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Fat Metabolism in the Exercising Thoroughbred Horse

by

Catherine Elisabeth Orme BSc (Hons)

January 1995

*A thesis submitted in fulfilment for the degree of doctor of philosophy
with the Open University*

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Abstract

Fat Metabolism in the Exercising Thoroughbred Horse

The thoroughbred horse has been selectively bred for speed and has a high capacity for carbohydrate metabolism. The following series of studies investigated the relative contribution of fat and carbohydrate to energy production during exercise of varying intensity. Furthermore the work assessed the capacity of the horse to increase the contribution of fat to energy production as the result of either an acute increase in the availability of plasma free fatty acids (FFA) or as the result of chronic fat supplementation. Finally an adaptational response to feeding a fat supplemented diet was described.

The variation in plasma long chain FFA over a 24 hour period was described. The early hours of the morning represented the period of greatest variability in plasma FFA concentration. This period was characterised by a significant increase in total and individual FFA concentration, which was unrelated to feed intake. As a result of the reported circadian rhythm in plasma FFA all subsequent exercise studies were performed during the period of least variability in plasma FFA concentration.

A model for the pre-exercise elevation of plasma FFA, using a combination of a triglyceride emulsion and the heparinoid type substance pentosan polysulphate, was used to investigate the effect of increased FFA availability on fat utilisation during prolonged low intensity exercise. Pentosan polysulphate was used in preference to heparin following an investigation of their relative lipolytic and anticoagulative properties. Pentosan polysulphate when administered at 3 times the dose of heparin resulted in a comparable increase in plasma total lipase activity. When co-administered with a triglyceride emulsion, pentosan polysulphate resulted in a similar increase in plasma FFA concentration relative to that produced with the same triglyceride emulsion and heparin. The anticoagulative effect of pentosan polysulphate, however, was approximately 9 times less than that of heparin, as measured by activated partial thromboplastin time.

The contribution of fat and carbohydrate to energy production during exercise was influenced by both the intensity and duration of exercise, as indicated by measurements of respiratory exchange ratio (RER). The inter-horse variability in RER was greatest during low intensity exercise. An increase in the contribution of carbohydrate to energy production occurred at the onset and during the early stages of prolonged exercise and as the result of an increase in exercise intensity. A proportion of horses exhibited an increase in the utilisation of fat during low intensity prolonged exercise as a result of a pre-exercise elevation in plasma FFA

concentration. RER was consistently lower during exercise in 5 out of the 7 horses studied following a pre-exercise elevation of plasma FFA. Furthermore, plasma glucose concentration was elevated above that observed during the control session in 4 of these 5 horses for at least the first 15 minutes of exercise.

A prolonged period of fat supplementation resulted in an improved management of the fat load. Following 10 weeks of dietary treatment a significant increase in plasma cholesterol concentration and a significant decrease in plasma triglyceride concentration was reported. The decrease in plasma triglyceride concentration was associated with a mean 50% increase in post pentosan polysulphate plasma total lipase activity. It is suggested that the increase in the post pentosan polysulphate plasma total lipase activity may have reflected an increase in muscle lipoprotein lipase activity. A significant increase in the activity of muscle citrate synthase was observed during the period of fat supplementation. No significant change occurred in muscle β -hydroxyacyl CoA dehydrogenase activity or in the concentration of resting muscle glycogen and triglyceride as a result of fat supplementation. RER was significantly lower in the latter stages of prolonged low intensity exercise, during the period of fat supplementation, relative to the same exercise performed before the introduction and following 5 weeks of withdrawal of the fat supplemented diet. The reduction in RER during the period of fat supplementation was associated with a greater exercise induced increase in plasma FFA concentration. The above differences were also apparent during moderate intensity exercise, although, examination of the individual horse data revealed that the effect was not as clear as that observed during low intensity exercise. No significant differences were reported in either RER or plasma FFA concentration in response to moderate/high intensity exercise during the period of fat supplementation. Neither were any significant differences observed in either RER or plasma FFA concentration in the control group at any exercise intensity. An increased availability of plasma FFA and an increase in the oxidative capacity of muscle, as well as an enhanced ability to utilise plasma triglycerides may have contributed to the increase in fat utilisation, observed during low and moderate intensity exercise, in response to fat supplementation. The effect of differences in the hormonal response to a fat supplemented diet as a precipitant of the observed adaptational responses in these studies requires further investigation.

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Chapter 1

Introduction and Literature Review

The modern horse, *Equus Caballus*, has evolved as a natural athlete. Its indigenous qualities of strength, power and speed, coupled with its grace and agility, lend it to excel at many athletic activities, including: racing on the flat and over obstacles, show jumping, 3-day eventing, endurance racing, polo, dressage, carriage driving and trotting and pacing. Originally a wild grazing animal, Man's domestication of the horse began in Neolithic times. The earliest use of horses for riding was by the Nomads of central Asia. Contact between the horse-riding Nomads and the Sumarians, the developers of the ox cart, probably resulted in the use of horses for driving. This was the beginning of the chariot driving era characteristic of the classical civilisations of Mediterranean Europe. The use of the ridden horse progressed much later firstly for hunting and then for its use in battle.

Some of the earliest records of the use of horses in sporting activities were in races dating back as far as the second millennium BC. Chariot racing was popular with the Greeks and Romans and was introduced into the Greek Olympic games in 648 BC. Modern flat-racing originated in England during the seventeenth century. During the reign of Henry VIII, races were held at Chester and James I established Newmarket as a racing capital by building the first permanent racecourse there. Today, horses compete in a range of sporting activities that vary considerably in terms of both intensity and duration of exercise as well as the amount of skill and aptitude required.

Equine Sporting Activities

Flat-racing, hurdling and steeple chasing

Flat-racing represents high intensity exercise of short duration. Races are run over distances in Britain ranging from 5 furlongs or 1000 m to 21 furlongs or 4200 m. The shortest races are run in America over 1/4 mile or 400 m by the American quarter horse. Races are run at maximal or near maximal speed, up to 17-20 m/s (Table 1.1). As the distance of the race increases, however, there is a greater reliance on tactical advantage and races are not run "against the clock".

Table 1.1 Peak heart rate, estimated oxygen uptake (VO_2) and estimated relative work intensity ($\% \text{VO}_{2 \text{ max}}$) in horses competing in a range of sporting activities. Estimated VO_2 was calculated using the equation relating heart rate to VO_2 as described by Evans and Rose (1987). A theoretical $\text{VO}_{2 \text{ max}}$ of 150 ml/kg/min was used in the calculation of $\% \text{VO}_{2 \text{ max}}$.

Activity	Exercise Intensity	Peak Heart Rate beats/min	Approximate speed (m/s)	VO ₂ ml/kg/min	% VO ₂ max
¹ Flat racing (1600 - 1800 m)	High	223	15.5	134	89
² Trotting race	High	226	-----	138	92
³ Cross country phase (3-day event)	Moderate / High	200	9.0	105	70
⁴ Polo (low goal)	High	215	13.0	124	83
⁵ Show jumping	Moderate	189	-----	92	63
⁶ Endurance racing	Low / Moderate	100 - 150	4.2	43	40
⁷ Vaulting	Low	113	-----	<40	<27

¹(Krzywanek *et al* 1970)

⁴(Allen 1993)

⁷(Oldruitenborgh-Oosterbaan *et al* 1991)

²(Asheim *et al* 1970)

⁵(Lekeux *et al* 1991)

³(Amory *et al* 1993)

⁶(Pollitt 1992)

Hurdling and steeplechase races are generally run over longer distances than flat-races, typically between 3-7 km. The speed at which the races are run is generally slower than that of flat-races. The introduction of fences, however, increases the demands on the equine athlete. Steeplechase fences tend to be much larger than those of hurdle races and the race distance are up to 7.5 km. Probably the most well known and most testing steeplechase, the Grand National, is run over the latter distance and includes fences of 1.5 m (5 ft) high often with large ditches on either side.

Show jumping

During a typical show jumping competition horses exercise for about 2 minutes, jumping 10-12 fences. Although the horse is not travelling at a great speed it requires agility and power to clear the fences which may be up to 1.6 m (5 ft 3 in) in height. The addition of fences therefore increases the intensity of this type of exercise substantially, (Table 1.1).

Dressage

Dressage is possibly the most aesthetically pleasing of all the equine events but represents a relatively low intensity of exercise. The horse and rider perform an exercise test lasting approximately 5-10 minutes, which includes performance of 3 of the 4 basic paces (walk, trot and canter) at different levels of collection*. The exercise intensity of a dressage test is comparable to that of vaulting, (Table 1.1). Although the duration of the actual dressage test is short and the intensity of exercise relatively low, the discipline requires a high level of basic fitness. Competitors may undergo several hours of warm-up exercise prior to the competition.

* *Collection refers to the subdivision of the basic paces (walk, trot and canter) with respect to speed.*

Horse trials or 3-day eventing

Three day eventing probably represents the greatest challenge to both horse and rider alike. Horses perform a multi-phase test of speed, endurance, power and versatility combining dressage show jumping, steeple chasing and a course of often very demanding cross-country fences. The dressage, and show jumping phases are held on the first and last day, respectively. The second day includes four phases A, B, C, and D which are undertaken immediately following each other. There is a 10 minute rest period between phases C and D. Phases A and C are classified as 'roads and track phases' and consist of 16-20 km of trotting or slow cantering. Phase B is a steeplechase course of 9 or 10 fences over 3-3.5 km which must be covered at a speed of about 11.5 m/s. Finally phase D is a cross-country jumping course of up to 8 km which must be completed within 13-14 minutes at an average speed of about 9.5 m/s. The intensity of this multiphase event varies considerably from the dressage to the steeple chase and cross country disciplines, (Table 1.1).

Endurance racing

Endurance racing is a relatively new equestrian sport that is rapidly gaining popularity. Typical races are held over distances of 35-170 km, at speeds of up to 5-6 m/s. The duration of a 170 km race is on average just over 8 hours. Although the major part of the race is carried out at comparatively low speeds the terrain has a great bearing on the intensity of the exercise, (Table 1.1).

Polo

Polo is a fast and explosive sport that resembles hockey on horseback. It involves constant changes in exercise intensity as hard galloping is interrupted by stopping and turning with constant changes in speed. Each match is divided into periods called chukkas of which at the highest level there are 6. Each chukka is 7 1/2 minutes long and is interspersed by 3 minute rest intervals. Each player changes pony every chukka, since the game is very physically demanding (Table 1.1). It is permissible for a pony to play two chukkas per match provided that they are not consecutive.

Trotting and pacing

Trotting and pacing races are typically run over 1600-3200 m, at near maximal to maximal intensity (Table 1.1). These races are very popular in America and throughout Europe but not so popular in Britain.

There is a large variation in the intensity and duration of equine performance events. This will effect fuel selection and the predominance or contribution of the different energy generating pathways to energy production during exercise. The following review briefly describes the major energy generating pathways, with particular reference to the use of fat as a fuel source. It also discusses the factors that influence the utilisation of fat during exercise and its integration with carbohydrate metabolism. Finally, the published literature investigating the inclusion of fat into the equine diet and its effect on exercise metabolism is reviewed.

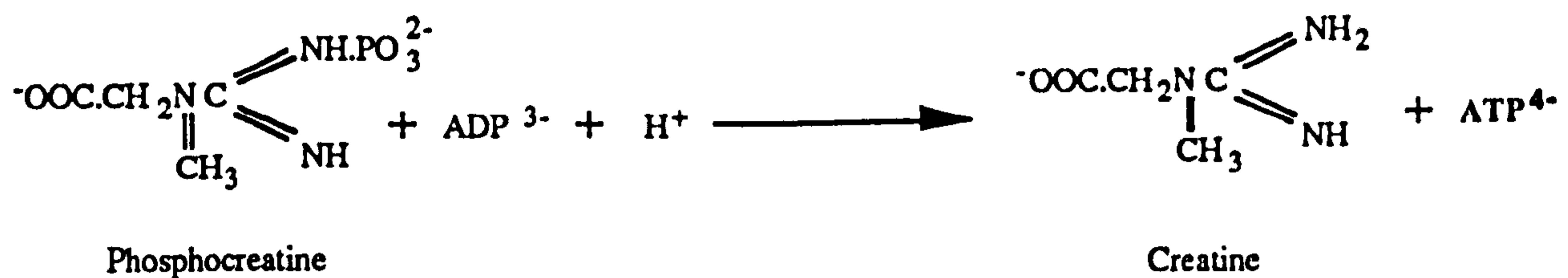
Fuel Utilisation

Muscular contraction requires adenosine triphosphate (ATP) which is ultimately produced from a number of fuel sources. The main fuel sources available for energy production by the horse, both at rest and during exercise, are carbohydrate in the form of muscle glycogen or blood glucose, fat in the form of muscle or plasma triglyceride, plasma free fatty acids (FFA) or ketones and muscle stores of ATP and phosphocreatine (PCr). Fat is by far the largest energy store in the horse, as in man. A typical 500 Kg horse has about 640 000 KJ of energy stored in the form of triglyceride, 75 300 KJ stored in the form of glycogen, 188 KJ stored in the form of PCr and 38 KJ of endogenous muscle ATP (McMiken 1983).

Phosphocreatine shuttle

PCr provides a reservoir of high-potential phosphoryl groups. The conversion of PCr to creatine involves the transfer of a phosphate group from the former to adenosine diphosphate (ADP), with the resultant formation of ATP. Generation of ATP by the PCr pathway occurs

more rapidly than by any other means and hence affords the greatest power output (Sahlin 1985). The stores of PCr, however, are small and can sustain exercise in Man for 10-20 seconds only (Miller *et al* 1988). PCr serves to prevent large decreases in muscle ATP concentration during periods of energy stress, e.g. prior to the stimulation of glycolysis and the tricarboxylic acid cycle (TCA). PCr is important at the onset of exercise or during exercise where the demand for energy is exceeded by that which can be produced by the anaerobic lactic acid pathway.



Aerobic and anaerobic carbohydrate utilisation

The thoroughbred horse has considerably larger muscle and liver glycogen stores in comparison to Man (Table 1.2) and hence has a greater potential for carbohydrate utilisation. Complete oxidation of carbohydrate occurs as a result of anaerobic glycolysis (Fig 1.1) and oxidation via the TCA cycle (Fig 1.2). The maximum rate of ATP production or power output by this pathway is, however, low (Table 1.3). A greater power output is achieved through the lactic acid pathway. During sprinting the thoroughbred horse produces and accumulates a large amount of lactic acid in skeletal muscle (Harris *et al* 1991). The greater capacity for proton buffering exhibited by the thoroughbred horse in contrast to man (Harris *et al* 1990) may be attributed to the high carnosine content of skeletal muscle in the former (Marlin *et al* 1989; Sewell *et al* 1991).

Table 1.2 Resting concentrations of carbohydrate and lipid fuel sources in Man and the horse.

Metabolite	Horse	Man
Liver Glycogen (mmol/kg)	*1606 ¹	†270 ²
Muscle Glycogen (mmol/kg dw)	550 - 600 ^{3,4}	300 - 400 ^{5,6}
Plasma Glucose mmol/l	4.47, 5.8 ⁸	5.0 ⁹
Plasma Free Fatty Acids $\mu\text{mol/l}$	47 ⁸ , 120 - 350 ^{7,10,11,12}	300 ⁹
% Body Fat	1.1 - 2.0 ¹³	5 - 15 ^{14,15}
Muscle Triglyceride (mmol/kg dw)	36 - 74 ^{16,17}	24 - 68 ^{18,19,20}
Adipose Triglyceride (g)	40000 ²¹	7800 ²² , 9000 ²³

* dry weight † wet weight

(Pagan *et al* 1987)¹
(Snow and Harris 1991)³
(Harris *et al* 1974)⁵
(Lucke and Hall, 1980a)⁷
(Newsholme and Leech 1983)⁹
(Lucke and Hall 1980b)¹¹
(Gunn 1987)¹³
(Mackova *et al* 1986)¹⁵
(Essen-Gustavsson *et al* 1991)¹⁷
(Gorski 1990)¹⁹
(Snow and Vogel 1987)²¹
(Davidson *et al* 1979)²³

(Nilsson 1973)²
(Snow *et al* 1985)⁴
(Hultman 1976)⁶
(Rose 1982)⁸
(Lucke and Hall, 1978)¹⁰
(Snow *et al* 1982)¹²
(Wilmore *et al* 1977)¹⁴
(Essen-Gustavsson *et al* 1984)¹⁶
(Hurley *et al* 1986)¹⁸
(Essen-Gustavsson and Tesch 1990)²⁰
(Wilmore and Costill 1994)²²

Carbohydrate in the form of plasma glucose or muscle glycogen is initially converted to glucose-6-phosphate (G6P) which then enters the glycolytic pathway (Fig 1.1). Muscle glycogen is cleaved to produce mainly glucose-1-phosphate (G1P) molecules by the active form of *glycogen phosphorylase (a)*. *Phosphoglucomutase* then converts G1P to G6P. A small number of the glucose residues in glycogen are released as glucose by *amylase*, *6-glucosidase*. Liver glycogenolysis is important for the supply of glucose to extra-hepatic tissue and plays a major role in the maintenance of blood glucose concentration during exercise. The rate of liver glycogenolysis is related to exercise intensity (Wahren *et al* 1971; Ahlborg *et al* 1974; Hultman 1976; Ahlborg and Felig 1982). As a result of strenuous exercise up to 35-40% of the liver glycogen stores are mobilised (Bjorkman and Wahren 1988)

Anaerobic glycolysis occurs in the cell cytosol producing pyruvate and potentially a net yield of 2 or 3 moles of ATP, per mole of glucose or G1P respectively (Hultman and Harris 1988). The fate of pyruvate is two fold and depends on the energy requirement of the cell and the local availability of oxygen. Pyruvate may firstly be converted to acetyl CoA by the action of the *pyruvate dehydrogenase* enzyme complex. It is then completely oxidised to carbon dioxide and water via the TCA cycle, (Fig 1.2). Alternatively pyruvate may undergo conversion to lactic acid anaerobically.

LACTATE DEHYDROGENASE



Complete oxidation of one mole of glucose results in the formation of 38 moles of ATP. Conversely the conversion to lactic acid produces only 2 moles of ATP, as reviewed by Hultman and Harris (1988). Therefore although an increased power output is achieved by conversion to lactic acid (Sahlin 1985) it occurs at the expense of ATP production.

Table 1.3 **The available energy and its rate of production from the different energy generating pathways in Man (Sahlin 1985).**

Energy Generating Pathway	Available Energy (Mol ATP)	Maximum Power (mmol ATP/kg dry muscle/sec)	Time to Reach Maximum Power
Anaerobic Processes			
ATP	0.02	11.2	<1 sec
PCr	0.34	8.6	<1 sec
CHO → Lactate	0.7 to 5.2	5.2	<5 sec
Aerobic Processes			
CHO → CO ₂ + H ₂ O	70	2.7	3 min
FFA → CO ₂ + H ₂ O	8000	1.4	30 min

Fig 1.1 **A summary of the main pathways of anaerobic glycolysis - adapted from Stryer (1981)**

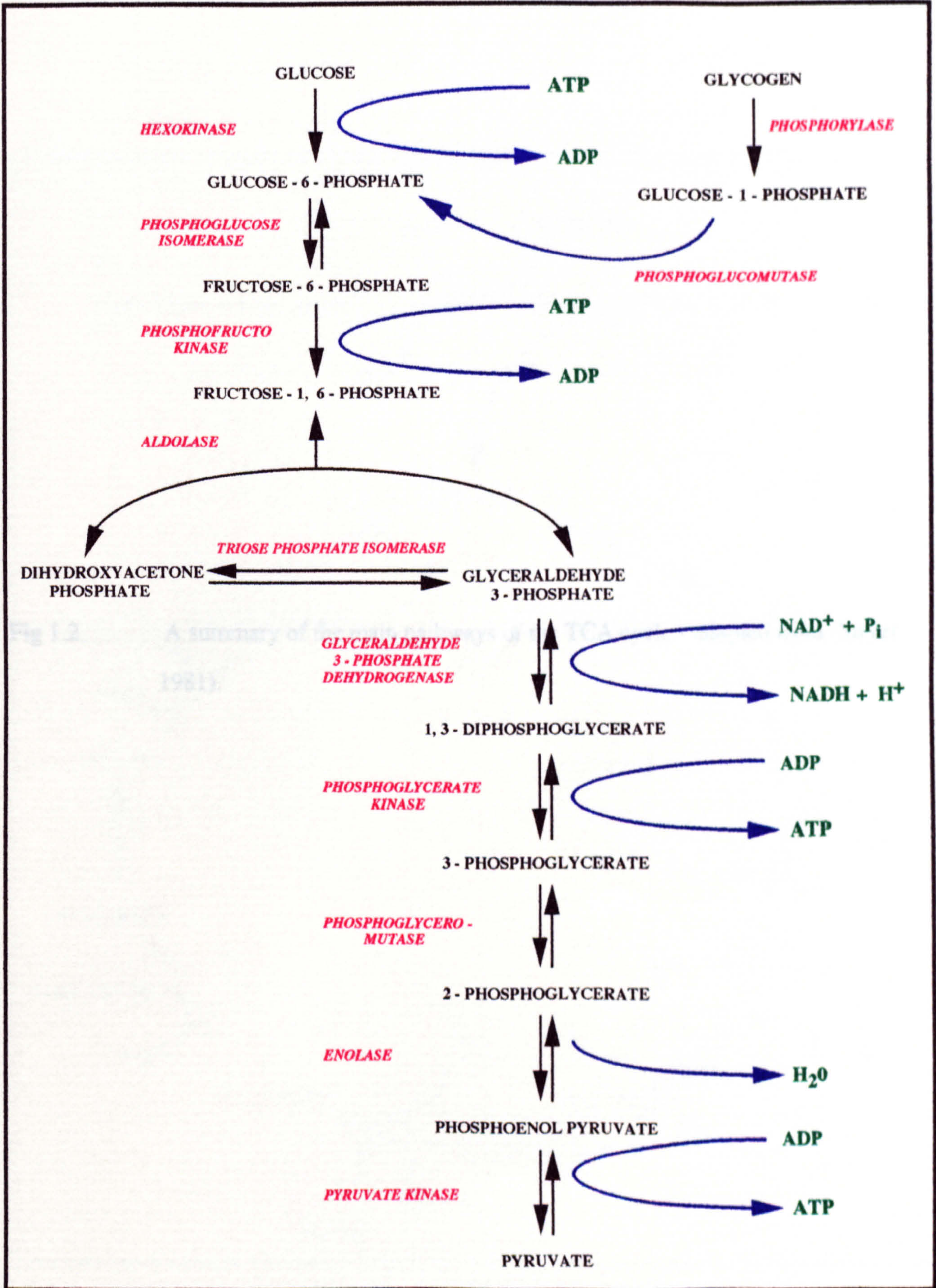
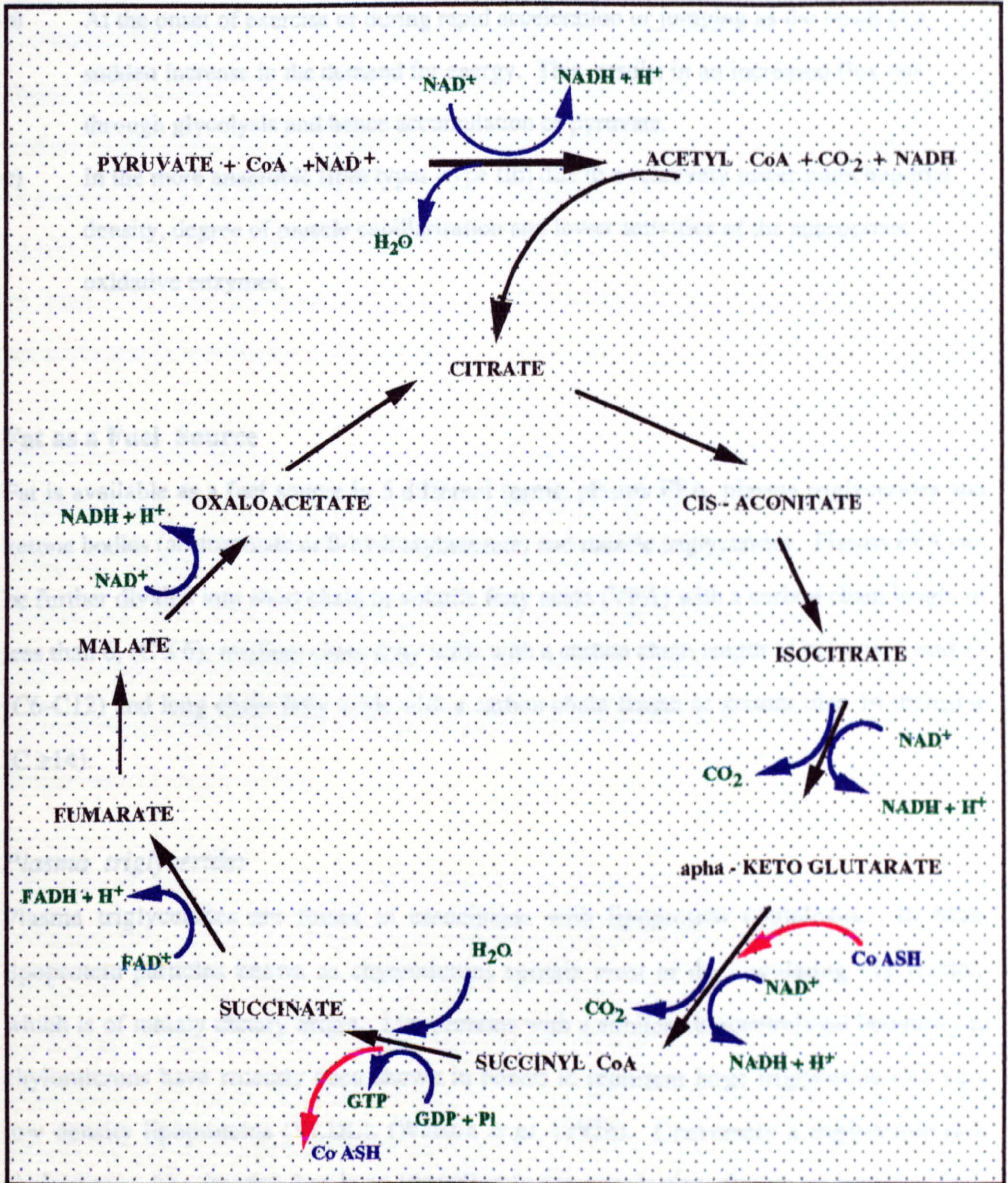


Fig 1.2 **A summary of the main pathways of the TCA cycle - adapted from (Stryer 1981).**

At least two lactic acid production occurs in the following situations:

- a) When the rate of demand for ATP exceeds the rate of ATP production by aerobic metabolism causing a shift in the intracellular redox potential.



(Warren et al. 1991). The absence of malic enzymes upregulates lactate dehydrogenase as a reflection of more anaerobic metabolism. In addition, the absence of malic enzymes is associated with a shift in the intracellular redox potential (Warren 1991). In adult horses the major intracellular coenzyme upregulates lactate dehydrogenase.

Accelerated lactic acid production occurs in the following situations:

- a) When the rate or demand for ATP exceeds the rate of ATP production by aerobic metabolism causing a shift in the intracellular redox potential.
- b) At the onset of exercise or during rapid acceleration or jumping as the result of a sudden increase in the demand for energy. This results in an increased flux rate through glycolysis and hence accumulation of pyruvate.
- c) In the more anaerobic fibre types (type IIb) due to a relatively lower mitochondrial density, degree of muscle capillarisation and lower activities of the appropriate oxidative enzymes.

Fat as a Fuel Source

Fat is available as a fuel source in 5 different forms: plasma FFA, plasma triglycerides and ketone bodies (acetoacetate or β -hydroxybutyrate) and muscle triglycerides. Plasma FFA can be further divided into short chain or volatile fatty acids (VFA) with a carbon chain length of less than 6, ($C \leq 6$); medium chain fatty acids with a carbon chain length of between 6 and 12 (C_6 - C_{12}) and long chain fatty acids with a carbon chain length of greater than 14 inclusive ($C \geq 14$).

Plasma triglycerides

Plasma triglycerides are found in association with lipoprotein complexes. In Man, lipoprotein particles known as chylomicrons contain most of the circulating triglyceride, which is of mainly dietary origin. Lipoproteins with a similar ultracentrifugal buoyancy to chylomicrons have recently been shown in horses to represent large triglyceride-rich very low density lipoproteins (VLDL), (Watson *et al* 1992b). Conversely a triglyceride-rich chylomicron like fraction has been identified in the non-fasting plasma of suckling foals (Watson *et al* 1991). The absence of a chylomicron lipoprotein fraction in adult horses may be a reflection of their diet, which is normally low in fat and lacking in long chain fatty acids (Watson 1991). In adult horses the major triglyceride containing lipoprotein fraction is

VLDL, followed by low density lipoproteins (LDL) and high density lipoproteins (HDL), (Watson *et al* 1993b).

Plasma triglycerides are not taken up directly by tissues but are hydrolysed first to their constituent FFA and glycerol by the enzyme *lipoprotein lipase* (LPL), (Cryer 1987). LPL is found in association with sulphated glycosaminoglycans. They are present on the luminal surface of the blood vessels of extra-hepatic tissue, predominantly adipose tissue and skeletal or cardiac muscle (Robinson 1987). After the administration of heparin (HEP), the activity of LPL assessed by its release into the plasma, is lower in ponies (3.65 $\mu\text{mol FFA/ml/hr}$) than in Man (7.81 $\mu\text{mol FFA/ml/hr}$), (Chan *et al* 1984; Watson *et al* 1992a). This may reflect the traditionally low fat diet of the former.

FFA entering the cell may be completely oxidised via β -oxidation (Fig 1.3) and the TCA pathway or may be re-assimilated into triglyceride. FFA re-esterification does not represent a complete reversal of lipolysis, since glycerol cannot be re-utilised within the adipocyte (Dixon and Webb 1979). The inability to re-utilise glycerol in extra hepatic tissue is due to low activities or absence of the enzyme glycerol kinase (Newsholme and Taylor 1969). The co-substrate for triglyceride resynthesis is glycerol-3-phosphate which is mainly derived from dihydroxyacetone phosphate.

The role of plasma triglyceride as a fuel source during exercise is unresolved. Most estimates, however, suggest that it represents a minor fraction of the total substrate oxidised. Olsson *et al* (1975) were unable to detect the uptake of plasma triglycerides, by the exercising forearm in Man, using either arterio-venous differences or radio labelled triglyceride techniques. Similarly, it has been demonstrated that the rate of removal of an infused exogenous triglyceride emulsion in the horse is not affected by low intensity exercise (Moser *et al* 1989). Conversely, an exercise induced reduction in plasma chylomicron concentration and a simultaneous increase in their uptake by skeletal muscle has been reported in dogs (Jones and Havel 1967; Mackie *et al* 1980). Although the contribution of

plasma triglycerides as a fuel source may be limited during exercise it has been proposed that they may be important for the post-exercise repletion of fat depots in muscle and adipose cells (Mackie *et al* 1980).

Adipose tissue triglycerides

Adipose tissue triglyceride represent the largest potential source of energy in the body, in the form of FFA. A typical 500 kg horse may store approximately 40 000g or 1 529 MJ (372 Mcal) in the form of adipose tissue triglycerides (Snow and Vogel 1987). Adipose tissue lipolysis and FFA mobilisation, occur in the horse as in Man, in situations of stress (Anderson and Aitken 1977) and sustained exercise or starvation (Rose 1982). Lipolysis is under hormonal control and is inextricably linked to carbohydrate metabolism. Triacylglycerol lipase or hormone sensitive lipase (HSL) is the enzyme responsible for the breakdown of triglycerides to diglycerides and then to monoglycerides, with the net release of a fatty acid. A specific monoglyceride lipase then acts on monoglycerides to produce further FFA and glycerol. As the name suggests, triacylglycerol lipase is under the influence of a number of hormones including the catecholamines, adrenaline and nor-adrenaline, cortisol, growth hormone (somatostrophin), the thyroid hormones and insulin (Hales *et al* 1978).

Muscle triglycerides

The amount of triglyceride stored in skeletal muscle varies between species, (Table 1.2). The triglyceride content of human muscle is comparable to that found in dogs but higher than that found in rats and monkeys (Gorski 1990). Concentrations of between 24-68 mmol/kg dry weight (dw) have been reported in human muscle (Hurley *et al* 1986; Essen-Gustavsson and Tesch 1990; Gorski 1990). The concentration of triglyceride found in equine muscle is variable. Concentrations of as low as 9 and as high as 138 mmol/kg dw have been reported (Essen-Gustavsson *et al* 1991), (Table 1.2). The triglyceride content of heart muscle is present in three different forms, as interstitial adipocytes or free floating cytosolic lipid droplets, lipid filled vacuoles and membrane bound particles. Skeletal muscle also contains

lipid droplets (Oscai *et al* 1990). The measurement of muscle triglyceride is notoriously variable, due to difficulties in isolation of 'true' endogenous muscle triglyceride as opposed to inter-fibre fat droplets. The removal of phospholipids is also essential (Frayn and Maycock 1980). Furthermore, direct measurement of pooled individual muscle fibres and histological data shows that triglyceride is not evenly distributed between the different fibre types, but is found mainly in type I fibres followed by types IIa and IIb fibres (Essen *et al* 1975; Essen 1977). The muscle triglyceride concentration will therefore reflect the fibre composition of the biopsy specimen.

The role of muscle triglycerides as a fuel source during exercise is not unequivocal. Early calculations using measurements of respiratory quotient (RQ) suggested that plasma FFA could not solely account for the total contribution of fat to energy expenditure during prolonged exercise (Havel *et al* 1967; Issekutz and Paul 1968). Furthermore, studies employing electron microscopy reported reductions in the size of fat vacuoles in muscle suggesting that muscle triglycerides were utilised during exercise (Oberholzer *et al* 1976). Direct determination of muscle triglyceride has confirmed their role during exercise, although their relative contribution remains essentially unresolved. Human studies using mixed muscle biopsy samples have revealed reductions in muscle triglyceride concentration of 36, 41 and 25% in response to relative exercise intensities of 55, 64 and 67% of $\text{VO}_2 \text{max}$, respectively (Carlson *et al* 1971; Essen 1977; Hurley *et al* 1986). Gorski, (1990) proposed that muscle triglycerides may be more important during moderate as opposed to low intensity exercise. Gorski, (1990) suggests that a failure to show a reduction in muscle triglyceride concentration in some studies may be due to the exercise being of low intensity, or the muscle sampled having a high percentage of type IIb fibres.

Another approach used in the quantitation of muscle triglyceride utilisation is to assess the rate of glycerol release from contracting muscle. Assuming that the utilisation of plasma triglyceride is small, it has been estimated using this technique that the contribution of muscle triglyceride to overall energy production, during prolonged exercise of 1-2 hours, is

only about 5 or at the most 10% (Saltin *et al* 1986; Kiens *et al* 1993). Muscle triglyceride may provide an important fuel source during exercise recovery when incoming glucose is channelled into glycogen resynthesis.

Ketones

Ketone bodies (acetoacetate and β -hydroxybutyrate) are formed in the liver during degradation of FFA, whence they are released into the circulation. The concentration in plasma is normally low but may increase in situations of increased FFA delivery to the liver, e.g. during fasting. Although they probably represent an important fuel source in the fasting state, their use during exercise is estimated to be very small (Rennie *et al* 1976; Hagenfeldt 1979).

Short chain or volatile fatty acids

The horse, although classified as a non-ruminant has the capacity for hind gut fermentation of structural carbohydrates such as cellulose and hemi-cellulose (Bergman 1990). The caecum and colon serve as chambers for microbial action with the production of mainly acetic, propionic and butyric acids, which represent a further potential energy source (Tappeiner 1884). Gluconeogenesis from propionate may account for up to 60% of available blood glucose in horses maintained on a mainly roughage diet (Simmons and Ford 1991). Acetate may contribute approximately 32% to hind limb oxidation at rest, in the thoroughbred horse maintained on a 100% roughage diet or 21% when maintained on a 52% oat and grain diet, respectively (Pethick *et al* 1993).

Medium chain fatty acids

Medium chain fatty acids (MCFA) are more water soluble than long chain fatty acids and as a result are absorbed much more quickly from the small intestine. Unlike long chain fatty acids they are not re-esterified in the intestinal epithelial cells. MCFA are absorbed intact and are transported from the intestine to the liver, via the portal circulation, in association with albumin (Isselbacher 1968). Oxidation of MCFA occurs with or without the

involvement of the carnitine dependant mitochondrial transport system. MCFA have the ability to traverse the mitochondrial membrane, activation occurring in the mitochondrial matrix. Medium chain containing triglyceride emulsions are more rapidly and completely oxidised in comparison to those containing long chain triglycerides (Johnson *et al* 1990; Metges and Wolfram 1991).

Plasma long chain free fatty acids

Plasma long chain fatty acids ($C \geq 14$), referred to as FFA, make the largest contribution to energy production during exercise of any of the available lipid fuel sources. Oxidation of FFA may provide up to 90% of the total energy demand in the latter stages of prolonged exercise in man (Bulow 1988). Plasma FFA concentration reflects adipose tissue lipolysis and FFA release, as well as uptake by peripheral tissues. The resting concentration of plasma FFA reported in the horse is variable, (Table 1.2). Concentrations of as low as 47 $\mu\text{mol/l}$ have been reported (Rose 1982), although they are generally much higher, ranging between 120-350 $\mu\text{mol/l}$ (Lucke and Hall 1978; Lucke and Hall 1980a; Lucke and Hall 1980b; Snow *et al* 1982). These variations may reflect differences in the feeding state and anxiety level of the horses when sampled as well as the method of analysis. At the onset of sub-maximal exercise there is often a characteristic fall in plasma FFA concentration, prior to a steady increase as exercise continues (Ahlborg *et al* 1974; Hagenfeldt 1979). This is generally assumed to be the result of a delay in adipose tissue lipolysis coupled with an increase in skeletal muscle blood flow enabling increased extraction of FFA by muscle.

Uptake of free fatty acids by muscle

FFA are found in the plasma in association with albumin which has a finite number of binding sites and facilitates their transport to their sites of oxidation or storage (Ashbrook *et al* 1975). On arrival at peripheral tissue, e.g. skeletal muscle, FFA molecules are released from the albumin complex and taken up by muscle cells. The mechanism for this process is presently unclear. A close relationship exists between the plasma FFA concentration and the amount oxidised. The uptake of plasma FFA by tissue cells is dependent on the FFA

concentration of the intracellular compared to the extracellular fluid and hence the arterial FFA concentration (Hagenfeldt 1979). It is the 'free' FFA concentration, i.e. that which has dissociated from the albumin complex which affects their cellular uptake. Hence the relative affinity of albumin and the plasma membrane binding sites for FFA probably also affects the rate of FFA uptake (Noy *et al* 1986).

The FFA flux through the plasma membrane has previously been described as a passive concentration-dependent process, uptake being facilitated by simple diffusion or partitioning with the lipid bilayer of the plasma membrane (DeGrella and Light 1980; Noy *et al* 1986). Noy *et al* suggested that a physico-chemical partitioning of FFA between albumin and binding sites or binding proteins in the plasma membrane may exert a certain regulatory control to prevent the uptake of FFA exceeding the oxidative capability of the cell. A specific fatty acid binding protein (mw 40 000) has been isolated in skeletal muscle (FABP_m), localised both in the plasma membrane and the cytosol (Said and Schultz 1985). Expression of FABP_m is correlated to muscle oxidative capacity and is greater in red compared to white skeletal muscle (Carey *et al* 1994; Veerkamp and Van-Moerkerk 1994). It has recently been demonstrated that FFA uptake by muscle follows saturation kinetics which suggests that a transport mechanism is involved (Turcotte *et al* 1991). Furthermore, it has been suggested that FABP_m may be involved in the regulation of FFA utilisation by muscle. The expression of FABP_m is increased in the more oxidative muscles of rats in response to fasting, when FFA utilisation is increased (Turcotte *et al* 1994). The expression of FABP_m may therefore provide a link between FFA uptake by muscle cells and the plasma concentration.

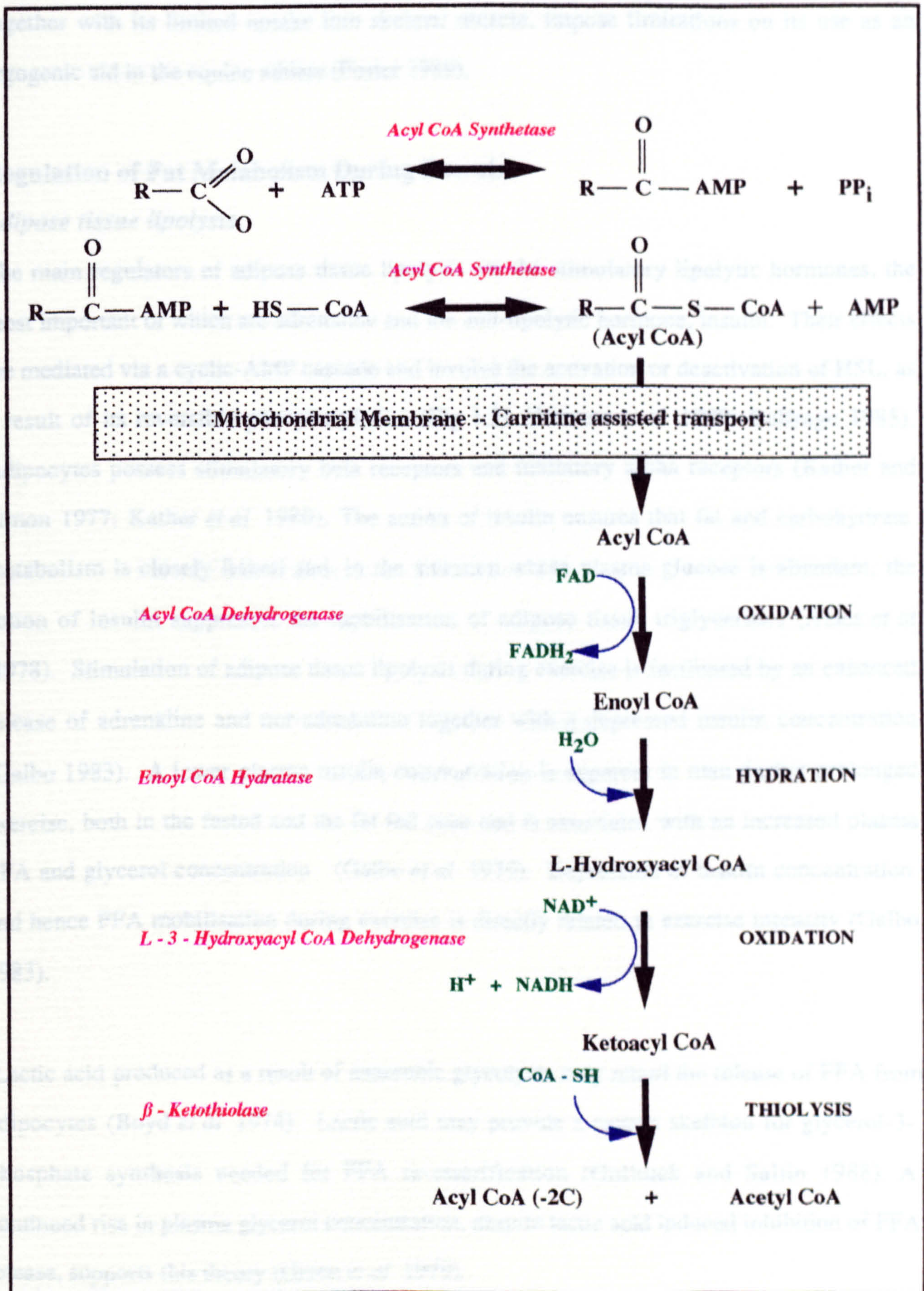
Fat Oxidation

The use of fat as a fuel source is facilitated by the oxidation of FFA whether from adipose tissue, plasma or muscle triglycerides. Oxidation of FFA occurs in the mitochondrial cytosol and is termed β -oxidation (Fig 1.3). Once inside the cell, fatty acids are initially converted to fatty acyl CoA. Fatty acyl CoA then proceeds across the mitochondrial membrane, using a

specific carnitine dependant transport system (Fritz 1963, Fig 1.4). Carnitine is found complexed to *carnitine acyl transferases* which have maximal affinity with either short, medium, or long chain fatty acids (Bulow 1988). Those located on the outer mitochondrial membrane are responsible for the conversion of fatty acyl CoA to fatty acyl carnitine. The latter traverse the mitochondrial membrane and a second *carnitine acyl transferase*, located on the inner mitochondrial membrane, facilitates the conversion of fatty acyl carnitine back to fatty acyl CoA. Fatty acyl CoA is then oxidised via β -oxidation and the TCA pathway. Oxidation of FFA occurs predominantly in the more oxidative muscle fibres, i.e. type I fibres followed by IIa fibres (Saltin 1982). This is due to a number of factors including:

- a) Greater mitochondrial density and hence endowment of oxidative enzymes (Hoppeler *et al* 1983; Snow 1983)
- b) Increased oxygen and FFA delivery due to enhanced muscle fibre capillarisation (Henckel 1983).
- c) Increased expression of FABP_m in the muscle sarcolemma (Carey *et al.* 1994; Veerkamp and Van-Moerkerk 1994)
- d) Increased carnitine content and activities of associated enzymes (Cederblad 1976; Foster 1989)
- e) Increased activity of LPL (Borensztajn *et al* 1975; Jacobs *et al* 1982)

Fig 1.3 **A summary of the main pathways of β -oxidation - adapted from (Stryer 1981).**



Carnitine supplementation has been used in the horse in an attempt to facilitate an increase in fat oxidation during exercise. However, poor absorption of carnitine from the equine gut together with its limited uptake into skeletal muscle, impose limitations on its use as an ergogenic aid in the equine athlete (Foster 1989).

Regulation of Fat Metabolism During Exercise

Adipose tissue lipolysis

The main regulators of adipose tissue lipolysis are the stimulatory lipolytic hormones, the most important of which are adrenaline and the anti-lipolytic hormone, insulin. Their effects are mediated via a cyclic-AMP cascade and involve the activation or deactivation of HSL, as a result of its reversible phosphorylation (Fig 1.5), (Nilsson *et al* 1980; Belfrage 1985). Adipocytes possess stimulatory beta receptors and inhibitory alpha receptors (Kather and Simon 1977; Kather *et al* 1980). The action of insulin ensures that fat and carbohydrate metabolism is closely linked and, in the situation where plasma glucose is abundant, the action of insulin suppresses the mobilisation of adipose tissue triglycerides (Hales *et al* 1978). Stimulation of adipose tissue lipolysis during exercise is facilitated by an enhanced release of adrenaline and nor-adrenaline together with a depressed insulin concentration (Galbo 1983). A lower plasma insulin concentration is apparent in man during prolonged exercise, both in the fasted and the fat fed state and is associated with an increased plasma FFA and glycerol concentration (Galbo *et al* 1979). Depression of insulin concentration and hence FFA mobilisation during exercise is directly related to exercise intensity (Galbo 1983).

Lactic acid produced as a result of anaerobic glycolysis may retard the release of FFA from adipocytes (Boyd *et al* 1974). Lactic acid may provide a carbon skeleton for glycerol-3-phosphate synthesis needed for FFA re-esterification (Gollnick and Saltin 1988). A continued rise in plasma glycerol concentration, despite lactic acid induced inhibition of FFA release, supports this theory (Green *et al* 1979).

