussed on its potential negative impact on the body, specifically oxidative damage to body tissues with the potential for pathogenesis (e.g. Jain, 2006; Singh & Jialal, 2006) and accelerated ageing (Harman, 1956). More recently interest has shifted to the potential impact of reactive species on training adaptation (Powers et al., 2010). It has been postulated that reactive species may act as signaling molecules which trigger adaptive responses to exercise, such as mitochondrial biogenesis and enhanced antioxidant enzyme capacity (Gomez-Cabrera et al., 2008a,b). Specifically, it has been reported that exercise causes activation of nuclear factor-κB in rats exposed to exhaustive exercise, leading to upregulation of the expression of the antioxidant enzyme SOD, and enhanced expression of nitric oxide synthase (Gomez-Cabrera et al., 2005) which may be important in skeletal muscle hypertrophy and fibre type transformation (Smith et al., 2002). Crucially, these responses were prevented when allopurinol was administered to inhibit the formation of reactive species, indicating this was a key component in the adaptive process. Further work by the same group suggested that vitamin C
supplementation throughout a period of exercise training in rats and humans interfered with the adaptive processes of mitochondrial biogenesis and antioxidant enzyme upregulation (Gomez-Cabrera et al., 2008a). Similarly, a combination of vitamin C and vitamin E supplementation prevented a rise in insulin sensitivity and enhanced antioxidant enzyme capacity in comparison to a non-supplemented group (Ristow et al., 2009). The magnitude and time course of the oxidative stress response may be critical, since it has been suggested that a moderate response over a short time period may signal adaptation, whereas a higher magnitude of response over a longer time period may lead to cell damage (Powers et al., 2010). Thus, information regarding a potential threshold intensity for the oxidative stress response, and how the response could be affected by exercise duration may be beneficial.

8.2.2 Antioxidant supplementation

The potential of antioxidant supplementation to interfere with training adaptation calls into question whether it may, in the balance, be detrimental to athletic performance. Antioxidant supplementation is widespread, with an estimated 70 % of American citizens utilising antioxidant supplements at least occasionally, and 40 % utilising them regularly (Hathcock et al., 2005). The use of antioxidant supplementation in the context of performance improvement has been studied by several authors. Some evidence suggests that vitamin deficiency may be detrimental to exercise performance. Davies and colleagues (1982) reported a 40 % lower endurance time for vitamin E deficient rats in comparison to rats fed a control diet over a period of 6 months. However, in general, performance does not appear to be enhanced when vitamin requirements are met (Clarkson & Thompson, 2000; Evans, 2000; Singh et al., 1992). For example, no effect on swimming performance was observed following a 6 month period of vitamin E supplementation in non-deficient individuals (Lawrence et al., 1975). Thus, evidence seems to suggest that antioxidant supplementation does not improve performance in the absence of pre-existing deficiency.
Antioxidant supplementation has been studied in the context of oxidative stress as well as that of sporting performance. The joint position statement from the American College of Sports Medicine, the American Dietetic Association and the Dieticians of Canada (2000) states that vitamin and mineral supplementation should not be required by any athlete who is consuming an adequate caloric intake from a balanced diet. Dietary antioxidant supplements are consumed by some athletes in an attempt to counteract the oxidative stress associated with strenuous exercise; however, it is unclear whether this supplementation is actually required or beneficial (Urso & Clarkson, 2003). For example, Watson and colleagues (2005) observed that although oxidative stress was lower in a group of healthy individuals consuming a normal diet, oxidative stress was not shown to rise significantly above baseline in a group consuming a diet restricted in antioxidant content. Thus, the authors concluded that antioxidant supplementation was likely not required. In fact, consumption of antioxidants in excess of requirements may actually be detrimental. McAnulty and colleagues (2005a) provided vitamin E supplements to a group of triathletes for a period of two months. It was found that lipid peroxidation was actually higher following a triathlon event in the vitamin E supplemented group in comparison to the placebo group. It was suggested that a high vitamin E concentration may have a pro-oxidant rather than an antioxidant effect. Evidence for reduced oxidative stress following supplementation is equivocal. However, the interpretation of findings in antioxidant supplementation studies is somewhat confounded by a lack of standardisation between studies in terms of supplements consumed, duration of supplementation, varied exercise protocols and differing means of assessment of oxidative stress. All factors could potentially effect the outcome of any supplementation regime.