Fig 1.4 **Fatty acid transport across the mitochondrial membrane via the carnitine dependent transport system.**

CPT I **Carnitine palmitoyl transferase I**

CPT II **Carnitine palmitoyl transferase II**

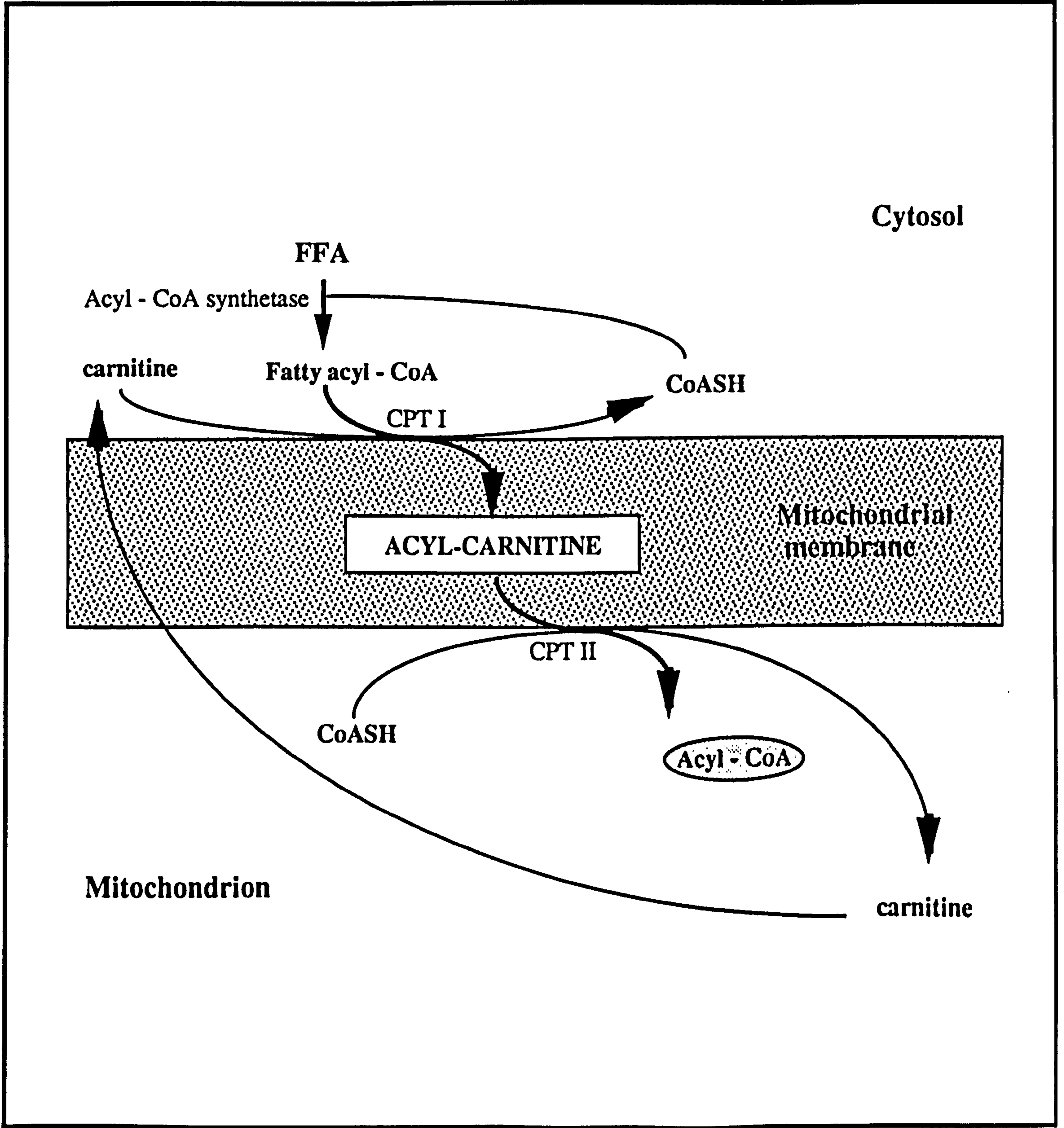
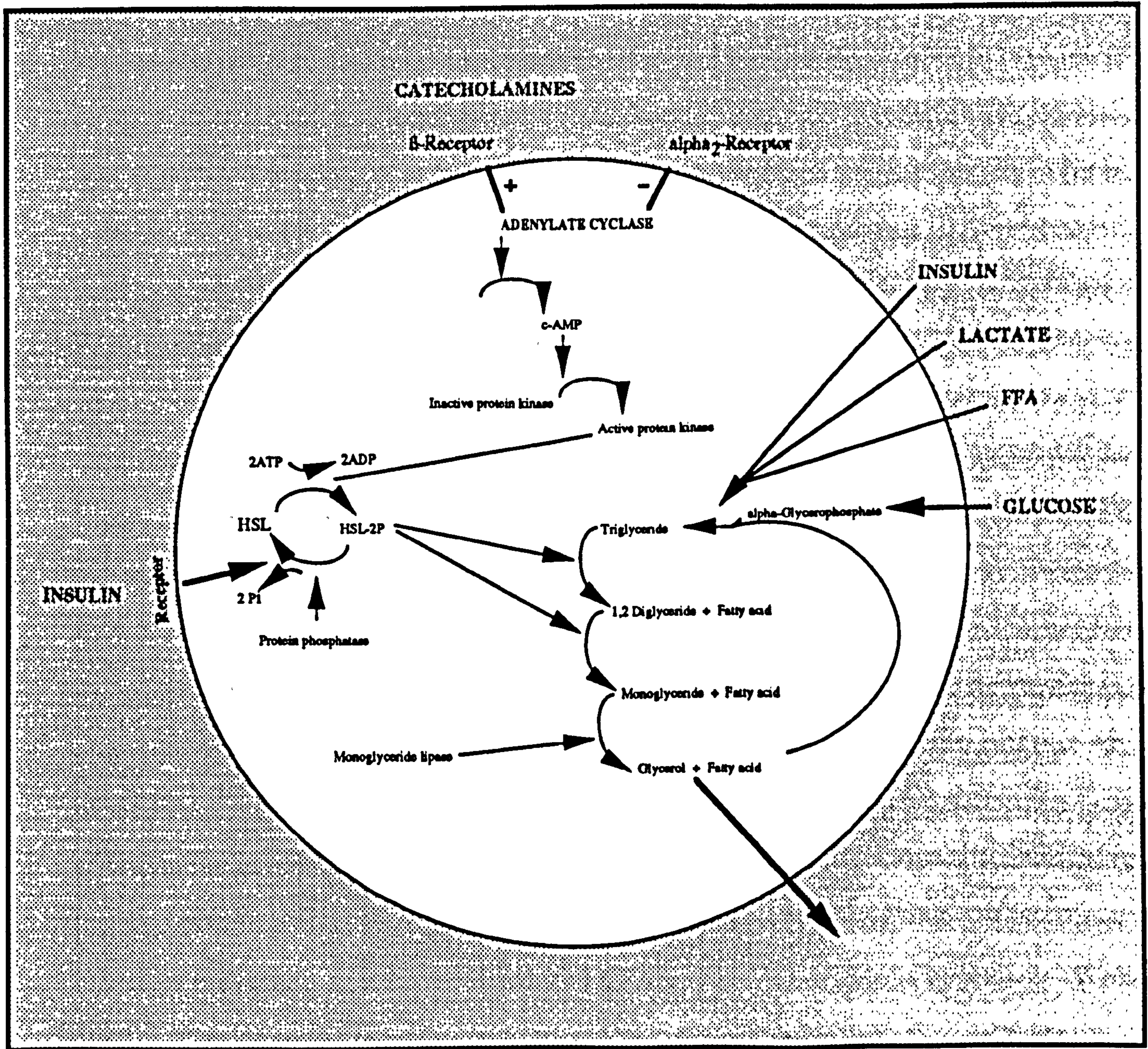


Fig 1.5 **Principal regulation of lipolysis in adipose tissue - adapted from (Bulow 1988)**



Other hormones that may potentiate the stimulatory effect of the catecholamines on lipolysis include, growth hormone, the thyroid hormones, glucagon and corticotrophin. With the exception of growth hormone, no evidence exists for their lipolytic effect during exercise in the normal healthy man, although effects can be demonstrated in vitro (Hales *et al* 1978.)

Free fatty acid recycling

Lipolysis is not regulated by the substrate needs of the working muscles since FFA release far exceeds that oxidised (Romijn *et al* 1993). Romijn *et al* (1993) reported that lipolysis was stimulated maximally at low exercise intensities and was not affected by a further increase in exercise intensity. Conversely, FFA release decreased as exercise intensity increased. A negative feedback mechanism involving FFA recycling, may therefore exist. FFA recycling or re-esterification may occur intracellularly, (i.e. in the tissue of origin), e.g. adipose tissue. Alternately it may occur extracellularly, i.e. the FFA are taken up and re-esterified by a tissue other than that of their origin, e.g. liver. Increased FFA re-esterification occurs as a result of an increase in the 'free' FFA concentration in the tissue water phase, as indicated by an increase in the plasma FFA/albumin ratio (Madsen *et al* 1986). When the FFA / albumin ratio exceeds 3 there may be a simultaneous increase in the vascular resistance of adipose tissue, which opposes adipose tissue blood flow and hence the removal of mobilised FFA (Bulow *et al* 1985; Madsen *et al* 1986). At rest, it is estimated that 70% of the FFA's released as a result of lipolysis are re-esterified (Wolfe 1992). The rate of re-esterification is reduced during prolonged exercise and may account for up to 50% of the increase seen in plasma FFA. During the subsequent recovery period FFA re-esterification increases drastically as a result of reduced FFA uptake and oxidation by muscle (Wolfe 1992). The increased recycling may therefore compensate for the sluggish response of adipose tissue to the cessation of exercise.

Integration of Fat and Carbohydrate Metabolism During Exercise

A reciprocal relationship exists between the rates of glucose and FFA utilisation during exercise. In the situation where an increase in the rate of FFA oxidation occurs during

exercise there is a simultaneous or reciprocal decrease in the utilisation of carbohydrate. This is evident from studies in which FFA utilisation was increased as a result of an artificial elevation of plasma FFA concentration (Costill *et al* 1977; Hickson *et al* 1977; Ravussin *et al* 1986). Conversely, when the availability of plasma FFA was reduced, as a result of the administration of nicotinic acid or any of its analogues, there was an increase in the utilisation of carbohydrate (Walker *et al* 1991; Heath *et al* 1993). Randle *et al* (1963) proposed a mechanism which partially explained the integration of fat and carbohydrate utilisation, the so called glucose-fatty acid cycle or Randle cycle (for review see Randle *et al* 1988). It was proposed that an increase in the level of citrate, produced as a result of an increased flux through the TCA cycle, causes inhibition of phosphofructokinase. The resultant accumulation of glucose-6-phosphate causing inhibition of hexokinase. The net result being an increase in the intracellular and extracellular glucose concentration. The early work of Randle, however, was carried out using heart muscle and a similar effect in skeletal muscle has not been demonstrated unequivocally. Further regulation of carbohydrate utilisation is mediated via the pyruvate dehydrogenase enzyme complex (PDH) which has an important function in generating acetyl CoA from pyruvate for further oxidation through the TCA cycle. In lipogenic tissues it has a further biosynthetic role, the provision of acetyl CoA for the synthesis of FFA or cholesterol. Regulation of PDH is achieved by its reversible phosphorylation. PDH kinase is the enzyme responsible for the conversion of the active dephosphorylated form to the inactive phosphorylated form. PDH kinase is itself activated by an increase in the ratio of acetyl CoA/CoA or NADH/NAD⁺. Thus in conditions of increased FFA oxidation PDH activity is inhibited providing a further link between fat and carbohydrate utilisation (for review see Sugden and Holness 1994). Malonyl CoA, an intermediary in fatty acid synthesis, is implicated in the regulation of carnitine palmitoyltransferase I which is involved in the transport of long chain FFA into the mitochondrial matrix (as reviewed by McGarry and Foster 1980). Inhibition of carnitine palmitoyltransferase I may contribute to the regulation of mitochondrial long chain FFA oxidation.

Factors Which Influence Fat Utilisation During Exercise

The intensity and duration of exercise, the hormonal milieu, as well as the training and nutritional status of the individual are all factors involved in the selection of substrate for muscular activity. Fat is utilised aerobically and although more efficient energetically in terms of ATP production than other fuel sources, the power output achieved as the result of fat oxidation is low (Table 1.4, Sahlin 1985).

In Man fat contributes approximately 50% of the total energy utilised at rest (Gollnick and Saltin 1988). During prolonged exercise of low to moderate intensity there is a progressive shift in fuel utilisation from carbohydrate to fat, as indicated by measurements of respiratory exchange ratio (RER), in Man (Edwards *et al* 1934; Bulow 1981), horses (Pagan *et al* 1987; Rose *et al* 1991) and dogs (Paul 1975). At the onset of exercise, however, there is an increased reliance on carbohydrate metabolism, even at low intensities. This is partly due to the rapid increase in energy demand and concomitant delay in FFA mobilisation (Table 1.3). The calculated contribution of fat to energy production, during exercise in the horse, is not documented. In the untrained human the absolute amount of fat oxidised during prolonged exercise is reported to reach a maximum at about 50% $\text{VO}_2 \text{max}$ (Gollnick and Saltin 1988). In the latter stages of prolonged exercise (4-8 hours), 80% or more of the energy could be supplied by fat (Edwards *et al* 1934; Bulow 1988). During relative exercise intensities of greater than 50% $\text{VO}_2 \text{max}$ the contribution of fat to energy production decreases. Maximal intensity exercise of short duration is reliant on the use of PCr and carbohydrate stores. The use of fat during this type of exercise is limited due to its low power output and probably forms a minimal part of the energy generating pathway.

Table 1.4 Biochemical and physiological adaptations to training in Man, the rat and the horse.

Adaptation	Man/Rat	Reference	Horse	Reference
Cardiac output	---		nc	1, 2, 3
Muscle capillarisation	inc	4, 5	inc	6
β -hydroxyacyl CoA dehydrogenase	inc	7	inc/nc	8, 9, 10, 11
Citrate synthase	inc	7	inc	10, 13, 14
Mitochondrial density	inc	4	inc	15
Muscle fibre IIa/IIb ratio	---		inc	8, 10, 12
Muscle carnitine	inc	16	---	
Carnitine acyl CoA transferase	inc	17	---	
<hr/>				
inc - increase		nc - no change		
1	(Skada <i>et al</i> 1976)		3	(Bayly <i>et al</i> 1983)
4	(Saltin and Gollnick 1983)	(Thomas <i>et al</i> 1983)	6	(Henckel 1983)
7	(Schantz <i>et al</i> 1983)	(Blomsqvist 1983)	9	(Essen-Gustavsson and Lindholm 1985)
10	(Lindholm <i>et al</i> 1983)	(Hodgson <i>et al</i> 1986)	12	(Essen-Gustavsson <i>et al</i> 1983)
13	(Cutmore <i>et al</i> 1985)	(Roneus 1994)	15	(Straub <i>et al</i> 1983)
16	(Sanguq <i>et al</i> 1984)	(Roneus <i>et al</i> 1991)		
		(Mole <i>et al</i> 1971)		

Effect of training

It is generally accepted that sub-maximal aerobic training increases the oxidative capacity of muscle in man (Mole *et al* 1971; Holloszy 1973) and the horse (Snow and Guy 1979; Hodgson *et al* 1986), (Table 1.4). The effects of training in the horse are not as clear since the majority of training studies have been carried out under 'field' conditions using conventional race training. Conventional race training involves both aerobic and anaerobic workloads. Typical training studies in the horse have previously monitored 2 year old animals through 1 or 2 seasons of race-training. It is difficult therefore to differentiate between the 'true effects of training' and the effects of age. Nevertheless many of the adaptational changes described in human and rodent studies have also been reported in the horse (Table 1.4).

An improved oxidative capacity increases the ability to utilise fat but also to use carbohydrate aerobically. A reduction in RER both in cross-sectional and longitudinal training studies in man (Bang 1936; Gollnick and Saltin 1988) and in longitudinal training studies in the horse (Thomas *et al* 1983) illustrates the increased ability to utilise fat during sub-maximal prolonged exercise. There are many biochemical and physiological adaptations that occur in response to training and which may contribute to the increased ability to utilise fat at sub-maximal work intensities. Increased muscle capillarisation may contribute to an increased uptake of oxygen and FFA. Increases in the activities of oxidative enzymes ensure increased utilisation. Other changes such as increases in the muscle carnitine and triglyceride content may further enhance fat oxidation overall.

The availability of plasma FFA to muscle cells in response to exercise may be altered as a result of training. Early studies suggested that plasma FFA concentration during exercise was lower in the trained compared to the untrained state, for a given exercise (Johnson *et al* 1969; Holloszy *et al* 1986). However, it is now accepted that for the same relative exercise intensity the plasma FFA concentration is the same or only slightly lower in the trained state, despite a blunted catecholamine response to exercise (Hartley *et al* 1972; Winder *et al*

1979). Studies in rats have reported an increased sensitivity of adipose tissue to adrenaline in the trained state (Bukowiecki *et al* 1980) which may represent a compensatory mechanism. The current consensus is that there is no change in the delivery of FFA to muscle in the trained state but that there is increased extraction from the blood (Kiens *et al* 1993). The uptake of FFA into muscle follows saturation kinetics (Turcotte *et al* 1991). A change in the FFA transport capacity of FABP_m may facilitate the increased extraction of plasma FFA by muscle in the trained state. This may alter the FFA concentration at which uptake into muscle becomes saturated (Kiens *et al* 1993).

Muscle LPL activity is increased by training (Cohen *et al* 1989; Kiens and Lithell 1989) thereby increasing its ability to extract FFA from circulating lipoprotein associated triglycerides. Endogenous muscle triglyceride storage is also reported to increase with training (Holloszy *et al* 1986). Increased muscle triglyceride utilisation during exercise, in the trained state, may contribute to the generalised increase in fat utilisation (Havel *et al* 1967).

Effect of age

Horses racing on the flat usually begin their athletic career as 2 year olds. However, horses competing in 3-day eventing, endurance racing, show jumping or national hunt racing tend to be much older (5-17 years). An increased oxidative capacity of equine muscle has been reported to occur with increasing age, illustrated by an increase in muscle *citrate synthase* (CS) and *β-hydroxyacyl CoA dehydrogenase* (β-HAD) activity and a concomitant decrease in *lactate dehydrogenase* (LDH) activity, as reviewed by Snow and Valberg (1994). These findings may reflect a combination of the effects of age and cumulative training.

Effect of diet

The early studies of Hultman (1967) and Bergstrom *et al* (1967) established that pre-exercise muscle glycogen concentration influenced its subsequent utilisation during exercise as well as the endurance time to fatigue. Endurance performance was consistently reported to be

compromised in subjects fed a high fat diet. Recently however, this notion has been challenged. An increase in the contribution of fat to energy production, with a concomitant reduction in carbohydrate utilisation, has been reported extensively in human and rodent studies as a result of feeding fat supplemented diets. Phinney *et al* (1983) reported that prolonged feeding of a low carbohydrate high fat diet (4-6 weeks) had no detrimental effect on exercise endurance in trained cyclists, despite significantly reduced resting muscle glycogen concentrations. Furthermore, Miller *et al* (1984) demonstrated that rats exposed to a high fat diet could run longer than ad-libitum fed animals. A similar finding has been reported in sledge dogs adapted to a 70% fat diet for a period of 20 weeks (Hammell *et al* 1977). Muoio *et al* (1994) suggested that the early findings of reduced endurance performance associated with high fat diets may have been influenced by a number of factors. The authors suggest that these studies used only moderately trained or untrained subjects who underwent a strenuous pre-test exercise bout before being introduced to the high fat diet. The diets were additionally severely carbohydrate restricted and the subjects were often glycogen depleted before the high fat diet was introduced.

Fat as Part of the Equine Diet

Traditionally the equine diet has contained very little fat, about 3-4% by weight of the concentrate portion. However, small amounts of oil, e.g. cod liver oil have been added to the diet for improvement in health and coat condition for many years. Recently much interest has been shown in the use of supplemental fat in the equine diet since it represents a very energy dense foodstuff. The gross energy content of a given weight of fat is over twice that of either carbohydrate or protein (Lentner 1981).

1 kg Fat , 9.3 Mcal

1 kg Carbohydrate, 4.1 Mcal

1 kg Protein, 4.1 Mcal

* *concentrate refers to the processed portion of the diet*

Fat supplemented diets have been suggested to offer an attractive alternative to high carbohydrate diets, since the latter have previously been implicated in conditions such as rhabdomyolysis (Harris 1989) and laminitis (Garner *et al* 1978) and in developmental orthopaedic diseases in foals (Biesik and Glade 1985). Furthermore, it has been reported that the addition of fat to the diet of the equine athlete may confer benefits at intensities of exercise ranging from prolonged low intensity to short duration high intensity exercise (Hintz *et al* 1987; Hambleton *et al* 1980; Duren *et al* 1987; Oldham *et al* 1990; Harkins *et al* 1992).

The FFA composition of equine tissues reflects that of the diet (Shoreland *et al* 1952) suggesting that the main site of digestion and absorption of fat is the small intestine, as it would therefore not be exposed to the actions of bacteria present in the large intestine. The digestion of fat is facilitated by its emulsification by bile salts present in the bile, which in the horse flows freely from the liver in the absence of a gall-bladder, and by the action of lipase enzymes. Lipase enzymes break down dietary triglycerides into FFA and glycerol. In Man triglycerides are re-assimilated in the epithelial cells of the small intestine and are pushed out into the circulation through the lymphatic system as part of chylomicrons, (for review see Thomsen 1978).

Horses have been shown to readily accept the addition of increased amounts of fat to their diet in the form of vegetable oils or animal fat (Bowman *et al* 1979; Rich *et al* 1981; Pagan *et al* 1993). Corn, soya, coconut and peanut oils, as well as rendered animal fat or inedible tallow, have been used in dietary studies with no reported palatability problems. The level at which fat can be fed in the diet is largely dependant on the physical problems of feed manufacture, if it is fed as part of a cube or coarse mix. The direct addition of oil to the feed allows much larger quantities to be added to the diet.

Previous studies have reported additions of fat to the equine diet of between 5-20% of the concentrate portion, on a dry matter (DM) basis (Scott *et al* 1989; Hollands and Cuddeford 1993). In the latter studies this was approximately equal to between 5-35% of the total digestible energy (DE) intake. Addition of excessive quantities of oil to the diet may cause digestive disturbances and diarrhoea due to a reduced transit time through the gut.

DE is the energy that is available to the animal as a consequence of digestion and is equivalent to the difference between the gross energy intake and the energy lost through undigested material in the faeces. Metabolisable energy (ME) is equivalent to the DE corrected for energy losses occurring in the urine. Both DE and ME have consistently been reported to increase as a result of the addition of fat to the equine diet (Rich *et al* 1981; McCann *et al* 1987; Hollands and Cuddeford 1993). However, the form in which the fat is fed, i.e. free in the form of oil or animal fat, or encapsulated in cereal grains may affect its digestibility (McCann *et al* 1987). Fat is digested predominantly in the small intestine, whereas cell wall material is digested in the hind gut. The encapsulation of oil in cereal grains, may effect its digestibility as some fibre may have to be digested to free the cereal fat. The situation is somewhat different in ruminants where free dietary oil may reduce fibre digestibility as a result of fibre coating during ruminal fermentation (Hollands and Cuddeford 1993).

Absorption of calcium and magnesium and digestion of fibre is unchanged by the addition of fat to the diet (Rich *et al* 1981; McCann *et al* 1987), although an increase in the digestibility of phosphorus has been reported (Hollands and Cuddeford 1993). In poultry and sheep, fat supplemented diets have been associated with reduced calcium absorption possibly due to the formation of calcium soaps in the small intestine (Hollands and Cuddeford 1993).

Fat supplemented diets have also been associated with a reduction in the thermal load or reduction in heat produced during digestion. McCann *et al* (1987) reported that heat production expressed as a percentage of total DE was lower during fat supplementation in

comparison to feeding a low fat control diet. Calculation of energy balance (metabolisable energy - heat production) by calorimetry revealed a significant increase over the control diet.

Little information is available concerning the optimum type of fat and FFA composition for inclusion into equine diets. The degree of saturation, FFA chain length, age of the animal, as well as the amount of fat included in the diet has been shown to affect its digestibility in other monogastric animals (Freeman 1983). The digestibility of corn oil has been reported to be higher than that of animal fat or blended fat in horses (Rich *et al* 1981; McCann *et al* 1987).

Rationale for undertaking the current studies and its relation to previous work carried out in the equine species.

The horse, a semi-ruminant herbivore, has traditionally eaten a 'low fat' diet. The thoroughbred horse has been bred for sprinting and has a high proportion of type II fibres in the middle gluteal muscle, one of the main hind limb locomotory muscles (Lindholm *et al* 1983). It has a high capacity for carbohydrate storage and a low body fat composition (Table 1.2). Furthermore, the horse exhibits relatively low activities of key enzymes of fat metabolism in relation to man (Chan *et al* 1984; Watson *et al* 1992a; Lindholm *et al* 1983). These findings suggest that the thoroughbred has evolved as a 'carbohydrate dependant' athlete. There are very few studies in the horse that have focused on fat metabolism during exercise, although many studies, some of which are anecdotal have investigated the effects of fat supplementation on exercise metabolism and performance. The following series of studies were carried out in order to increase the understanding of fat metabolism during exercise in the horse and furthermore, to elucidate the mechanisms involved in the metabolic response to fat supplementation.

The principal aims of the following series of studies were as follows:

- i) To investigate the relative contribution of fat and carbohydrate metabolism to energy production at various exercise intensities.**
- ii) To investigate the capacity of the horse to increase the contribution of fat to energy production as the result of :**
 - a) Acute increase in triglyceride derived plasma FFA.**
 - b) Addition of fat to the diet.**
- iii) To describe any adaptational changes occurring in plasma or muscle in response to feeding a fat supplemented diet.**

The studies carried out as part of this thesis were as follows:

- i) An investigation of the variation in plasma long chain FFA over 24 hours.**
- ii) A comparison of the anticoagulative and lipolytic effects of HEP and a related substance pentosan polysulphate, in order to develop a model for the pre-exercise elevation of plasma FFA.**
- iii) An investigation of the effect of a pre-exercise elevation of plasma FFA on fat utilisation during prolonged low intensity exercise.**
- iv) An investigation of the effect of fat supplementation on muscle and blood parameters at rest.**
- v) An investigation of the effect of fat supplementation on the metabolic response to low, moderate and moderate/high intensity exercise.**

Chapter 2

Materials and Methods

Sampling Techniques

Blood samples

Single blood samples were drawn by venepuncture of the left or right jugular vein using a sterile 19 g needle and syringe. Serial blood samples were drawn through an indwelling catheter introduced into the left or right jugular vein, under local anaesthesia.

Catheterisation technique

An area of skin over the right or left jugular vein was shaved and sterilised with a mixture of povidine and surgical spirit. The area was anaesthetised using local anaesthetic (Xylocaine, 2% with adrenaline, 1 ml) injected subcutaneously. A small incision was then made through the skin and underlying fascia through which a 13 g catheter was introduced. An extension tube with a 3-way tap was fitted to the catheter which was then sutured into place. The catheter was kept patent by flushing after each blood sample withdrawn, with heparinized saline (5-10 ml, 5000 IU/l).

Anticoagulants

Blood was dispensed into tubes containing ethylenediamine tetra acetic acid (EDTA) for the analysis of FFA, triglyceride, glycerol and cholesterol; fluoride oxalate for the analysis of lactic acid and glucose; citrate for the analysis of activated partial thromboplastin time (APTT) and into either tubes containing lithium heparin or no anticoagulant for the analysis of plasma total lipase activity (T. Lip). Gleeson (1987) reported an elevation in plasma FFA concentration during storage of samples that had previously been treated with lithium heparin. EDTA inhibits the action of bacterial lipase and prevents the auto oxidation of FFA (Bachorik 1982). All blood samples were centrifuged immediately (4000 rpm for 5 minutes at 4 °C) and the plasma was aspirated and frozen in liquid nitrogen. Plasma for the analysis of FFA, triglyceride, glycerol, cholesterol, lactic acid and glucose was stored at -20 °C. Plasma for the analysis of T. Lip activity was stored at -90 °C.

Muscle samples

Muscle biopsy samples were taken according to Snow and Guy (1976) using a 6 mm Bergstrom-Stille needle (Bergstrom 1962). All biopsy samples were taken from the middle gluteal muscle, one of the main hind-limb locomotory muscles in the horse, 1/3 of the distance between the tuber coxae and the head of the tail. Standardisation of the sampling site and sampling depth was carried out in order to minimise the variation in fibre composition of the biopsy sample. The sampling site was prepared by shaving and sterilising a small area of skin. Local anaesthetic (Xylocaine, 2% with adrenaline, 2 ml) was injected subcutaneously along the line of the proposed incision. An incision was made through the skin and underlying fascia using a sterile surgical scalpel blade. In the instance where a further biopsy was to be taken, (e.g. post exercise), a second incision was also made. The biopsy needle was inserted to a depth of 7 cm and a muscle sample taken under suction. Samples of approximately 50-100 mg (wet weight) were obtained. A sub-sample of the muscle biopsy was separated and mounted on filter paper, with the fibres orientated at 90° to the surface of the paper, for histochemical analysis. The sample was covered in talcum powder to prevent the formation of ice crystals and was then frozen and stored in liquid nitrogen. The remainder of the muscle sample was rapidly frozen and stored in liquid nitrogen for metabolite and enzyme analysis. All muscle analysis was carried out on freeze-dried powdered muscle that had been dissected free of any visible blood, fat or connective tissue.

Plasma analysis

Individual free fatty acids

Individual FFA (FFA_i), with a carbon chain length greater than C14 inclusive were analysed using high-performance liquid chromatography (HPLC), using a Constametric 1 HPLC pump (LDC/Milton Roy, Stone, Staffordshire, UK), with a Rheodyne 7125 injector and 50 µl sample loop (Rheodyne, Cotati, CA, USA) and a LC-UV variable wavelength ultraviolet spectrophotometric detector (Phillips Analytical, Cambridge, UK). An Apex 1 octadecylsilica (ODS) analytical column (4.6 mm id/150 mm) protected by an Apex ODS 1

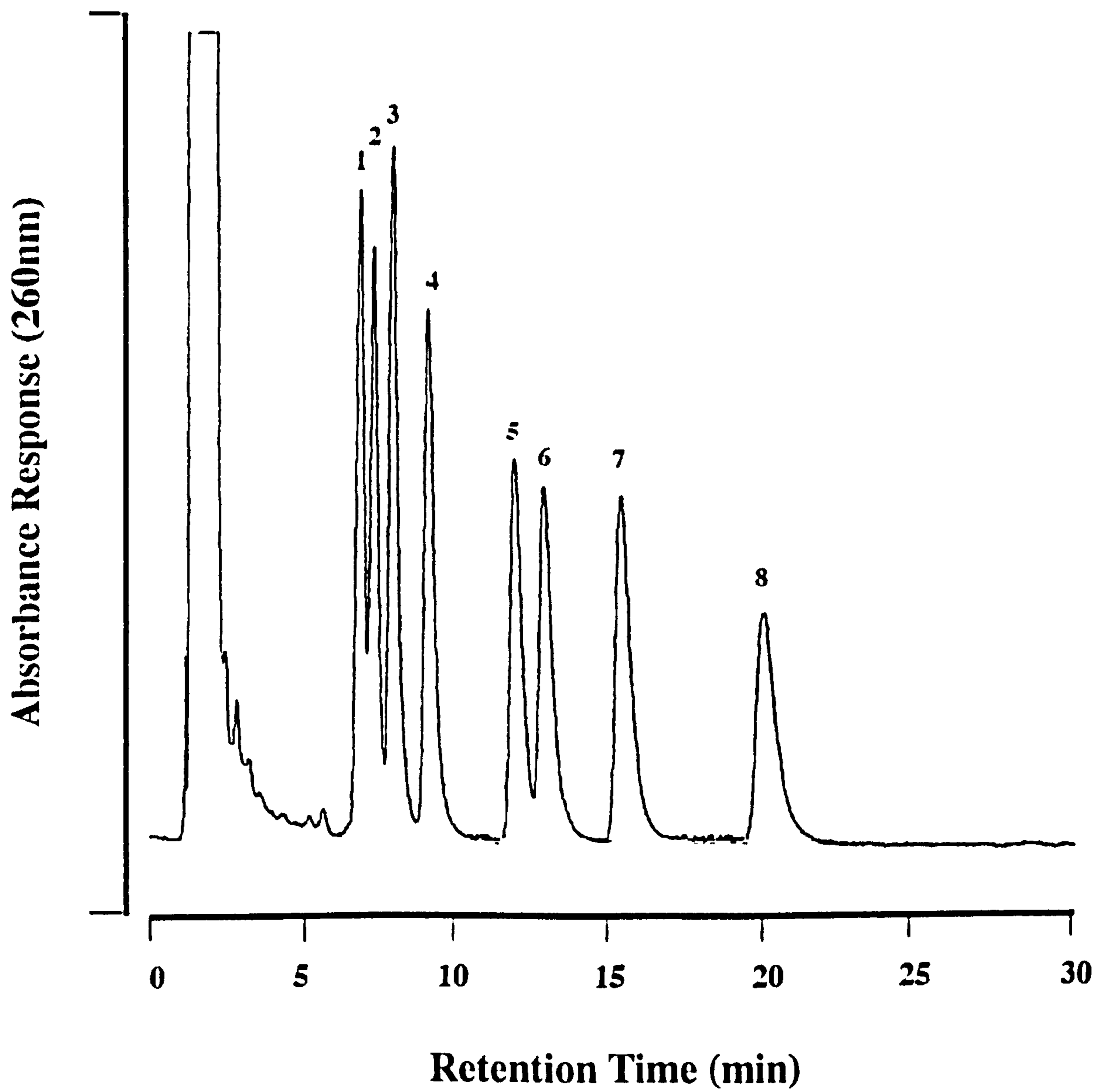
(4.6 mm id/20 mm) guard column, with packing material of 5 μm was used (Jones Chromatography Ltd, Hengoed, Mid-Glamorgan, UK).

Chloroform extracts of EDTA plasma were prepared according to Dawson *et al* (1986) Margaric acid (20 μl) was dried down under a stream of nitrogen and reconstituted with 500 μl of plasma. The margaric acid was present in order to provide an internal standard. Ethanol (2.5 ml) was added to the plasma before vortexing for 2 minutes. The extract was boiled (80°C) for 2 minutes after which chloroform (6 ml) was added. The extract was boiled for a further 2 minutes at 80°C and vortexed for 2 minutes. After cooling the extracts were centrifuged at 3000 rpm for 5 minutes. The upper chloroform layer was removed and dried down under a stream of oxygen free nitrogen.

Derivatisation

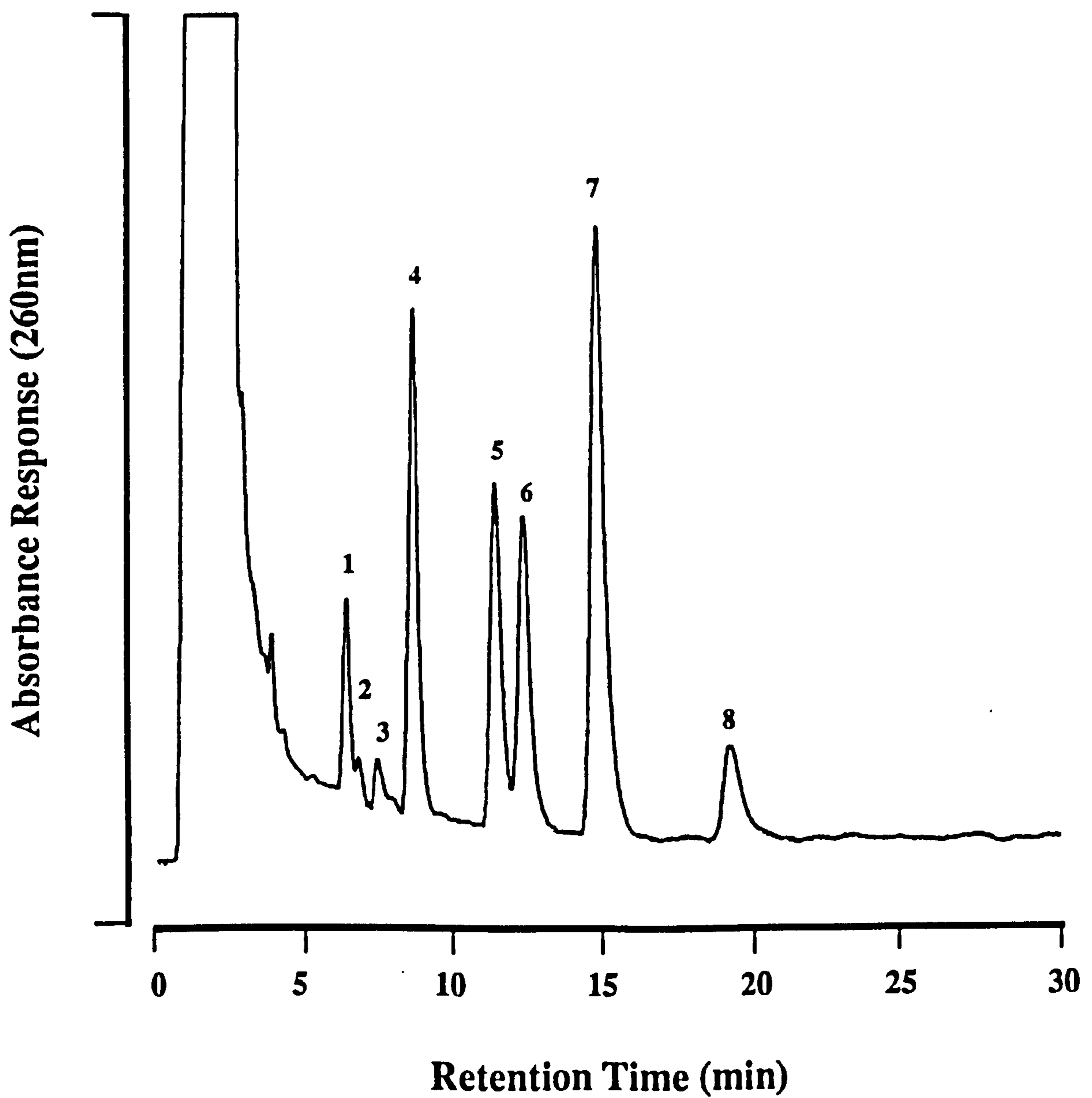
The dried extracts were derivatised at 80 °C for 30 minutes to produce *p* -bromophenacyl fatty acid esters using a modification of the method of Tracey (1986). The derivatisation mixture consisted of acetonitrile (560 μl), methanol (160 μl), methanolic KOH (0.16 M, 5 μl) and derivatisation reagent (30 μl). The derivatisation reagent consisted of *p*-dibromoacetophenone (100 mmol/l) and 1, 4, 7, 10, 13, 16, hexaoxacyclooctadecane (18 crown 6, 10 mmol/l) in acetonitrile. Derivatised FFA_{*i*} were eluted from the HPLC column using a mobile phase comprising methanol, acetonitrile and water (81:9:10) at a flow rate of 1.5 ml/minute. Derivatised FFA_{*i*} were detected by their ultra-violet absorption at 260 nm. The retention times of FFA_{*i*} were dependant on both the FFA chain length and the degree of saturation (Fig 2.1, 2.2). The concentration of FFA_{*i*} was calculated by comparison of sample peak heights to those of external standards of each acid (Fig 2.1, 2.2). An extraction correction was made using the internal standard (margaric acid). Total long chain FFA concentration ($C \geq 14$) was calculated by the addition of FFA_{*i*} concentrations.

Fig 2.1 HPLC chromatogram of individual *p*-bromophenacyl fatty acid esters (standard mixture).



1 - Linolenic acid	(C18:3)
2 - Myristic acid	(C14:0)
3 - Palmitoleic acid	(C16:1)
4 - Linoleic acid	(C18:2)
5 - Palmitic acid	(C16:0)
6 - Oleic acid	(C18:1)
7 - Margaric acid	(C17:0)
8 - Stearic acid	(C18:0)

Fig 2.2 HPLC chromatogram of individual *p*-bromophenacyl fatty acid esters (plasma sample).



1 - Linolenic acid	(C18:3)
2 - Myristic acid	(C14:0)
3 - Palmitoleic acid	(C16:1)
4 - Linoleic acid	(C18:2)
5 - Palmitic acid	(C16:0)
6 - Oleic acid	(C18:1)
7 - Margaric acid	(C17:0)
8 - Stearic acid	(C18:0)

Free fatty acid standards

FFA standards (1 mmol/l) of linolenic, myristic, palmitoleic, linoleic, oleic, palmitic, margaric and stearic acids were prepared in methanol. Mixed FFA standards in methanol, over the range of 25-125 $\mu\text{mol/l}$, were then prepared. The mixed FFA standards were derivatised as for the plasma samples, 100 μl of the mixed standard replacing the same volume of methanol in the derivatisation mixture.

Automated total free fatty acid analysis

Due to the large number of samples generated during the studies in Chapters 4, 5, 6 and 7 a rapid automated FFA method was used. A commercially available FFA kit (Wako NEFA C, Alpha Laboratories, Eastleigh, Hampshire) was adapted for use on a Kone specific autoanalyser (Labmedics, Stockport, Cheshire). The enzymatic colourimetric method is based on the formation of a purple coloured adduct which absorbs maximally at 550 nm (Fig 2.3). A sample blank was run for any samples that were visibly lipaemic, since hyperlipaemic plasma was reported in the methodology to result in artificially elevated FFA concentrations. This was especially relevant to the plasma samples taken during the studies in Chapters 4 and 5.

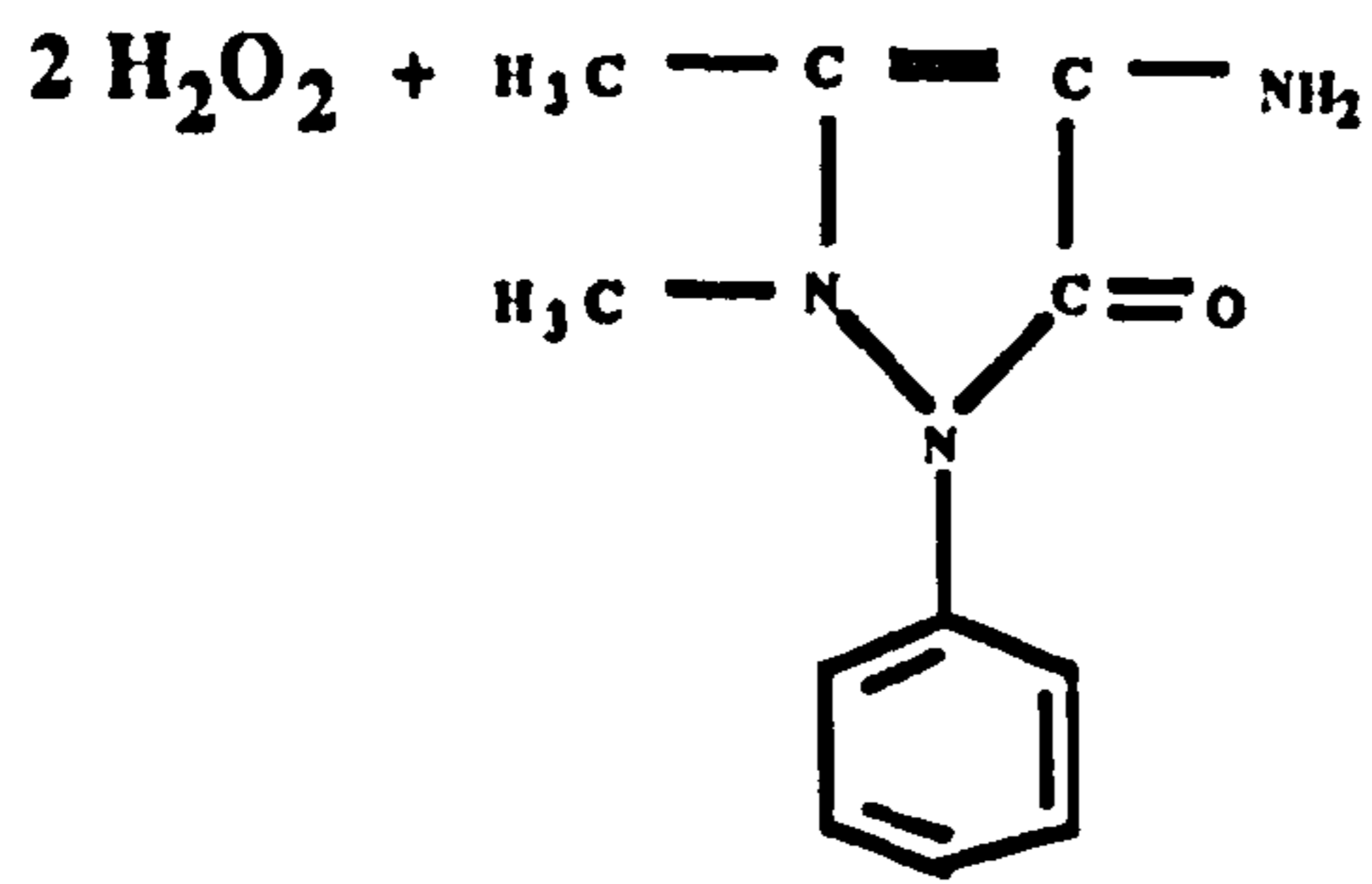
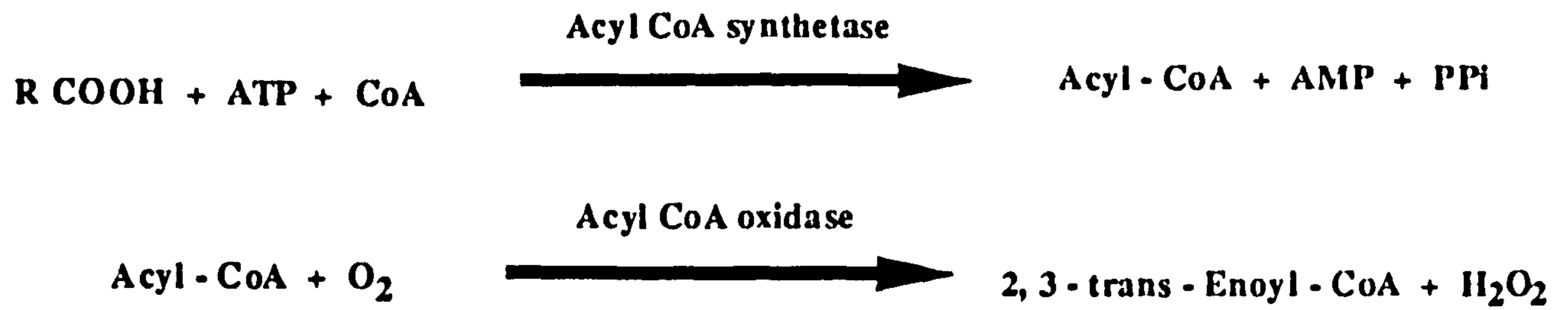
Glucose, lactic acid and cholesterol

Glucose (Randox Laboratories, Co. Antrim, N. Ireland), cholesterol (Randox Laboratories, Co. Antrim, N. Ireland) and lactic acid (Sigma Chemical Co. Poole, Dorset) were analysed using commercially available reagents on a Kone specific autoanalyser (Fig 2.4).

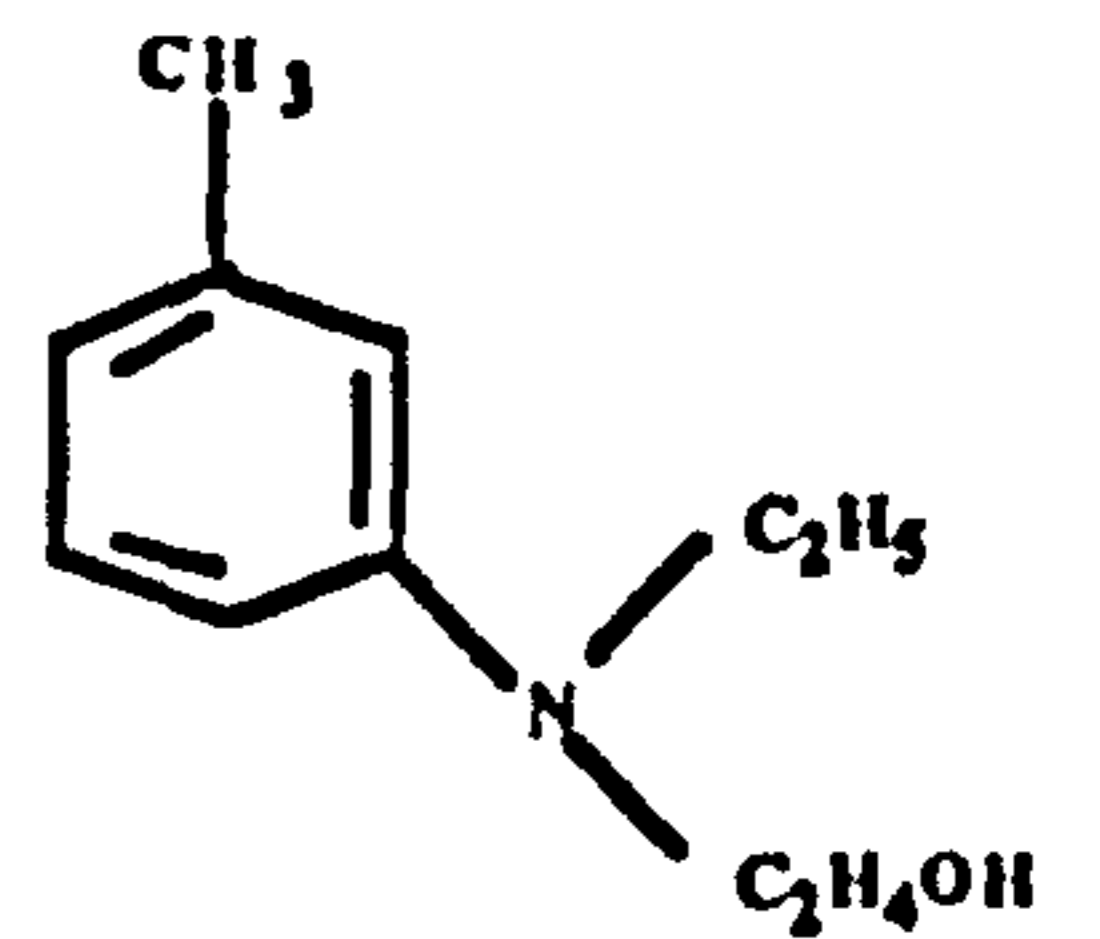
Activated partial thromboplastin time

APTT was measured using the method of Austen and Rhymes (1975), in order to assess clotting function. APTT measures the time required for the coagulation of platelet free plasma in the presence of kaolin, thromboplastin and calcium.