Vitamin deficiency in healthy individuals is rare (Kanter, 1998), and antioxidant supplementation may not be required in non-deficient individuals since, although acute exercise may reduce endogenous antioxidant reserves in the short term, it has been
reported often that exercise training can result in enhancement of antioxidant enzyme capacity (Finaud et al., 2006). For example, the effect of training on antioxidant enzyme activity in older adults was studied via a twelve week resistance training programme during which only one leg was trained (Parise et al., 2005). Biopsies of the vastus lateralis muscle taken at baseline and 48 hours following the final training session indicated a significant increase in CuZnSOD and CAT, but not in MnSOD following training. Enhanced antioxidant enzyme activity has also been reported following endurance training. Miyazaki and colleagues (2001) found increased activities of erythrocyte SOD and GPx but not CAT at rest following a twelve week training programme during which untrained subjects ran at 80% of maximum heart rate for 60 minutes, five days per week.

8.2.3 Assessment of oxidative stress

The other common aim of the current studies was the investigation of a novel technique for the non-invasive assessment of lipid peroxidation. Non-invasive assessment is appealing for reasons of subject comfort and safety. There is no “gold standard” invasive means of assessment of lipid peroxidation, and it has been suggested that more than one plasma marker should be utilised in any study (Halliwell & Gutteridge, 1999). This, of course, creates added financial burden and requires greater time for analysis. The novel technique investigated here would be cost effective and time efficient as well as more pleasing for the participant. It was not possible to show that ethane output as measured by laser spectroscopy is a viable technique for the assessment of oxidative stress in both dynamic and isometric exercise. Its use remains to be validated against traditional plasma markers of oxidative stress; however, the technique itself has been validated against absolute measures of known ethane concentration so it could be argued that this is sufficient validation and justification for its use.
8.3 Conclusions

The findings presented here provide some new insight into the nature of the oxidative stress response in both aerobic and isometric exercise. Although, due to severe adverse circumstances, it was not possible to firmly describe the time course of the response in any mode of exercise, some evidence was produced in support of previous studies.

The use of the novel non-invasive technique of laser spectroscopy was tested for the first time in relation to exercise situations and appears to be suitable for the study of exercise-induced oxidative stress. The availability of this technique may lead to a greater ability to describe oxidative stress events during exercise in future studies. However, inter-individual variability in the ethane output response was a feature in all studies and remains a relevant topic for further investigation.
References


Appendix A

Information sheets, consent forms and questionnaires
Appendix A.1  Information sheet and consent form

The effect of exercise intensity on ethane production during treadmill exercise

Institute of Biomedical and Life Sciences
University of Glasgow

INFORMATION SHEET

TITLE OF INVESTIGATION: A comparative study of the production of exhaled gases (carbon monoxide, hydrogen peroxide and ethane) following maximal exercise in human, canine and equine athletes subjects

We invite you to participate in an investigation which we believe to be of potential importance. In order to help you to understand what the investigation is about, please read the following information carefully. Be sure you understand it before you formally agree to participate. If there are any points that need further explanation, please ask a member of the research team. It is important that you understand what you are volunteering to do and are completely happy with all the information before you sign this form.

What is the purpose of the study? Exercise is thought to affect breath levels of certain gases (ethane, hydrogen peroxide and carbon monoxide) in human athletes. The purpose of this study is to compare the effect of exercise on breath carbon monoxide and ethane in dogs, in horses and in humans.