Fig 2.3 **Reaction sequence for the colourimetric analysis of plasma FFA.**

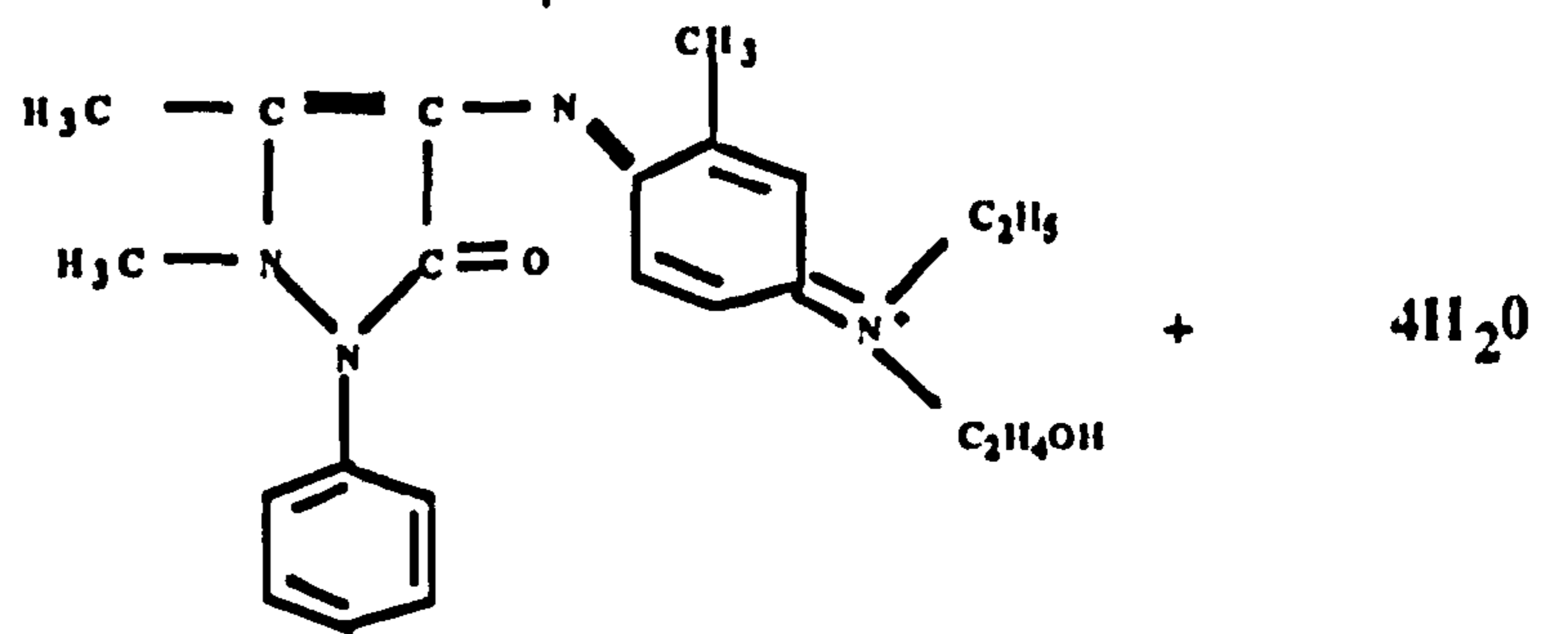


3 - methyl - N - ethyl - N - (β - hydroxyethyl) - aniline



4 - aminoantipyrine

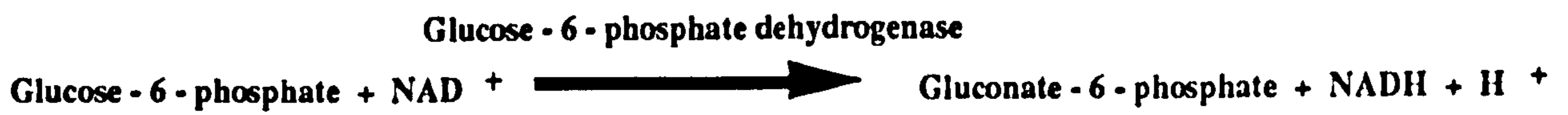
Peroxidase



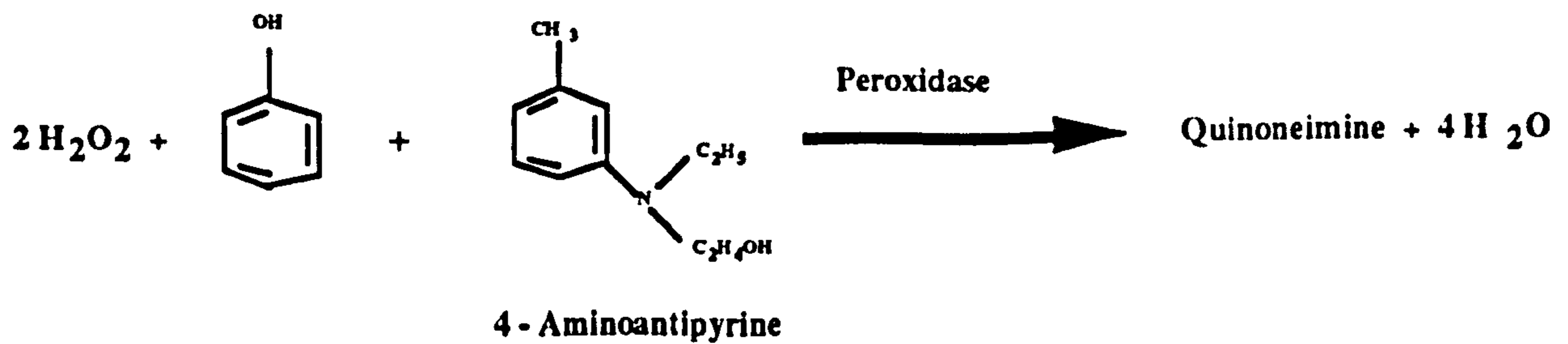
Final purple coloured adduct

Fig 2.4 **Reaction sequence for the analysis of plasma glucose and cholesterol concentration.**

GLUCOSE



CHOLESTEROL



Triglyceride and glycerol

Plasma triglycerides were saponified using alcoholic KOH. Plasma (200 μ l) was added to ethanol (500 μ l, 95%) and KOH (20 μ l, 8 M), vortexed and heated at 70°C for 20 minutes. After cooling MgSO₄ (1000 μ l, 0.15 M) was added, and the samples vortexed and centrifuged. The aspirate was analysed for glycerol, according to the method of Bergmeyer (1986), to give the plasma total glyceride-glycerol concentration. Neutralised perchloric acid extracts of plasma were prepared and were analysed for glycerol (Bergmeyer 1986). Plasma triglyceride concentration was then calculated by subtraction of the plasma glycerol concentration from the total glyceride concentration. The glycerol assay followed the conversion of NADH to NAD⁺ by monitoring the absorbance of NADH at 340 nm. Phosphoenol pyruvate, ATP, NADH, *pyruvate kinase* and *LDH* were incorporated into the reagent mixture. The reaction was initiated by the addition of *glycerol kinase*. The reaction sequence for the determination of triglyceride and glycerol was as follows:

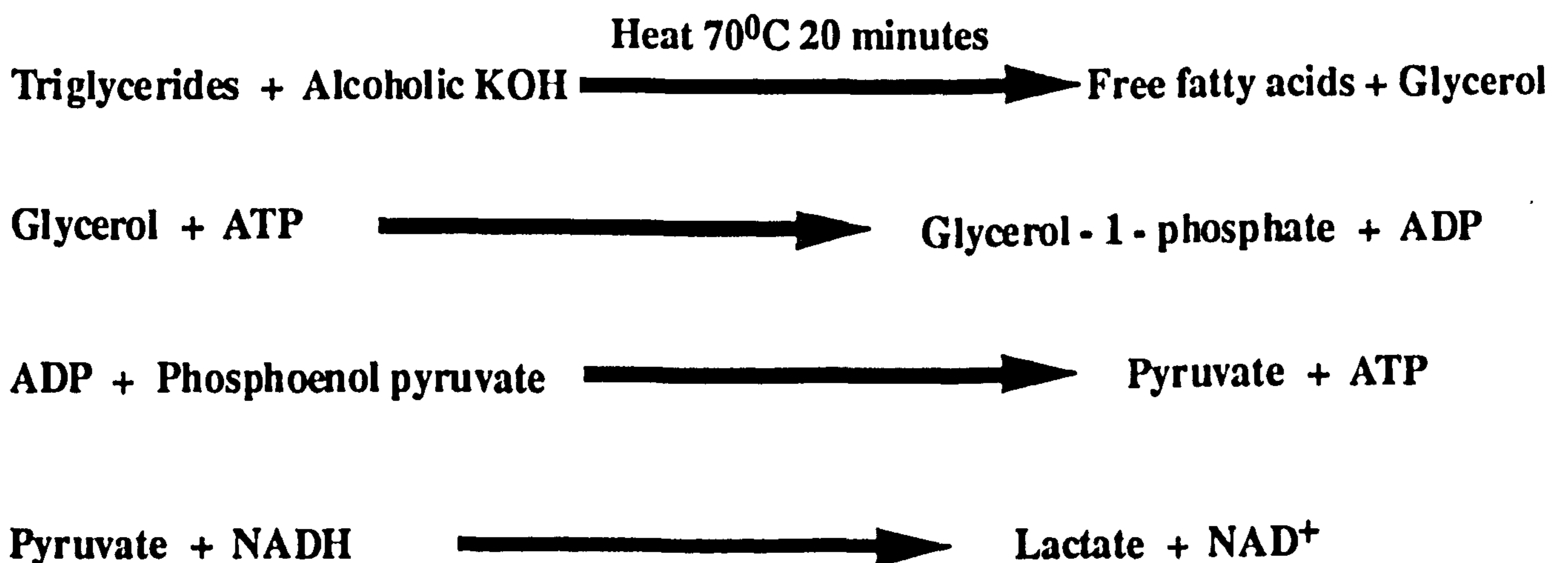


Table 2.1 Reagent solution for the analysis of plasma total glyceride and glycerol concentration.

TEA - Triethanolamine

ATP - Adenosine triphosphate

PEP - Phosphoenol pyruvate

NADH - Nicotinamide dinucleotide

PK - Pyruvate kinase

LDH - Lactate dehydrogenase

GK - Glycerol kinase

REAGENT	Volume	Final concentration mmol/l	Stock concentration mmol/l	Weight/volume
Buffer G1 pH 8.2	3200			
TEA		100	375	7.0 g/100ml
KOH		40	150	0.8 g/100ml
Mg(Ac)2		30	112.6	2.4 g/100ml
EDTA- Na		1	3.8	0.14 g/100ml
ATP	40	0.15	45	24.79 mg/ml
PEP	200	1.0	60	27.92 mg/ml
NADH	220	0.22	12	8.51 mg/ml
WATER	4140			
LDH 1/10	80		10mg/ml	
PK 1/10	80		10mg/ml	
GK 1/10 reaction initiator			10mg/ml	

Total lipase activity

Plasma T. Lip activity was analysed according to the method of Watson *et al* (1992a). Activity was measured in the presence of a low concentration of NaCl (0.1 M) without pre-incubation with sodium dodecyl sulphate. The principle of the method involves the incubation of plasma with a ¹⁴C labelled triglyceride emulsion, in the presence of gum arabic, bovine serum albumin and apolipoprotein C. FFA's released by lipase activity are captured by albumin and extracted into a suitable solvent (see later). The enzyme activity was calculated using the ratio of the radioactivity in the extracted fraction to the total radioactivity in blank incubations. Apolipoprotein C is present as a co-factor for LPL enzyme.

Preparation of apolipoprotein C source

A pooled blood sample (100 ml) was collected from a number of horses that had been fasted overnight. The blood sample was dispensed into tubes containing EDTA. The sample was centrifuged and the plasma aspirated. The pooled plasma was then incubated at 37 °C for 30 minutes, in the presence of calcium chloride (0.1% w/v) and bovine thrombin (1 IU/ml). A clot was formed as the result of fibrinogen coagulation. The clot was removed and the resulting serum dialysed against NaCl (0.15 M) for 12-15 hours. The serum was then heated at 56 °C for 30 minutes, in order to destroy any residual lipase activity. The serum was then dialysed (12-15 hours) against phosphate buffered saline. The concentrated apolipoprotein C source was stored in aliquots at -20 °C. A single batch of concentrated apolipoprotein C was used for all T. Lip analysis in the following studies.

Preparation of radioactive triolein

Cold triolein (0.5 g) was dissolved in toluene (25 ml). Radiolabelled triolein, glycerol tri (1-¹⁴C) oleate (50 µCi, 0.5 ml), was added to a separate volume of toluene (24.5 ml) and mixed before being separated into 7 x 3.5 ml aliquots in round bottom glass flasks. An aliquot of cold triolein (3.5 ml) was added to each flask. The flask contents were dried down under a stream of nitrogen in a hot water bath. Each flask was then washed in triplicate with heptane

(3 ml); between each washing stage the triolein was dried down under a stream of nitrogen. After the final washing stage the flasks were dried and then sealed and stored at -20°C.

Solutions of gum arabic (5%) and bovine serum albumin (10%) in a Tris-HCl buffer (0.2 M, pH 8.4) were prepared and filtered through cotton gauze. Both solutions were divided into 6 ml aliquots and stored at -20°C. An extraction solution consisting of methanol, chloroform and heptane (1.41 : 1.25 : 1.0) was freshly prepared daily. An extraction buffer consisting of potassium carbonate (0.14 M) and boric acid (0.14 M) at pH 10.5 was prepared.

Preparation of substrate mixture

The substrate mixture was freshly prepared approximately 30 minutes before use. Gum arabic (5.5 ml) was added to one of the pre-prepared triolein flasks. The mixture was vortexed and sonicated on ice using an ultrasonic disintegrator (Soniprep 150, MSE) at 18 microns for 4.25 minutes or until there were no visible fat droplets on the surface of the emulsion. Bovine serum albumin (5.5 ml) was added and the emulsion mixed and kept on ice.

Plasma (10 µl) was added to a 10 ml plastic (PTFE) conical tube containing NaCl (30 µl; 0.15 M). Substrate (200 µl), "low salt buffer" (0.2 M Tris, 0.1 M NaCl pH 8.4, 250 µl) and apolipoprotein C (50 µl) were added to the reaction tube on ice. The tubes were capped and vortexed before being incubated at 28 °C for 60 minutes. Two sample blanks were prepared which were treated in the same manner. Following the incubation period the reaction tubes were rapidly placed on ice and extraction solution (3.25 ml) and extraction buffer (750 µl) were added. The tubes were vortexed and then centrifuged at 3000 rpm for 30 minutes at 4 °C. One ml of the upper phase was dispensed into a scintillation vial containing scintillation fluid (10 ml) and acetic acid (200 µl). One ml of the upper and lower phases of the sample blank were dispensed separately into two scintillation vials and were treated in the same manner. The blank samples provided a background and a total C¹⁴ count. Samples were counted over a 5 minute period using a β-scintillation counter. Results were expressed as the

concentration of FFA released per ml of plasma per hour ($\mu\text{mol/ml/hr}$). All samples were analysed in duplicate and serial samples were analysed in the same run.

Muscle Analysis

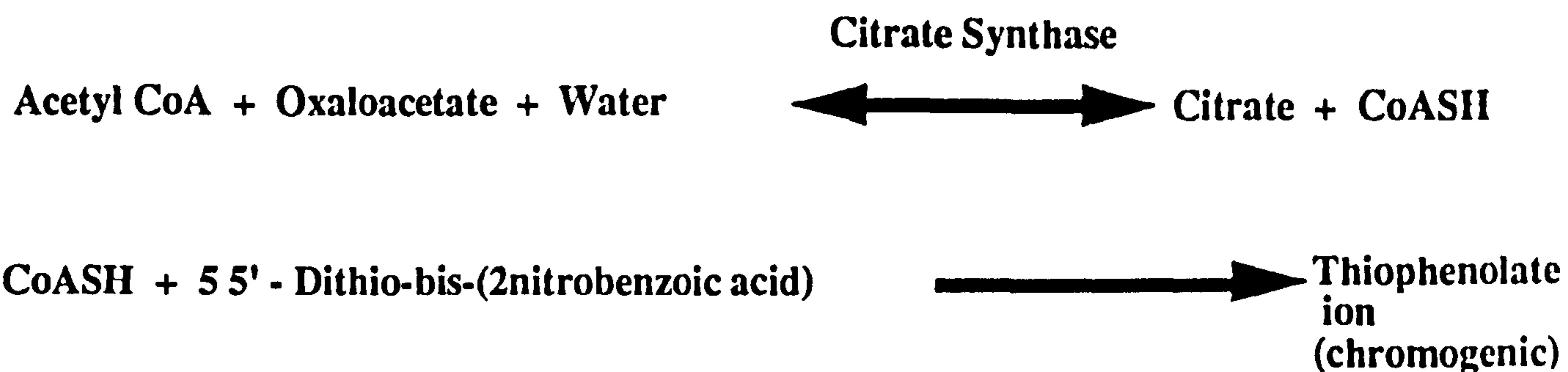
Glycogen

Glycogen was analysed according to Essen and Henriksson (1974). Approximately 3 mg of freeze-dried powdered muscle was weighed into a glass screw capped vial. HCl (500 μl , 1 M) was added and the vial vortexed. The vials were then heated at 100 °C for 60 minutes in order to release glycosyl residues from the glycogen polymer. After cooling, KOH (90 μl , 5 M) was added and the tubes centrifuged at 3000 rpm for 10 minutes. The aspirate was analysed for glucose as described earlier. In order to destroy any 'free glucose' present in post exercise samples the powdered muscle was pre-incubated with KOH (250 μl , 2 M) for 10 minutes at 50 °C. HCl (250 μl , 1 M) was then added as described previously and the sample treated as above.

Citrate synthase activity

CS activity was analysed using a method adapted from Alp *et al* (1976). Approximately 1.25 mg of freeze-dried powdered muscle was homogenised in phosphate buffer (50 mmol/l dipotassium hydrogenphosphate K_2HPO_4 and potassium dihydrogen phosphate KH_2PO_4 , pH 8.0) using an Ultra-Turrax homogeniser for 1 minute on ice. Semi-micro cuvettes containing reagent (575 μl), as described in (Table 2.2), were pre-incubated at 25°C. The muscle homogenate (20 μl) was added to the reagent and any initial change in absorbance measured at 405 nm. The reaction was initiated by the addition of oxaloacetic acid (5 μl) according to the reaction sequence overleaf. The absorbance change at 405 nm was recorded using a Vitatron spectrophotometer with a slit width of 1.0 mm, pre-incubated to 25 °C. The reaction was monitored over 3 minutes using a chart recorder with a X5 scale expansion. Muscle extracts were assayed within 3 minutes of homogenisation. Standard solutions of cysteine (0-0.7 mmol/l) were used to construct a standard curve from which the milimolar

extinction coefficient of acetyl CoA at 405 nm was determined. Enzyme activity was expressed in IU/kg muscle (dry weight, dw) at 25 °C.



β-Hydroxyacyl CoA dehydrogenase activity

Approximately 1.25 mg of freeze-dried powdered muscle was homogenised in phosphate buffer (50 mmol/l dipotassium hydrogenphosphate K₂HPO₄ and potassium dihydrogen phosphate KH₂PO₄, pH 7.5) using an Ultra-Turrax homogeniser for 1 minute on ice. Muscle homogenates were immediately frozen in liquid nitrogen and thawed immediately prior to analysis. Semi-micro cuvettes containing reagent (980 µl) as described in (Table 2.3) were pre-incubated at 25 °C. The muscle homogenate (20 µl) was added to the cuvette and the reaction sequence triggered by the addition of acetoacetyl CoA (20 µl). The absorbance change at 334 nm was recorded using a Vitatron spectrophotometer with a slit width of 0.5 mm, pre-incubated to 25 °C. The reaction was monitored over a 20 minute period using a chart recorder set with a X8 scale expansion.

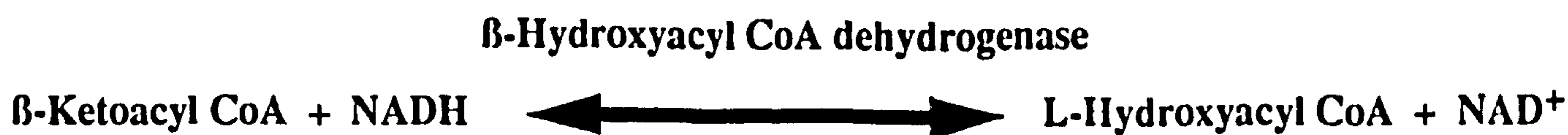


Table 2.2 Reagent mixture for the analysis of muscle CS activity

DTNB 5, 5' dithio-bis-2-nitrobenzoic acid

	Final concentration (mmol/l)	Cuvette volume (μ l)	Stock concentration (mmol/l)	Mass/Volume
Buffer				
Glycylglycine	100	160	375	4.95 g / 100 ml
EDTA pH 8.0	1		3.75	0.14 g
DTNB	0.2	10	12	4.76 mg / ml *
Acetyl CoA (Li salt)	0.1	40	1.5	1.46 mg / ml
Water		365		
Homogenate		20		
Oxaloacetate	0.625	5	75	9.9 mg / ml

* Dissolve in buffer

Table 2.3 Reagent mixture for the analysis of muscle β -HAD activity

NADH Nicotinamide adenine dinucleotide

TEA Triethanolaminutese

	Final Concentration (mmol/L)	Cuvette volume (μ l)	Stock Concentration. (mmol/l)	Mass/vol
TEA	80	340	240	4.45g/100 ml
pH 7.5				
EDTA	0.6	20	30	1.07g/100 ml
NADH	0.18	15	12	9.0mg/ml
Na. (2H ₂ O)				
Water		605		
Homogenate		20		
Acetoacetyl CoA		20	2.5	

Triglyceride

Lipid extracts of freeze-dried muscle (5 mg) were prepared by sonication in 3 mls of 2:1 chloroform : methanol on ice, using an ultrasonic disintegrator (Soniprep 150, MSE, 18 microns, 4 x 30s). NaCl (0.15M, 400 μ l) was then added and the samples centrifuged at 4000 rpm for 5 minutes. The lipid containing layer was aspirated and dried down under a stream of nitrogen before being reconstituted into 550 μ l of chloroform. Tri, di and monoglycerides were separated by solid-phase extraction using glass columns packed with aminopropyl bonded phase (Isolute, Jones Chromatography Ltd, Hengoed, Mid. Glamorgan), according to the method of Renuka-Prasad *et al* (1988). Glass columns were activated with 4 x 1 ml of hexane and 500 μ l of the lipid extract was then loaded onto the column. The column was then eluted with 4 x 1 ml of propan-2-ol : chloroform (2:1). The tri, di and monoglyceride containing eluate was dried down under nitrogen and reconstituted in ethanol (95%, 250 μ l) and KOH (8 M, 10 μ l), vortexed and heated at 70°C for 20 minutes. After cooling MgSO₄ (0.15 M, 500 μ l) was added and the extracts vortexed and centrifuged at 4000 rpm for 5 minutes. The supernatant was removed and analysed for glycerol according to the method of Bergmeyer (1986), as described earlier.

Histological staining of muscle biopsy sections

Muscle samples for histochemical analysis were mounted in OCT (Tissue Tek II, Miles Labs. Inc.) and serial 15 μ sections were cut using a Bright OFT motor-driven cryostat. Sections were stained for myofibrillar actinomyosin ATPase after pre-incubation at pH 4.5 as described by Brooke and Kaiser (1970), (appendix 1). Stained sections were mounted and photographed using a Leitz Dialux 20 stereo microscope with a Wild MPS 51S camera and MPS 45 automatic controller (X 10 magnification). Using the photographs all fibres within a given field were classified as either type I, IIA or IIB fibres. Fibres were then individually cut out and the total number of type I, IIA and IIB fibres weighed. Fractional fibre area occupied was calculated by expressing the weight of a given fibre type within a given field as a fraction of the total fibre weight within that same field.

Physiological Measurements

Heart rate

Heart rate was recorded during exercise using a telemetric system (HippocardTM PE200, Bioengineering Isler A G, Switzerland). Heart rate was recorded and displayed at 5 second intervals throughout exercise on an IBM compatible computer. Mean measurements of heart rate were then calculated over an appropriate time period.

Respiratory measurements

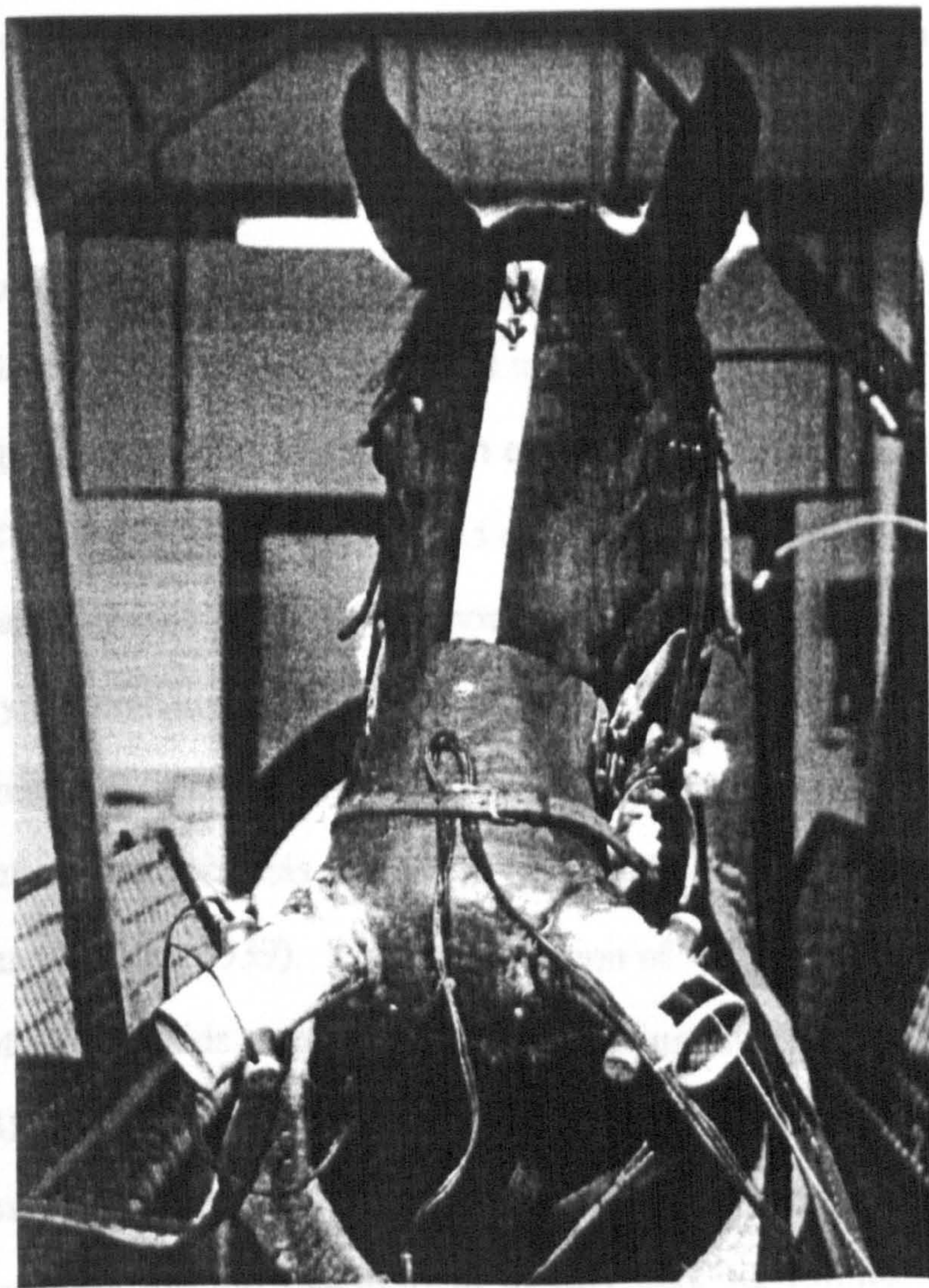
RER was measured using a respiratory system consisting of a lightweight fibreglass mask with two flow tubes, incorporating two pairs of ultrasonic transducers, and an on-line mass spectrometer, as described by Butler *et al* (1993) The mask fitted over the lower part of the head, covering the nostrils but leaving the mouth free Fig 2.5. The flow tubes incorporating the ultrasonic transducers, were mounted opposite each nostril, in line with the direction of air flow. Velocity of the airflow in the flow tubes was detected by phase shifts in beams of ultrasound transmitted in one and then the other direction.

Expired respiratory gas concentrations were monitored using a mass spectrometer (Airspec Ltd, MGA 2100 with MKII software, Chest Scientific Instruments Ltd, Westerham). Samples of expired air were obtained through a capillary tube (0.38 mm internal diameter) positioned centrally within the left flow tube. Changes in the gas composition of expired air were corrected to dry gas values normalised to 100% (O₂, CO₂, N₂ + Argon = 100%) and were displayed in real time on a CRT monitor. The mass spectrometer was calibrated using a certified 12% O₂, 5% CO₂, N₂ balance gas mixture (BOC Special Gases, Guildford).

Both respiratory airflow, O₂ and CO₂ concentrations were recorded on a Gould 6 channel recorder . The output was fed simultaneously to a DT2801, 16 channel, 12 bit analogue to digital converter (Data translation, Marlborough, Massachusetts, USA) housed in a Compaq DeskPro 386TM computer (Compaq Computer Corporation, Houston, Texas USA). The programme for data acquisition and analysis was written in ASYST (version 3.0, MacMillan

Fig 2.5 Horse G1 wearing the lightweight fibreglass mask with incorporated flow tubes and expired gas sampling capillery.

Software Company, New York). Data was reported over 1 minute periods averaged over a 30 minute period. During exercise data was collected every 5 minutes and not and continuously every minute during exercise. RER was calculated from the volume, over 1 minute periods using normalised expired gas concentrations (Bassett et al 1997). RER calculated by the volume method was calculated by the volume method using measurements of respiratory flow and volume (Bassett et al 1997). RER was not calculated due to difficulty in collection of expired gas. It was assumed that the RER is the ratio of gas exchange.



Limitations to RER is the ratio of gas exchange to be made. (Christenson et al 1983) amount of oxygen utilised for carbohydrate metabolism (Christenson et al 1983) contribution of RER to estimate

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consumption of skeletal muscle represents only about 20% of the total energy expenditure. The liver, brain and heart collectively consume about 10% of the total energy expenditure. During exercise when muscle blood flow is greatly increased and a large amount of oxygen is required, RER correlates well with measurements of respiratory quotient. (Bassett et al 1997) walking leg (Bassett et al 1997; Jansen 1982).

Software Company, New York). Data was recorded over 1 minute periods subdivided into 6 X 10 second subfiles. During exercise data was acquired every 5 minutes during walk and trot and continuously every minute during canter. RER was calculated independently of volume, over 1 minute periods using normalised expired gas concentrations (Hoffman *et al* 1993). RER calculated by the volumeless method was highly correlated to that calculated using measurements of respiratory flow and volume ($r = 0.95$, $p < 0.0001$). Non-protein RER was not calculated due to difficulties in obtaining timed urine collections in horses. It was assumed that protein utilisation was low and that the error due to protein utilisation small (for review see Goodman 1988).

Limitations to the use of respiratory exchange ratio measurements

RER is the ratio of carbon dioxide produced to that of oxygen consumed. The measurement of gas exchange at the lung level enables an estimation of gas exchange over the whole body to be made. The absolute value of RER is characteristic of the foodstuff being catabolised (Christenson and Hansen 1939); the amount of carbon dioxide produced relative to the amount of oxygen consumed is a function of the amount of carbohydrate and fat that is utilised for energy production. An RER of 1.0 indicates exclusive reliance on aerobic carbohydrate utilisation, whereas an RER of 0.7 is indicative of total reliance on fat oxidation (Christenson and Hansen 1939). The interpretation of RER is, however, complicated by the contribution of amino acids to fuel utilisation, yielding an RER value of between 0.80-0.82 (Kinney 1988). A further limitation of RER interpretation is that measurements of gas exchange across the lung represent a mean of that for all the body tissues. Therefore the use of RER to estimate muscle metabolism at rest may be limited. At rest, the oxygen consumption of skeletal muscle represents only about 20% of the total consumption whereas, the liver, brain and heart collectively consume about 60% (Jansson 1982). However, during exercise when muscle blood flow is greatly increased and muscle metabolism predominates, RER correlates well with measurements of respiratory quotient as measured across the working leg (Essen *et al* 1977; Jansson 1982).

Although RER estimates the relative contribution of total fat and carbohydrate to energy production it gives no insight into the specific sub-types of fuels used; i.e. FFA or triglyceride in the case of fat and glucose or glycogen in the case of carbohydrate. RER is also influenced by partial oxidation of substrates, e.g. formation of ketones or lactic acid, acid base effects and hyperventilation as discussed by Jansson (1982). Lactic acid formed during high intensity exercise, as the result of incomplete oxidation of carbohydrate, tends to increase RER. RER values above 1.0 are commonly observed during exercise with a high anaerobic component. The lactic acid formed during exercise dissociates to produce hydrogen ions and lactate. The bicarbonate system contributes to hydrogen ion buffering with the formation of carbonic acid; which then dissociates to carbon dioxide and water. Thus, the formation of lactic acid is associated with carbon dioxide production without a corresponding consumption of oxygen. Incomplete oxidation of FFA with the formation of ketones is associated with oxygen consumption without carbon dioxide production and hence results in a lowering of RER. Hyperventilation causes a lowering of alveolar PCO₂ by 'blowing off' carbon dioxide and hence an increase in measured RER.

Statistical Analysis

Where appropriate, initial statistical analysis was carried out using a 1-way or 2-way analysis of variance (ANOVA) for repeated measures. In the instance where significance was detected multiple comparison tests were then employed as appropriate and are described in the relevant chapters. Simple linear regression (least squares method) and correlation (Pearson's method) were also performed. The precision and coefficients of variation for the plasma and muscle analysis methods are shown in Tables 2.5 and 2.6. Precision was calculated as the square root of the sum of the square differences between duplicate determinations over 2n. Coefficient of variation (CV) was calculated as below.

$$S = \sqrt{\sum d^2 / 2n} \quad C V = (SD . 100) / \bar{X}$$

The significance level was declared at (p < 0.05). All values in the text and figures are presented as mean and standard deviation (mean ± SD), except where individual horse data is presented.

Table 2.5 Precision, mean of material and coefficient of variation (CV) for the plasma analysis methods (n refers to the number of paired analyses).

Table 2.6 Coefficient of variation (CV) for muscle metabolite and enzyme activity methods both within extract and on duplicate extracts of the same muscle sample. n refers to the number of times each extract was analysed (within extract) or the number of extracts analysed (between extracts).

Plasma analysis	Precision	mean	CV(%)	n
FFA (Kone)	13.9 (μmol/l)	295.5 (μmol/l)	4.7	20
Total glycerides	5.5 (μmol/l)	267.0 (μmol/l)	2.1	20
Glycerol	7.7 (μmol/l)	119.5 (μmol/l)	6.5	20
Glucose	0.1 (mmol/l)	5.5 (mmol/l)	1.8	20
Cholesterol	0.1 (mmol/l)	3.1 (mmol/l)	2.0	20
Lactic acid	0.1 (mmol/l)	2.2 (mmol/l)	3.1	20
FFA (HPLC)*	(0.2 - 0.3) (μmol/l)	(18.9 - 52.2) (μmol/l)	(0.7 - 2.0)	6
Total lipase	0.2 (μmol/ml/hr)	7.0 (μmol/ml/hr)	2.2	20

* Range of precision, mean and CV for linolenic, myristic, palmitoleic, linoleic, oleic, palmitic and stearic acids.

Muscle analysis	CV between extract (%)	n	CV within extract (%)	n
Glycogen	2.3	10	2.06	18
Triglyceride	8.5	10	----	---
Citrate synthase	4.3	10	2.46	20
β-HAD	6.4	10	0.29	20

Chapter 3

Variation in the Concentration of Long Chain Free Fatty Acids Over 24 Hours

Introduction

In man and other species certain biochemical parameters and hormones exhibit regular variations throughout the day. These changes may be related to food intake, e.g. glucose (Frape 1986) or may be independent of feeding and regulated by some other means, e.g. cortisol (Hoffsis *et al* 1970; Larsson *et al* 1979) and iron (Speck 1968; Ulvik and Romslo 1977). The day-night cycle usually imposes a diurnal rhythm on the feeding behaviour of an animal, sufficient fuel being stored during the active feeding period to provide for the metabolic demands of the period of sleep. Plasma FFA concentration is related to food intake in human subjects fed a mixed fat and carbohydrate diet (Hollister and Wright 1956). No previous investigations describing the within-day variation in the concentration of long chain FFA in equine plasma could be found in the literature.

The resting concentration of plasma FFA influences the rate of FFA oxidation during subsequent exercise (Costill *et al* 1977; Hickson *et al* 1977; Ravussin *et al* 1986). FFA mobilisation is influenced by many factors including the blood glucose concentration as well as circulating levels of insulin and catecholamines (Hales *et al* 1978). Variations in plasma FFA concentration may occur as a result of diurnal changes in any of the above. This study was undertaken in order to establish whether resting plasma FFA concentration remains stable throughout the day or whether it is influenced by feeding status in the horse. Additionally the study would contribute to the design of exercise and feeding studies, investigating fat metabolism, in order to take account of any diurnal changes or circadian rhythm in the plasma FFA concentration.

Materials and methods

The within day variation of *total* long chain ($C \geq 14$) FFA concentration (FFA_t) and *individual* long chain FFA concentrations (FFA_i) were measured in equine plasma over a 24 hour period during 3 experimental sessions. Six thoroughbred horses (Sn, Bd, He, Kj, Mr and Lb) were sampled during the first session and 6 others during sessions 2 (Bo, Co and Gl) and 3 (Hl, Pn and Li). The horses used consisted of 8 geldings (Sn, Bd, He, Kj, Mr, Bo, Co

and Li) and 4 fillies (Lb, Gl, Hl and Pn), weighing between 400-500 kg and between the ages of 2-8 years old. All horses were fed normally with a traditional low fat diet and were not exercised during the study. Prior to the study, 3 of the horses (Kj, Mr and He) had been grass fed and had undertaken little or no work, whilst the remaining 9 horses were stabled and were in moderate work. During the sampling period 11 of the 12 horses were fed *Spillers Stud cubes* (approximately 2 kg) and the twelfth, Bd, *Spillers racehorse cubes* (approximately 2 kg). Horses were fed at 7am, 12.30pm and 4.30pm. The oil content of the stud and racehorse cubes was 4.25 and 4.0% by fresh weight respectively. All horses were fed 2-3 kg of hay with the morning and evening feeds. Water was available *ad libitum* at all times.

Venous blood samples were drawn through a 13 g catheter, as previously described, at hourly intervals starting at 5pm. The horses were catheterised a minimum of 1 hour before the first sample was taken in order to discount the effects of any catecholamine release during catheterisation. Blood was dispensed into tubes containing EDTA as the anticoagulant and centrifuged immediately. The plasma was aspirated and stored at -20°C until analysis of FFA_t, FFA_i and glucose. Concentrations of FFA_i were measured by HPLC as previously described. FFA_t concentrations were calculated by the addition of FFA_i concentrations. Plasma glucose was analysed on a Kone Specific auto analyser (Labmedics, Stockport, Cheshire) using commercially available reagents.

Statistical analysis

The changes in FFA_t and glucose observed were analysed using a 1-way ANOVA for repeated measures. A multiple comparison test, Fisher's protected least significant difference (PLSD), was used where significance was detected. The FFA_t data was then divided into two periods: 5pm-1am, 10am-4pm (period 1) and 2am-9am (period 2) inclusive. This division was made on arbitrary grounds following analysis of the data by ANOVA and inspection of Figs 3.4 & 3.5. A pooled estimate of the within-horse variance S_p^2 was calculated using data from period 1 over the x_i values for each horse according to the following equation (Wonnacott & Wonnacott 1972) :-

$$S_p^2 = \sum_{i=1}^r \sum_{j=1}^{n_i} (x_{ij} - \bar{x}_i)^2 / \sum_{i=1}^r (n_i - 1)$$

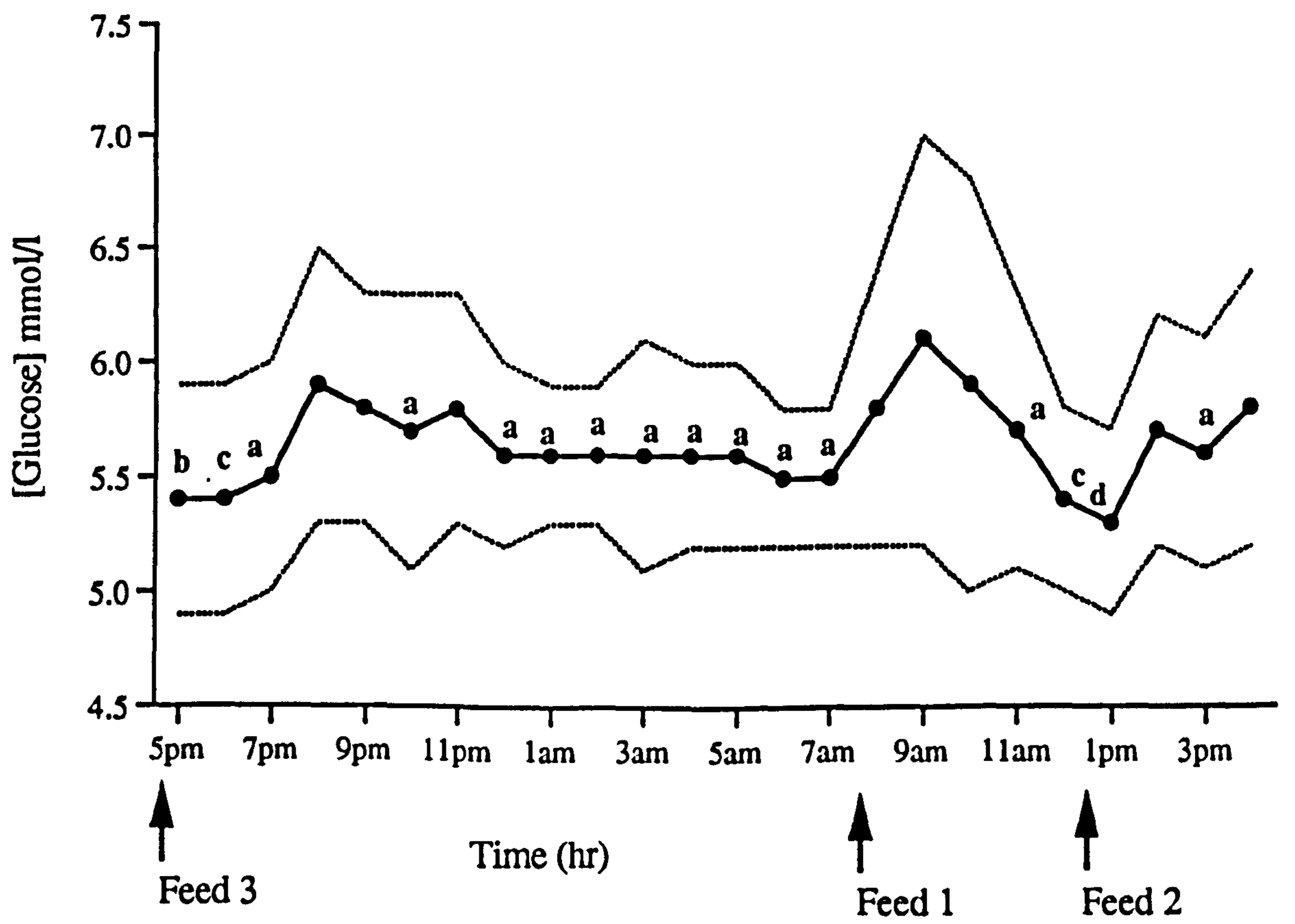
The estimate of S_p^2 was subsequently used to calculate confidence limits about individual horse means during the same period using $\sum_{i=1}^r (n_i - 1)$ degrees of freedom.

Results

Blood glucose concentration fluctuated throughout the 24 hour sampling period, (Fig 3.1). ANOVA indicated a significant effect of time of day ($p < 0.01$). Plasma glucose concentration was significantly increased between 8am-10am and at 2pm, 4pm and at 8pm ($p < 0.01$, Fig 3.1). Glucose concentration was at its highest 1.5 hours following the first feed (7.30am); 3.5 hours following the second feed (12.30pm) and 3.5 hours following the final feed (4.30pm). Glucose homeostasis was maintained overnight since the glucose concentration immediately before the first feed was not significantly different from the concentration at 5pm ($p > 0.05$).

Fig 3.1 **Variation in plasma glucose concentration (mmol/l, mean \pm SD) over 24 hours (n=11).**

Using Fisher's PLSD test the glucose concentration at 9am was $>$ than at time points marked a, b, c or d ($p < 0.01$); at 10am and 8pm was $>$ that at time points marked b, c or d ($p < 0.01$); and at 4pm was $>$ that at the time point marked d ($p < 0.01$).



—●— Mean
 Mean + SD
 - - - - - Mean - SD

The percentage contribution of FFA_i to the total concentration was calculated. As shown in Fig 3.2, the most abundant FFA_i was palmitic acid (C16:0). At 5pm, in decreasing order, the next most abundant acids were linoleic (C18:2), oleic (C18:1), stearic (C18:0), and linolenic acid (C18:3). Together these five acids constituted 94.5 % of the total concentration and individually 26.1, 24.9, 19.2, 17.0 and 7.3% respectively at 5pm. Other FFA detected included myristic (C14:0) and palmitoleic acids (C16:1), both of which individually constituted less than 5% of the total concentration. The predominance of the 5 main FFA was maintained over the 24 hours. There were however, small fluctuations in their respective contribution to the total concentration. Between 4am and 9am there was an increase in the percentage contribution of oleic acid to the total concentration, with a simultaneous decrease in that of stearic acid (Fig 3.2).

FFA_t concentration showed minimal variation during period 1 (Fig 3.3). ANOVA indicated a significant effect of time ($p < 0.001$) and revealed a period in which FFA_t concentration was significantly elevated ($p < 0.001$). Ten out of the 11 horses exhibited an increase in FFA_t in the early hours of the morning (Figs 3.4 & 3.5). The increase was localised around 7am and represented a mean 4.5 fold increase (range 2.0-8.5) above individual horse means over period 1. Changes in FFA_i concentrations showed the same trend as that of FFA_t (Table 3.1, Fig 3.3).

The magnitude and the duration of the elevation in FFA_t varied between horses (Figs 3.4 & 3.5). ANOVA indicated that the mean FFA_t concentration at 5am, 6am and 7am was significantly increased ($p < 0.001$). This statistical analysis, however, implies that all horses showed an increase in FFA concentration and does not illustrate the variation between horses. For this reason further statistical analysis was carried out as described previously.

Table 3. 1 Individual FFA concentrations (mean \pm SD, $\mu\text{mol/l}$) over 24 hours (n=11).

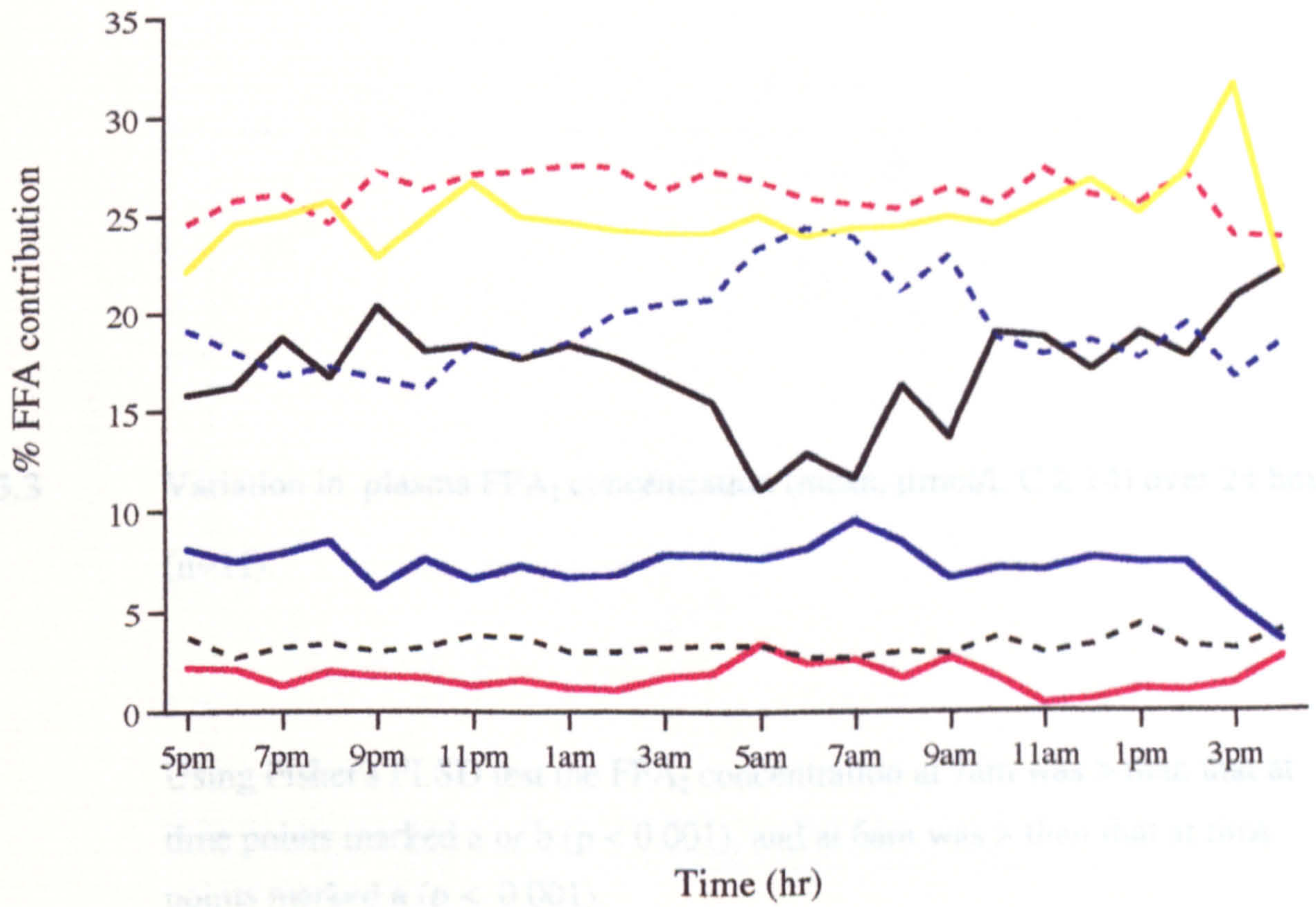
*** Using Fisher's PLSD test FFA concentration was significantly different ($p < 0.05$) at any given time when the numerical difference between two sample times exceeded 1.5, 0.6, 0.7, 3.9, 4.2, 4.2 or 1.7 $\mu\text{mol/l}$ for linolenic, myristic, palmitoleic, linoleic, palmitic, oleic or stearic acids respectively. The bold type highlights the period of greatest variation in FFA concentration.**

Time (hr)	Linolenic	Myristic	Palmitoleic	Linoleic	Palmitic	Oleic	Stearic
5pm	2.2 ± 2.0	1.0 ± 0.6	0.6 ± 0.6	6.0 ± 2.0	6.7 ± 3.4	5.2 ± 2.8	4.3 ± 1.8
5pm	1.8 ± 1.0	0.7 ± 0.4	0.5 ± 0.8	5.9 ± 1.2	6.2 ± 2.7	4.3 ± 1.2	3.9 ± 1.7
7pm	1.7 ± 1.1	0.7 ± 0.3	0.3 ± 0.3	5.4 ± 1.4	5.7 ± 2.3	3.6 ± 1.2	4.1 ± 2.3
8pm	1.9 ± 1.3	0.8 ± 0.2	0.4 ± 0.8	5.6 ± 1.5	5.4 ± 1.5	3.8 ± 0.8	3.7 ± 1.0
9pm	1.4 ± 0.8	0.7 ± 0.4	0.4 ± 0.4	5.3 ± 1.2	6.3 ± 2.1	3.9 ± 0.8	4.7 ± 2.6
10pm	1.6 ± 1.1	0.7 ± 0.3	0.4 ± 0.4	5.3 ± 1.1	5.6 ± 1.3	3.4 ± 0.8	3.8 ± 1.0
11pm	1.4 ± 1.0	0.8 ± 0.3	0.3 ± 0.3	5.5 ± 1.4	5.6 ± 1.4	3.8 ± 1.1	3.8 ± 0.9
12pm	1.5 ± 0.8	0.8 ± 0.3	0.3 ± 0.5	5.1 ± 0.7	5.6 ± 1.0	3.6 ± 0.7	3.6 ± 0.9
1am	1.5 ± 1.1	0.7 ± 0.4	0.3 ± 0.3	5.6 ± 1.5	6.3 ± 1.8	4.2 ± 1.7	4.2 ± 0.7
2am	1.8 ± 1.3	0.8 ± 0.3	0.3 ± 0.3	6.3 ± 2.5	7.2 ± 3.2	5.2 ± 2.9	4.6 ± 1.4
3am	2.3 ± 2.1	0.9 ± 0.4	0.5 ± 0.5	6.9 ± 4.1	7.6 ± 4.2	5.9 ± 4.3	4.8 ± 1.7
4am	2.3 ± 1.6	1.0 ± 0.4	0.6 ± 0.6	7.0 ± 3.4	7.9 ± 4.0	6.0 ± 3.7	4.5 ± 1.0
5am	4.4 ± 3.5	1.9 ± 2.0	2.0 ± 2.5	14.5 ± 11.4	15.6 ± 12.1	13.5 ± 11.6	6.4 ± 2.3
6am	4.4 ± 4.1	1.5 ± 0.9	1.3 ± 1.3	12.8 ± 8.2	13.9 ± 9.8	13.1 ± 9.7	6.9 ± 3.7
7am	6.5 ± 5.2	1.8 ± 1.2	1.8 ± 1.5	16.7 ± 10.5	17.5 ± 12.1	16.3 ± 12.4	8.0 ± 4.0
78am	2.8 ± 1.4	1.0 ± 0.7	0.6 ± 0.7	8.1 ± 4.4	8.4 ± 3.9	7.0 ± 4.0	5.4 ± 2.2
9am	2.5 ± 1.8	1.1 ± 0.9	1.0 ± 1.5	9.3 ± 9.7	9.9 ± 9.7	8.6 ± 11.2	5.1 ± 3.3
10am	1.7 ± 0.7	0.9 ± 0.4	0.4 ± 0.4	5.8 ± 1.9	6.1 ± 2.9	4.4 ± 2.8	4.5 ± 1.8
11am	1.3 ± 0.6	0.5 ± 0.4	0.1 ± 0.2	4.7 ± 1.0	5.0 ± 1.0	3.3 ± 0.5	3.4 ± 0.8
12am	1.6 ± 1.0	0.7 ± 0.3	0.1 ± 0.3	5.6 ± 2.8	5.4 ± 1.6	3.8 ± 2.1	3.5 ± 0.8
1pm	1.4 ± 0.7	0.8 ± 0.8	0.2 ± 0.4	4.9 ± 1.8	5.0 ± 1.2	3.4 ± 1.2	3.7 ± 1.1
2pm	1.6 ± 1.6	0.7 ± 0.5	0.2 ± 0.4	5.8 ± 3.6	5.8 ± 4.5	4.2 ± 4.2	3.8 ± 2.0
3pm	1.3 ± 0.9	0.8 ± 0.4	0.4 ± 0.3	8.0 ± 5.6	6.1 ± 2.7	4.2 ± 2.2	5.2 ± 2.7
4pm	1.3 ± 0.7	0.9 ± 0.3	0.4 ± 0.4	4.7 ± 1.4	5.8 ± 2.2	3.5 ± 0.8	4.6 ± 2.7

The estimated within subject variance for period 1 was 4.5 $\mu\text{mol/l}$. Ten out of the 11 horses studied showed a rise in FFA_t between 2am and 9am. In all of these horses the peak concentration exceeded the 99% confidence limits calculated about the individual horse means during period 1, i.e. \bar{X}_i (period 1) and in 7 out of the 10 horses exceeded the 99.9% confidence limits. In 5 out of 10 horses the FFA concentration remained outside the 99.9% confidence limits for 4 or more consecutive hours (Fig 3.4). The increases in FFA_t observed were much greater than could be accounted for by random variation recorded during period 1.

The data from the twelfth horse (Kj) has not been included in the statistical analysis and has been reported separately (Fig 3.6a & 3.6b). Difficulties were experienced during catheterisation and the FFA_t and FFA_i for this horse were elevated for the first 12 hours of sampling. The increase in FFA concentration following catheterisation probably resulted from adrenaline release. Adrenaline stimulates adipose tissue lipolysis (Anderson and Aitken 1977) and could explain the increases in plasma FFA observed in this horse

Fig 3.2 **Variation in the mean percentage contribution of FFA_i to the FFA_t concentration over 24 hours (n=11).**



— Linolenic - - - - Oleic — Linoleic - - - - Myristic
 — Stearic — Palmitoleic - - - - Palmitic

Fig 3.3 **Variation in plasma FFA_t concentration (mean, μmol/l, C ≥ 14) over 24 hours (n=11).**

Using Fisher's PLSD test the FFA_t concentration at 7am was > than that at time points marked a or b (p < 0.001), and at 6am was > than that at time points marked a (p < 0.001).

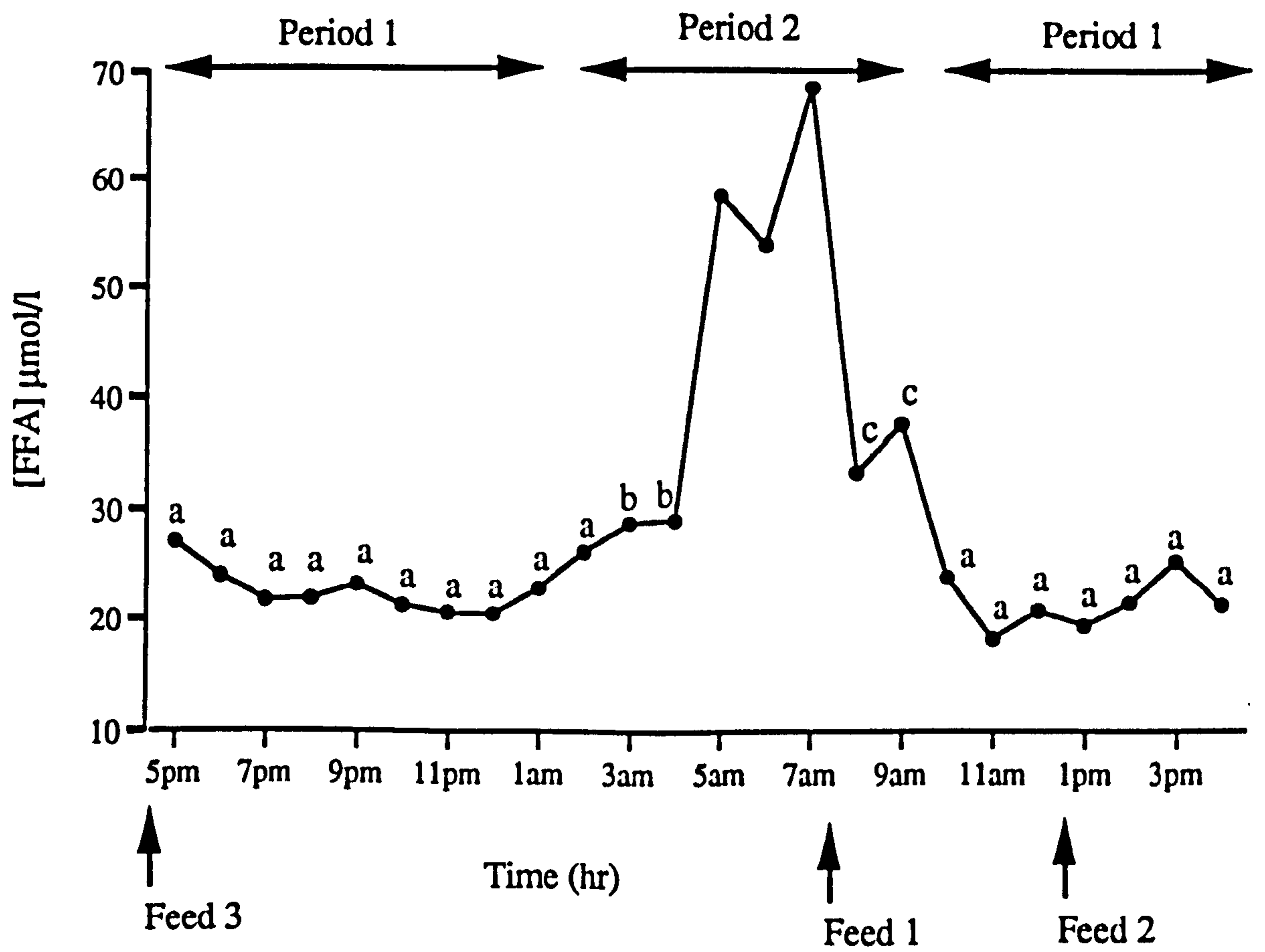
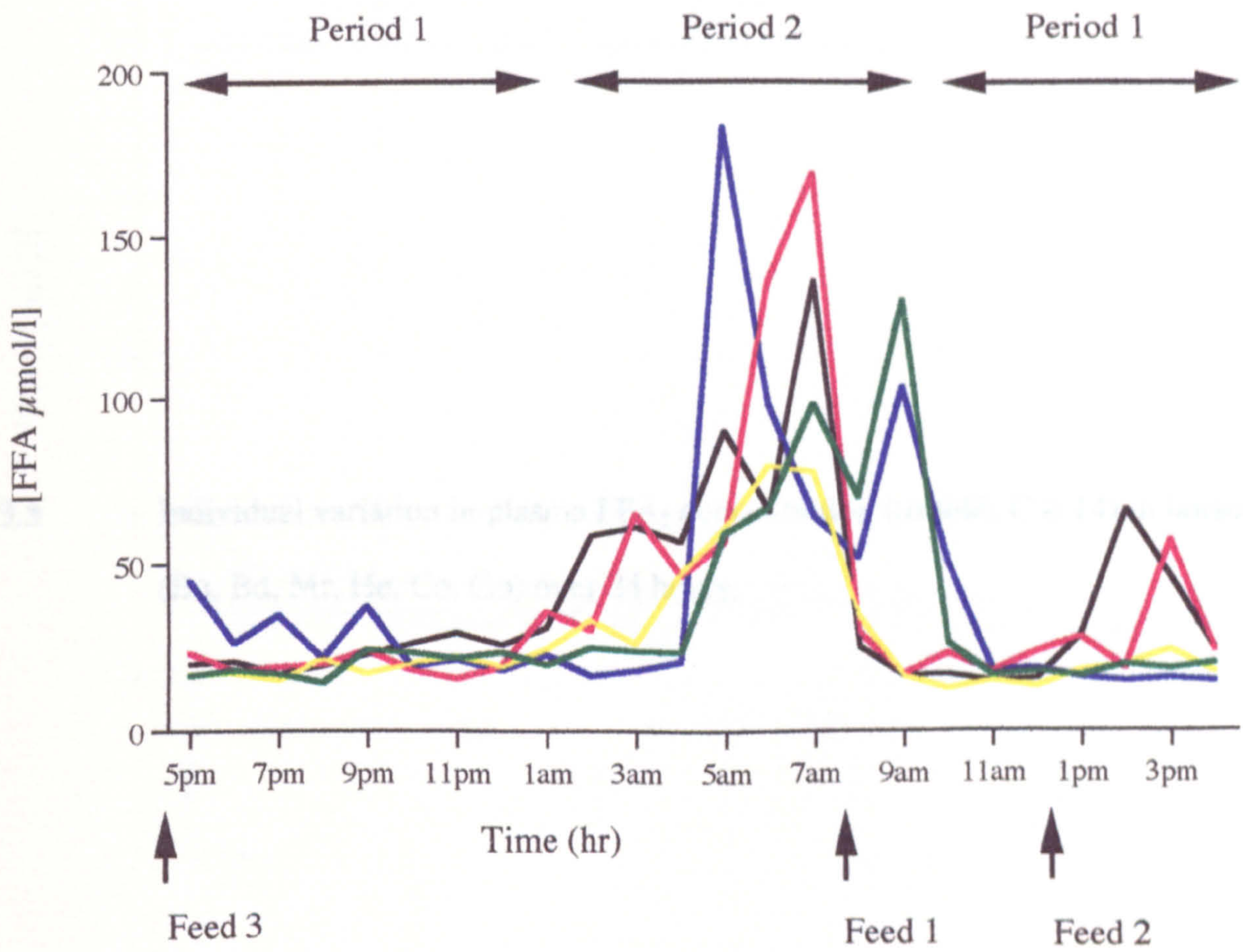


Fig 3.4 Individual variation in plasma FFA_t concentration (μmol/l, C ≥ 14) in horses (Lb, Li, Hl, Sn, Pn) over 24 hours.



— Li — Sn — Ls
— Pn — Ho

Fig 3.5 Individual variation in plasma FFA_t concentration (μmol/l, C ≥ 14) in horses (Bo, Bd, Mr, He, Co, Go) over 24 hours.

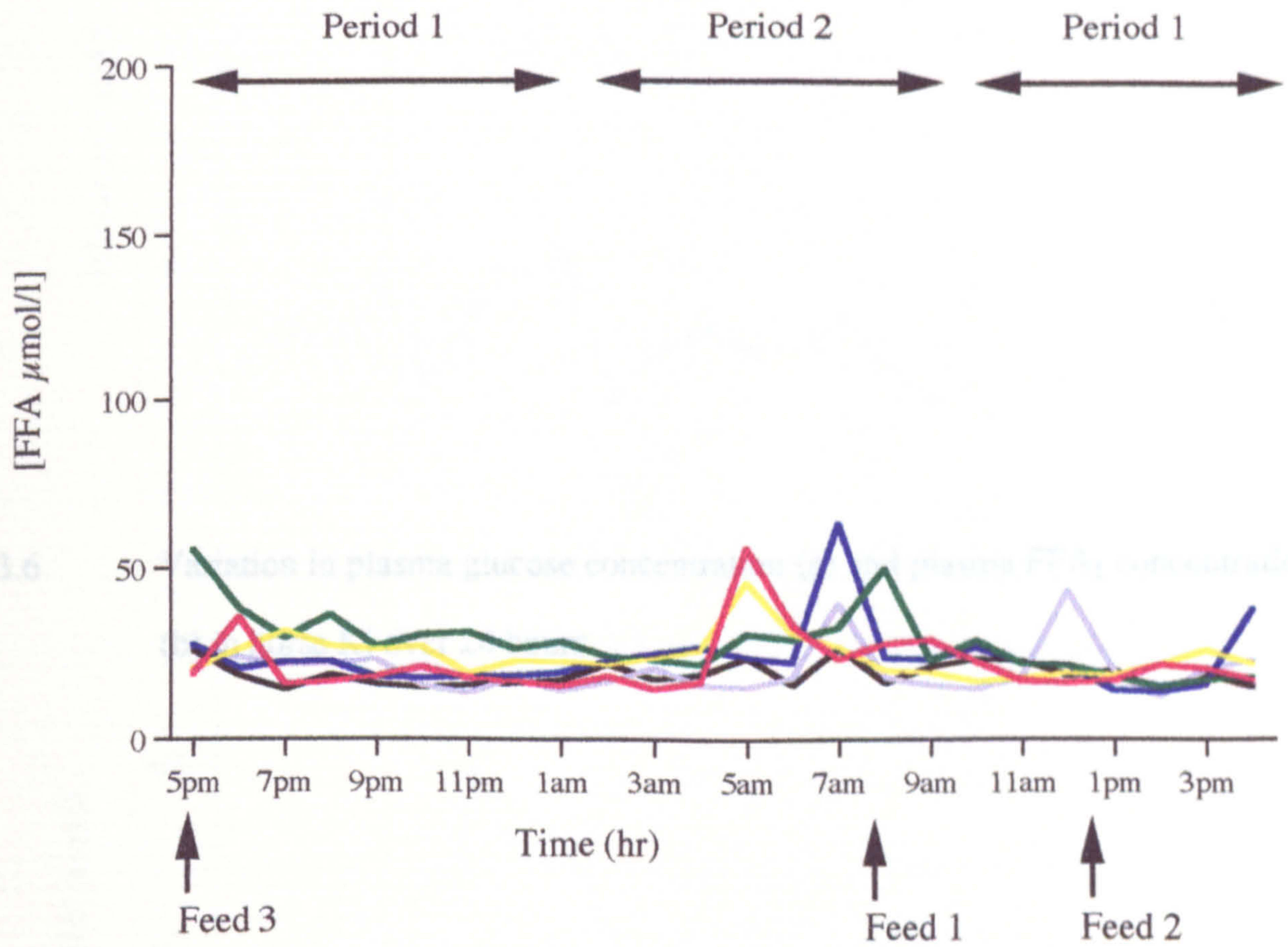
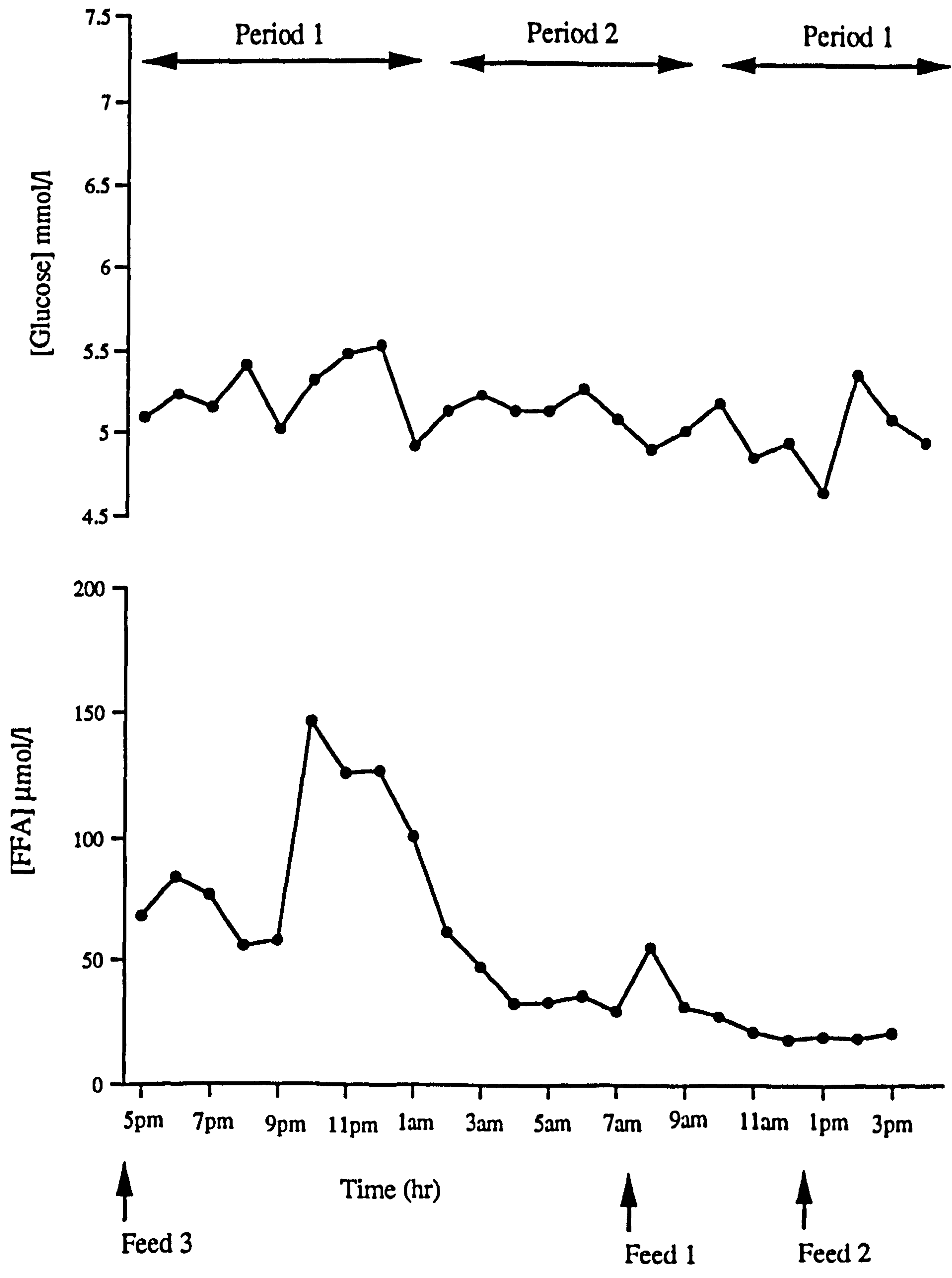


Fig 3.6

— Bo	— Bd	— Mr
— He	— Co	— Gl

Fig 3.6 **Variation in plasma glucose concentration (a) and plasma FFA_t concentration (b) in horse Kj over 24 hours**



Discussion

During normal feeding the most abundant FFA found in this group of horses were palmitic, linoleic, oleic, stearic and linolenic acids in decreasing order of abundance. These results differ slightly from those of previous authors. Palmitic, oleic, stearic and myristic acids were reported by Luther *et al* (1981) to be the most abundant FFA in equine plasma in decreasing order of abundance, constituting 96.1% of the total plasma concentration. In contrast, Rose (1982) reported that during exercise and food deprivation the predominant FFA, in order of abundance were, oleic, palmitic, linoleic and linolenic acids, which together constituted 88% of the total. It is probable that the minor differences observed in the plasma FFA profile between these studies reflects differences in the respective diets of the animals used. The plasma FFA profile is largely influenced by that of adipose tissue, which in the non-ruminant, is affected by the dietary FFA composition. The FFA profile of adipose tissue in grass or oat fed horses reflects the FFA composition of the relative diet (Shoreland 1962; Shoreland *et al* 1952). The adipose tissue of grass fed horses contained 17% linolenic and 4% linoleic acid as compared with 2% linolenic and 22% linoleic acid in the adipose tissue of horses fed oats.

Blood glucose concentration showed a significant increase 1.5-3.5 hours post feeding. Plasma glucose concentration has previously been reported to peak in horses between 4-8 hours following a meal (Frape 1986). The time at which the peak in blood glucose following a meal is observed will depend on the nature of the carbohydrate component of the diet, i.e. the relative amounts of complex carbohydrate to simple sugars, since the latter are digested and absorbed much more rapidly (Frape 1986). The observed peak in plasma glucose concentration, following all feeds in this study, may have been slightly earlier than previously reported due to a higher soluble carbohydrate content of the diet.

The sharp rise in plasma FFA_t concentration observed in the early hours of the morning was much greater than could be accounted for by random variation recorded during period 1. Zanzinger *et al* (1994) similarly reported that during a 24 hour sampling period plasma FFA

concentration increased significantly, in the early hours of the morning before feeding, in draught zebu and simmental oxen. It is possible that the increase in FFA_t concentration occurred as a result of a reduction in plasma glucose concentration during the night. Mobilisation of FFA may have occurred as a result of reduced inhibition of lipolysis by a lower circulating level of insulin. Insulin is a potent anti-lipolytic hormone (Hales *et al* 1978), which probably exerts its effect by influencing cellular levels of c-AMP. This in turn leads to dephosphorylation of HSL (Belfrage 1985; Nilsson *et al* 1980). Fig 3.1, however, indicates that an adequate supply of forages ensured that glucose homeostasis was maintained overnight. Forages which are rich in fibre extend the absorptive period. Horses although essentially non-ruminants have the ability to digest structural carbohydrates such as cellulose and hemicellulose in the large intestine, mainly in the caecum. Symbiotic bacterial colonisation of the caecum enables the digestion of fibre with the subsequent production of VFA (Bergman 1990). VFA provide a substantial energy source which ensures that glucose homeostasis is maintained overnight (Hintz *et al* 1971; Pethick *et al* 1993).

An increased plasma cortisol concentration may have influenced FFA mobilisation. Cortisol increases the sensitivity of the adipose tissue to the lipolytic hormones. For a given concentration of lipolytic hormone, the rate of lipolysis is higher in the presence of cortisol (Fernandez and Saggerson 1978). Cortisol has been reported to exhibit a diurnal rhythm in the horse; its concentration reaching a maximum in the early hours of the morning, prior to waking (Hoffsis *et al* 1970, Larsson *et al* 1979). The time at which the peak in cortisol concentration is reported to occur however, varies. Larsson *et al* (1979) reported that the peak in cortisol occurred at 6am and Hoffsis *et al* (1970) at 8am. In Man, cortisol has a latent effect on fat mobilisation with the response taking several hours to be elicited (Guyton 1992). A similar effect on fat mobilisation in horses would suggest that it is unlikely that cortisol was responsible for the observed increase in plasma FFA. At 5am there was already a significant increase in plasma FFA in most of the horses, which if due to an earlier rise in cortisol would have meant an increase in cortisol at about 3am. However, cortisol cannot be dismissed as the cause of the increase in FFA since it was not measured.

It is likely that the increase in plasma FFA in the early hours of the morning occurred in preparation for the increased energy demands of wakefulness as a result of the energy mobilising actions of the lipolytic hormones. Mobilisation of adipose triglycerides resulting in an increased plasma FFA concentration occurs in situations of food deprivation and hypoglycaemia (Rose 1982), sustained exercise (Lucke and Hall 1978) and stress (Anderson and Aitken 1977). Adipose triglyceride lipase or HSL catalyses the flux generating step in the release of FFA from adipose tissue (Bulow 1988). In man, the main lipolytic hormones are adrenaline and nor-adrenaline, although thyroid stimulating hormone, parathyroid hormone, cortisol and thyroxin may also exert an effect (Hales *et al* 1978).

The findings of this study suggest that a proportion of horses exhibit an early morning increase in plasma FFA concentration, which cannot be explained by normal variation in the plasma FFA concentration throughout the rest of the day. The magnitude of the increase is highly variable between individuals and its cause is unknown and requires further investigation.

Chapter 4

A Comparison of the Lipolytic and Anticoagulative Effects of Heparin and Pentosan Polysulphate

Introduction

Triglyceride emulsions or fat loaded meals have previously been used in other species in conjunction with heparin (HEP), to elevate plasma FFA prior to exercise (Costill *et al* 1977; Hickson *et al* 1977; Ravussin *et al* 1986). They have provided a useful tool for the investigation of fuel utilisation during exercise and for studying the effects of FFA chain length and degree of saturation upon fat utilisation (Vukovich *et al* 1993). The aim of the present study was to develop a model for the pre-exercise elevation of plasma FFA in the horse; in order that it could be used in investigations of fat metabolism during exercise.

The rationale for the use of HEP in previous studies is that it has the ability to cause the release of LPL and hepatic lipase (HL) into the circulation (Fischer *et al* 1982; Barrowcliffe *et al* 1986, Barrowcliffe *et al* 1988). The presence of LPL and to a lesser extent HL in the plasma, facilitates the hydrolysis of infused exogenous triglyceride or triglyceride of dietary origin. A pilot study carried out in two horses suggested that HEP administration, at a dose that had previously been shown to result in release of LPL and HL in ponies (Watson *et al* 1992a), had an unacceptably pronounced effect on blood coagulation. HEP has previously been advocated for the treatment of hyperlipaemia in ponies, ostensibly to increase intravascular lipolysis (Schotman and Kroneman 1969; Field 1988). However, at the doses used it is reported to result in large alterations in blood coagulation, which may cause haemorrhage (Pearson and Maas 1990).

The following study was undertaken to compare the lipolytic and anticoagulative properties of both HEP and a related substance, pentosan polysulphate (PP) in the thoroughbred horse. When compared to HEP on a weight for weight basis, PP has been reported in other species to be less effective at clearing lipaemic plasma but to have a markedly reduced effect upon clotting function, as measured by APTT (Cucurachi *et al* 1967 ; Scully *et al* 1983). The aim of the study was therefore to investigate whether the latter could be used in conjunction with a triglyceride emulsion, to achieve increases in pre-exercise plasma FFA concentration with reduced effects upon clotting function compared to HEP in the horse.

PP is a low molecular weight (4000-6000 Dalton) synthetically manufactured HEP analogue and is a polymer of β -D-xylopyranose-residues. HEP and PP are structurally and biochemically very similar (Brunaud *et al* 1967). In human and rat studies, PP has been shown to exhibit similar biological properties to HEP (Brunaud *et al* 1967; Maffrand *et al* 1991). Both substances exhibit anticoagulative properties and have the ability to cause the release of LPL and HL into the circulation (Fischer *et al* 1982; Barrowcliffe *et al* 1986, Barrowcliffe *et al* 1988). LPL and HL are responsible for the lipolysis of lipoprotein associated triglycerides. LPL acts on triglyceride-rich lipoproteins (Robinson and French 1953), whilst HL is involved in the intravascular remodelling of low and high density lipoproteins. Although the exact function of HL is unclear it is probably responsible for the removal of cholesterol and phospholipids from low and high density lipoproteins as they pass through the liver (Jansen *et al* 1980). LPL is located on the luminal surface of blood vessels of extra-hepatic tissue, predominantly adipose tissue and skeletal and cardiac muscle (Robinson and French 1953). It is bound in association with sulphated glycosaminoglycans. A structural similarity between HEP and the endogenous molecules to which LPL is bound facilitates its release into the plasma as a result of HEP administration (Robinson 1963). It is likely that the injected HEP competes with the sulphated glycosaminoglycans for the LPL enzyme (Robinson 1963). Similarly HL is mainly found bound to endothelial cells in the liver (Jansen 1980). It is assumed that the ability of PP to release both LPL and HL into the circulation occurs by a similar mechanism to that of HEP.

Both HEP and PP prolong the clotting time of blood. HEP in the presence of a co-factor (anti-thrombin III) delays the thrombin-fibrinogen reaction, but also acts as an anti-prothrombin, preventing the conversion of prothrombin to thrombin. HEP is also a powerful inhibitor of Factor Xa, (for review see Biggs 1972). The mode of action of PP is similar to that of HEP in that it inhibits Factor Xa and its precursors in the endogenous coagulation cascade. However, unlike HEP this effect is independent of antithrombin III (Fischer *et al* 1982b).

Materials and Methods

A comparison between HEP and PP was carried out using a group of 6 thoroughbred horses during four experimental sessions, separated by a minimum period of one week. Horses were weighed on the morning of each session and normal feed and water was supplied. No exercise was undertaken on the day of any of the sessions. A dose of HEP that had previously been shown to release LPL and HL in ponies (Watson *et al* 1992a) was used. Since PP on a weight for weight basis is reported to be less effective at clearing lipaemic plasma a dose of PP which was approximately 3 times that of HEP was given, in order to achieve a suitable increase in plasma FFA.

The lipolytic and anticoagulative effects of HEP and PP were assessed by:

- a) Administration of HEP or PP in isolation.
- b) Co-administration of HEP or PP with an exogenous triglyceride emulsion.

In all instances HEP and PP were administered intravenously on a dry weight basis in relation to body weight (bw).

Session a

Effect on total lipase activity and clotting function

Horses were given a single bolus injection of either HEP (0.39 mg/kg bw; CP Pharmaceuticals Ltd, Wrexham) or PP (1.3 mg/kg bw; Fibrizym, Bene Arzneimittel GmbH, Germany). The order of treatment was randomised and was separated by a period of at least one week. The injections were given through an 18 g catheter inserted into the right jugular vein under local anaesthesia. Venous blood samples were obtained by venepuncture of the left jugular vein. Blood was dispensed into tubes containing either sodium citrate or lithium heparin for the analysis of APTT and plasma T. Lip activity, respectively. Only 5 of the 6 horses underwent this session since the 6th horse was receiving unrelated medication.

Session b

Effect on triglyceride clearance and FFA increase

Horses were given either a bolus injection of HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw) immediately followed by an infusion of a triglyceride emulsion (400 mg/kg bw) over approximately 6 minutes. The order of treatment was again randomised and was separated by a period of at least one week. The triglyceride emulsion (Iverlip 20; Clintec, France) consisted of soya oil (20%), egg phospholipids (1.2%), glycerol (2.5%), sodium oleate (0.03%) and water. The solutions were introduced through an 18 g catheter inserted into the right jugular vein whilst venous blood samples were withdrawn through a catheter inserted into the left jugular vein. Separate aliquots of the plasma were stored for the analysis of FFA, triglyceride and glycerol in order to prevent lipolysis of triglycerides during freeze-thawing cycles.

FFA were analysed on a Kone Specific autoanalyser using a commercially available kit. Sample blanks were run for each sample to correct for interference resulting from lipaemia. Triglyceride, glycerol, T. Lip activity and APTT were analysed as described previously (Chapter 2). All samples were analysed in duplicate for all analyses.

Statistical analysis

A 2-factor ANOVA for repeated measures was used to identify differences in T. Lip, APTT and FFA concentration both within and between treatments. Multiple comparison tests using Fisher's PLSD test were then undertaken in instances where significance was detected. Significance was declared at a level of ($p < 0.05$). A logarithmic transformation of the APTT data was undertaken due to marked differences in the individual clotting response to HEP and thus inequality of variance between the two treatment groups. ANOVA and multiple comparison tests were then applied as before. The clearance of infused triglyceride from the plasma, between 10-30 minutes post infusion, was compared between sessions using a paired Student's t test. A paired Student's t test was also used to compare the peak FFA concentration achieved between sessions and the time taken to achieve peak concentration.

Results

Effect on total lipase activity and clotting function

Pre-injection T. Lip activity was negligible ($< 0.1 \mu\text{mol/ml/hr}$). Post-injection T. Lip activity was significantly increased 10 minutes following either HEP or PP administration ($p < 0.001$) and remained elevated for up to 2 hours (Figs 4.1, 4.2). There was no significant difference in post-injection T. Lip activity between the HEP and PP sessions ($p > 0.05$). Mean peak T. Lip activities were 6.9 (range 3.6-9.3) and 7.8 (range 5.6-12.0) $\mu\text{mol/ml/hr}$ for the HEP and PP administrations, respectively. The variation in peak T. Lip activity between horses was large, though relatively constant between treatments (Fig 4.2).

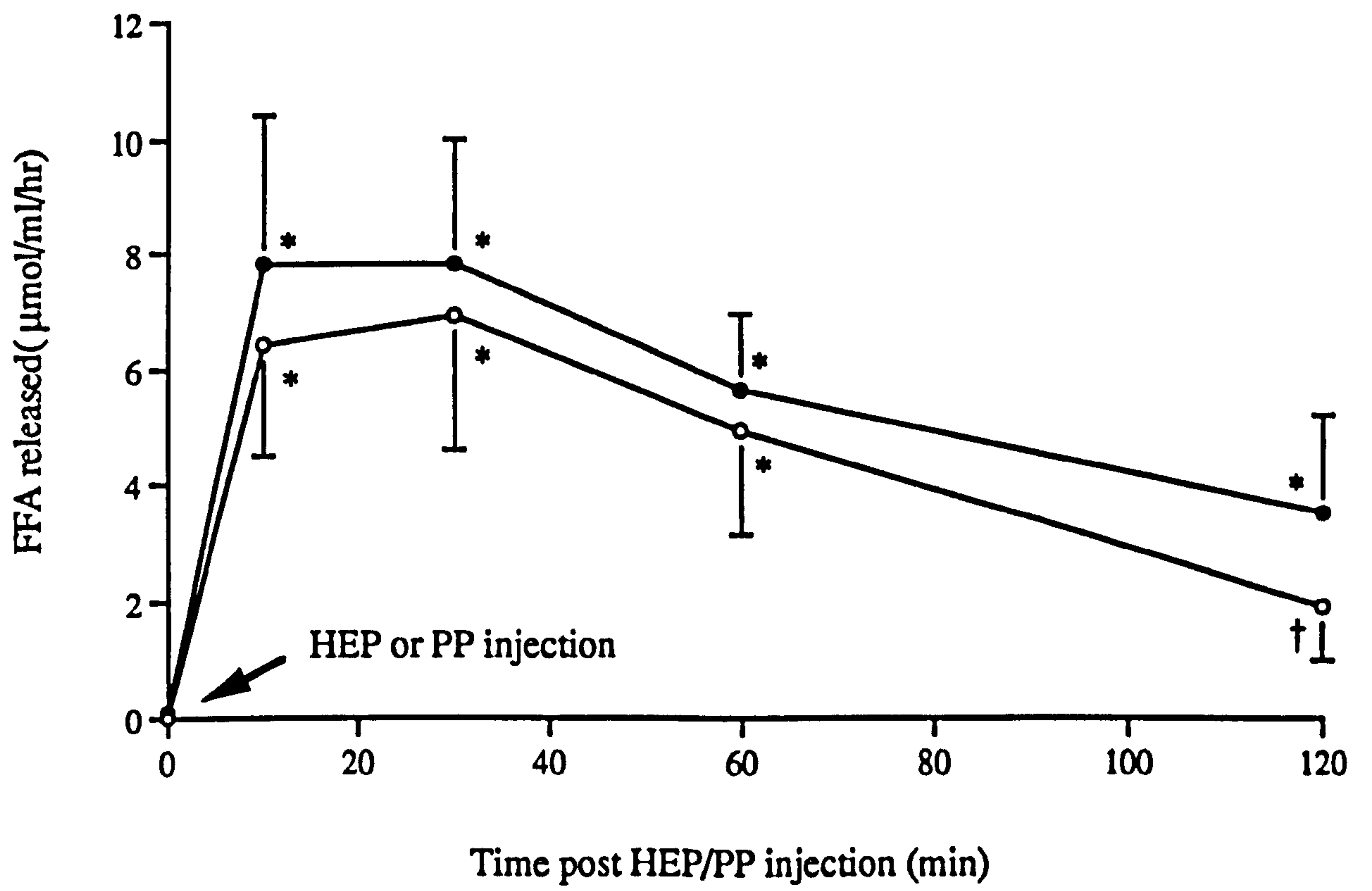
APTT was significantly increased following either injection of HEP or PP ($p < 0.001$). The APTT remained significantly elevated throughout the 2 hour sampling period after the administration of HEP ($p < 0.05$). APTT was, however, no longer significantly different from pre-administration levels after 1 hour during the PP session. APTT was significantly higher following the administration of HEP compared to PP throughout the 2 hour sampling period ($p < 0.05$), (Table 4.1). HEP resulted in a mean 14.0 ± 6.5 fold increase in APTT compared to a mean 1.5 ± 0.1 fold increase with PP.

Lipolytic effect in the presence of raised plasma triglycerides

Plasma triglyceride concentration increased immediately following the infusion of Iverlip 20 ($p < 0.001$), returning to pre-infusion levels after 90 minutes (Fig 4.3). Mean peak triglyceride concentrations were 1272 and 1767 $\mu\text{mol/l}$ for HEP and PP, respectively when co-administered with Iverlip 20. The removal of triglyceride from the plasma appeared to be linear between 10 and 30 minutes post-infusion. During this period there was a marked variation in the triglyceride removal rate between horses and a marginal difference ($p < 0.05$) between sessions (Table 4.2).

Fig 4.1 Mean T.Lip activity (\pm SD, $\mu\text{mol FFA released/ml/hr}$) following an injection of either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw).