Why have I been chosen? You have been selected as a possible participant in this investigation because you are aerobically trained and in good health. Before you become a subject, you will be medically screened by a trained physician and complete a medical questionnaire. People who have asthma, heart-related and/or circulatory problems, hypertension or any other contraindicated condition will not be allowed to take part in the study.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part? You will be asked to visit the laboratory on no more than three separate days over a one-month period. Each visit will last no more than 2 hours.

On your first visit, you will be introduced to the laboratory personnel and familiarised with all the equipment used. You will be asked to complete two confidential questionnaires: the first will allow us to obtain information related to your general health; and the second will allow us to quantify your past exercise/activity involvement. You will also undergo a medical screening to ensure you are in good health to participate.
On your second visit you will be asked to perform what is called a “progressive exercise test” on a treadmill. During this test, the running speed and gradient will start at a very low level and increase gradually every minute. The idea is that you keep going until you can do no more. The test will be stopped when you decide you can go no longer. You may be asked to repeat this test once more on a separate occasion.

In all tests we will ask you to breathe through a mouthpiece similar to that used for snorkelling, and use a noseclip. This is so we can monitor the air you breathe in and out. Normally your expired air (the air you breathe out) will be analysed to determine how hard your muscles are working, but at intervals throughout the test some of the air will be collected in small sample containers to analyse for the gases of interest (ethane, hydrogen peroxide and carbon monoxide).

A small harmless device will be placed on your finger to measure the amount of oxygen in your blood. This is achieved by shining light into your finger and measuring how much is absorbed by the blood.

Finally, you will not be able to consume any alcohol 48 hours prior to each lab visit. You will be excluded from participating in this study if you take drugs (recreational or performance-enhancing drugs).

**What are the side effects of taking part?** There are none.

**What are the possible disadvantages and risks of taking part?** Exercise has a negligible risk in healthy adults, although maximal exercise does carry a small risk of inducing myocardial ischaemia (“heart attack”). The primary symptom of myocardial ischaemia is chest pain on exertion. If you experience any unusual sensations in your chest during the experiment, you should cease exercising immediately. Your heart rate will be monitored via adhesive electrodes placed at points on the chest (an “electrocardiogram” or ECG). In the unlikely event you experience serious problems during the exercise, medically-qualified personnel are on call at all times during the test and approved emergency procedures are in place.

At the end of the tests you will be very tired (exhausted), your legs will be very heavy and you will be out of breath. It is also not uncommon to feel a little light-headed and nauseous.

You may experience difficulty swallowing while breathing through a mouthpiece and wearing a nose-clip; this is due to a slight but transient pressure build up in your ears. Also, you may experience increased salivation while breathing through a mouthpiece. There are no specific risks associated with collection of breath samples.

**What are the possible benefits of taking part?** The results from the exercise tests will give you a good idea of how fit you are. The research team will take the time to explain these results to you. The information from these tests will provide us with valuable information on the effect of exercise on production of certain gases (carbon monoxide, hydrogen peroxide and ethane).
What if something goes wrong? If you are harmed by taking part in this research project, there are no compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. The principal investigators, although not medically qualified, are fully trained in Basic Life Support. In the event of an untoward incident, the principal investigator(s) will provide basic life support including chest compressions and ventilation, and will apply an advisory defibrillator (if necessary) until emergency medical staff are on hand.

Will my taking part in this study be kept confidential? All information which is collected about you during the course of the research will be kept strictly confidential.

What will happen to the results of the research study? Results will be published in a peer-reviewed scientific journal once the study is completed. You will automatically be sent a copy of the full publication. You will not be identified in any publication.

If you are worried about any unwanted side effects from any of the above procedures, you should contact:

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Phone: 0141 330 2416  
Fax: 0141 330 2915  
E-mail: A.Cathcart@bio.gla.ac.uk
Title: A comparative study of the production of exhaled carbon monoxide, hydrogen peroxide and ethane following maximal exercise in human, canine and equine athletes subjects (Approved: 19/05/04)

Consent Form

I .................................................................

give my consent to the research procedures which are outlined above, the aim, procedures and possible consequences of which have been outlined to me.

Signature ......................................................

Date .........................................................
Appendix A.2  Information sheet and consent form

A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains

Institute of Biomedical and Life Sciences  
University of Glasgow

INFORMATION SHEET

TITLE OF INVESTIGATION: A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains

We invite you to participate in an investigation which we believe to be of potential importance. In order to help you to understand what the investigation is about, please read the following information carefully. Be sure you understand it before you formally agree to participate. If there are any points that need further explanation, please ask a member of the research team. It is important that you understand what you are volunteering to do and are completely happy with all the information before you sign this form.

What is the purpose of the study? Oxidative stress is thought to be a common feature of exercise and refers to damage to body tissues through chemical reactions which occur during exercise. The purpose of this study is to investigate the effect of different intensities of exercise on oxidative stress.

Why have I been chosen? You have been selected as a possible participant in this investigation because you are aerobically trained and in good health. Before you become a subject, you will be medically screened by a trained physician and complete a medical questionnaire. People who have asthma, heart-related and/or circulatory problems, hypertension or any other contraindicated condition will not be allowed to take part in the study.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part? You will be asked to visit the laboratory typically on four separate days and on no more than seven separate days over a one-month period. Each visit will last no more than 2 hours.

On your first visit, you will be introduced to the laboratory personnel and familiarised with all the equipment used. You will be asked to complete two confidential questionnaires: the first will allow us to obtain information related to your general health; and the second will allow us to quantify your past exercise/activity involvement. You will also undergo a medical screening to ensure you are in good health to participate.
On your second visit you will be asked to perform what is called a “progressive exercise test” on a cycle ergometer. During this test, the work rate (a measure of how hard you are exercising) will start at a very low level and increase gradually every minute. The idea is that you keep going until you can do no more. The test will be stopped when you decide you can go no longer. You may be asked to repeat this test once more on a separate occasion.

On all other visits you will be asked to perform what is called a “constant workrate exercise test” on a cycle ergometer. During this test, the work rate (a measure of how hard you are exercising) will start at a very low level and then be increased to either a light level which you will be able to perform comfortably or a hard level which you will only be able to sustain for less than 30 minutes.

Ideally we will ask you to perform only three exercise tests: one progressive exercise test and two constant work rate tests. However, we may ask you to repeat any, or all, of these tests if we are unable to interpret the results. You will not be asked to repeat any test more than once.

In all tests we will ask you to breathe through a mouthpiece, similar to that used for snorkelling, and use a nose-clip. This is so we can monitor the air you breathe in and out. Normally your expired air (the air you breathe out) will be analysed to determine how hard your muscles are working.

We will take blood from you at various times during these tests. With the use of a surgical needle, a small plastic tube (called a catheter) will be inserted into a vein on the inside of your elbow. This is common practice and will cause only a mild “prick” of the skin. A small tap is connected to the inserted tube which allows repeated samples of blood to be taken without having to use a needle each time. Typically there is no discomfort associated with the presence of the catheter during the test. No more than 100 ml (about 10 dessert spoons) of blood is taken in any one test.

A small harmless device will be placed on your finger to measure the amount of oxygen in your blood. This is achieved by shining light into your finger and measuring how much is absorbed by the blood.

Finally, you will not be able to consume any alcohol 48 hours prior to each lab visit. You will be excluded from participating in this study if you take drugs (recreational or performance-enhancing drugs).

What are the side effects of taking part? There are none.