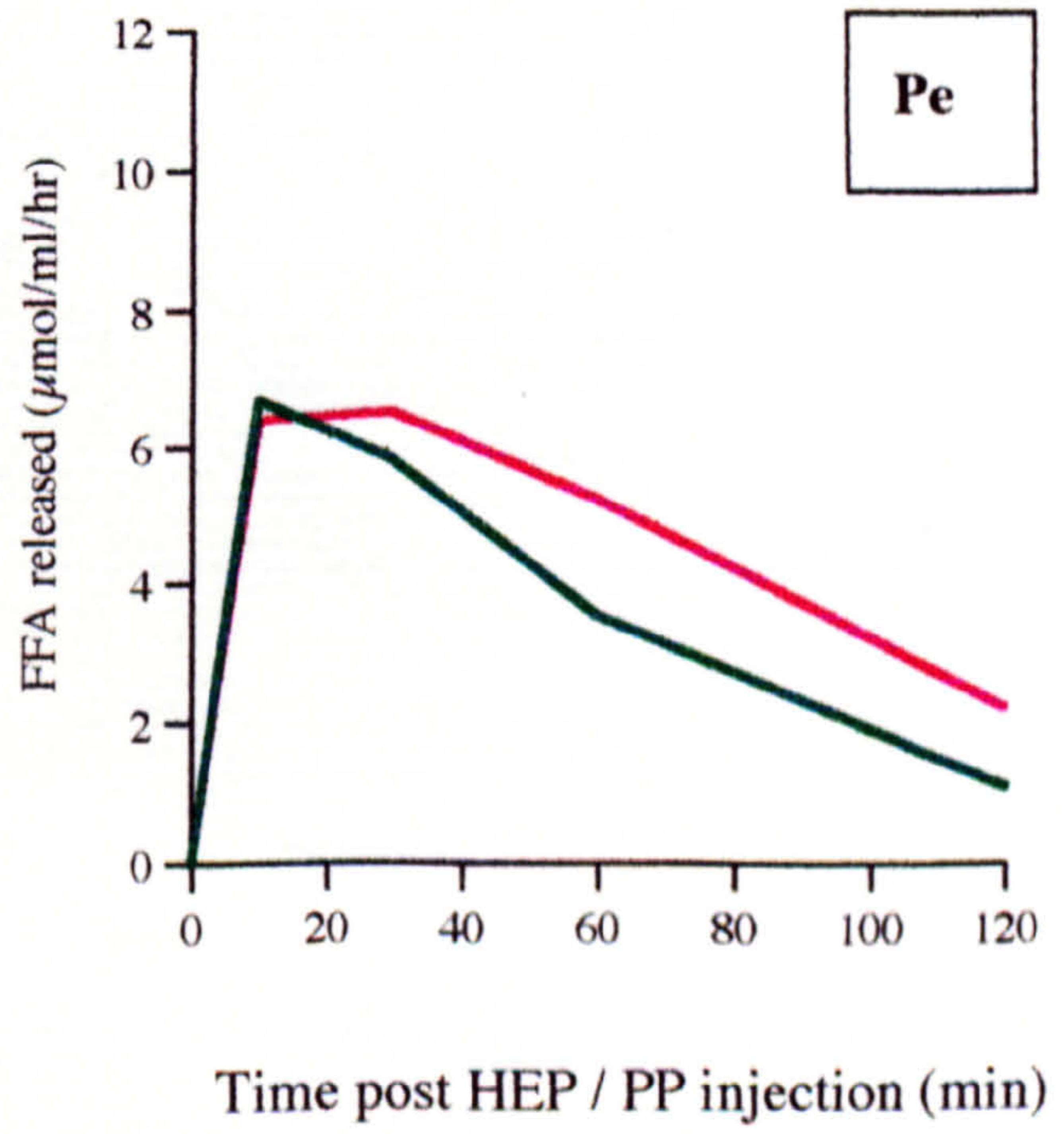
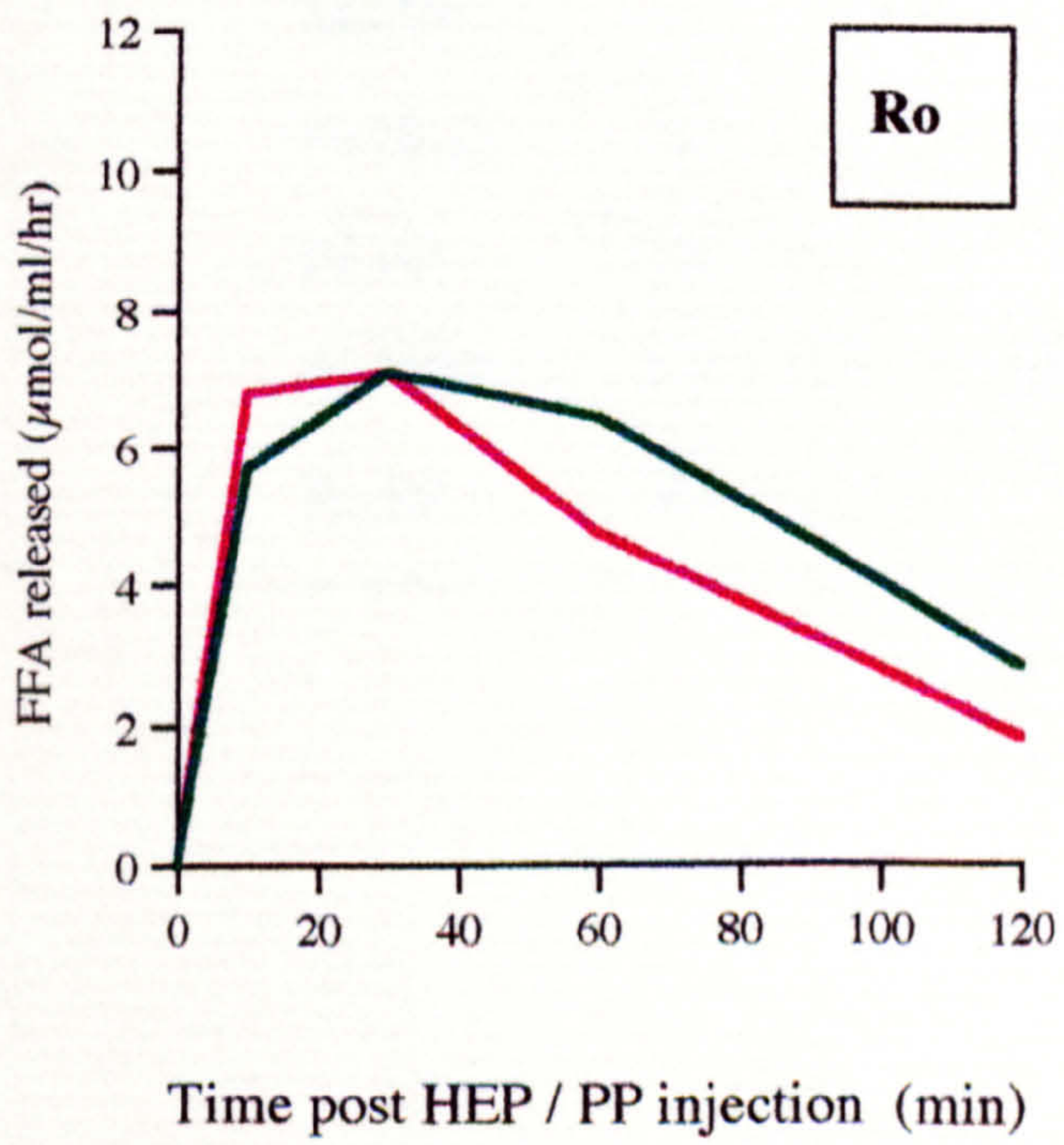
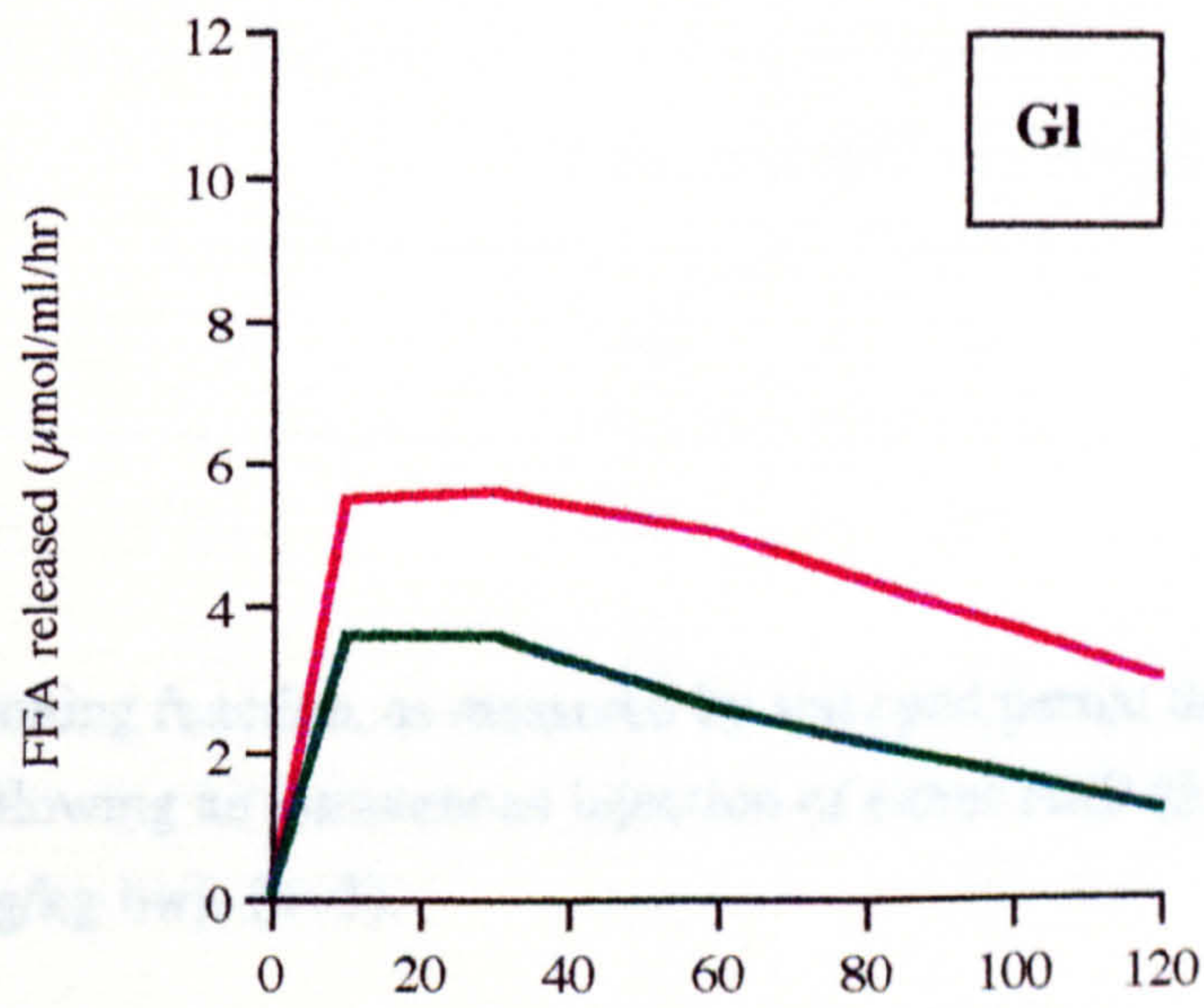
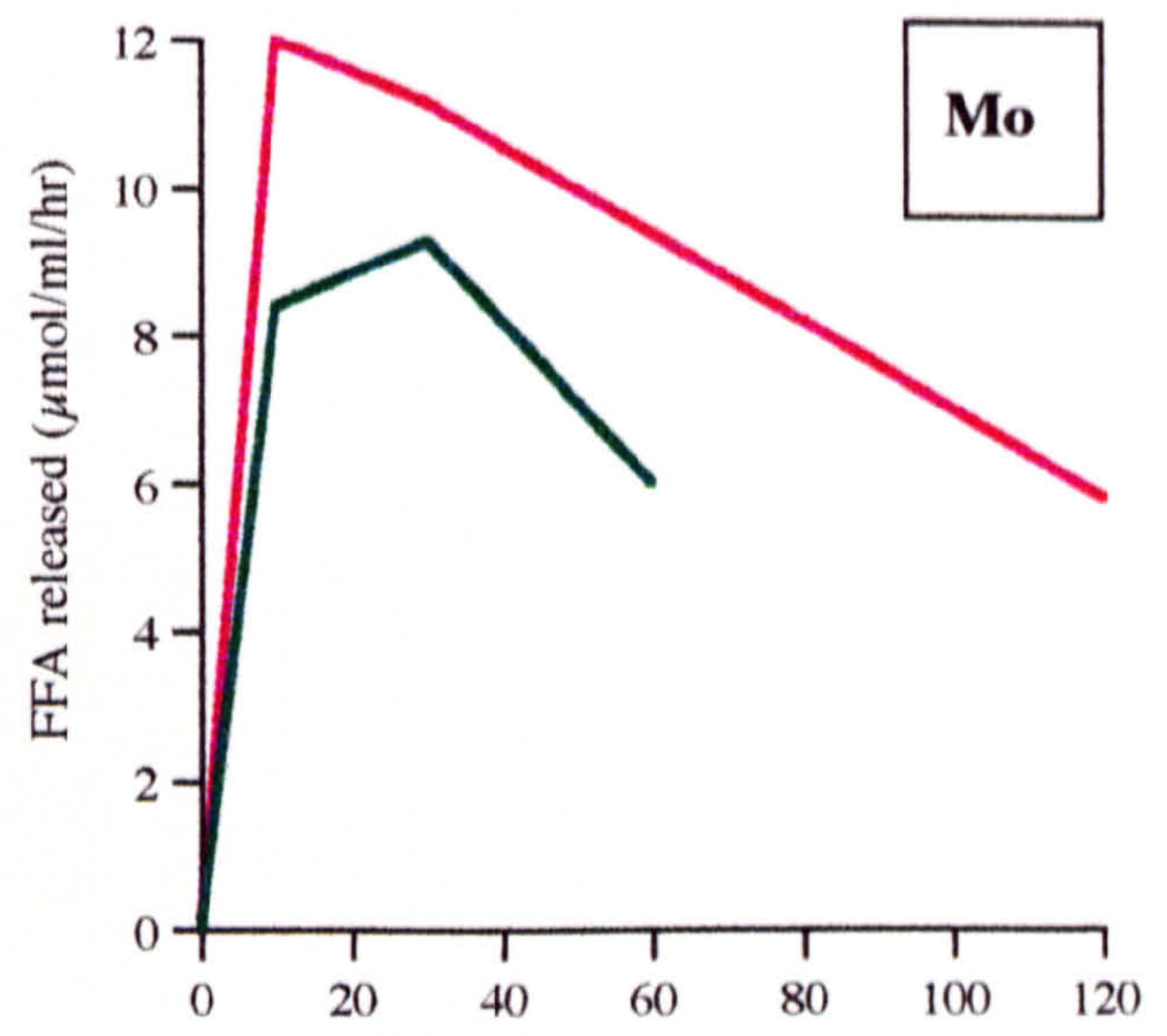
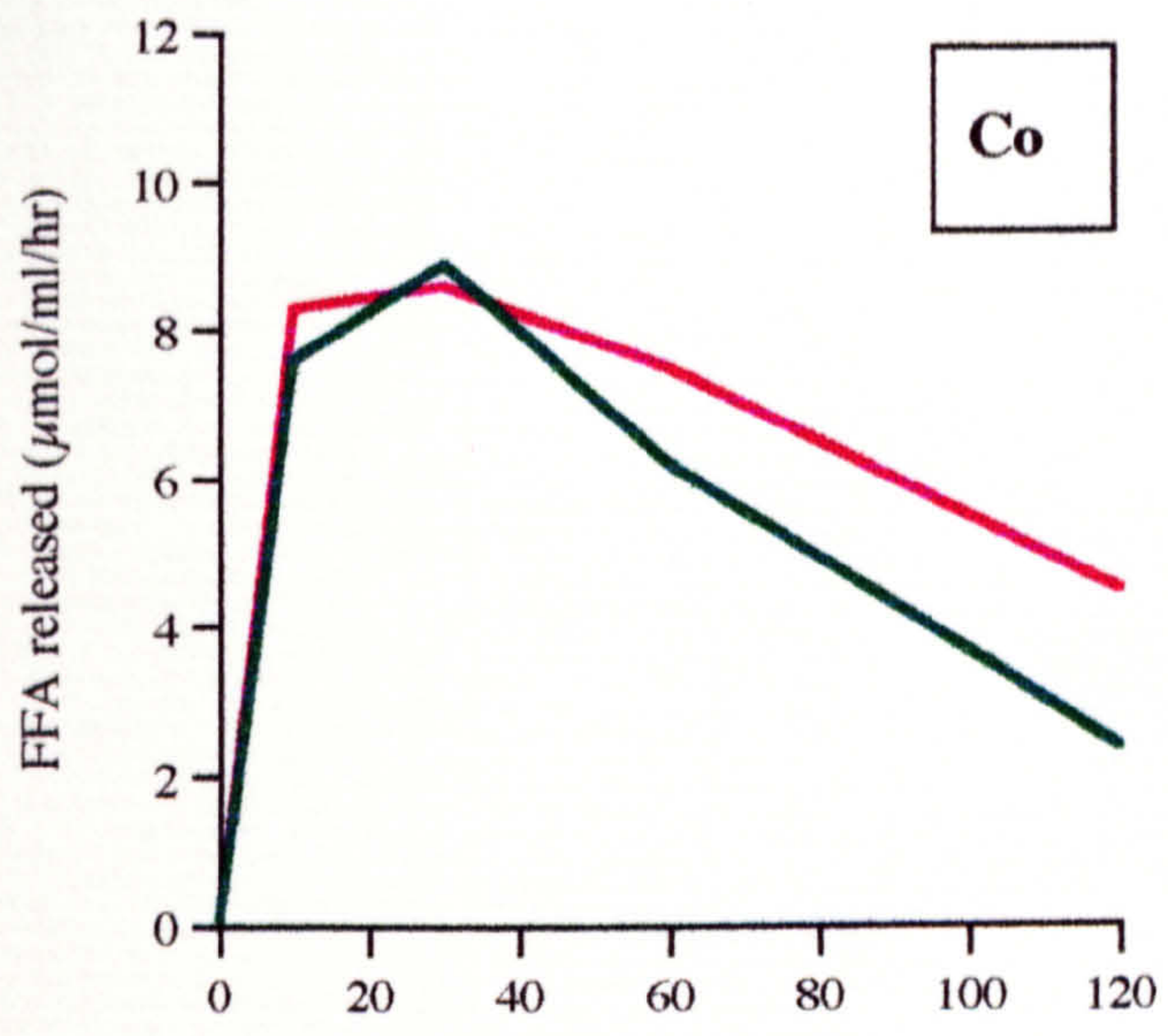
T. Lip activity at time points marked * and † respectively, were significantly different ($p < 0.001$), ($p < 0.05$) from pre-infusion activities, (n=5).



—●— PP

—○— HEP

Fig 4.2 Individual activities of plasma T. Lip activity ($\mu\text{mol FFA released/ml/hr}$) following an injection of either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw) in horses Co, Mo, Gl, Ro and Pe.



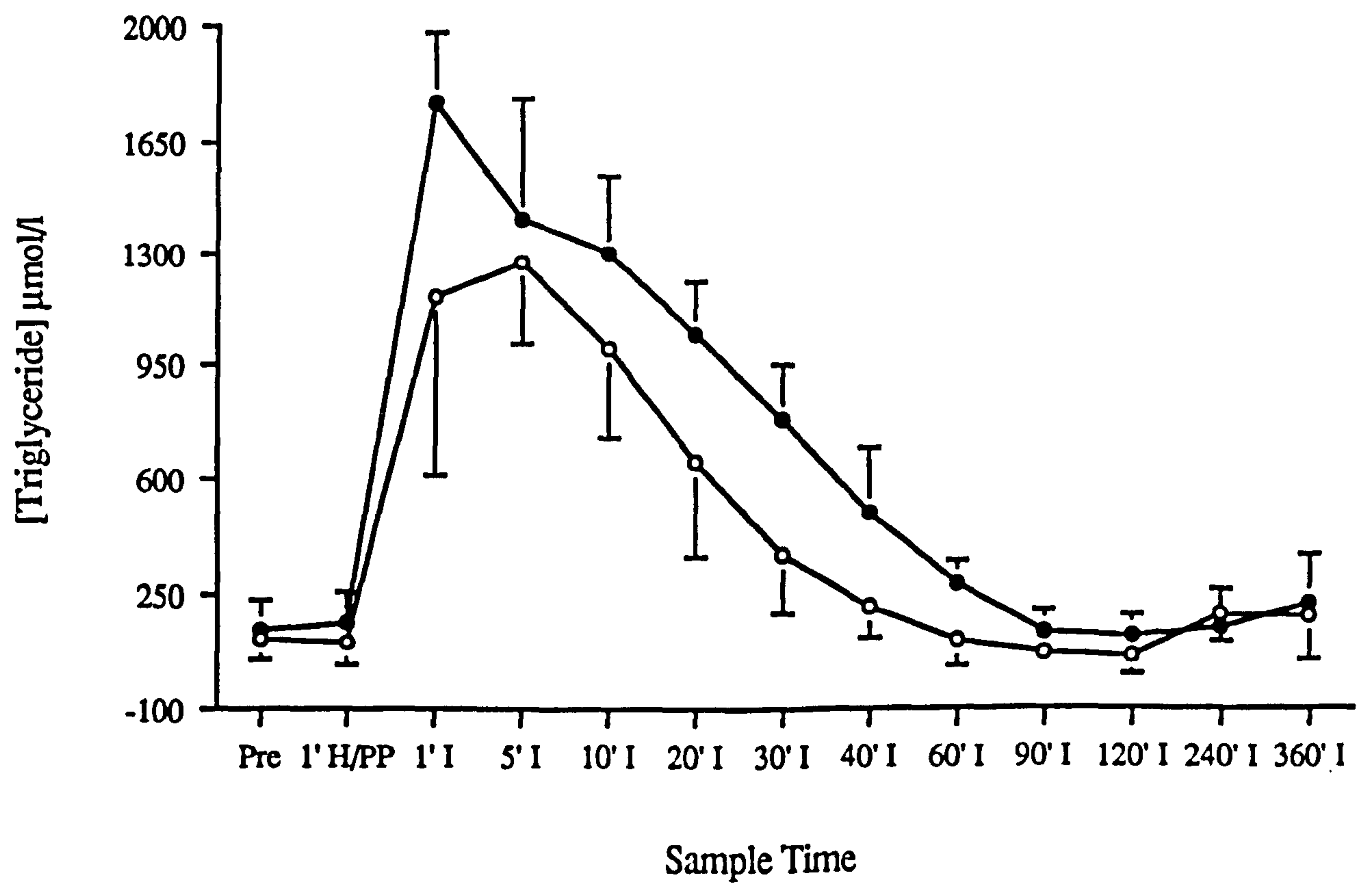
— HEP — PP

Table 4.1 **Clotting function, as measured by activated partial thromboplastin time (sec) following an intravenous injection of either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw), (n=5).**

Horse	Time post HEP or PP infusion (min)				
	0	10	30	60	120
Co HEP	34	229	171	156	75
Co PP	50	75	69	64	53
GI HEP	43	712	265	136	53
GI PP	50	85	73	64	51
Mo HEP	34	373	224	170	76
Mo PP	45	69	63	56	48
Pe HEP	33	363	314	189	78
Pe PP	46	66	61	55	49
Ro HEP	45	1000	844	520	141
Ro PP	48	79	81	72	62

Fig 4.3 Mean increase in plasma triglyceride concentration (\pm SD, $\mu\text{mol/l}$) following co-administration of Iverlip 20 and either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw), (n=6)

Pre	Pre administration
' H/PP	minutes post injection of HEP or PP
' I	minutes post infusion of Iverlip 20



—○— HEP
—●— PP

Table 4.2 Triglyceride removal rate between 10 and 30 minutes post-Iverlip infusion ($\mu\text{mol}/\text{min}$), (n=6).

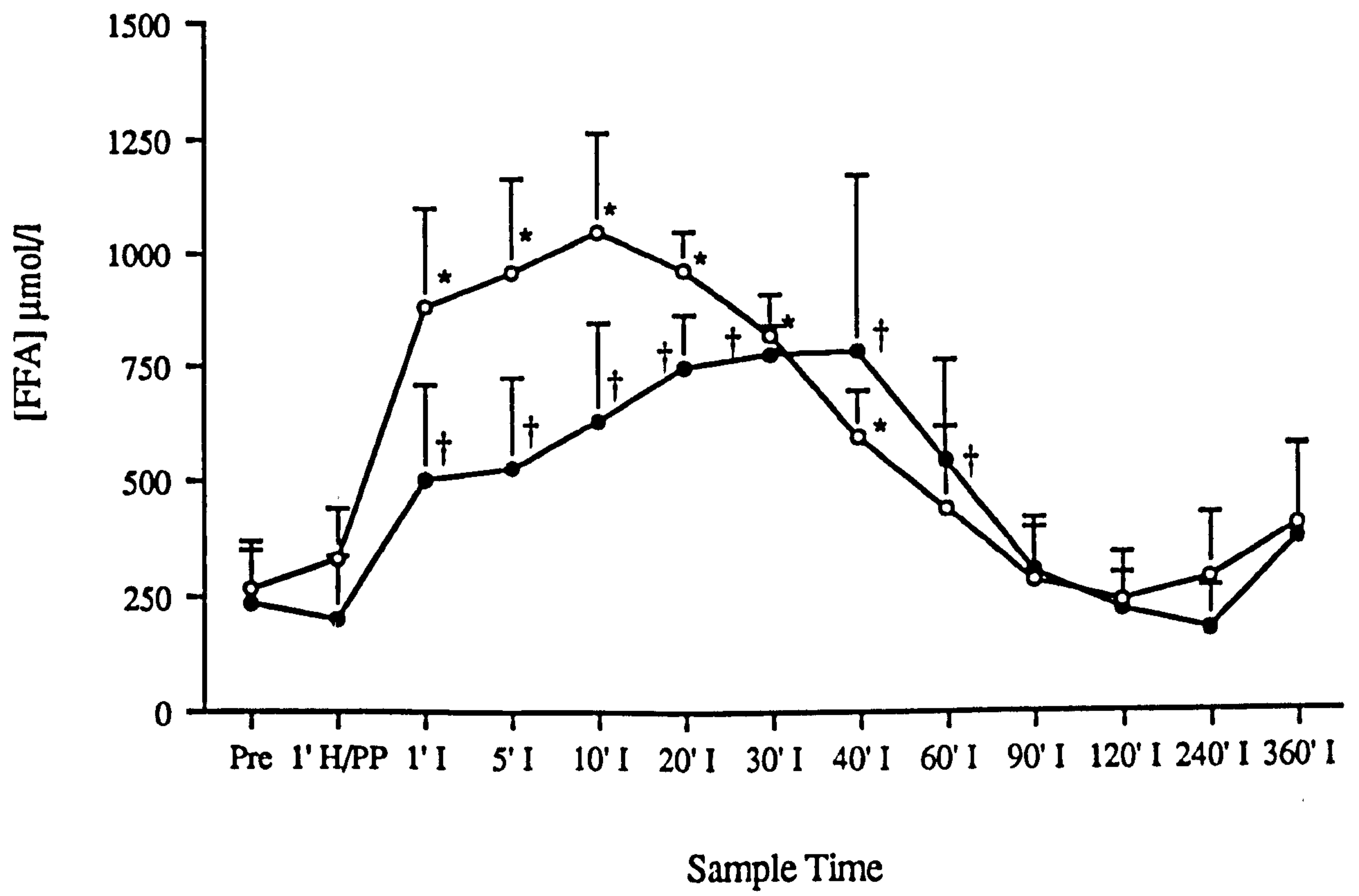
HORSE	Triglyceride removed ($\mu\text{mol}/\text{min}$)	
	HEP	PP
Co	19.9	21.4
Gl	48.3	38.7
Mo	29.6	27.7
Pe	35.2	24.5
Ro	31.6	26.6
Br	25.6	18.8
Mean \pm SD	31.7 \pm 9.7	26.3 \pm 6.9

Plasma FFA concentration increased significantly following the infusion of Iverlip in association with either HEP or PP ($p < 0.001$, Fig 4.4). However, there was no significant difference in the peak concentration reached between sessions. Mean peak concentrations were 1055 and 922 $\mu\text{mol/l}$ for the HEP and PP sessions, respectively. The increase in plasma FFA concentration was similar between horses (Fig 4.5). Peak FFA concentration was reached significantly earlier after the administration of HEP and Iverlip ($p < 0.05$). Peak FFA concentrations were reached in a mean time of 13 and 23 minutes for HEP and PP, respectively.

Changes in plasma glycerol concentration followed the same pattern of increase as the triglyceride concentration. The decline in glycerol concentration, however, was not as rapid as that of triglyceride (Fig 4.6).

Fig 4.4 Mean increase in plasma FFA concentration (\pm SD, $\mu\text{mol/l}$) following co-administration of Iverlip 20 and either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw). Time points marked * and † were significantly different from pre Iverlip and HEP or PP administration values, (n=6).

Pre	Pre administration
' H/PP	minutes post injection of HEP or PP
' I	minutes post infusion of Iverlip 20

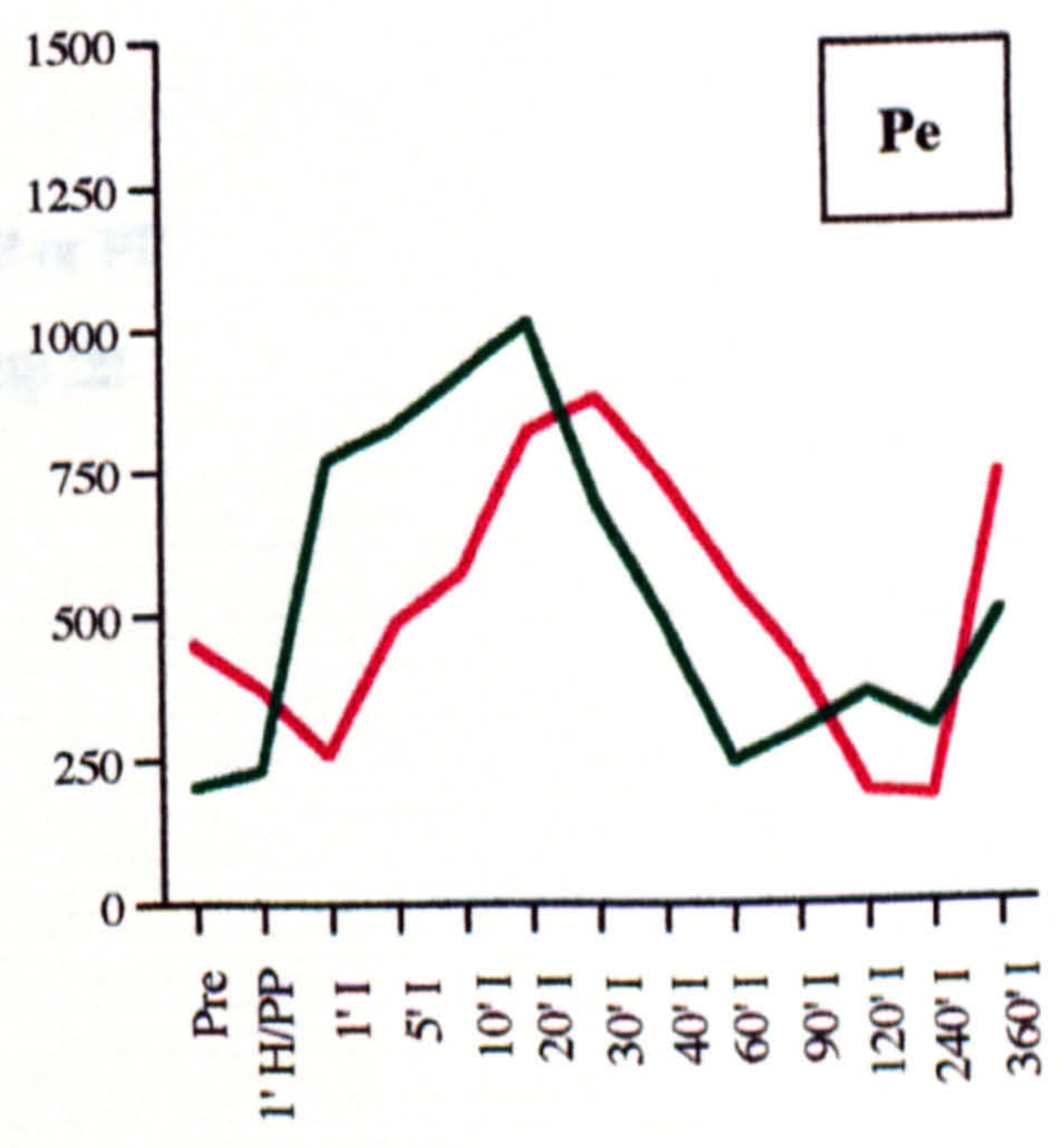
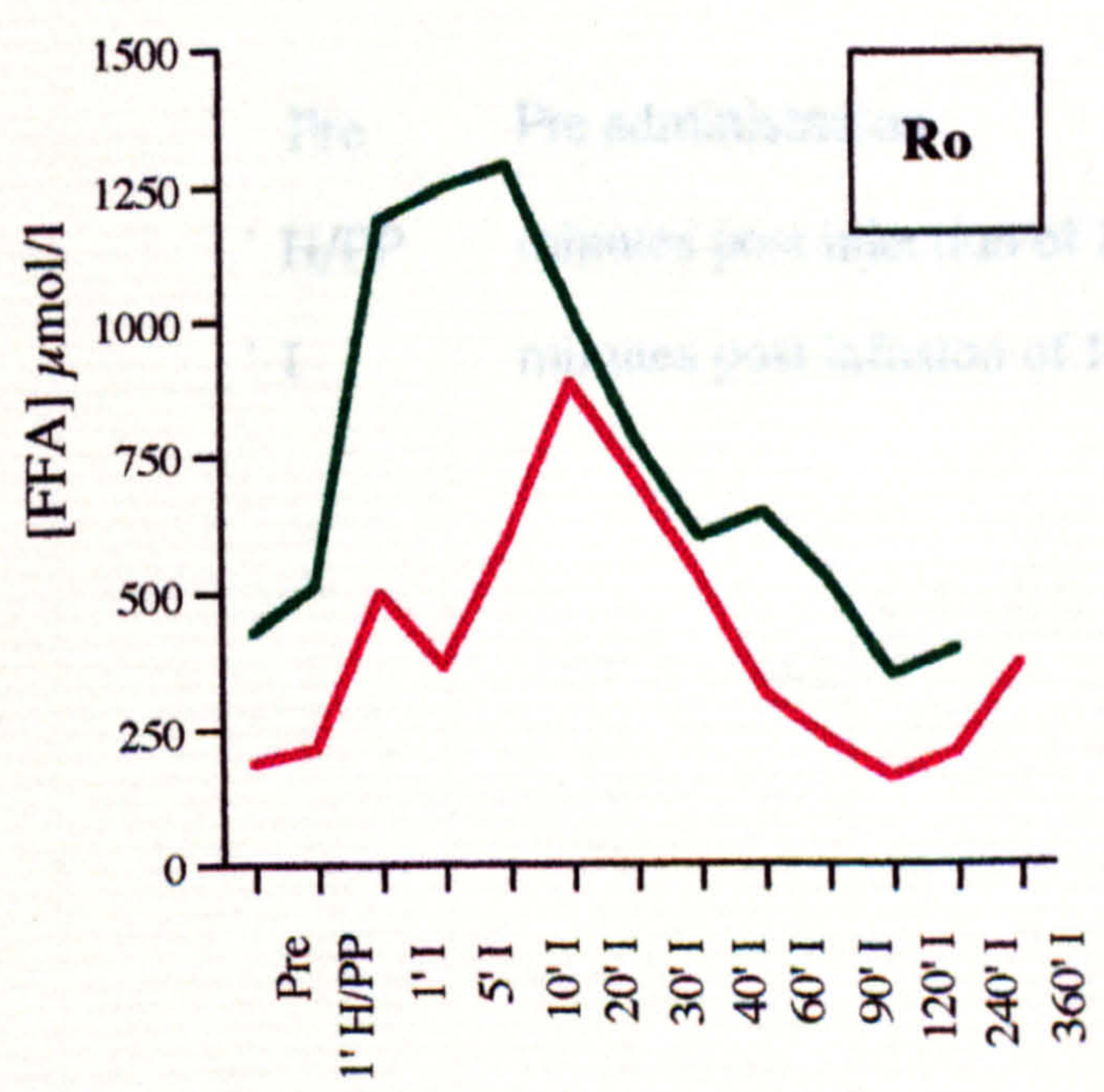
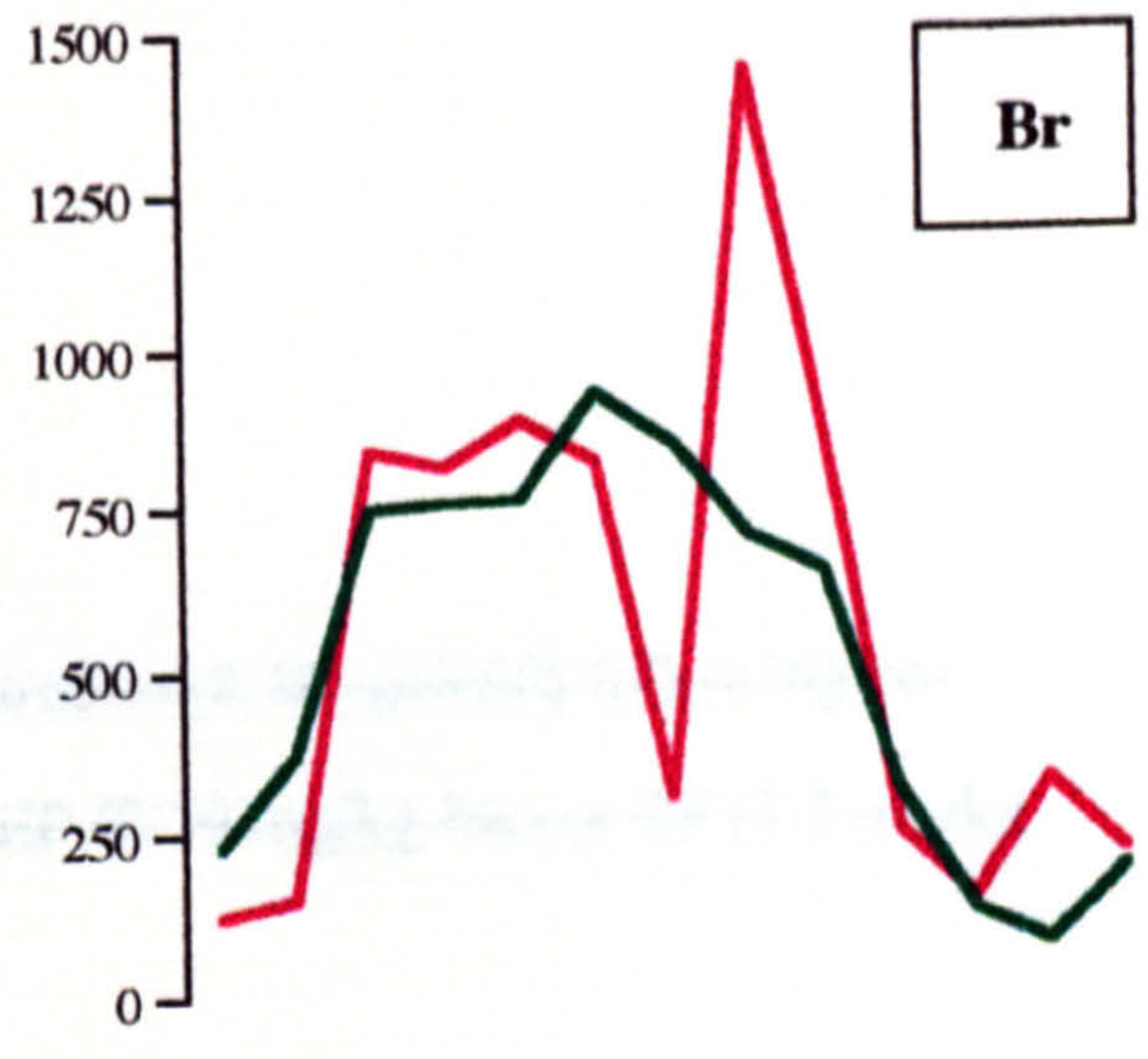
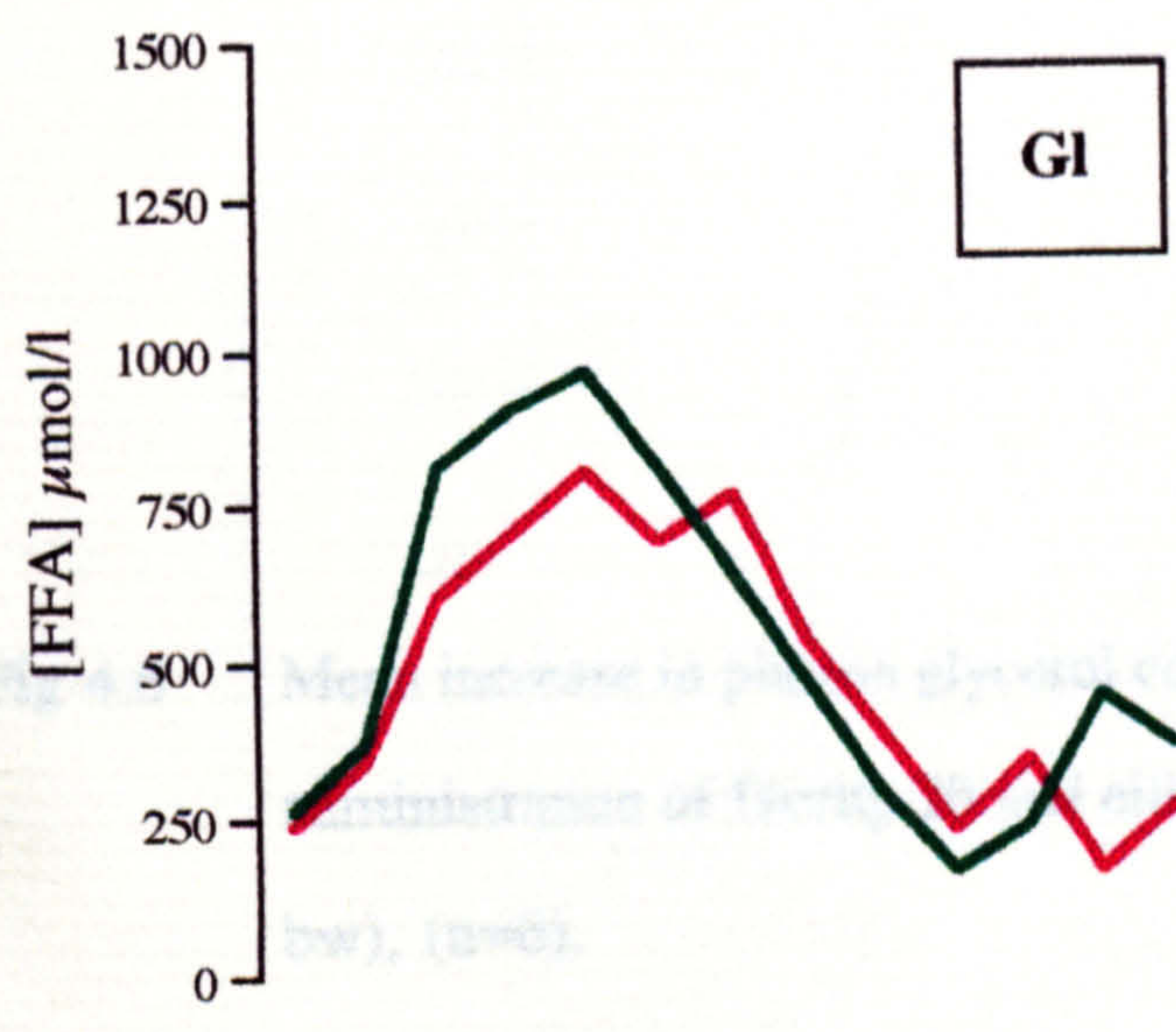
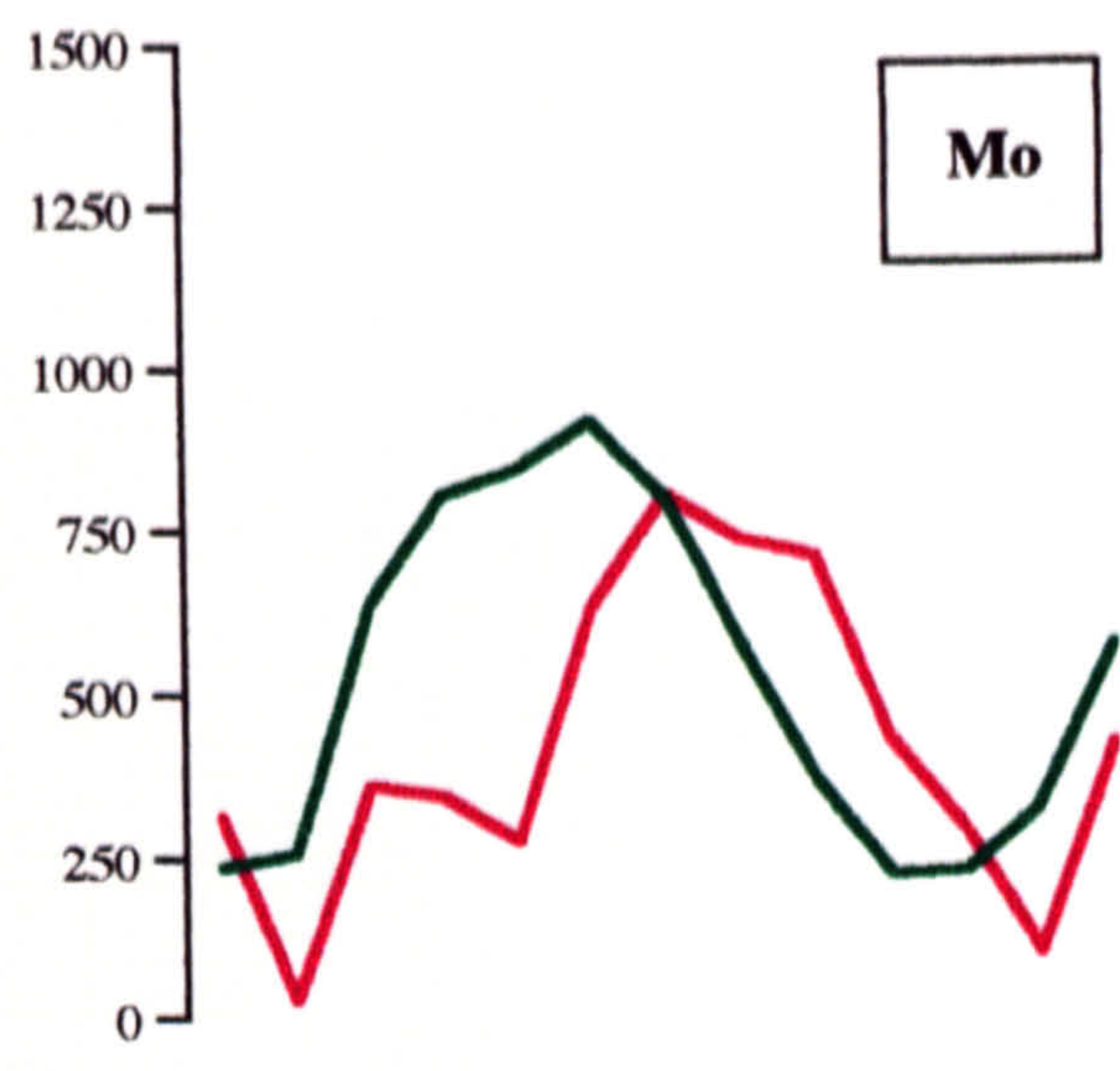
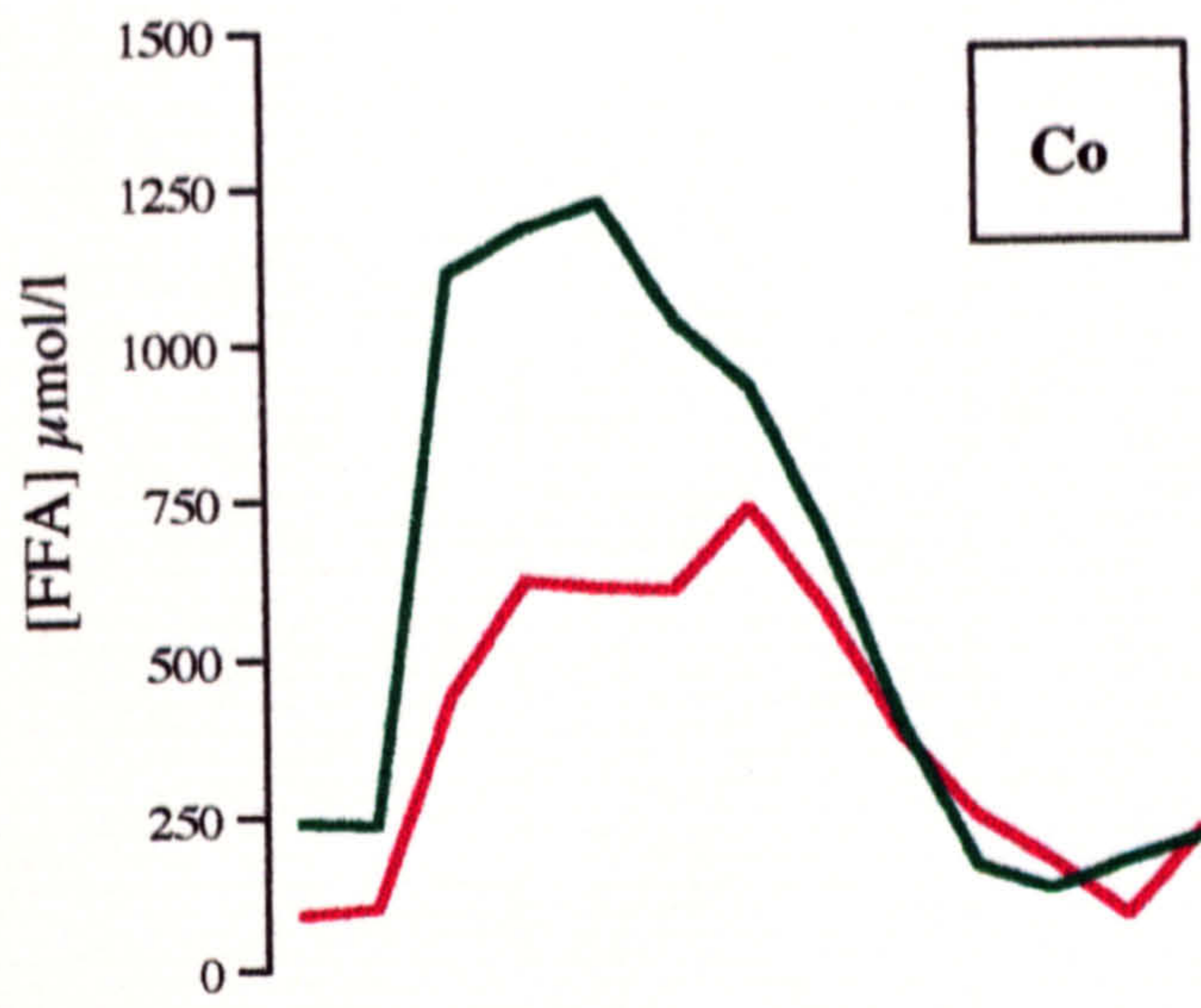


—○— HEP

—●— PP

Fig 4.5 Individual increase in plasma FFA concentration ($\mu\text{mol/l}$) following co-administration of Iverlip 20 and either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw) in horses Co, Mo, Gl, Br, Ro and Pe.