What are the possible disadvantages and risks of taking part? Exercise has a negligible risk in healthy adults, although maximal exercise does carry a small risk of inducing myocardial ischaemia (“heart attack”). The primary symptom of myocardial ischaemia is chest pain on exertion. If you experience any unusual sensations in your chest during the experiment, you should cease exercising immediately. Your heart rate will be monitored via adhesive electrodes placed at points on the chest (an "electrocardiogram" or ECG). In the unlikely event you experience serious problems during the exercise, medically-qualified personnel are on call at all times during the test and approved emergency procedures are in place.
At the end of the tests you will be very tired (exhausted), your legs will be very heavy and you will be out of breath. It is also not uncommon to feel a little light-headed and sometimes nauseous.

You may experience difficulty swallowing while breathing through a mouthpiece and wearing a nose-clip; this is due to a slight but transient pressure build up in your ears. Also, you may experience increased salivation while breathing through a mouthpiece. There are no specific risks associated with collection of breath samples.

You may experience mild discomfort during the placement of the catheter and/or from the sampling of blood from the catheter. When the catheter is removed, a little bruising may occur. A small wound may also be evident. This may take a few days to heal.

**What are the possible benefits of taking part?** The results from the exercise tests will give you a good idea of how fit you are. The research team will take the time to explain these results to you. The information from these tests will provide us with valuable information on the effect of different exercise intensities on oxidative stress.

**What if something goes wrong?** If you are harmed by taking part in this research project, there are no compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. The principal investigators, although not medically qualified, are fully trained in Basic Life Support. In the event of an untoward incident, the principal investigator(s) will provide basic life support including chest compressions and ventilation, and will apply an advisory defibrillator (if necessary) until emergency medical staff are on hand.

**Will my taking part in this study be kept confidential?** All information which is collected about you during the course of the research will be kept strictly confidential.

**What will happen to the results of the research study?** Results will be published in a peer-reviewed scientific journal once the study is completed. You will automatically be sent a copy of the full publication. You will not be identified in any publication.

If you are worried about any unwanted side effects from any of the above procedures, you should contact:

Dr Andy Cathcart  
Institute of Biomedical and Life Sciences,  
West Medical Building,  
University of Glasgow,  
Glasgow G12 8QQ  
Phone: 0141 330 3736  
Fax: 0141 330 2915  
E-mail: A.Cathcart@bio.gla.ac.uk
Title: A study of the dynamics of the oxidative stress response to aerobic exercise in different intensity domains (Approved: 24/11/05)

Consent Form

I ..................................................

give my consent to the research procedures which are outlined above, the aim, procedures and possible consequences of which have been outlined to me.

Signature ..................................

Date .....................................
Appendix A.3 Information sheet and consent form

The effect of varying the intensity of isometric exercise on oxidative stress

Institute of Biomedical and Life Sciences
University of Glasgow

INFORMATION SHEET

TITLE OF INVESTIGATION: The effect of varying the intensity of isometric exercise on oxidative stress

We invite you to participate in an investigation looking at the effect of varying the exercise intensity during handgrip exercise on oxidative stress. In order to help you to understand what the investigation is about, please read the following information carefully. Be sure you understand it before you formally agree to participate. If there are any points that need further explanation, please ask a member of the research team. It is important that you understand what you are volunteering to do and are completely happy with all the information before you sign this form.

What is the purpose of the study? Oxidative stress is thought to be a common feature of exercise and refers to damage to body tissues through chemical reactions which occur during exercise. This study aims to investigate the effect of different intensities of handgrip exercise on oxidative stress levels. We also aim to clarify, to some extent, the cause of oxidative stress during this type of exercise, which can be done by sampling blood during post-exercise recovery. In addition, oxidative stress during this type of exercise has traditionally been measured by analysing blood samples taken during exercise. We wish to investigate the utility of using breath measures of oxidative stress as a more pleasant alternative for future studies in this area.

Why have I been chosen? You have been selected as a possible participant in this investigation because you are in good health. Before you become a subject, you will complete a confidential medical questionnaire. People who have asthma, heart-related and/or circulatory problems, hypertension or any other contraindicated condition will not be allowed to take part in the study.