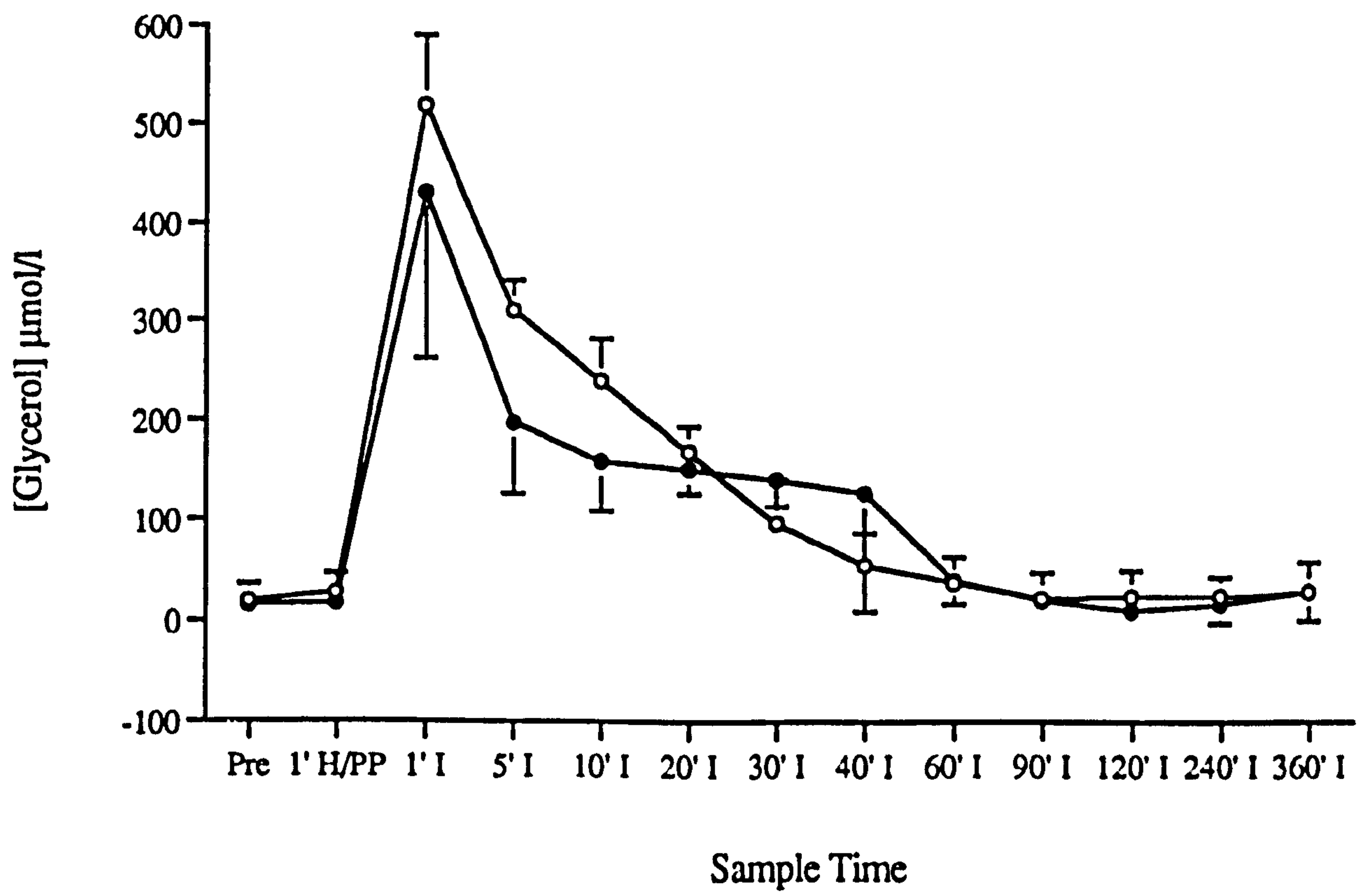
Pre	Pre administration
' H/PP	minutes post injection of HEP or PP
' I	minutes post infusion of Iverlip 20



— HEP — PP

Fig 4.6 Mean increase in plasma glycerol concentration (\pm SD, $\mu\text{mol/l}$) following co-administration of Iverlip 20 and either HEP (0.39 mg/kg bw) or PP (1.3 mg/kg bw), (n=6).

Pre	Pre administration
' H/PP	minutes post injection of HEP or PP
' I	minutes post infusion of Iverlip 20



—○— HEP

—●— PP

Discussion

The ability of HEP and PP to cause the release of LPL and HL into the circulation is well documented in Man (Barrowcliffe *et al* 1986; Chan *et al* 1984) and the rabbit (Barrowcliffe *et al* 1988). Additionally HEP administration has previously been reported to cause the release of LPL and HL into the circulation in horses (Watson *et al* 1992a; Watson *et al* 1992b; Watson *et al* 1993a). T. Lip activity ($\mu\text{molFFA/ml/hr}$) measured in this study 10 minutes following the injection of HEP, was in agreement with that calculated by the addition of the activities of LPL and HL, which were selectively measured after the same dose of HEP in normal healthy ponies (Watson *et al* 1992a). There was considerable inter-horse variation in post HEP and PP T. Lip activity. However, the magnitude of the response to both HEP and PP injections was very similar within horses. The large individual biological variation in LPL and HL release in response to HEP has been previously reported in other species; as has the reproducibility of the magnitude of enzyme release with repeated doses of HEP (10-15%), (Nilsson-Ehle 1987). The lipase release was comparable after administration of HEP or PP at their respective doses. Although there was a trend for the lipase release to be higher following PP injection, this was not significant. PP would therefore be expected to show a reduced ability to release LPL and HL in the horse, in comparison to HEP at the same dose, as in other species.

The infusion of both HEP and PP resulted in a significant increase ($p < 0.001$) in APTT for all horses. The increase remained significant for longer (up to 60 minutes post infusion) during the HEP session and was much greater.

The triglyceride concentration was significantly increased after the infusion of Iverlip ($p < 0.001$). The infused triglyceride was rapidly metabolised and removed from the circulation. A session in which Iverlip was administered in isolation was not undertaken in this study. However, a previous study by Moser *et al* (1989) reported that the removal of a 10% triglyceride emulsion (0.2 ml/kg) administered without the co-administration of either HEP or PP, was very slow in the horse and was not affected by exercise. Since the removal of the triglyceride emulsion in this study was very rapid it can be attributed to the lipase release as the

result of HEP or PP administration. The main removal sites for infused triglyceride emulsions are skeletal muscle, adipose tissue, myocardium, subcutaneous tissue and the splanchnic viscera (excluding the liver), (Rossner 1974). It was estimated that in the supine position up to 50% of the triglyceride emulsion infused in Man was removed by the skeletal muscle and that this may be further increased during exercise (Rossner 1974).

Plasma glycerol concentration represented the sum of the glycerol infused as part of the emulsion (2.5% of the total volume infused) and that produced from the breakdown of infused triglycerides. Immediately following the Iverlip infusion, there was a large increase in plasma glycerol concentration followed by a gradual decline. There was no apparent increase in glycerol concentration corresponding to the increase in FFA concentration. This was probably due to the rate of its removal from the plasma greatly exceeding that for FFA.

A significant increase in plasma FFA concentration was achieved following the administration of both HEP and PP in conjunction with Iverlip. A trend for the lipase release to be slightly higher after the administration of PP was not reflected in the corresponding increase in plasma FFA concentration. The increase in plasma FFA concentration was marginally higher following the administration of Iverlip and HEP, however, this was not significant. Peak FFA concentration occurred earlier when Iverlip was given in conjunction with HEP compared to PP. This effect has been previously described in other species (Cucurachi *et al* 1967). Thus whilst a higher dose of PP (x3) was required to elicit a comparable lipolytic effect to HEP in the horse, this was associated with a greatly reduced effect upon clotting function. In view of these findings PP represents a suitable alternative to HEP, for use in combination with a triglyceride emulsion, to produce an elevation in pre-exercise plasma FFA concentration. In conclusion, PP (1.3 mg/kg bw) and Iverlip (400 mg/kg bw), administered approximately 25 minutes before the onset of exercise, would produce a substantial increase in plasma FFA for use in the investigation of FFA utilisation during exercise in the horse.

Chapter 5

Effect of Elevated Plasma Free Fatty Acids on Fat Utilisation During Low Intensity Exercise

Introduction

Both fat and carbohydrate are utilised as fuel sources during low to moderate intensity prolonged exercise. Their relative contribution to energy production is dependent on a number of factors, including the duration of exercise. Studies in Man have indicated that during the early stages of prolonged exercise up to 90% of the energy used is provided by carbohydrate, with a minimal contribution from fat, whereas the situation is reversed during the latter stages (Costill 1972). The intensity of exercise, as well as the training (Costill *et al* 1979) and nutritional status (Janson 1982; Pagan *et al* 1987) of the individual, may also influence the relative contribution of the two fuel sources. The contribution of fat to energy production is higher the lower the intensity of exercise and the longer its duration as shown by a decreasing RER (Gollnick and Saltin 1988). Aerobic training increases the capacity for fat oxidation, at a given exercise intensity, due to increases in muscle oxidative enzyme activities (Lindholm *et al* 1983; Essen-Gustavsson and Lindholm 1985; Hodgson and Rose 1987), increased muscle capillarisation (Henckel 1983; Saltin and Gollnick 1983; Hodgson and Rose 1987) and mitochondrial density (Saltin and Gollnick 1983; Straub *et al* 1983). It has been suggested that the inclusion of fat into the equine diet increases its use during low to moderate intensity exercise (Duren *et al* 1987; Pagan *et al* 1987).

The plasma concentration of FFA at rest is low and characteristically falls at the onset of exercise due to increased muscle blood flow and a delay in adipose tissue lipolysis (Carlson and Pernow, 1959). Stimulation of adipose tissue lipolysis results in a steady increase in plasma FFA concentration as the exercise proceeds. Studies which have used nicotinic acid or its analogues to block FFA release confirm their role as an energy source during exercise at intensities of 60% $\text{VO}_2 \text{ max}$ or less (Walker *et al* 1991; Heath *et al* 1993). The rate of FFA oxidation is influenced by its extra-cellular concentration and is thus related to the arterial FFA concentration, as reviewed by Bulow (1988). Studies in other species have reported that a pre-exercise elevation of plasma FFA concentration stimulates the rate of fat oxidation during the early stages of sub-maximal exercise. Increases in fat oxidation with reduced utilisation of carbohydrate have been reported in both trained and untrained human

subjects (Costill *et al* 1977; Ravussin *et al* 1986). Furthermore, a delay in the fall of blood glucose concentration and the onset of exhaustion has also been reported as a result of a pre-exercise elevation of plasma FFA in rats (Hickson *et al* 1977).

The aim of the present study was to evaluate the capacity of the horse to increase the contribution of fat oxidation to energy production, as a result of a pre-exercise elevation of triglyceride derived plasma FFA, during low intensity exercise.

Materials and Methods

During two randomised exercise sessions (test, T and control, C), separated by a period of 1 week, 7 thoroughbred horses (3-7 years) were exercised on a treadmill (Sato, Sweden) for 91 minutes, at 18°C and 60% relative humidity. A fan was placed in front of the horses generating a wind speed of approximately 3.2 m/s. Prior to the study the horses had been trained for an 8 week period and had been maintained on a commercially available pelleted diet "low fat" (Spillers, Dalgety Agriculture) with additional forages. The exercise protocol consisted of a 1 minute walk (1.6 m/s) followed by 60 minutes of trot (3.2 m/s) and a further 30 minutes walk. All exercise was carried out on a 0° incline. The trotting phase of the exercise protocol was calculated to represent a mean of $13 \pm 3\%$ of $\text{VO}_2 \text{max}$ (Table 5.1).

Twenty-five minutes before the onset of exercise during session T, horses were injected with 1.3 mg/kg bw of PP and were infused with a triglyceride emulsion (Iverlip 20, 400 mg/kg bw) over 6 minutes in order to raise plasma FFA, as described in the previous chapter. The same procedure was followed during session C with the exception that no triglyceride emulsion or PP was administered. All exercise tests were carried out at the same time of day and followed a 12 hour period of food restriction. The horses were fed normally at approximately 4.30pm on the previous day and then any remaining pelleted feed or hay was removed at 10pm that evening. Exercise was started as near to 10am the following morning as was possible.

Table 5.1 Individual $\text{VO}_{2\text{max}}$ values and relative exercise intensity of individual horses during the trotting phase of the control and test exercise sessions.

Horse	VO _{2max} (ml/kg/min)	% of VO _{2max}
Br	130	15
Co	144	17
Gl	166	8
Mo	(see text)	(see text)
Pe	133	14
Ro	196	14
Ri	132	13
Mean ± SD	150 ± 26	13 ± 3

Individual maximum oxygen uptake

Individual $\text{VO}_{2\text{max}}$ was determined within 5 days of the second exercise test (T or C). A stepwise exercise protocol similar to that described by Rose *et al* (1987) was used. The horses were walked at 1.6 m/s for 10 minutes on a 0° incline. They then undertook a series of canters each of 1 minutes duration, on a 5° incline, at speeds of 6, 8, 9, 10, 11 and 12 m/s or until the time of fatigue. Fatigue was defined as the point at which the horse could no longer keep pace with the treadmill despite humane encouragement.

Blood samples

Venous blood samples were taken through an 18 g catheter inserted into the left jugular vein under local anaesthesia, contra-lateral to the side used for the introduction of PP and Iverlip. Blood samples were separated into tubes containing EDTA for the analysis of FFA, triglyceride and glycerol; fluoride oxalate for the analysis of glucose and lactic acid and into tubes containing no anticoagulant for the analysis of T. Lip activity. Samples were treated as previously described (Chapter 2).

Muscle samples

Muscle biopsy samples were taken and treated, as previously described (Chapter 2), 40 minutes before the start of exercise and immediately after the completion of 60 minutes of trotting. The muscle samples were analysed for glycogen, triglyceride and CS activity. Muscle triglyceride concentration was expressed as a ratio to the CS activity in an attempt to correct for differences in the fibre composition of serial biopsy samples, as previously described by Foster and Harris (1992). CS was correlated to muscle triglyceride concentration in resting muscle biopsy samples ($r = 0.66, p < 0.001$).

RER was analysed using an open-flow system as described in Chapter 2. Heart rate was recorded every 5 seconds throughout the exercise period. Mean heart rate was calculated over a minute period during rest and the initial walk and then every 5 minutes thereafter.

Statistical Analyses

Where appropriate, a 1 factor ANOVA for repeat measures was applied to identify significant differences within sessions. Student's t test for paired data was used to analyse differences between sessions with each horse serving as its own control. All horses were included in the statistical analysis reported. However, in the case of RER and plasma glucose concentration there appeared to be more than 1 response during subsequent exercise to the elevation of plasma FFA. No significant effect of treatment, with respect to RER and plasma glucose concentration, was identified using ANOVA. However, as there appeared to be more than 1 response to the elevation of plasma FFA the results have been discussed on an individual horse basis.

Results

Mean heart rates were 94 ± 10 and 96 ± 14 beats/minute for the C and T sessions, respectively ($p > 0.05$). Mean $VO_2 \text{ max}$ was calculated to be 150 ± 26 ml/kg/min, range (130-196 ml/kg/min), (Table 5.1). The intensity of the trotting phase of the exercise protocol was therefore calculated to represent a mean of $13 \pm 3\%$ of $VO_2 \text{ max}$, (Table 5.1). Horse Mo did not undertake the $VO_2 \text{ max}$ test since he had a previous clinical history of tendon injury. A decision was taken not to risk jeopardising his use in the low intensity protocols by carrying out a $VO_2 \text{ max}$ test.

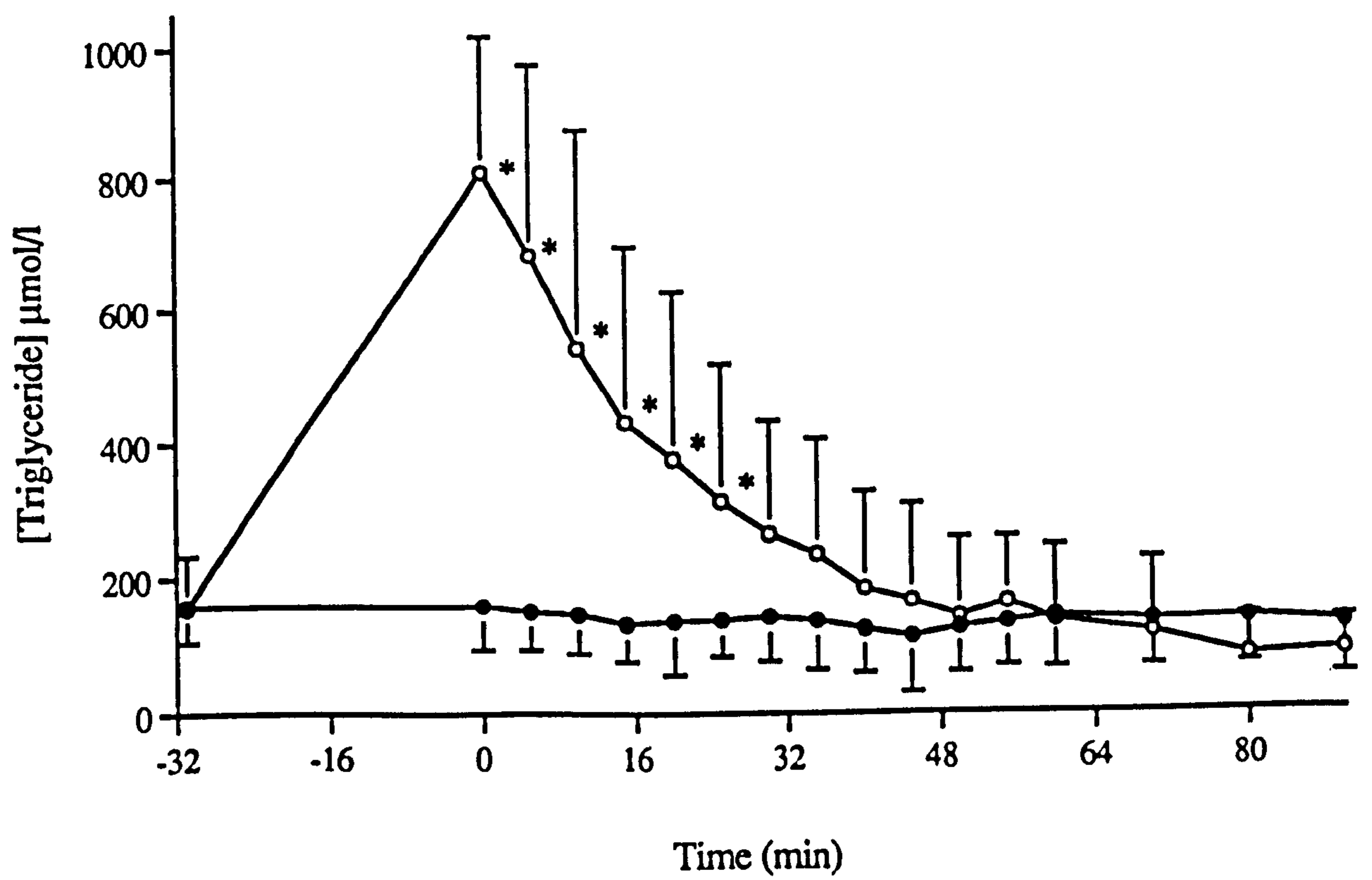
Plasma triglyceride concentration remained unchanged during exercise session C ($p > 0.05$). During session T plasma triglyceride concentration was significantly higher than the pre-infusion concentration, up to 25 minutes into the trot ($p < 0.05$, Fig 5.1). Plasma T. Lip activity was unchanged during session C but was significantly increased immediately prior to the onset of exercise, i.e. 25 minutes following the co-administration of PP and Iverlip, in session T ($p < 0.05$, Fig 5.2). Plasma T. Lip activity was further increased, although non-significantly, following 30 minutes of exercise (i.e. 55 minutes post PP and Iverlip administration) in all horses except Ri during session T. Plasma T. Lip activity remained significantly elevated above the pre-administration level throughout exercise in session T (p

< 0.05, Fig 5.2). Plasma FFA and glycerol concentration was significantly higher during session T compared to C ($p < 0.05$, Fig 5.3, Table 1 and 2). Plasma FFA concentration initially declined at the onset of exercise during both sessions. There was a significant increase in both FFA and glycerol concentration compared to pre-exercise values regardless of exercise session ($p < 0.001$, Fig 3, Table 1 and 2).

RER initially increased during the first 5-10 minutes of trot regardless of exercise session. RER was negatively correlated to plasma FFA concentration between 10 and 60 minutes of trotting, during both exercise sessions (Fig 5.4, 5.5). The degree of correlation between plasma FFA concentration and RER during exercise, varied between horses. Similarly the magnitude of the decrease in RER for a given increase in FFA concentration was also different between horses. With the exception of horses Ro and Br the decrease in RER for a given increase in FFA concentration was similar within horses between the two exercise sessions. This is illustrated by the singular line of regression about the combined RER and FFA data for the two exercise sessions in Fig 5.4. Both horses Ro and Br exhibited a much more rapid decrease in RER during exercise session T compared to C (Fig 5.5). The change in RER for a given change in plasma FFA concentration was similar between the two sessions with respect to horse Ri. RER was, however, slightly higher during the test exercise session in this horse. There was a significant reduction in RER during both sessions ($p < 0.01$), however, this was manifested earlier during session T, (Fig 5.6). There was a large inter-horse variation in RER during both exercise sessions, although it was consistently lower throughout session T compared to C in 5 out of 7 horses (Br, Ro, Mo, Pe, Gl), (Fig 5.7). In the remaining 2 horses, RER was unchanged (Co) or higher (Ri) during session T (Fig 5.8).

Fig 5.1 Plasma triglyceride concentration (mean \pm SD, $\mu\text{mol/l}$) during 91 minutes of low intensity exercise during the control and test exercise sessions.

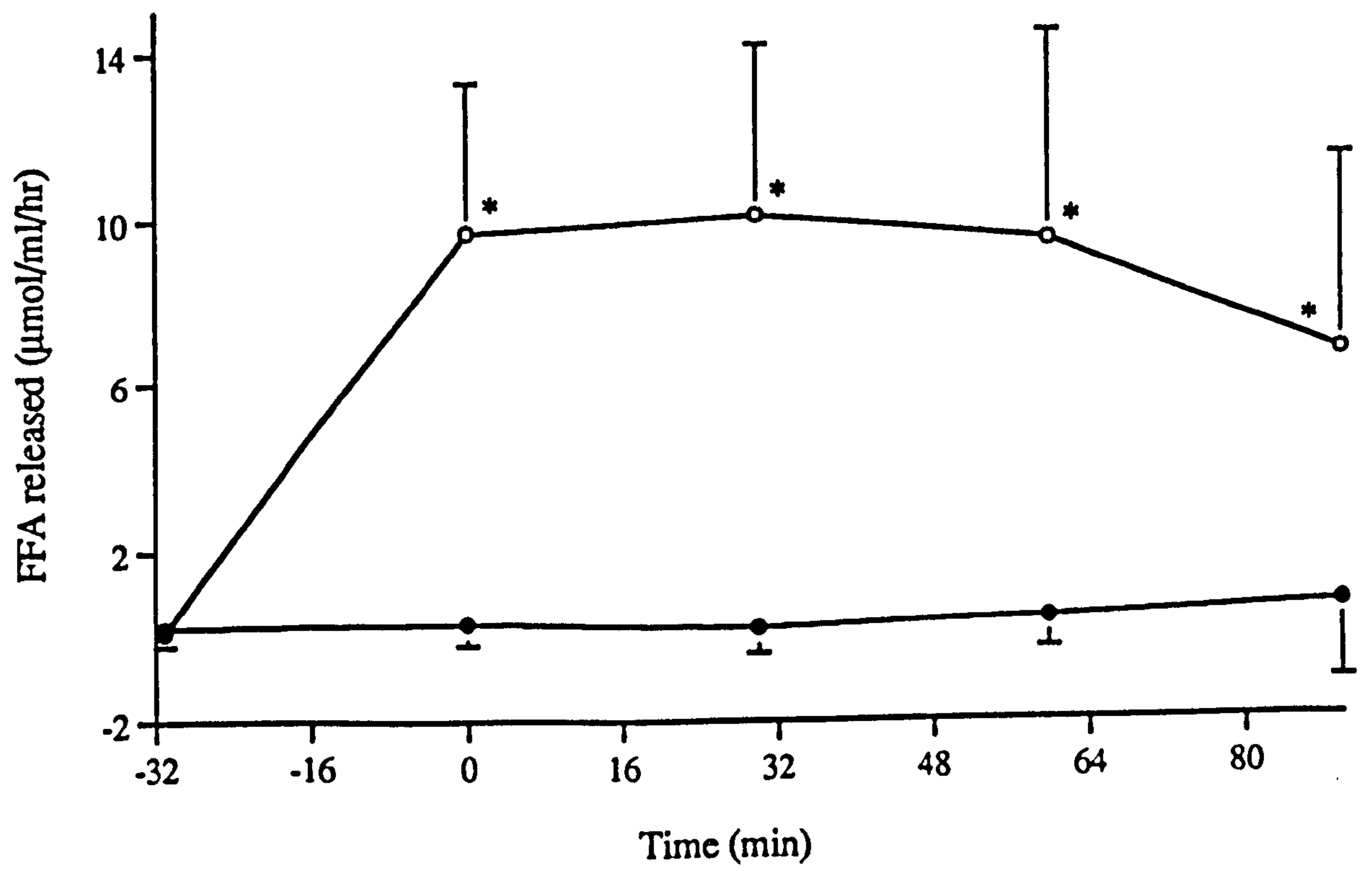
* Significantly different from the triglyceride concentration at time point -31 minutes ($p < 0.05$)



—●— Control
—○— Test

Fig 5.2 Plasma T. Lip activity (mean \pm SD, FFA released $\mu\text{mol/ml/hr}$) during 91 minutes of low intensity exercise during the control and test exercise sessions.

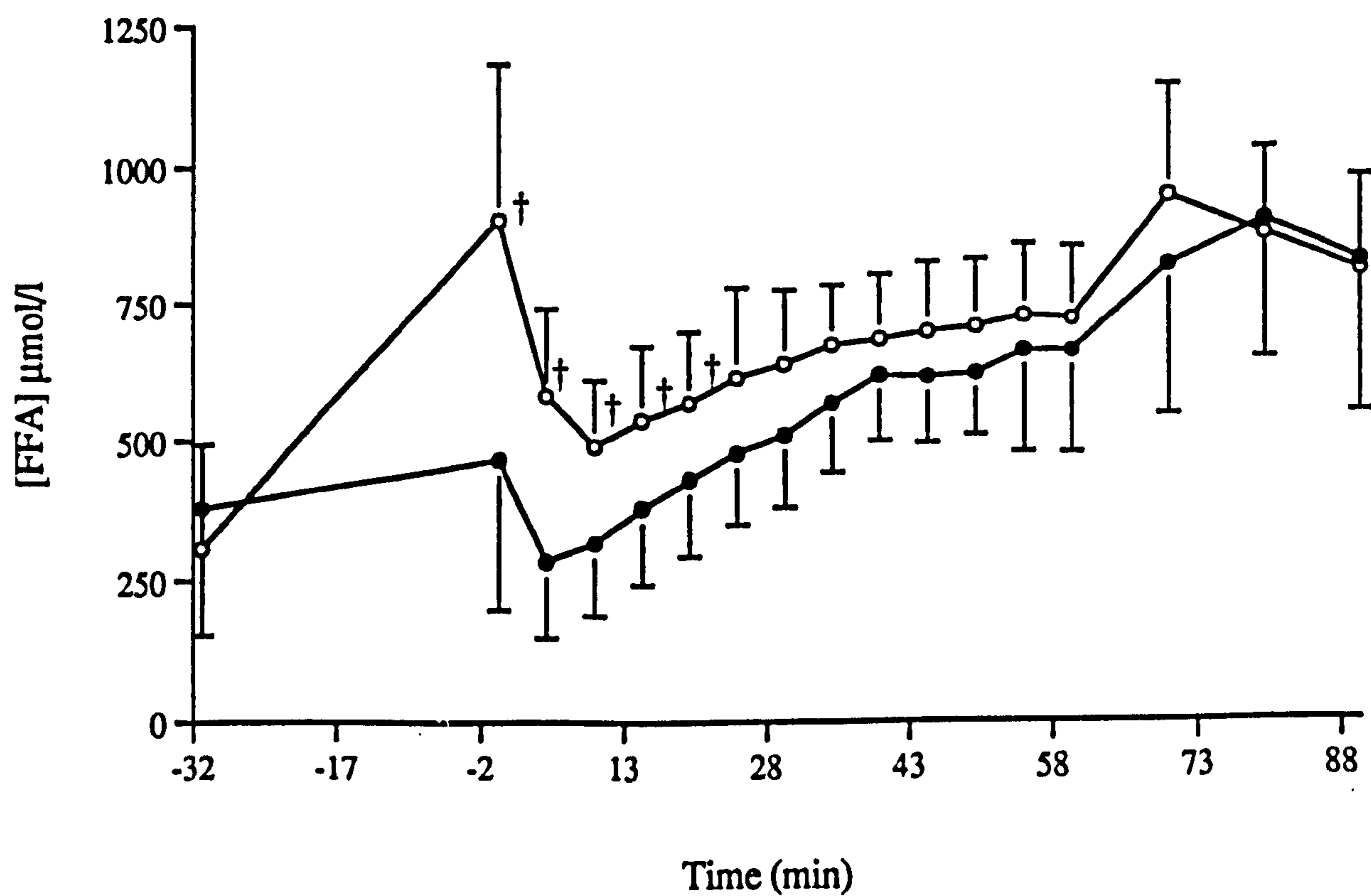
* Significantly different from the plasma T. Lip activity at time point -31 minutes ($p < 0.05$)



—●— Control
—○— Test

Fig 5.3 Plasma FFA concentration (mean \pm SD, $\mu\text{mol/l}$) during 91 minutes of low intensity exercise during the control and test exercise sessions.

† Significantly different from the plasma FFA concentration at the corresponding sample time during the control session ($p < 0.05$)



—●— Control
—○— Test

Fig 5.4 **Correlation between plasma FFA concentration and RER during the trotting phase (10 - 60 minutes) of the control and test exercise sessions (combined data) in horses (Gl, Mo, Co and Pe).**

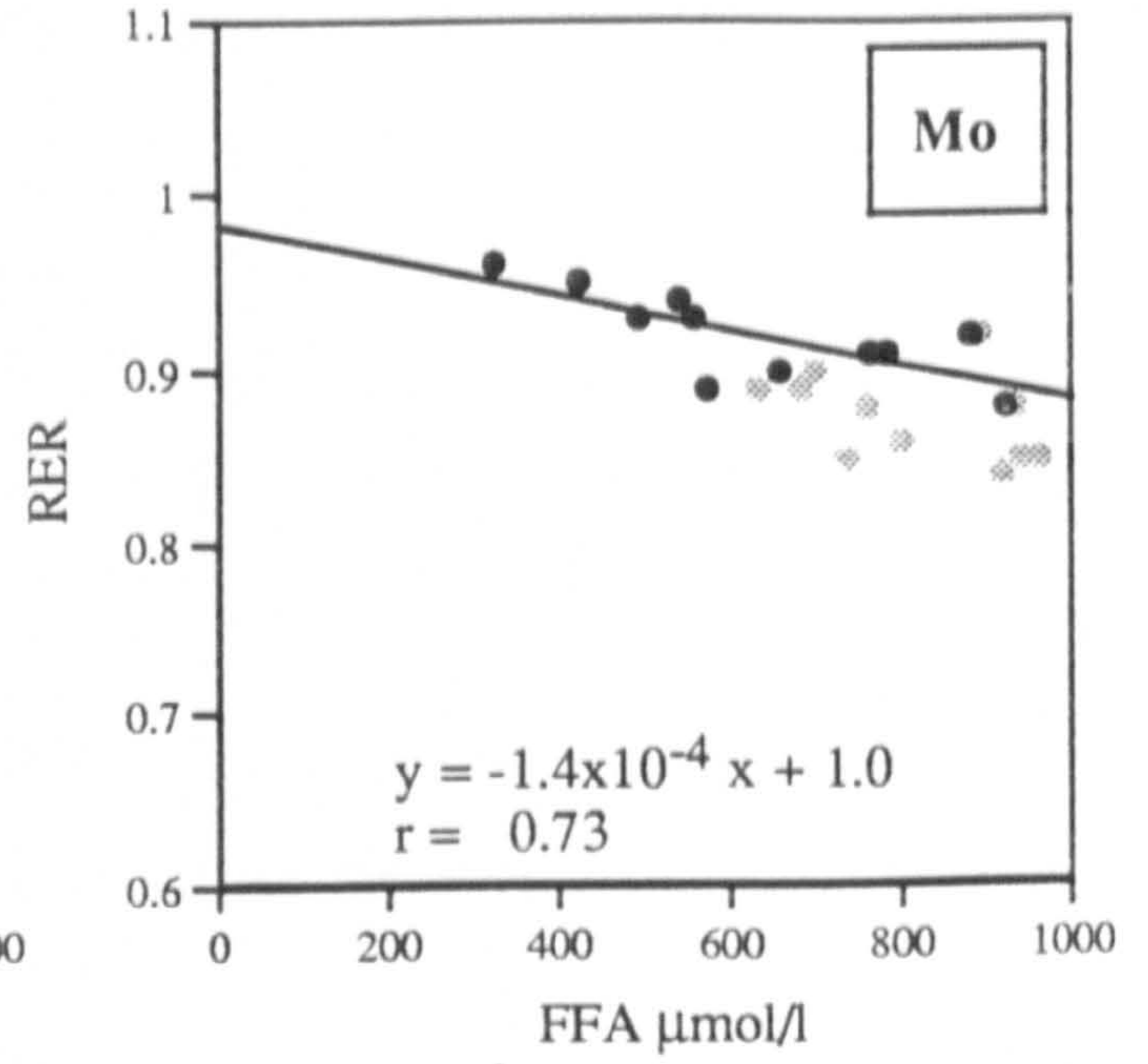
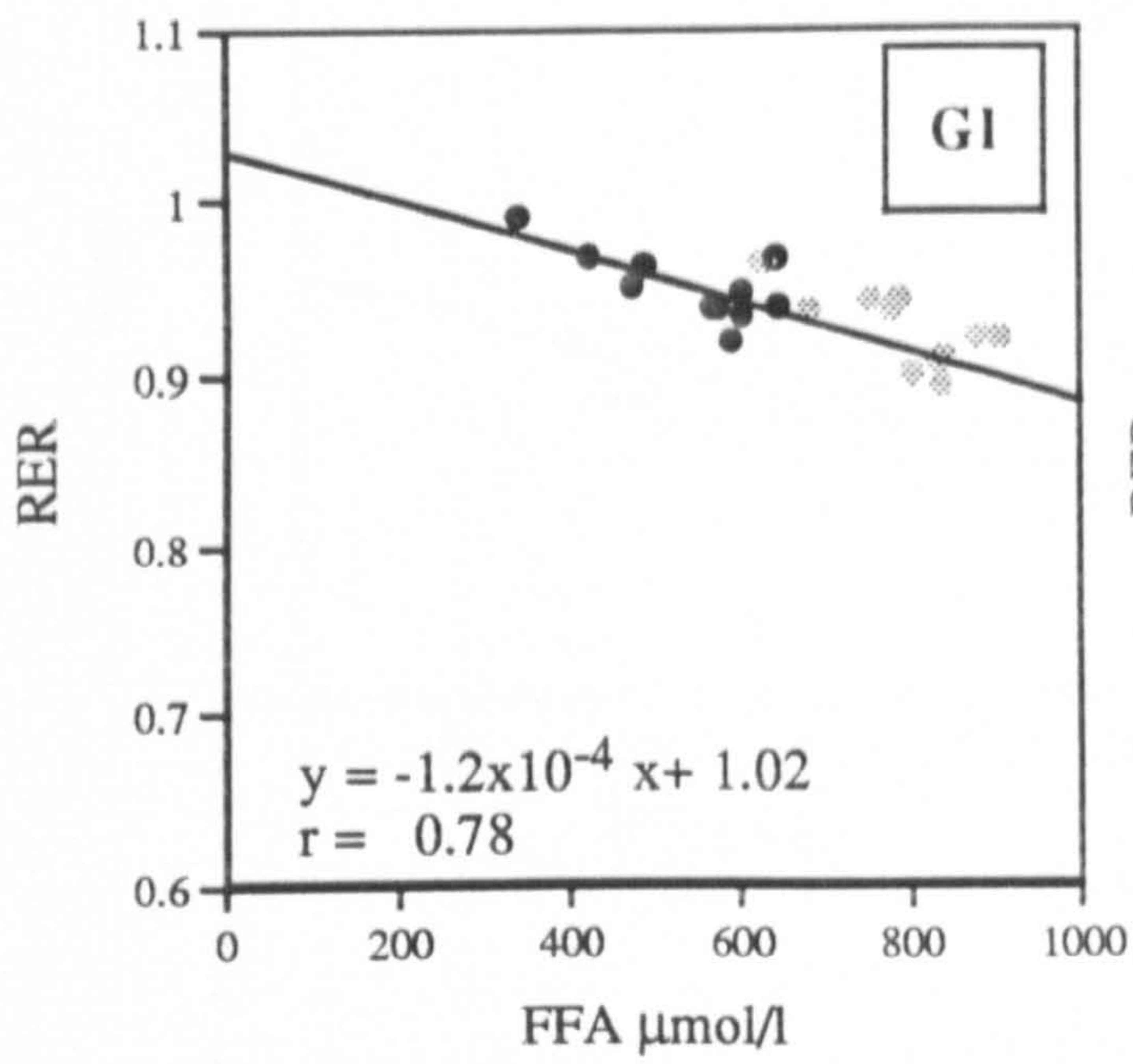
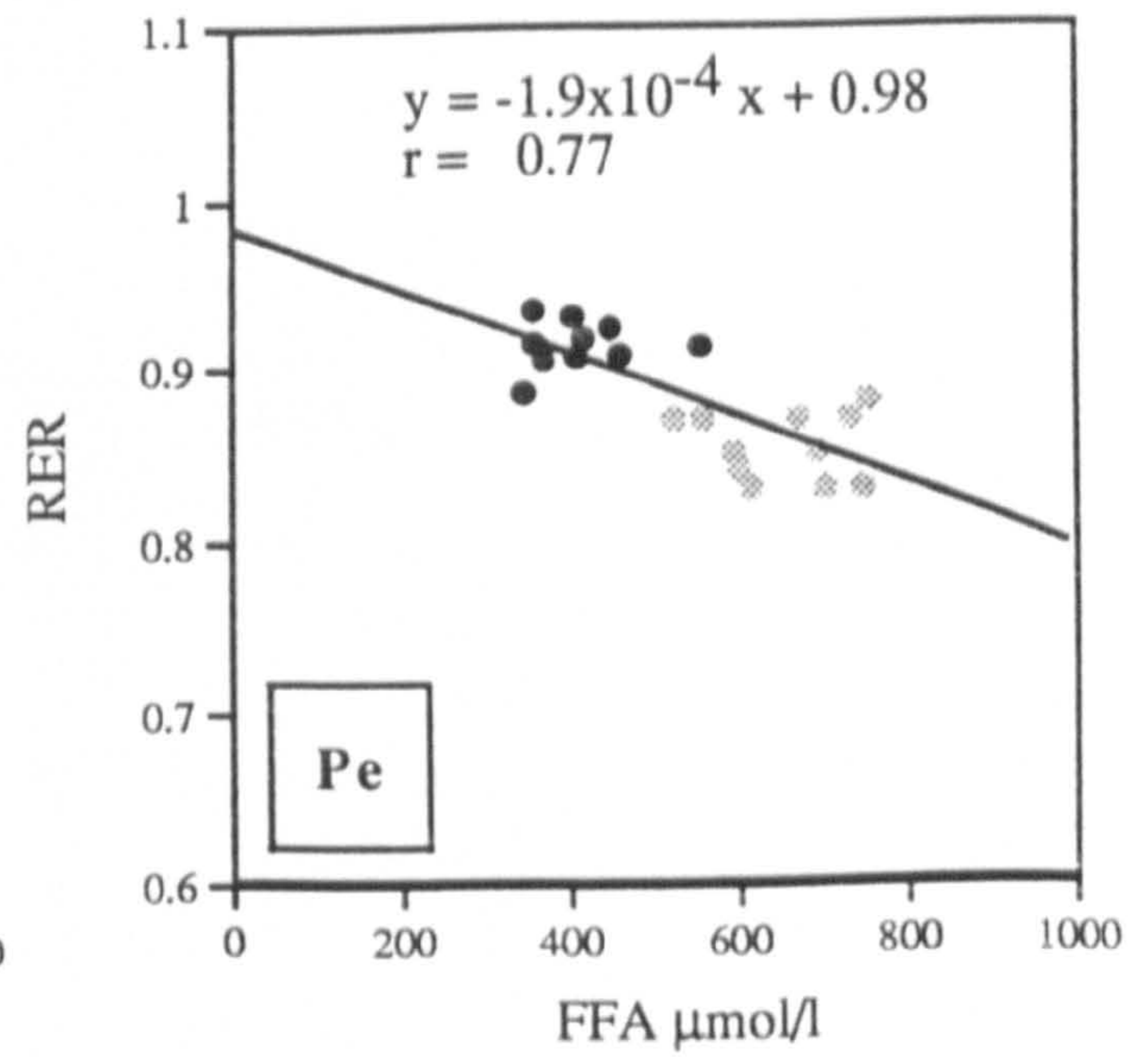
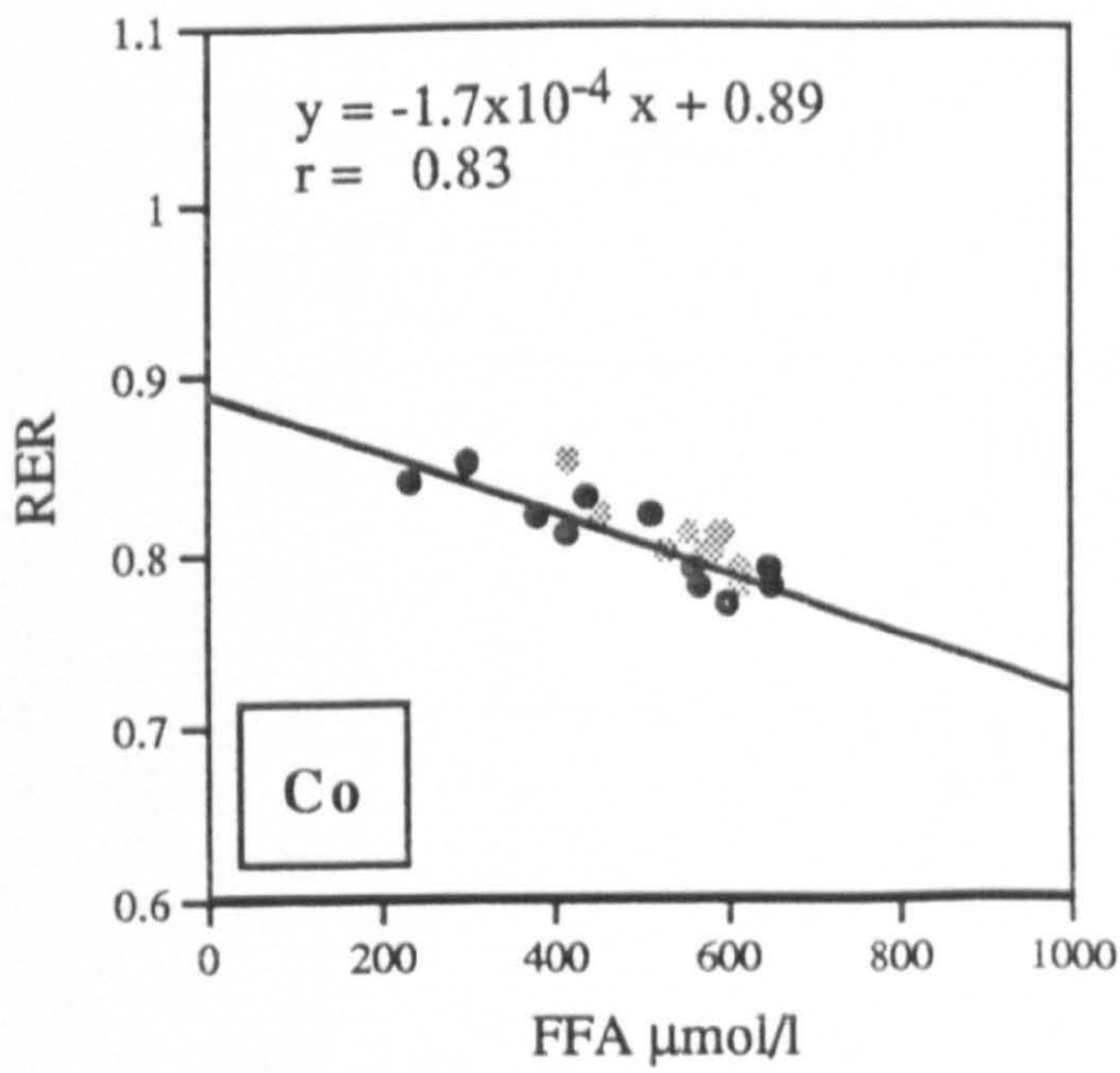
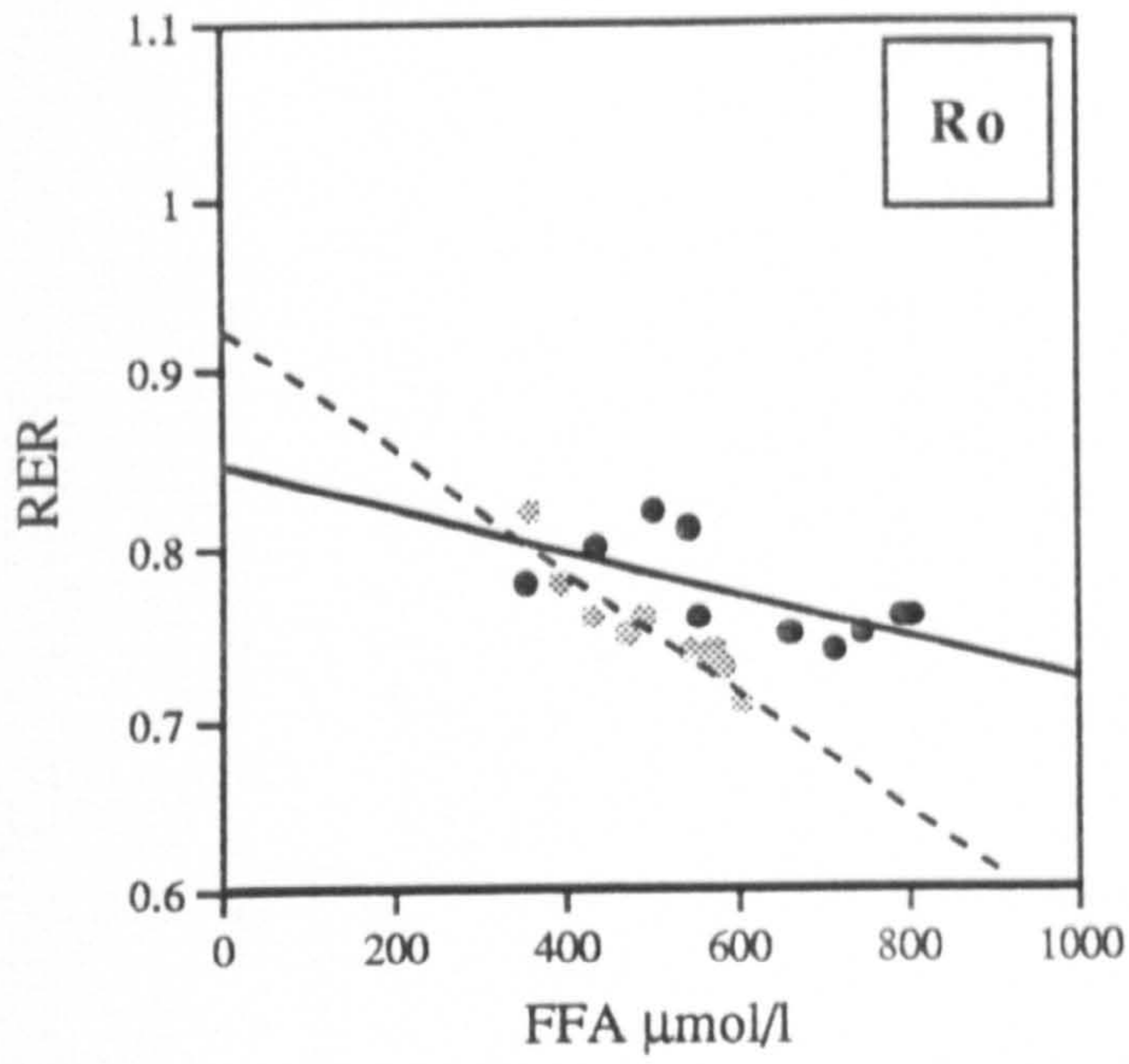


Fig. 3.3 Correlation between plasma FFA concentration and RER for the steady phase (70–120 minutes) of the steady-state test (see Fig. 3.1 for raw data) in horses (G1, G2, Mo and Pe)