Do I have to take part? It is up to you to decide whether or not to take part. If you decide to take part, you will be given this information sheet to keep and you will be asked to sign a consent form. If you decide to take part, you are still free to withdraw at any time and without giving a reason.

What will happen to me if I take part? You will be asked to visit the laboratory on no more than three separate occasions, typically within a two-week period. All visits will take place in Lab 327 of the Kelvin Building and will take place at the same time of day whenever possible.
At the first visit, you will be introduced to the people who will carry out the tests and you will be familiarised with all equipment and procedures to be used. You will be given the opportunity to have any queries about the study answered and will be asked to sign a consent form. You will be asked to complete a confidential medical questionnaire to ensure that you are suitable as a participant for the study. You will then be asked to perform three to five maximal voluntary muscle contractions with your dominant hand using a handgrip dynamometer which is a device for measuring grip strength (similar to gripping the handle of a suitcase as hard as possible). This will determine your maximum grip strength for that hand. Five minutes rest will be given between each maximal contraction. This visit should last for no longer than one hour.

At your second and third visits, you will be asked to sustain a voluntary muscle contraction, using the handgrip dynamometer, for as long as possible (up to a maximum of 30 minutes) at four specified intensities. The intensities will be 20%, 40%, 60% and 80% of your previously measured maximum grip strength. Two of these trials will be performed at the second visit and two at the third visit. A recovery period of 30 minutes will be given after each contraction.

During each trial, we will ask you to breathe through a mouthpiece, similar to that used for snorkelling, and use a nose-clip. This is so we can monitor the air you breathe out. The air you breathe out will be analysed to detect ethane gas, which is a product of oxidative stress.

We will take blood from you at various times during these tests. With the use of a surgical needle, a small plastic tube (called a catheter) will be inserted into a vein on the inside of your elbow. This is common practice and will cause only a mild “prick” of the skin. A small tap is connected to the inserted tube which allows repeated samples of blood to be taken without having to use a needle each time. No more than 160 ml (about 16 dessert spoons) of blood is taken in any one test.

We will continue to monitor the air you breathe out and your blood during recovery periods. These visits should last for no longer than 3 hours each.

Finally, you will be asked to refrain from all strenuous exercise in the 24 hour period prior to each test and you will not be able to consume any alcohol 48 hours prior to each lab visit. You will be excluded from participating in this study if you take drugs (recreational or performance-enhancing drugs).

**What are the side effects of taking part?** There are none.

**What are the possible disadvantages and risks of taking part?** Isometric exercise has a negligible risk in healthy adults. You may experience mild discomfort during the placement of the catheter in a vein on the inside of the elbow and/or from the sampling of blood from the catheter. When the catheter is removed, a little bruising may occur. A small wound may also be evident. This may take a few days to heal.

**What are the possible benefits of taking part?** The information from these tests will provide us with valuable information on the effect of different exercise intensities on oxidative stress during isometric exercise but will provide no information of personal benefit other than your maximum grip strength.
**What if something goes wrong?** If you are harmed by taking part in this research project, there are no compensation arrangements. If you are harmed due to someone's negligence, then you may have grounds for a legal action but you may have to pay for it. The principal investigators, although not medically qualified, are fully trained in Basic Life Support. In the event of an untoward incident, the principal investigator(s) will provide basic life support including chest compressions and ventilation, and will apply an advisory defibrillator (if necessary) until emergency medical staff are on hand.

**Will my taking part in this study be kept confidential?** All information which is collected about you during the course of the research will be kept strictly confidential.

**What will happen to the results of the research study?** Results will be published in a peer-reviewed scientific journal once the study is completed. You will automatically be sent a copy of the full publication. You will not be identified in any publication.

If you are worried about any unwanted side effects from any of the above procedures, you should contact:

Dr Niall MacFarlane  
Institute of Biomedical and Life Sciences,  
West Medical Building,  
University of Glasgow,  
Glasgow. G12 8QQ  
Phone: 0141 330 5965  
Fax: 0141 330 4612  
E-mail: N.MacFarlane@bio.gla.ac.uk
Title: The effect of varying the intensity of isometric exercise on oxidative stress
(Approved: 24/05/05)

Consent Form

I .................................................................

give my consent to the research procedures which are outlined above, the aim,
procedures and possible consequences of which have been outlined to me.

Signature  .....................................................

Date  .............................................................
Appendix A.4  Medical Questionnaire

CENTRE FOR EXERCISE SCIENCE AND MEDICINE

MEDICAL HISTORY

(CONFIDENTIAL)

Please read.

It is important to take a record of your medical history. You may have, or may have once had a condition that would make this type of testing unsuitable for you. For this reason we ask you to be as truthful and detailed as possible. At no point will this information be made available to any one other than the principal investigators for this study. If you have any doubts or questions, please ask.
SUBJECT DETAILS:

NAME: 

AGE: D.O.B: 

SEX (M/F): 

GP NAME & ADDRESS: 

SMOKING: 

Never Smoked 
Not for >6 months 
Smoke <10 per day 
Smoke >10 per day 

ILLNESSES: 

ALLERGIES: 

HOSPITALISATIONS: 

MUSCULO-SKELETAL DISORDER: 
(Arthritis, Joint Pain, Fractures, Sports injury, Others) 

CARDIOVASCULAR DISORDER: (Fever, Heart Murmurs, Chest Pain, Palpitations, High Blood Pressure, Others) 

RESPIRATORY DISORDER: (Asthma, SOB, Cough, URTI, Others) 

GASTROINTESTINAL DISORDER: (Jaundice, Bleeding, Others) 

DIABETES: 

CNS DISORDER: (Fits, Blackouts, Tremor, Paralysis, Epilepsy, Other) 

PSYCHIATRIC TREATMENT: 

FAMILY HISTORY: (Sudden death in a first degree relative under the age of 35 years)
ARE YOU CURRENTLY TAKING ANY MEDICATION?    No / Yes*

(*Please specify)_________________________________________________________

ARE YOU CURRENTLY TAKING ANY SUBSTANCES TO HELP IMPROVE YOUR TRAINING OR CONTROL YOUR WEIGHT i.e. CREATINE, PROTEIN SUPPLEMENT?    No / Yes*

(*Please specify)_________________________________________________________

ARE YOU CURRENTLY TAKING ANY OTHER SUPPLEMENTS i.e. FOOD SUPPLEMENTS, VITAMINS?    No / Yes*

(*Please specify)_________________________________________________________

CAN YOU THINK OF ANY OTHER REASON WHY YOU SHOULD NOT TAKE PART IN ANY OF OUR TESTS?

________________________________________________________

SYMPTOMS:

Do you experience any of the following, particularly on exercise?

<table>
<thead>
<tr>
<th>Symptom</th>
<th>No / Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breathlessness</td>
<td></td>
</tr>
<tr>
<td>Chest Pain</td>
<td></td>
</tr>
<tr>
<td>Dizzy Fits/Fainting</td>
<td></td>
</tr>
<tr>
<td>Palpitations</td>
<td></td>
</tr>
</tbody>
</table>

Please note that if you feel unwell on the day of the proposed test, or have been feeling poorly over the preceding day or two, please inform the investigators and DO NOT TAKE PART in the exercise test.
DECLARATION:

I have completed this questionnaire fully and truthfully. I have not kept any information from the investigators that may put myself at risk during high-intensity exercise, or affect the results that they obtain. I understand that I may withdraw from any one test or the study as a whole if I feel unwell, or feel uncomfortable with any part of the testing procedure.