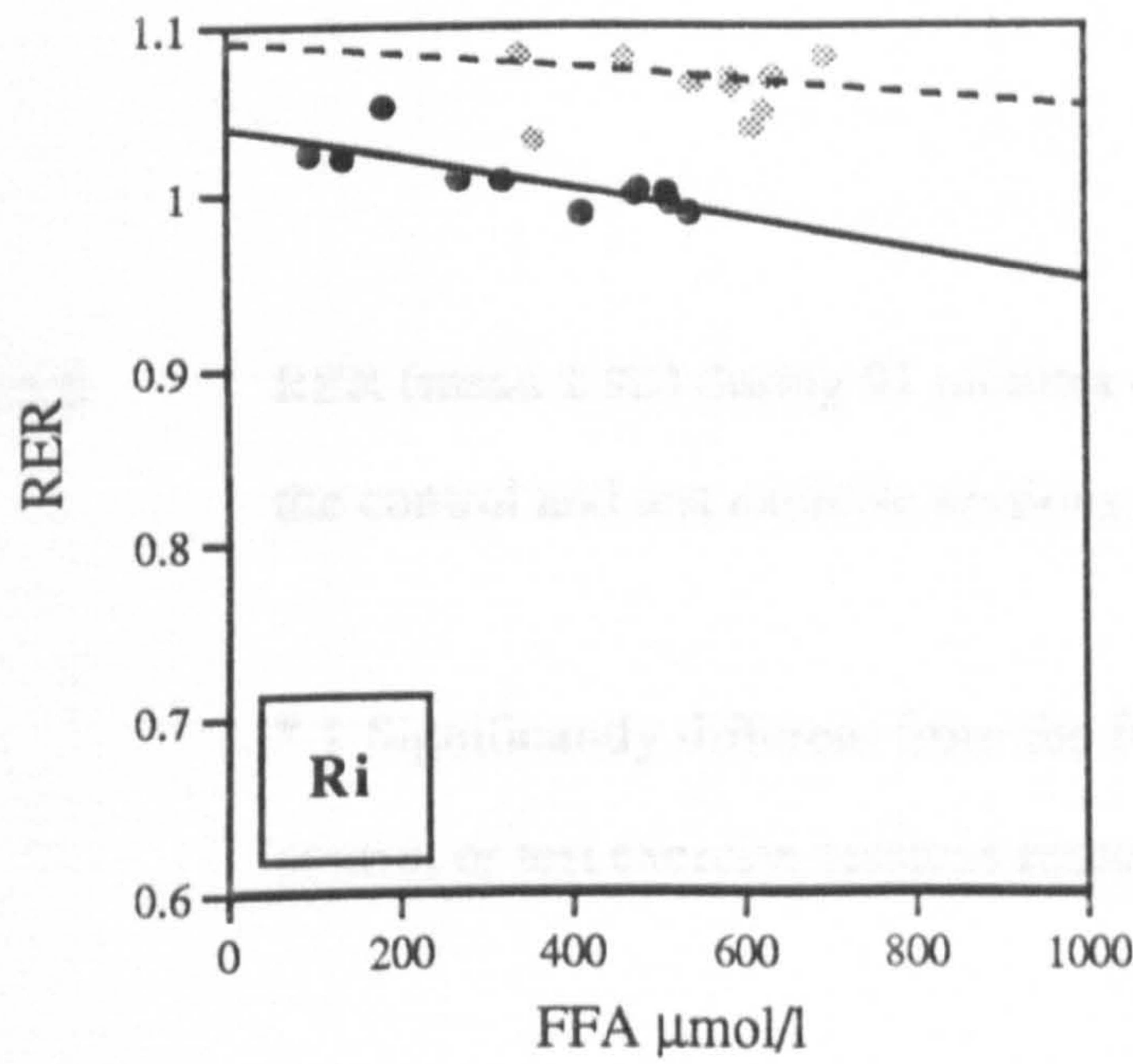
● Control ※ Test

Fig 5.5 **Correlation between plasma FFA concentration and RER during the trotting phase (10 - 60 minutes) of the control and test exercise sessions (combined data) in horses (Br, Ri and Ro).**



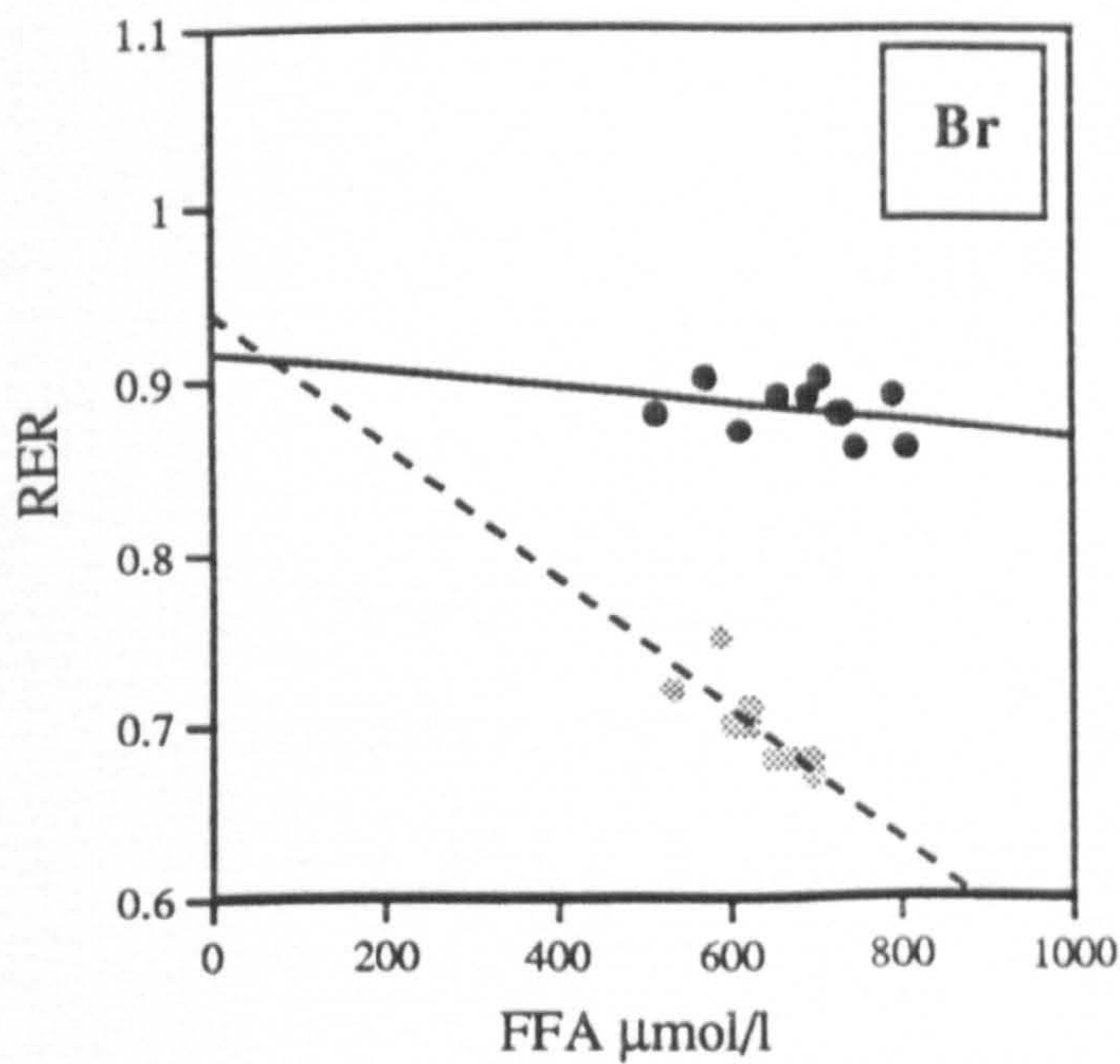
— $y = -1.2 \times 10^{-4}x + 0.85$
 $r = 0.66$

- - - $y = -3.3 \times 10^{-4}x + 0.92$
 $r = 0.93$



— $y = -0.9 \times 10^{-4}x + 1.04$
 $r = 0.79$

- - - $y = -0.4 \times 10^{-4}x + 1.09$
 $r = 0.66$



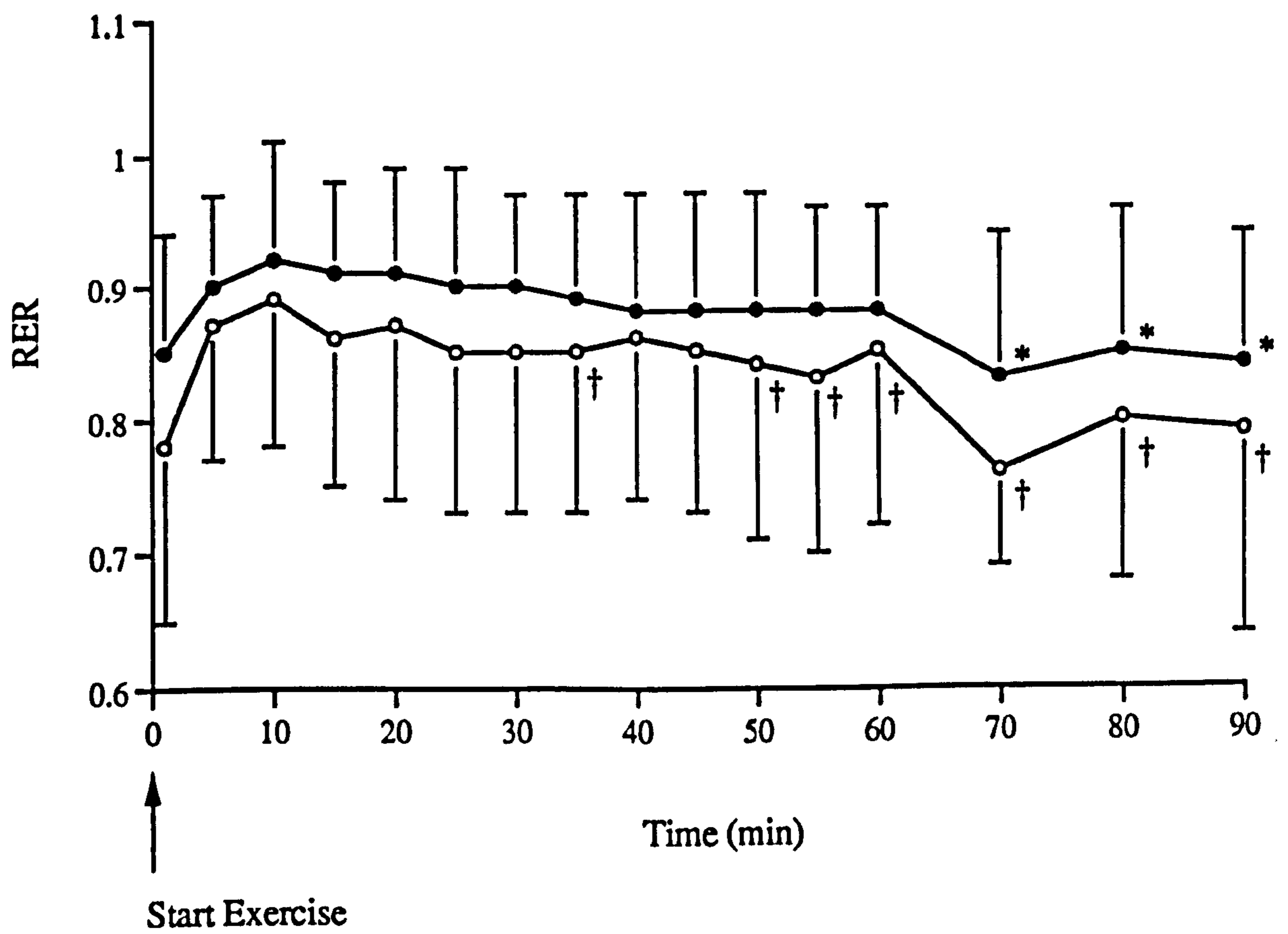
— $y = -0.5 \times 10^{-4}x + 0.92$
 $r = 0.32$

- - - $y = -3.8 \times 10^{-4}x + 0.94$
 $r = 0.81$

● Control ♦ Test

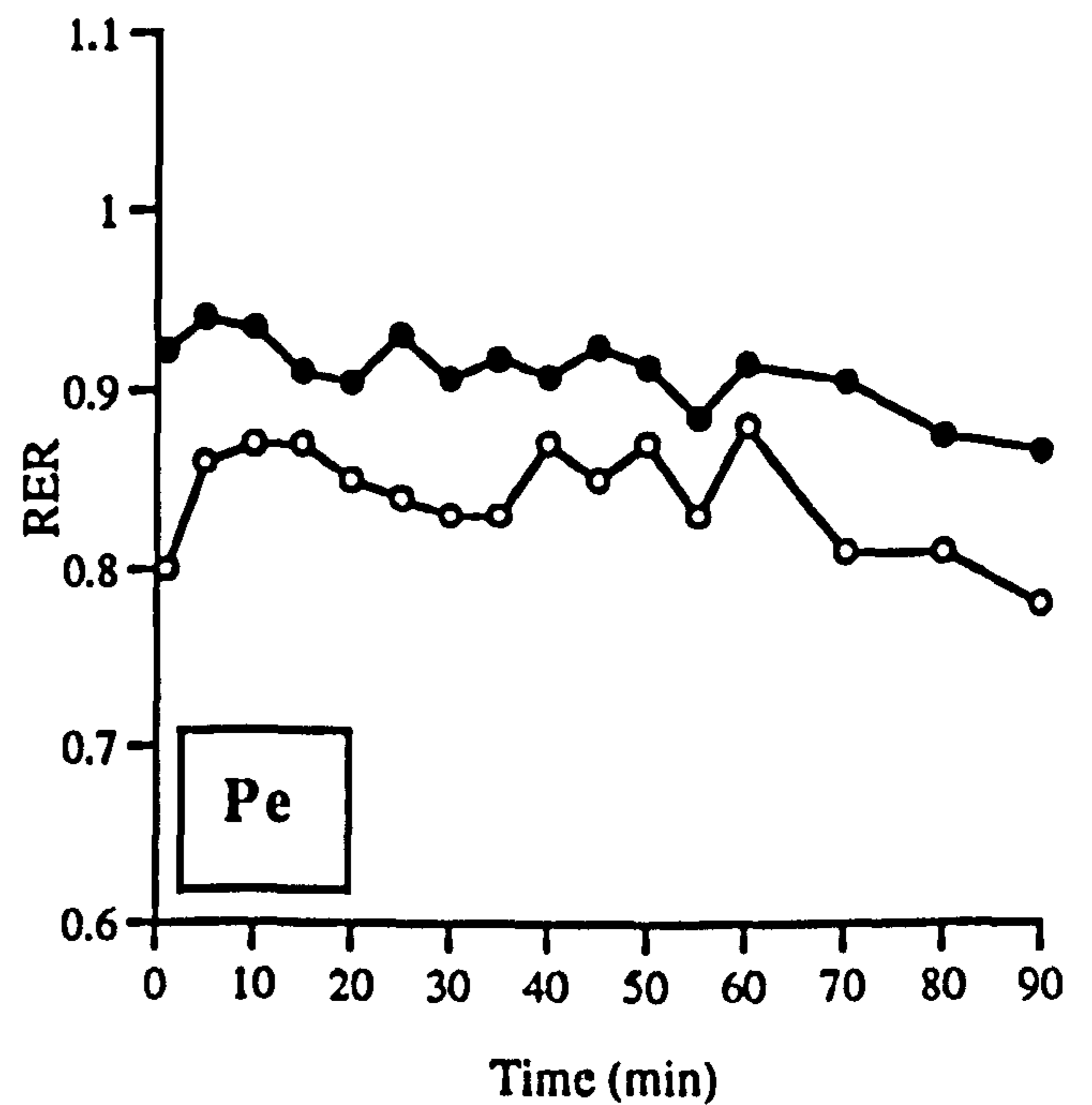
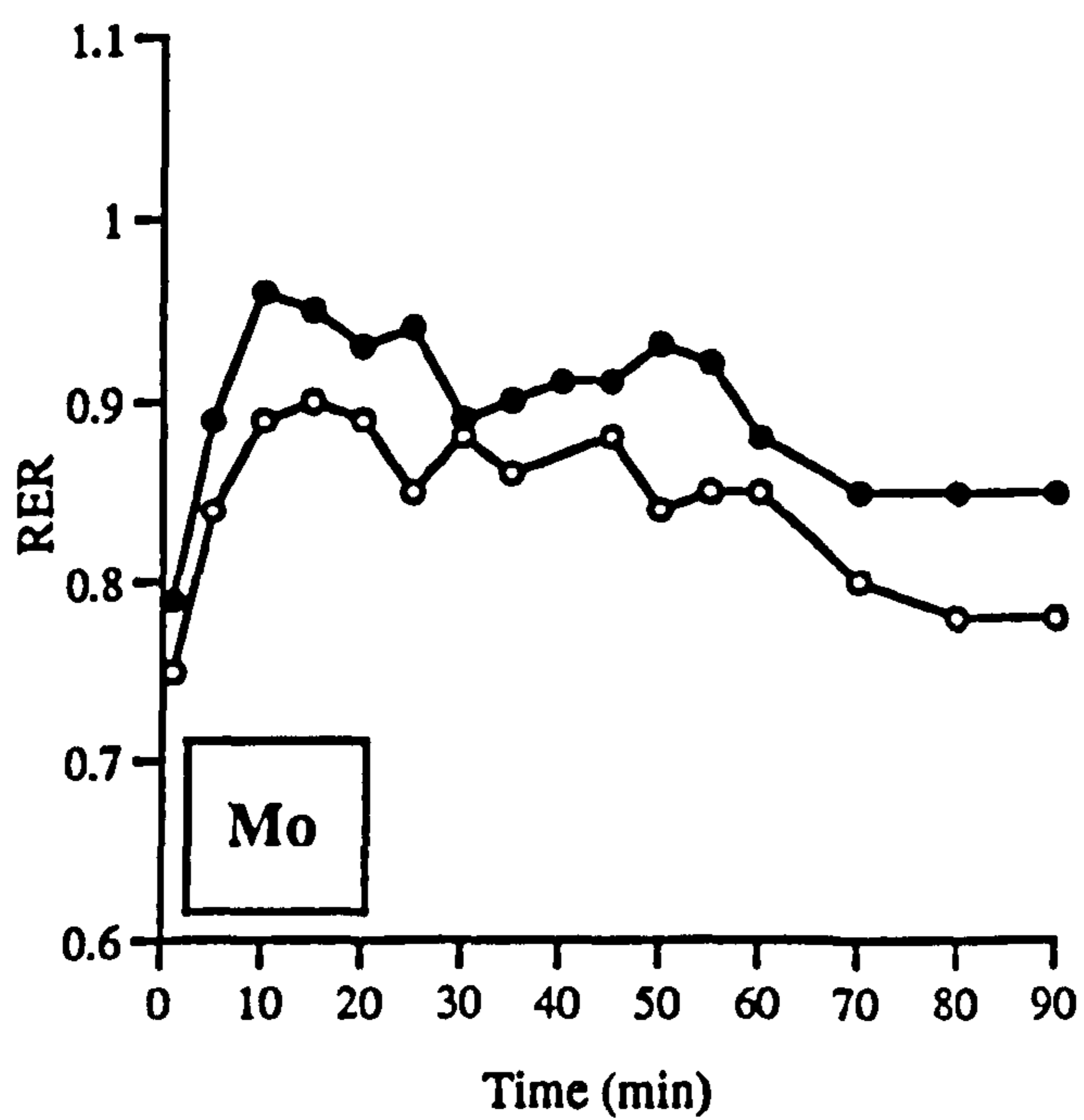
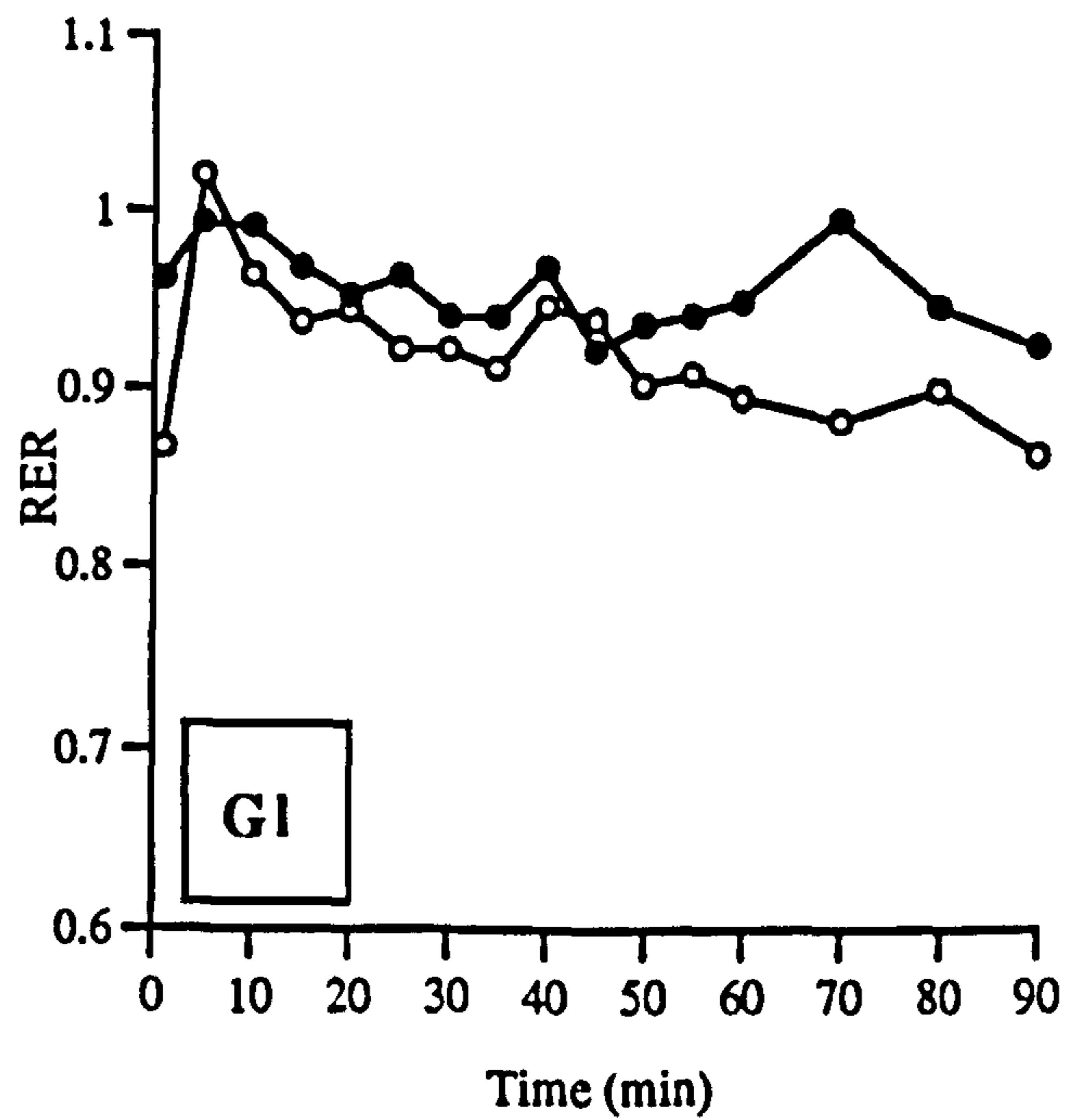
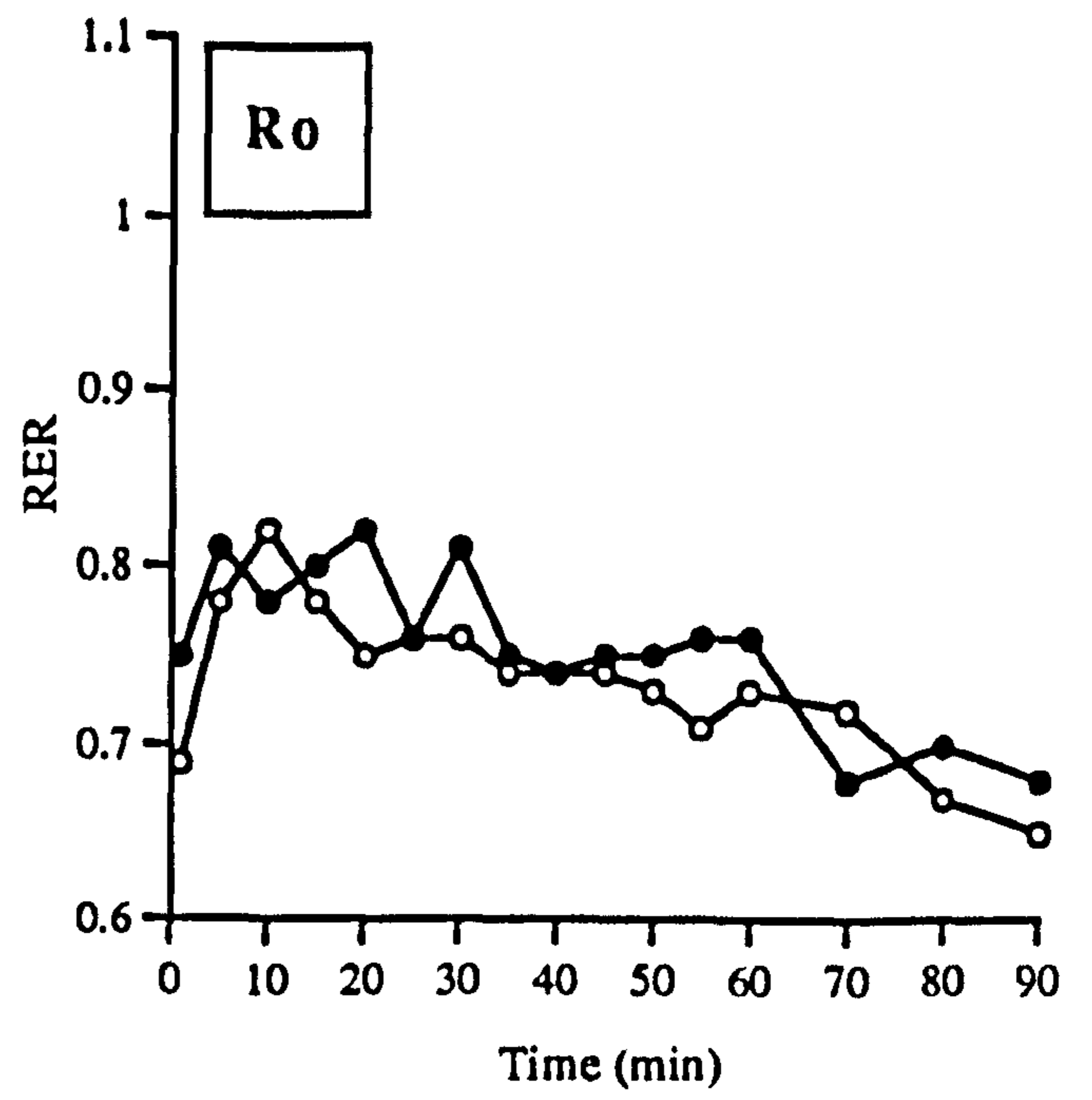
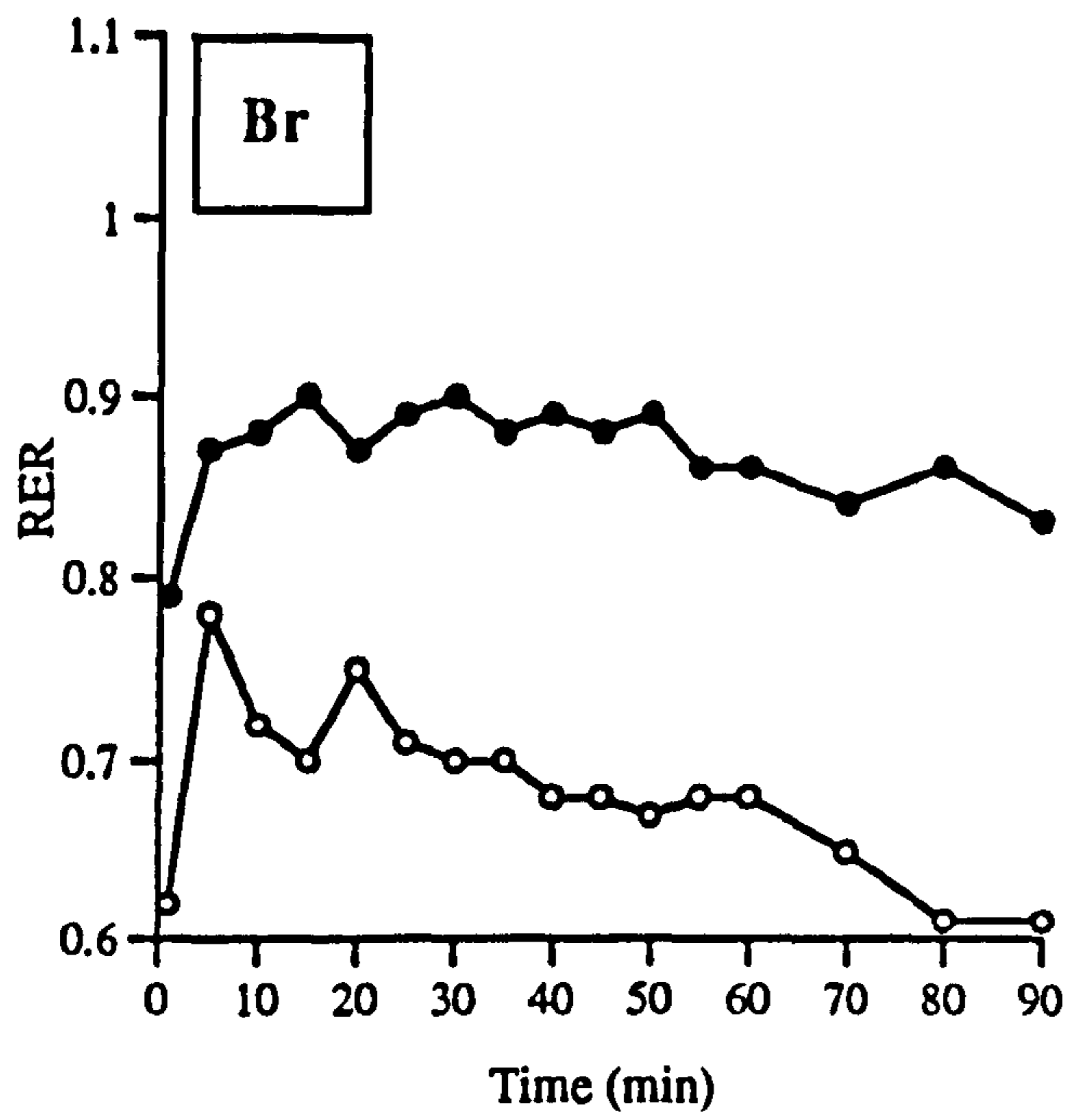
Fig 5.6 RER (mean \pm SD) during 91 minutes of low intensity exercise during the control and test exercise sessions.

* † Significantly different from the RER after 5 minutes of trotting during the control or test exercise sessions respectively ($p < 0.05$).



—●— Control
 —○— Test

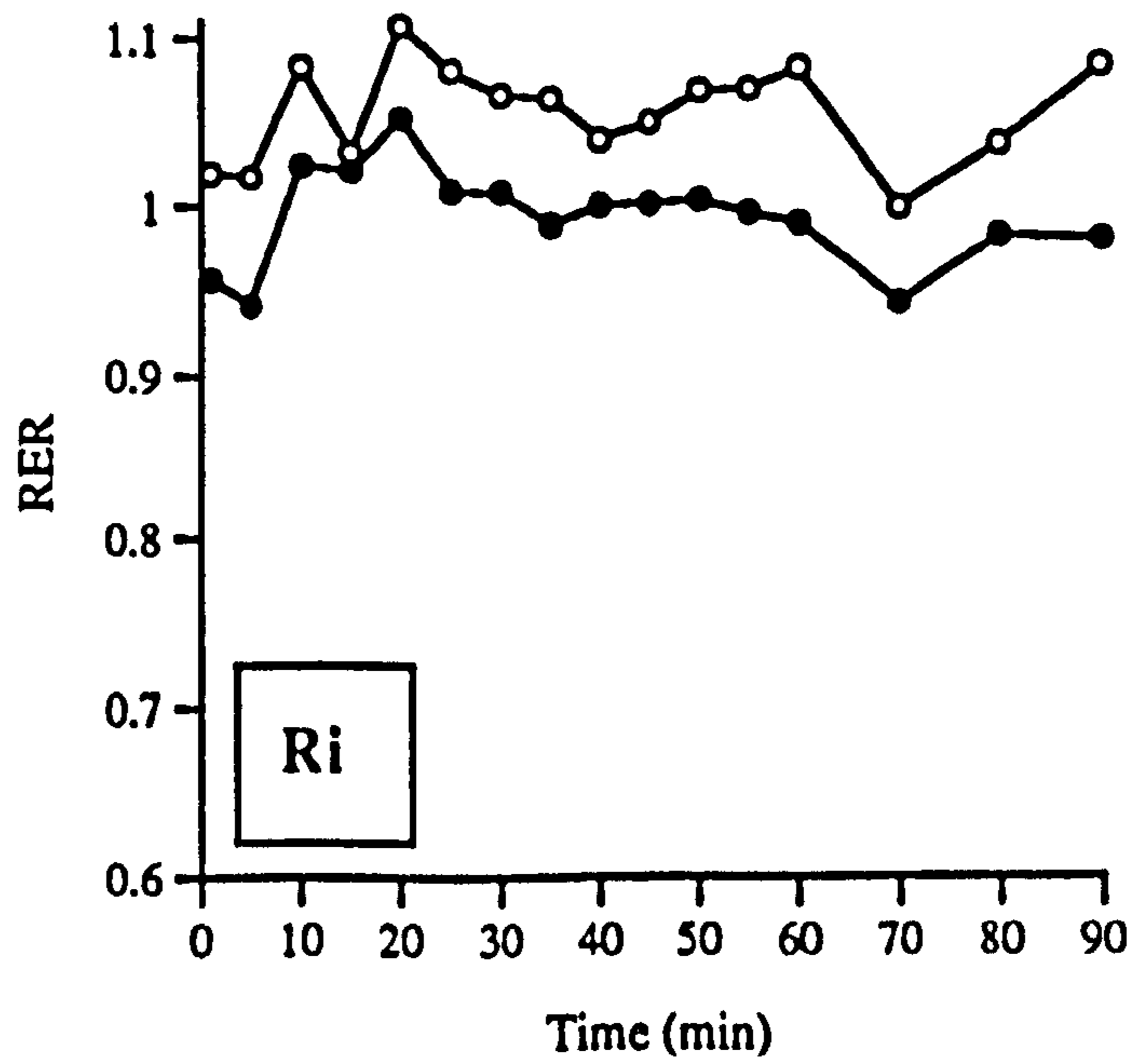
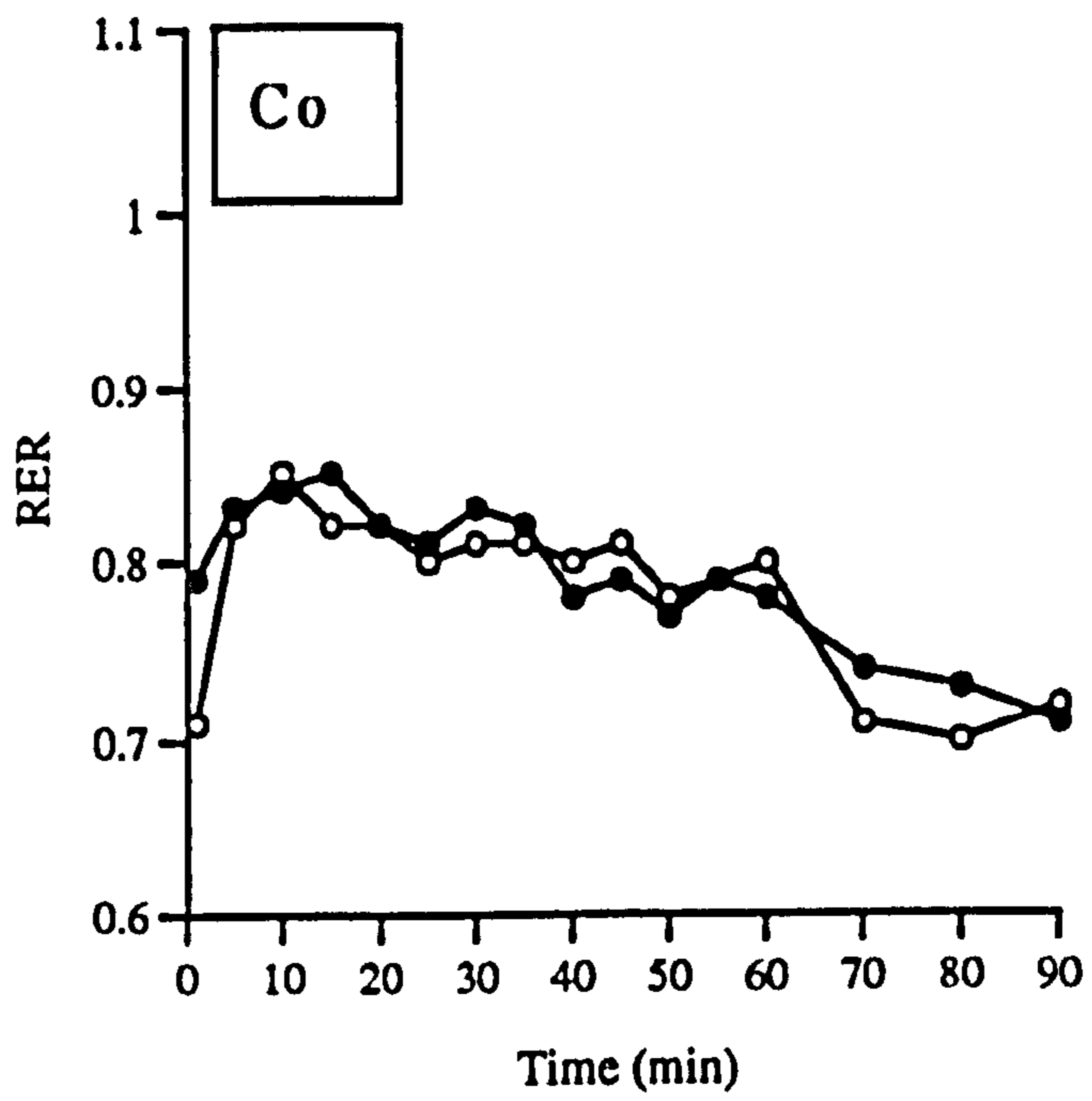
Fig 5.7 RER during 91 minutes of low intensity exercise during the control and test exercise sessions in horses (Br, Ro, Mo, Pe and Gl).



—●— Control

—○— Test

Fig 5.8 RER during 91 minutes of low intensity exercise during the control and test exercise sessions in horses (Co and Ri).



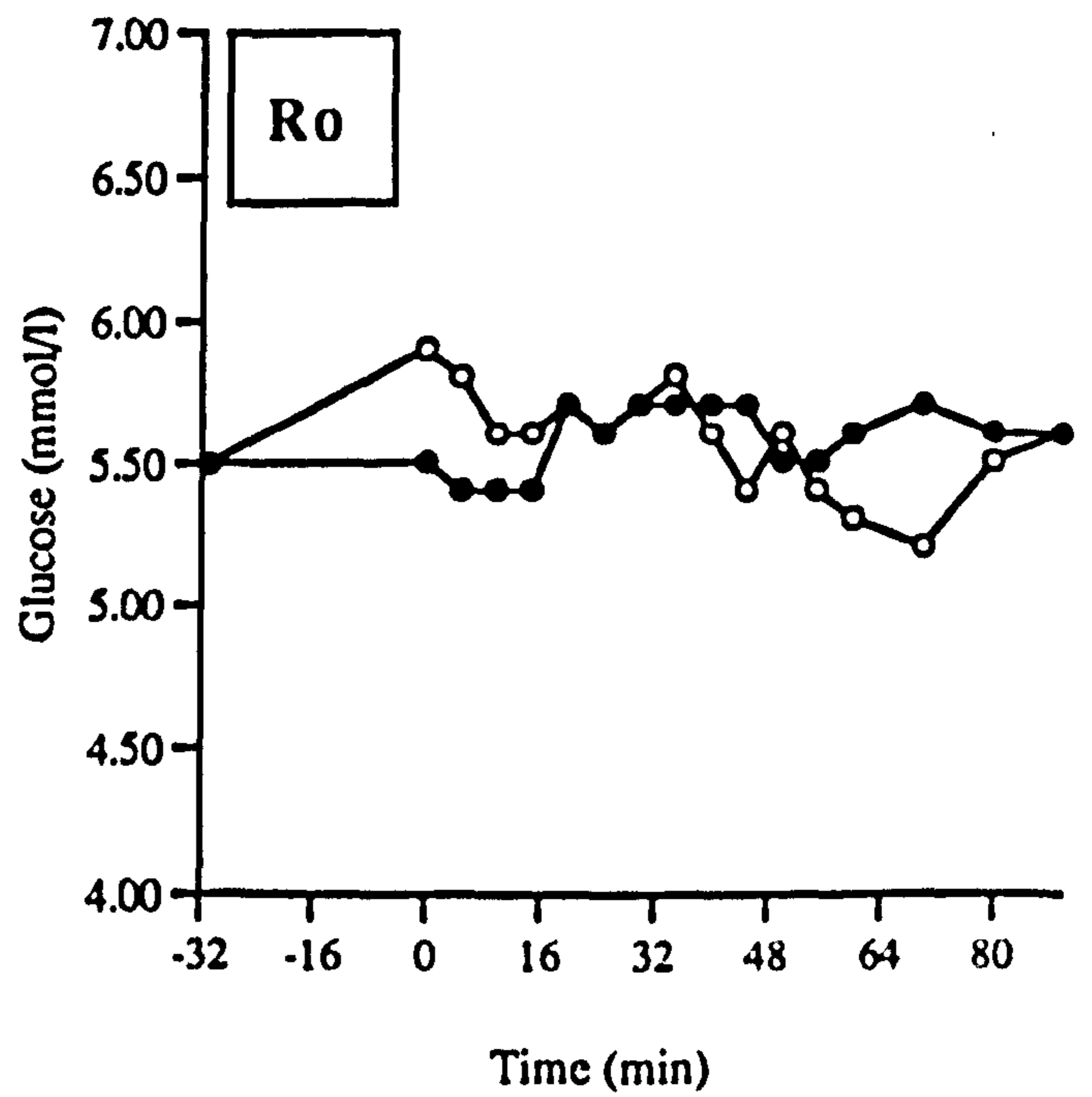
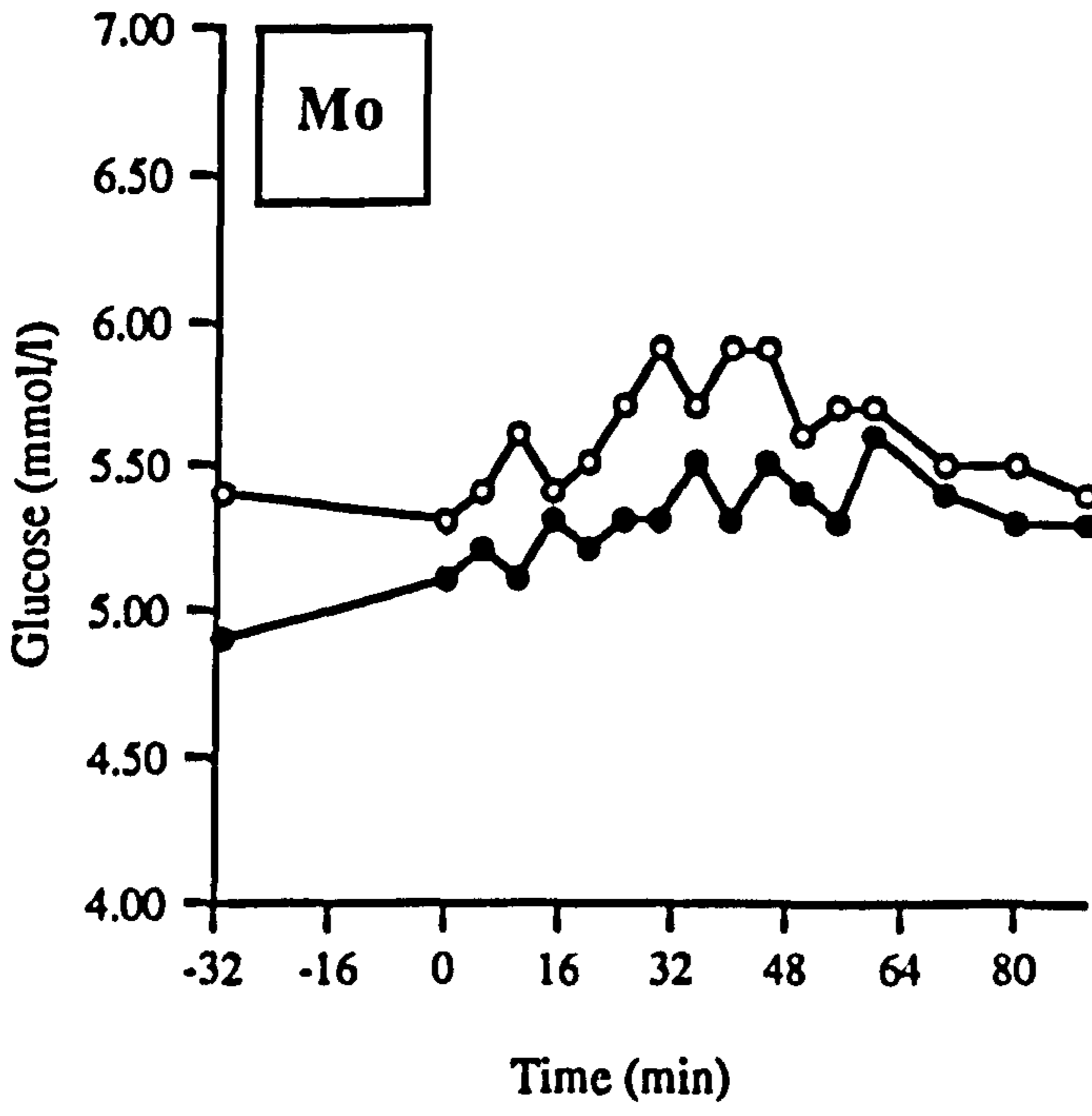
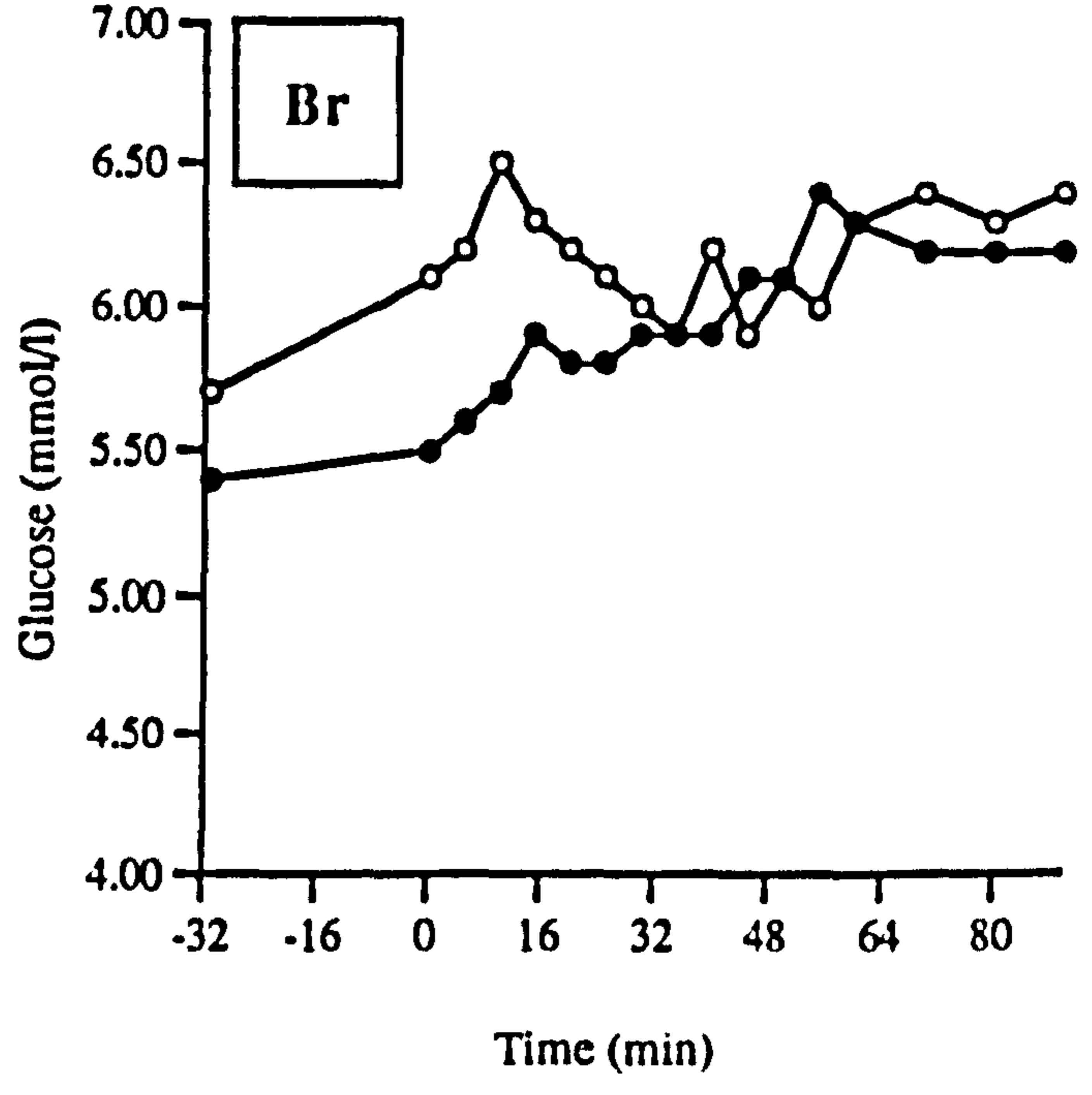
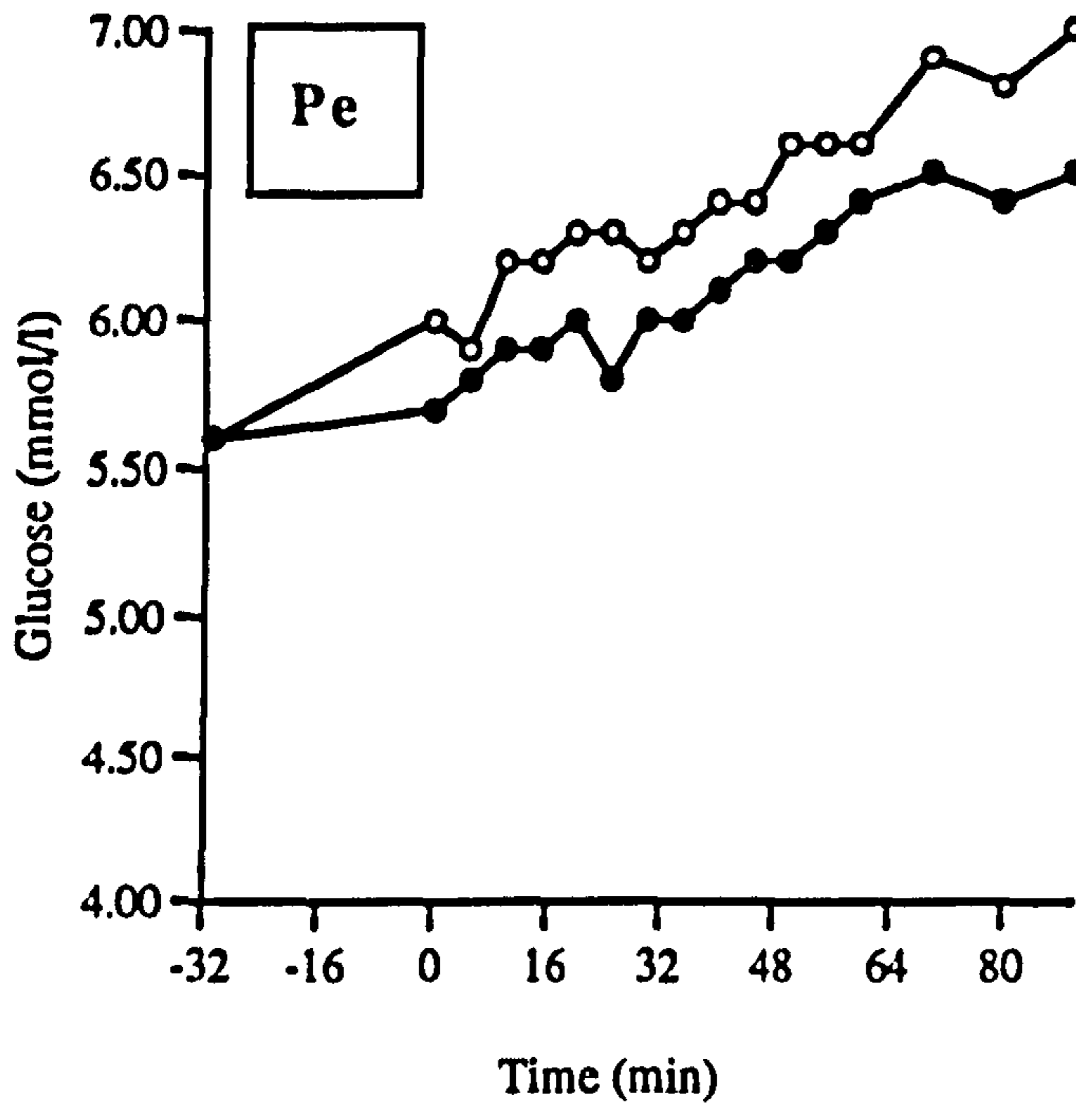
—●— Control

—○— Test

There was no significant difference between sessions in the plasma glucose concentration 31 minutes prior to the onset of exercise, i.e. before the administration of Iverlip and PP during session T ($p > 0.05$). However, at the onset of exercise 25 minutes after the administration of Iverlip and PP, plasma glucose concentration was higher during session T ($p < 0.01$). There was no significant change in the plasma glucose concentration during the control exercise session, (Figs 5.9, 5.10). During the test session plasma glucose concentration remained higher in 4 out of the 5 horses (Br, Ro, Mo, Pe) which exhibited a lower RER, for at least the first 15 minutes of exercise, during session T compared to C (Fig 5.9). Plasma lactate concentration was low during both exercise sessions, mean < 1.0 mmol/l. There was no significant change in plasma lactate concentration during either session ($p > 0.05$) and there was no difference in the concentration between the sessions ($p > 0.05$, Tables 1 and 2).