(Signature)……………………………………

(Date) ………………………

PHYSICAL EXAM:

WEIGHT:  __________  HEIGHT:  __________
PULSE (Resting):  __________  BP (Resting):  __________

Screened by: ……………………………

(Signature) ………………………………  (Date) ………………………..
If you feel unwell on the day of a proposed test, or have been feeling poorly within the last two weeks, you are excluded from taking part in an exercise test. The considerations that follow apply to people who have been feeling well for the preceding two weeks.

NAME: ………………………………………………………

SEX: M/F AGE: ……. (yr)

HEIGHT: ………. (m) WEIGHT: …….. (kg)

Details of last medical examination (where appropriate):

Date: …………………… Location: ……………………………………..
(day/mo/yr)

Exercise lifestyle:
What kind(s) of exercise do you regularly do (20 min or more per session), and how often?
(Please circle the number of times per average week):

Walking 1 2 3 4 5
Running 1 2 3 4 5
Cycling 1 2 3 4 5
Swimming 1 2 3 4 5
Skiing 1 2 3 4 5
Rowing 1 2 3 4 5
Gymnastics 1 2 3 4 5
Martial Arts 1 2 3 4 5
Tune Up 1 2 3 4 5
Popmobility 1 2 3 4 5
Sweat Session 1 2 3 4 5
Weight Training 1 2 3 4 5
Field Athletics 1 2 3 4 5
Racket sports 1 2 3 4 5
Rugby/soccer/hockey 1 2 3 4 5
Others*
* (Please specify) ………………………………………………………

How long have you been exercising at least twice/week for at least 20 min/session? …………... years
**Smoking:** *(Please tick one)*

- Never smoked ........
- Not for > 6 months ........
- Smoke < 10 per day ........
- Smoke > 10 per day ........

**Illnesses:** Have you ever had any of the following? *(Please circle NO or YES)*

<table>
<thead>
<tr>
<th>Illness</th>
<th>NO/YES</th>
<th>NO/YES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anaemia</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Heart Disease</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Other*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Asthma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Epilepsy</td>
<td></td>
<td></td>
</tr>
<tr>
<td>High Blood Pressure</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*(Please specify) .............................................................................................................*

**Symptoms:**
Have you ever had any of the following symptoms to a significant degree at rest or during exercise? That is, have you had to consult a physician relating to any of the following?

<table>
<thead>
<tr>
<th>Symptom</th>
<th>Rest</th>
<th>Exercise</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breathlessness</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Chest Pain</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Dizzy Fits/Fainting</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Heart Murmurs</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Palpitations</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Tightness in chest, jaw or arm</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
<tr>
<td>Other*</td>
<td>NO/YES</td>
<td>NO/YES</td>
</tr>
</tbody>
</table>

*(Please specify) .............................................................................................................*

**Muscle or joint injury:**
Do you have or have you had any muscle or joint injury which could affect your safety in performing exercise (e.g. cycling or running), strength testing or strength training?

* NO/YES*

*(Please specify) .............................................................................................................*

**Medication:**
Are you currently taking any medication?

* NO/YES*

*(Please specify) .............................................................................................................*

**Family History of Sudden Death:**
Is there a history of sudden death in people under 40 years in your family?  NO/YES*
The following exclusion and inclusion criteria will apply to this study:

**Exclusion criteria**
If you have any of the following, you will be excluded from the study:

(a) Asthma, diabetes, epilepsy, heart disease, a family history of sudden death at a young age, fainting bouts, high blood pressure, anaemia and muscle or joint injury.

(b) If you are taking any medication that may adversely affect your performance or health in this study, you will not be allowed to take part in the study.

(c) If you take recreational drugs, you will not be allowed to take part in the study.

(d) If you have ingested alcoholic drinks in the previous 48 hours, you will not be allowed to take part in the study.

**Inclusion criteria**

(a) Male or female subject, aged at least 18 years and normally no more than 35 years.

(b) In good health at the time of testing.

Signature …………………………………………………………… Date ………………