The post exercise muscle triglyceride/CS ratio was not significantly different from pre-exercise values during either session C or T. However, there was a trend towards an increase in the triglyceride/CS ratio after exercise during exercise session T (Tables 5.2 and 5.3). Muscle glycogen utilisation was low during both exercise sessions and there was no difference between sessions ($p > 0.05$). Mean muscle glycogen utilisation was 40.8 ± 44.1 and 40.8 ± 78.9 mmol/kg dw for the C and T exercise sessions, respectively (Tables 5.1 and 5.3). There was a large inter-horse variation in muscle glycogen utilisation.

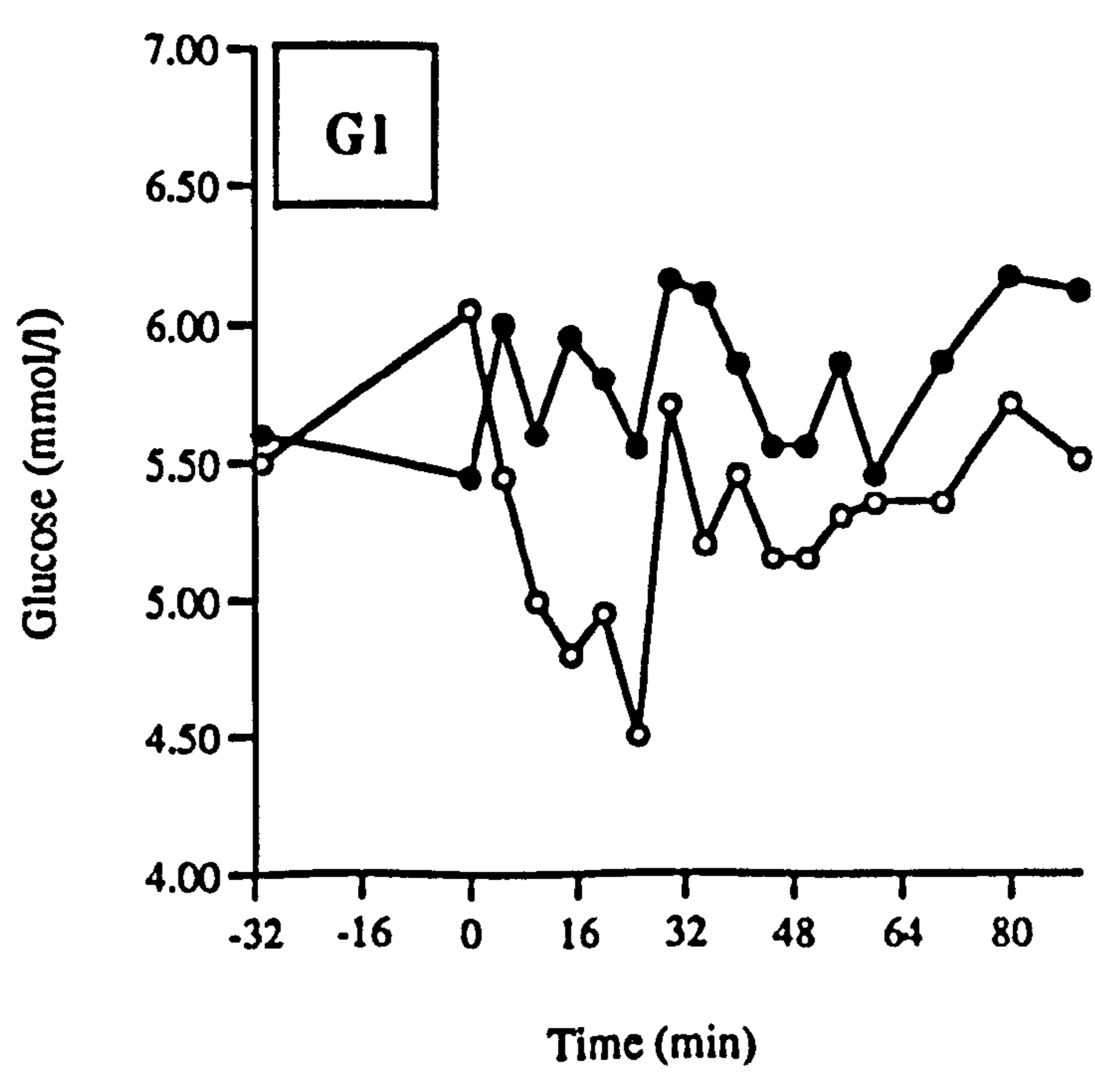
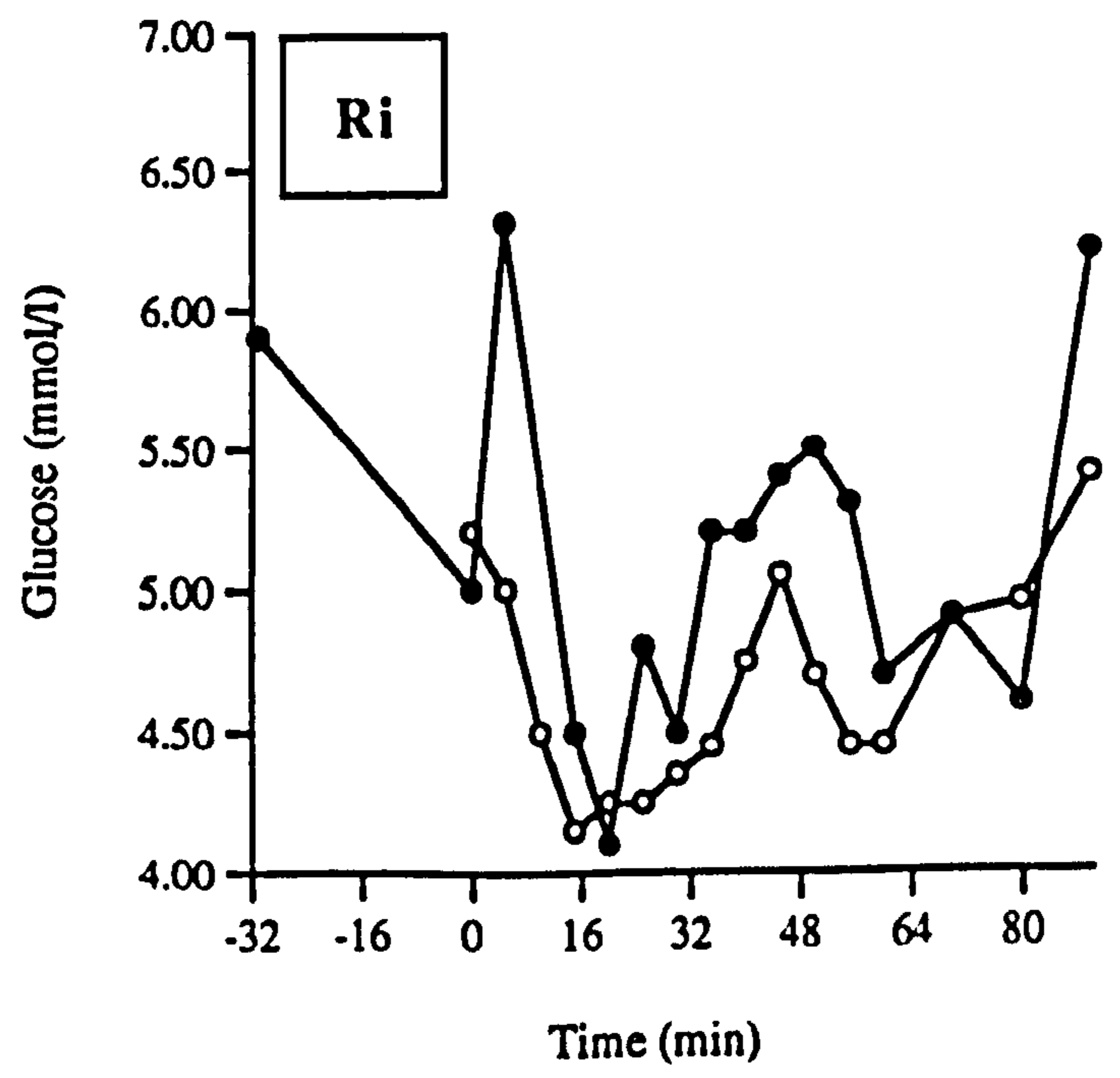
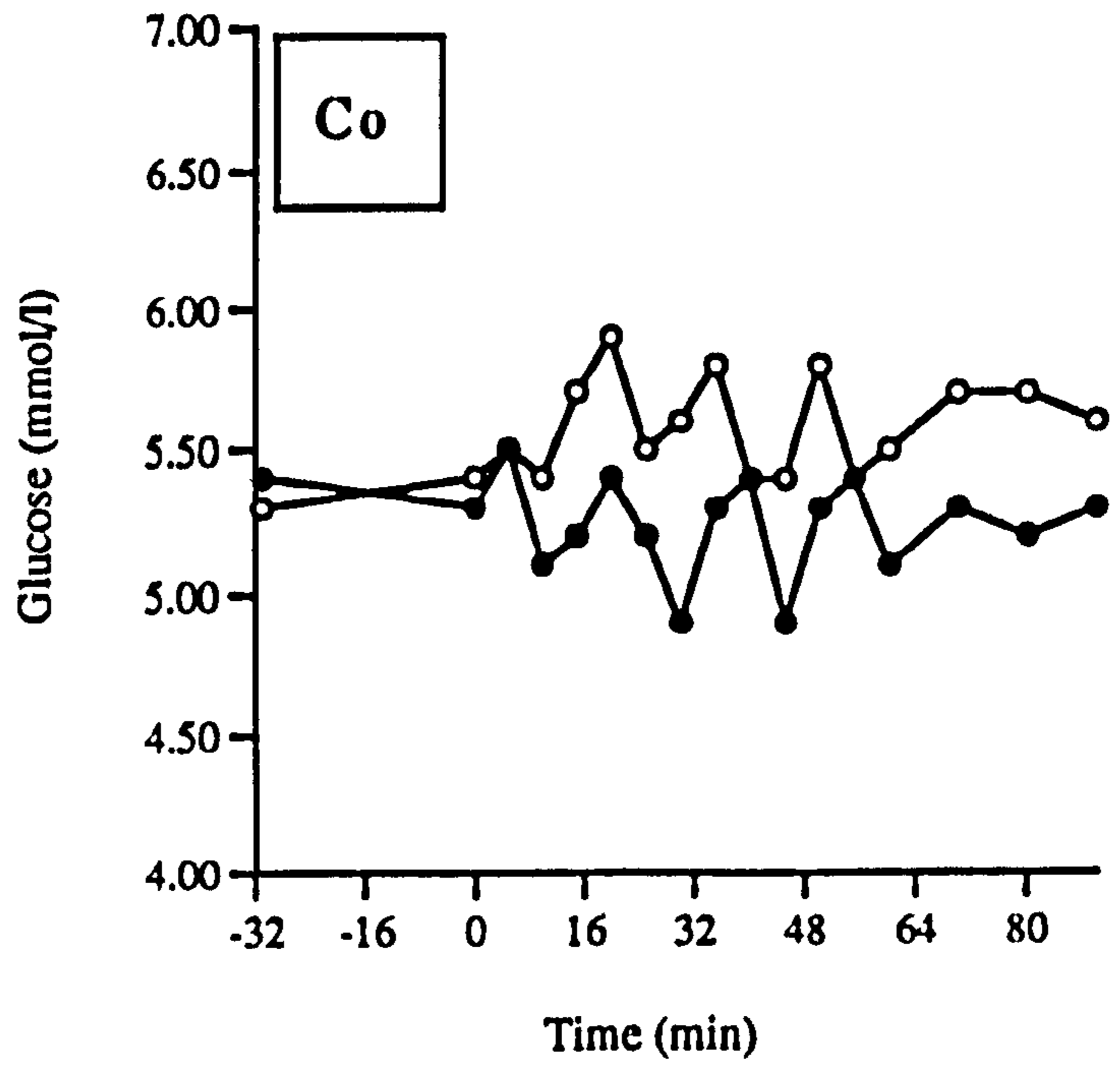
Fig 5.9 Plasma glucose concentration during 91 minutes of low intensity exercise during the control and test exercise sessions in horses (Br, Ro, Mo and Pe).



—●— Control

—○— Test

Fig 5.10 Plasma glucose concentration during 91 minutes of low intensity exercise during the control and test exercise sessions in horses (Gl, Co and Ri).



—●— Control —○— Test

Table 5.2 Mean concentration (\pm SD) of plasma glycerol; mean muscle glycogen concentration and mean muscle triglyceride content (expressed as a ratio with CS activity) during the control and test exercise sessions in horses (Br, Ro, Gl, Mo, Pe).

Table 5.3 **Concentration of plasma glycerol; muscle glycogen and muscle triglyceride content (expressed as a ratio with CS activity) during the control and test exercise sessions in horses (Co and Ri).**

Parameter		Pre infusion	Pre exercise	10' trot	20' trot	30'trot	40' trot	60' trot	30'
MUSCLE Glycogen (mmol/kg dw)	Co	493.2							
	C	503.0							
	Co	538.5							
	T	541.2							
	Co	23.2							
	C	23.6							
	Co	20.5							
	T	9.4							
CS (IU/kg dw)	Co	81.1							
	C	81.2							
	Co	141.3							
	T	109.7							
TRIG/CS (mmol/IU)	Co	0.286							
	C	0.291							
	Co	0.145							
	T	0.086							
PLASMA Glycerol (μ mol/l)	Co	34.0	41.3	57.8	110.1	123.2	161.3	194.6	198.6
	C	16.8	188.0	110.4	137.0	151.9	157.7	176.4	122.7
	Co	<20	<20	<20	22.3	46.6	65.3	77.9	59.5
	T	<20	13.7	33.8	52.3	103.6	94.7	44.8	91.5

C - control
TRIG - Triglyceride
T - test

Discussion

During session C there was no significant change in plasma triglyceride concentration, which as in other species (Gollnick and Saltin 1988), suggests that it does not represent a significant energy source during this type of exercise. At the onset of exercise during session T, (i.e. 25 minutes following the administration of Iverlip and PP), plasma triglyceride concentration remained significantly elevated. Triglyceride clearance from the plasma proceeded rapidly, the concentration returning to resting levels in all but one horse (Ri), following 30 minutes of trotting. During session T the plasma of horse Ri was visibly lipaemic and the triglyceride concentration failed to reach pre-infusion levels until 80 minutes of exercise had been completed. The slow clearance of plasma triglyceride in this horse may be partially explained by the post PP plasma T. Lip activity measured. Horse Ri exhibited a relatively low post PP plasma T. Lip activity ($7.8 \mu\text{mol/ml/hr}$) which increased only transiently in comparison to the other horses.

Plasma T. Lip activity was unchanged as the result of exercise during session C. During session T, post PP plasma T. Lip activity was significantly increased immediately before the onset of exercise (i.e. 25 minutes following the injection of PP). The plasma enzyme activity continued to rise in all horses with the exception of Ri (as indicated earlier), reaching a peak after completion of 30 minutes of exercise (i.e. 55 minutes following the injection of PP). As previously reported in Chapter 4, peak plasma T. Lip activity following the same dose of PP administered in isolation, occurred 30 minutes after the PP injection in the resting state. The co-administration of Iverlip and or the exercise undertaken in this instance, may have potentiated the increase in plasma T. Lip activity. Exercise has previously been reported to increase the activity of cardiac and skeletal muscle HEP releasable LPL (Ladu *et al* 1991; Lithell *et al* 1979). Muscle HEP releasable LPL resides in association with muscle capillary endothelium and can therefore be released into the plasma following injection of HEP or any of its analogues. Muscle HEP releasable LPL may have been elevated during exercise session C. However in the absence of PP, LPL was not detectable in the plasma.

The plasma FFA concentration was significantly raised prior to exercise as the result of co-administration of Iverlip and PP ($p < 0.001$) and was significantly higher during exercise ($p < 0.05$). There was considerable inter-horse variation in the rate of increase in plasma FFA concentration during session C and consequently the length of time during which the concentration remained below that of session T. After 60 minutes of trotting, plasma FFA concentration was higher during session T compared to session C in 5 out of the 7 horses (Mo, Pe, Gl, Co, Ri). This may partially explain the individual horse response to the exercise in terms of RER.

RER initially increased during the first 5-10 minutes of trot during both exercise sessions. This occurred, during the test exercise session despite a high plasma FFA concentration. This may be a reflection of the relative insensitivity of the β -oxidative and TCA pathways to a rapid change in the plasma FFA concentration. Furthermore, a transient increase in the rate of glycolysis and change in the pH of the cell may have depressed the rate of FFA oxidation at the start of exercise. RER was negatively correlated to plasma FFA concentration during both sessions illustrating the interdependence of the two parameters. The rate of change in RER, for a given increase in plasma FFA concentration, was similar during both exercise sessions. This suggests that FFA uptake and oxidation by skeletal muscle and hence RER was linearly related to plasma FFA concentration. Turcotte *et al* (1991) reported that the uptake of palmitate by intact perfused skeletal muscle followed saturation kinetics, when FFA concentration was expressed in terms of the 'free' unbound form. The concentration range, however, to which the perfused muscle was exposed in the latter study was much higher than the plasma FFA concentration observed in the present study. Over the total palmitate concentration range of 750-1250 $\mu\text{mol/l}$, which is equivalent to an unbound concentration of 0-0.25 $\mu\text{mol/l}$, palmitate uptake by the isolated muscle was not saturated and increased rapidly with increasing palmitate concentration (Turcotte *et al* 1991). The rate of decrease in RER during exercise session T was greater than that observed during session C in horses Ro and Br. Both of these horses exhibited a large initial increase in plasma FFA concentration at the start of exercise, as a result of Iverlip and PP administration. Equally,

however, both horses exhibited an extremely rapid decrease in plasma FFA concentration during the first 5-10 minutes of exercise. It is possible that the rapid uptake of FFA by muscle during this period meant that the actual FFA concentration to which muscle was exposed was not reflected by the plasma concentration during the remainder of the exercise period.

RER was not significantly different between sessions C and T. However, RER was consistently lower throughout exercise session T compared to C in 5 out of the 7 horses (Br, Ro, Mo, Pe, Gl), indicating an increased reliance on fat metabolism during the T exercise session. Of these 5 horses, 4 also exhibited an elevated plasma glucose concentration during session T compared to session C. Increased FFA utilisation in these horses may have resulted in a glucose sparing effect, as has been previously reported in other species (Costill *et al* 1977; Hickson *et al* 1977). Inhibition of glucose oxidation may have been mediated by inhibition of hexokinase or PDH. In cardiac muscle elevated levels of citrate, resulting from an increased flux through the TCA cycle, inhibit phosphofructokinase. This leads in turn to an accumulation of glucose-6-phosphate and hence, hexokinase inhibition, i.e. the so called Randle Cycle (Randle *et al* 1963). A similar effect in skeletal muscle has not as yet been demonstrated unequivocally during exercise. Similarly, inactivation of PDH may occur as the result its phosphorylation by pyruvate kinase, due to an increase in the ratios of acetyl CoA / CoA and NADH / NAD⁺, as a result of increased FFA oxidation, as reviewed by Randle *et al* (1988).

The plasma glucose concentration immediately before the start of exercise during session T, 25 minutes following the administration of Iverlip and PP, was significantly higher than at the corresponding time during the control session. This occurred despite no difference in plasma glucose concentration between the two sessions, before the administration of Iverlip and PP. It suggests that inhibition of glucose utilisation at rest may have occurred as a result of increased FFA utilisation. An increase in skeletal muscle citrate concentration at rest has

been demonstrated in conditions of elevated plasma FFA concentration, which may facilitate this glucose sparing effect (Spriet 1994).

There was no apparent muscle glycogen sparing effect as the result of the pre-exercise elevation in plasma FFA concentration, although the total amount utilised was low. Differences in fibre composition of pre and post muscle biopsies make whole biopsy muscle glycogen data notoriously difficult to interpret. This is due to patterns of fibre recruitment and therefore inter-fibre differences in glycogen utilisation during exercise.

There was no significant difference between pre and post exercise muscle triglyceride content, (expressed as a ratio with CS), during either exercise session. However, there was a trend towards an increase in muscle triglyceride content during session T, in some horses. This may be the result of extra-cellular triglyceride cycling. Extra-cellular triglyceride cycling is the result of re-esterification of plasma FFA which have been transported to a tissue other than adipose tissue, mainly the liver (Wolfe 1992). The rate of appearance of FFA in the plasma and the total rate of fat oxidation influences the fraction of FFA that are recycled (Wolfe 1992). During session T, an increase in the availability of FFA for re-esterification due to an increase in muscle blood flow and plasma FFA concentration, may have resulted in a net accumulation of muscle triglyceride, in the situation where a further increase in muscle FFA oxidation was not possible. It has been reported that during the recovery phase of an exercise bout when availability of FFA is high but energy requirement and hence FFA oxidation is reduced, there is an increase in the proportion of the FFA flux that is re-esterified (Wolfe 1992).

Horses Ri and Co showed no reduction in RER in response to the pre-exercise elevation in plasma FFA. Horse Ri exhibited a very slow clearance of infused triglyceride and a correspondingly low increase in pre-exercise plasma FFA and glycerol. This may be related to the relatively low post PP plasma T. Lip activity which was unaffected by exercise. RER was comparatively high in this horse at around 1.0 during both exercise sessions and showed

little decrease with the duration of exercise. Post-exercise muscle triglyceride content was also increased during session T in this horse. Horse Ri may have an inherent high dependence on carbohydrate utilisation and an inability to utilise fat to a great extent during exercise, even in the presence of elevated plasma FFA. This may be a reflection of the fibre composition of the muscle mass in this horse. Horse Co exhibited a relatively low RER during both exercise sessions in comparison to the other horses and may have a naturally high dependence on fat utilisation during low intensity exercise. This may again be a reflection of the fibre composition of the muscle mass in this horse.

Whilst the number of horses used in this study was limiting, 5 out of the 7 horses exhibited a lower RER in response to the pre-exercise elevation in plasma FFA; and 4 of these 5 also presented a glucose sparing effect during the test session. These results present evidence to suggest that the thoroughbred horse has the capacity to increase the utilisation of fat during prolonged low intensity exercise. Increased fat utilisation was apparent only in a proportion of the horses studied. However, this was probably influenced by the individual biological and metabolic variation in the response to exercise, including the individual's inherent ability to utilise fat.

Chapter 6

Changes in Resting Muscle and Blood Parameters Associated with a Fat supplemented Diet

Introduction

Previous research has suggested that the inclusion of fat into the equine diet may have beneficial effects on exercise performance, either through direct substrate effects or through some other indirect mechanism. Shifts in substrate utilisation from carbohydrate to fat, during both low intensity prolonged (Hambleton *et al* 1980; Hintz *et al* 1987) and moderate (Oldham *et al* 1990) or high intensity exercise (Duren *et al* 1987; Harkins *et al* 1992) have been suggested. However, the mechanism involved in the suggested increase in fat utilisation during exercise, as a result of fat supplementation in the horse, is unresolved.

An alteration in exercise performance during prolonged exercise, attributed to an increase in the relative contribution of fat as a fuel source, has been reported in other species. Endurance exercise performance was preserved, despite dietary carbohydrate restriction and subsequent reduction in resting muscle glycogen concentration, in Man and rats fed a high fat diet (Phinney *et al* 1983; Conlee *et al* 1990). Increased endurance capacity has also been described in trained cyclists (Lambert *et al* 1994), sledge dogs (Hammell *et al* 1977) and rats (Miller *et al* 1984) fed diets containing 38, 70 and 78% fat respectively, when compared to a control group fed an isocaloric high carbohydrate diet. It has been proposed that under conditions of carbohydrate restriction, a shift occurs in the carbon source for the TCA cycle from carbohydrate to FFA. This results in a rise in the oxidative capacity and enhancement of the β -oxidising capacity (Simi *et al* 1991). An increase in the activity in muscle of both CS and β -HAD has been reported in response to diets containing 78% of the caloric content in the form of fat (Miller *et al* 1984; Simi *et al* 1991). The supply of FFA to exercising muscle may also be increased as a result of feeding a high fat diet. Kiens *et al* (1987) and Conlee *et al* (1990) reported an increase in the concentration of muscle triglyceride in response to feeding a high fat diet. Furthermore, an increase in the activity of muscle LPL in response to fat supplementation suggests an increase in the capacity of muscle to utilise FFA derived from lipoprotein associated triglyceride (Delorme and Harris 1975; Jacobs *et al* 1982; Kiens *et al* 1987).

In previous equine studies the amount of fat contained in the fat supplemented diets, when expressed as a percentage of the total energy intake, is much lower when compared to that used in other species (Delorme and Harris 1975; Jacobs *et al* 1982; Kiens *et al* 1987). Previously used fat supplemented diets have typically contained between 5-35% of the total energy content of the diet (DE) in the form of fat (Hambleton *et al* 1980; Pagan *et al* 1993; Pagan *et al* 1987; Hintz *et al* 1987; Harkins *et al* 1992; Hollands and Cudderford 1993). The expression of the fat content of the diet in previous equine studies has not been standardised, which makes comparisons between studies more difficult. The fat content of the diet has been expressed as either a percentage of the weight or energy content (DE) of the concentrate portion or of the whole diet.

There are few reports which describe the effect of fat supplementation on the resting concentration of plasma and muscle metabolites. An increase in resting muscle glycogen concentration has been associated with fat supplementation in the horse (Meyers *et al* 1989; Oldham *et al* 1990; Harkins *et al* 1992; Scott *et al* 1992). Equally several authors have failed to show a similar increase in resting muscle glycogen concentration (Pagan *et al* 1987; Greiwe *et al* 1989; Hodgson *et al* 1994).

The aims of the following study were as follows:

- a) To further investigate the effect of fat supplementation on resting muscle glycogen concentration, in horses at a stable level of fitness and energy intake.
- b) To describe any changes in muscle triglyceride concentration in response to feeding a fat supplemented diet.
- c) To assess the capacity for uptake of FFA derived from plasma lipoprotein associated triglyceride, by muscle and other tissues, in response to a fat supplemented diet using measurements of post pentosan polysulphate total lipase activity (post PP T.Lip).
- d) To describe any changes in resting plasma metabolite concentrations in response to feeding a fat supplemented diet compared to a high carbohydrate control diet.
- e) To Investigate the effect of fat supplementation on key oxidative enzymes in muscle.

Concurrently the effect of fat supplementation on exercise metabolism was also investigated. The methodology and the results of which are discussed in Chapter 7.

Materials and Methods

Seven thoroughbred horses weighing between 405-543 kg (3 fillies and 4 geldings) aged between 3-8 years were trained for a period of 10 weeks. A regimen of walking and trotting was employed for the first 6 weeks of training, for approximately 1 hour per day, except on Sundays. On the 7th week of training, and throughout the remainder of the feeding study, all horses additionally underwent a 10 furlong (1200 meters) canter (10-12 m/s) 3 times a week.

During the last 7 weeks of the training period, designated the baseline period, all horses were fed a low fat pelleted control diet (C, Table 6.1). Following the baseline period the horses were randomly divided into a control group (C: horses Co, Ho & Je; 2 fillies and 1 gelding) and a fat supplemented group (F), (horses Bo, Li, Mo & Pe; 1 filly and 3 geldings). Horses in group C were fed diet C throughout the study. Horses in group F were fed a pelleted fat supplemented diet (F, Table 6.1) for a period of 10 weeks, designated the feeding period. Diets C and F were calculated to be isocaloric and isonitrogenous with an ME of 9.8 MJ/kg which relates to an approximate DE of 12.4 MJ/kg (as calculated using a computer programme, Format International, UK). Both diets were fed at 1.37% of body weight together with hay at 1% of body weight. Diets C and F supplied 0.22 and 1.0 g of fat/kg body weight, respectively. The total amount of energy (DE) supplied by the fat content of diets C and F, including hay, were calculated to be 3 and 20 % respectively. The pelleted diets were analysed for oil (petroleum ether extract), protein, crude fibre, calcium, phosphorus and starch and the hay for oil (petroleum ether extract), protein, dry matter and calcium and phosphorus by Spillers, Dalgety Agriculture who manufactured the diets (Table 6.2). Following the 10 week period of fat supplementation the horses in group F were reverted to the control diet for a further 5 weeks, designated the washout period.

Table 6.1 **Percentage of ingredients used in the manufacture of the control (low fat) and the fat supplemented diets, on a dry matter basis.**

Ingredient	% Dry Matter	
	Control	Fat Supplemented
Barley	30.00	----
Wheat	3.00	----
Wheatfeed	29.30	33.40
Straw	1.00	14.20
Grass	15.0	10.30
Sugar beet pulp	7.50	----
Soya Oil	----	8.0
Lysine	0.07	0.05
Molasses	8.80	----
Limestone	1.30	2.40
Dicalcium phosphate	2.46	1.05
Salt	0.71	0.88
Minerals & Vitamins	0.80	0.90
Mould Inhibitor	0.08	0.08

Table 6.2 Percentages of oil, protein, fibre, calcium, phosphorus, salt, sodium, methionine, lysine, sugar, starch and vitamins A, D3 and E in the control and fat supplemented diets (calculated and actual)*.

*Calculated values were estimated from the raw ingredients using a computer programme (Format International, UK) and actual values were obtained by direct analysis of the feed.

% Dry Matter Basis				
Constituent	Control		Fat Supplemented	
	Calculated	(Actual)	Calculated	(Actual)
Oil	2.0	(2.8)	10.0	(9.5)
Protein	12.0	(11.4)	12.0	(12.9)
Fibre	9.2	(8.9)	18.0	(15.4)
Calcium	1.7	(1.6)	1.7	(1.7)
Phosphorus	0.90	(0.82)	0.65	(0.68)
Salt	1.2		1.2	
Sodium	0.4		0.4	
Total Lysine	0.44		0.44	
Methionine	0.15		0.16	
Starch	22.3		8.0	
Sugar	11.6		5.0	
Vitamin A	8000		8000	
Vitamin D3	1000		1000	
Vitamin E	237		237	

Feed intake

The pelleted feed and hay ration were divided into two meals that were weighed separately. Hay and pelleted feed were initially fed together, after exercise in the morning, at approximately 9.30am and in the afternoon at 4.30pm. Any residual feed that remained after the morning meal was carried over and added to the evening meal. Any feed that had then not been eaten by the following morning was removed and weighed. The actual feed intake and energy intake were calculated on a daily basis. No palatability problems or significant feed refusals were encountered with either diet. The forage component of the diet was fed approximately 2 hours before the pelleted portion after the 6th week of the baseline period, in order to encourage the horses to eat all of their hay. Forage intake was increased in all horses as a result of this adjustment.

Blood samples

At the end of the 7th week of the baseline period (Sunday) and at the end of every week thereafter, blood samples were obtained from all horses by venepuncture. Blood samples were analysed for plasma glucose, FFA, triglyceride and cholesterol. All blood samples were taken in the morning before feeding the concentrate portion of the diet.

Body weight

At the end of the 1st week of the baseline period (Sunday) and each week thereafter all horses were weighed in the morning before feeding.

Muscle samples

Muscle biopsy samples were obtained from the middle gluteal muscle, as described previously, at the end of the baseline period, after 3, 6 and 10 weeks of the feeding period and at the end of the washout period. The biopsy samples were taken alternately from the right and left middle gluteal muscle. A sub-sample of the biopsy was retained for histochemical fibre analysis and the remainder was used for the analysis of triglyceride and glycogen concentration and CS and β -HAD activities. All muscle samples were obtained at

least 24 hours following exercise on the previous day. In order to account for differences in the fibre composition of serial muscle biopsy samples triglyceride concentration and CS and β -HAD activities were expressed as a ratio to the combined fractional area occupied by type I and IIa fibres.

Post pentosan polysulphate total lipase activity

Plasma samples were obtained for the analysis of T. Lip activity, both before and 10 minutes after the intravenous injection of PP (1.3 mg/kg bw). Samples were taken at the end of the baseline period, after 3, 6 and 10 weeks of the feeding period and at the end of the washout period.

Digestion trial

Between weeks 7 and 8 of the feeding period a digestion trial was conducted. All faeces were collected throughout 7 consecutive 24 hour periods. Individual faeces were collected into separate bags mixed and a sub-sample of constant size taken. Total weight of faeces produced each day was recorded. Sub-samples of each 24 hour collection were combined to produce a single mixed sample per horse. The samples were freeze-dried and were analysed for percentage dry matter, protein, fibre, ether extract and acid insoluble ash by Dalgety Agriculture Ltd. Using the hay, pelleted feed and faecal analysis the digestibility of the total diet was determined. A predicted value for the fibre content of the hay and the dry matter of the pelleted feed was used.

Statistical analysis

A 2-factor ANOVA for repeated measures was used to identify significant effects of diet or time. Dietary treatment was designated as factor 1 with 2 levels: control and fat supplemented. The second factor, the repeat measure, was time in weeks. Significance was declared at ($p < 0.05$). In the instance where significance was established a multiple comparison test (Fisher's PLSD test) was applied, and again significance reported at ($p < 0.05$). An unpaired Student's t test was used to identify differences between diets.

Results

The intake of hay was increased in horses Bo, Li and Mo once it was fed before the concentrate portion of the diet. This was reflected in the calculated total energy intake during the baseline, feeding and washout periods (Table 6.3). Feed intake was sufficient for maintenance of body weight at the level of work undertaken and was therefore maintained at initially designated levels in all horses, except horse Li. Mean digestibility of ether extract, protein and fibre was 67 ± 7 , 85 ± 14 and $79 \pm 10\%$ respectively for the control diet. The corresponding digestibilities for the test diet were 85 ± 3 , 81 ± 4 and $122 \pm 13\%$ respectively. The digestibility of ether extract and fibre was significantly higher in the fat supplemented horses ($p < 0.01$) whilst there was no significant difference in protein digestibility between dietary groups.

A slow decline in body weight was observed in horse Li, a 2 year old gelding, after 5 weeks of the feeding period. As a result, the pelleted portion of the feed of this horse was increased by 1.2 kg and the hay by 0.8 kg. Horse Li had previously been described as a horse that was difficult to maintain weight when in full training. Body weight was restored once the ration of this horse was increased and at the end of the study his weight was no different to that prior to the study. In all other horses body weight fluctuated randomly with no apparent effect of diet (Fig 6.1). There was no significant interaction between diet and time with regard to body weight during the feeding period. There was no significant change in body weight throughout the feeding study in horses in group F. Horses in group C, however, showed a small but significant increase in body weight by the 4th week of the washout period with respect to week 1 ($p < 0.05$).

Plasma glucose and FFA

The plasma concentration of both FFA and glucose fluctuated significantly with time ($p < 0.001$) but there was no significant effect of diet (Table 6.4)

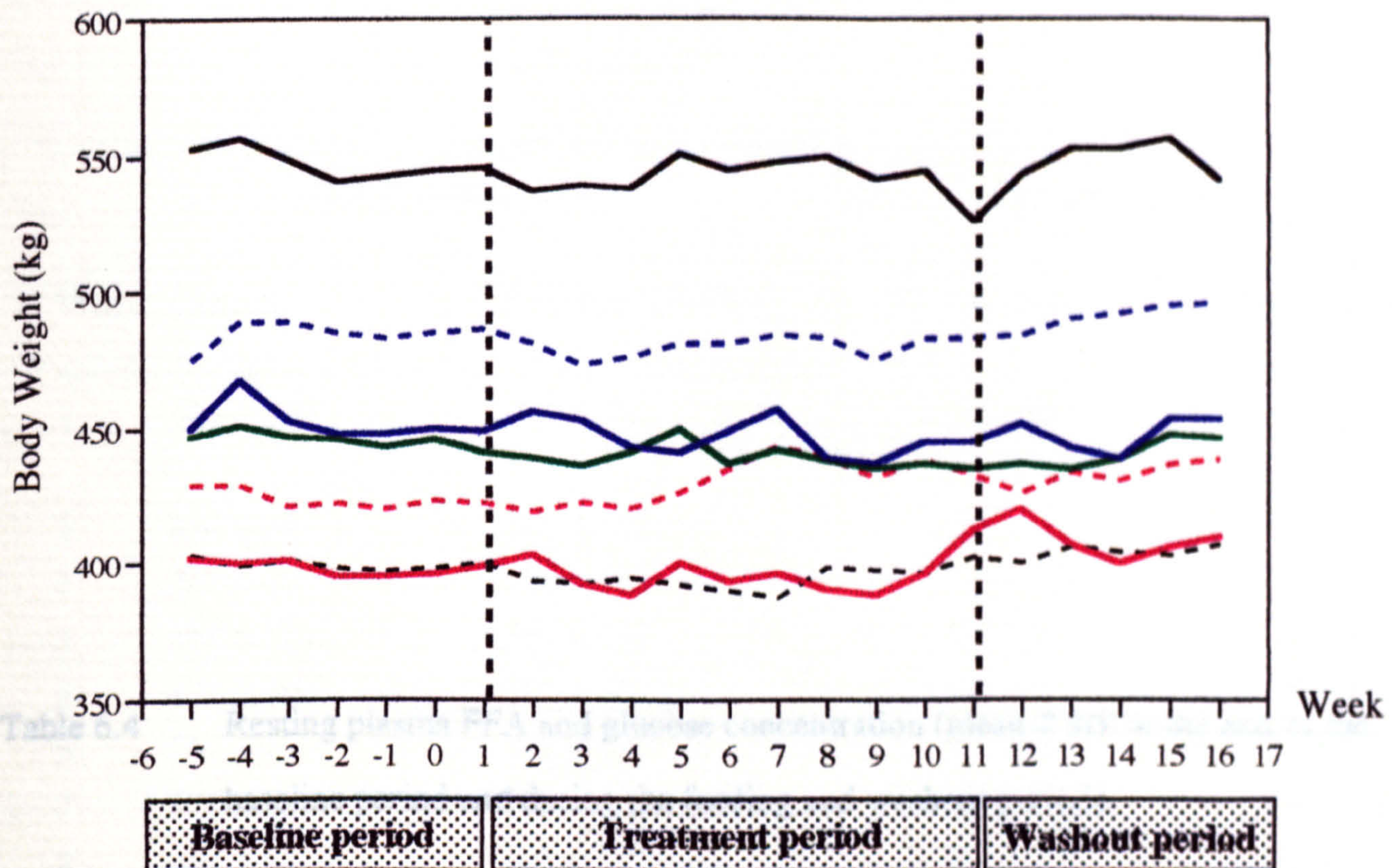
Table 6.3 Mean total energy intake (MJ) and energy intake per kg body weight in individual horses during the baseline, feeding and washout periods in horses maintained on either a control low fat or fat supplemented diet.

Total Energy Intake (MJ)

Energy Intake MJ/kg bw

Horse	Control Group				Fat Supplemented Group				Control Group				Fat Supplemented Group			
	Co	Ho	Je	Pe	Bo	Li	Mo	Pe	Co	Ho	Je	Bo	Li	Mo	Pe	
Baseline	118.8	92.9	86.0	102.1	93.8	127.6	111.4	0.25	0.23	0.2	0.23	0.23	0.24	0.23	0.25	
Feeding	118.9	89.0	100.2	110.1	99.5	132.0	112.6	0.25	0.25	0.23	0.25	0.25	0.25	0.24	0.25	
Washout	118.6	99.0	104.4	110.6	99.1	132.3	112.6	0.24	0.25	0.24	0.25	0.25	0.24	0.24	0.25	

Fig 6.1 **Variation in body weight (kg) during the baseline, feeding and washout periods of the feeding study in individual horses.**



Week 1: end of baseline period Weeks 2-11: baseline period
 Weeks 12-16: washout period

- | Fat Group | Control Group |
|-----------|---------------|
| — Mo | - - - Co |
| — Pe | - - - Je |
| — Br | - - - Ho |
| — Li | |

Table 6.4 Resting plasma FFA and glucose concentration (mean \pm SD) at the end of the baseline period and during the feeding and washout periods.

Week 1 end of baseline period

Weeks 2-11 feeding period

Weeks 12-16 washout period

Week	FFA ($\mu\text{mol/l}$)		Glucose (mmol/l)	
	Control	Fat Supplemented	Control	Fat Supplemented
1	594 \pm 220	604 \pm 16	5.6 \pm 0.3	5.9 \pm 0.2
2	240 \pm 44	336 \pm 122	5.6 \pm 0.7	5.4 \pm 0.2
3	397 \pm 81	495 \pm 168	5.7 \pm 0.2	5.5 \pm 0.2
4	464 \pm 201	390 \pm 132	5.5 \pm 0.1	5.4 \pm 0.3
5	434 \pm 103	583 \pm 163	5.2 \pm 0.2	5.2 \pm 0.1
6	235 \pm 57	372 \pm 227	5.6 \pm 0.4	5.4 \pm 0.1
7	201 \pm 60	282 \pm 79	5.2 \pm 0.2	5.2 \pm 0.1
8	241 \pm 30	321 \pm 107	5.1 \pm 0.3	5.4 \pm 0.4
9	481 \pm 55	435 \pm 116	5.3 \pm 0.4	5.6 \pm 0.5
10	251 \pm 25	267 \pm 139	5.5 \pm 0.4	4.8 \pm 0.2
11	342 \pm 158	322 \pm 169	5.6 \pm 0.3	5.1 \pm 0.2
12	120 \pm 5	109 \pm 24	5.3 \pm 0.2	5.3 \pm 0.5
13	201 \pm 79	266 \pm 88	4.7 \pm 0.4	5.2 \pm 0.3
14	120 \pm 30	169 \pm 110	5.3 \pm 0.5	5.5 \pm 0.2
15	177 \pm 84	235 \pm 139	5.4 \pm 0.5	5.4 \pm 0.2
16	182 \pm 112	126 \pm 79	5.2 \pm 0.2	5.3 \pm 0.1

Plasma cholesterol

Plasma cholesterol concentration was unchanged throughout the sampling period in the horses in group C ($p < 0.05$). A significant interaction between sampling week and diet ($p < 0.001$) was identified within group F. Plasma cholesterol concentration increased significantly in horses in group F during the period of fat supplementation ($p < 0.05$, Fig 6.2). The cholesterol concentration was significantly greater than the baseline concentration after 1 week of fat supplementation but was no longer significantly different 3 weeks after withdrawal of the fat supplemented diet (Fig 6.2). Although the plasma cholesterol concentration was increased during fat supplementation, the concentration remained within the normal range at all times (1.9-3.9 mmol/l) and there was no significant difference between the two dietary groups ($p > 0.05$).

Plasma triglyceride

Plasma triglyceride concentration was unchanged throughout the sampling period in horses in group C ($p > 0.05$). A significant interaction between sampling week and diet ($p < 0.002$) was identified within dietary group F. Triglyceride concentration decreased significantly during the period of fat supplementation of the horses in group F and was significantly lower than the baseline concentration after 5 weeks of fat supplementation. The plasma triglyceride concentration of the horses in group F was also significantly lower than those of group C during this period ($p < 0.05$, Fig 6.3)

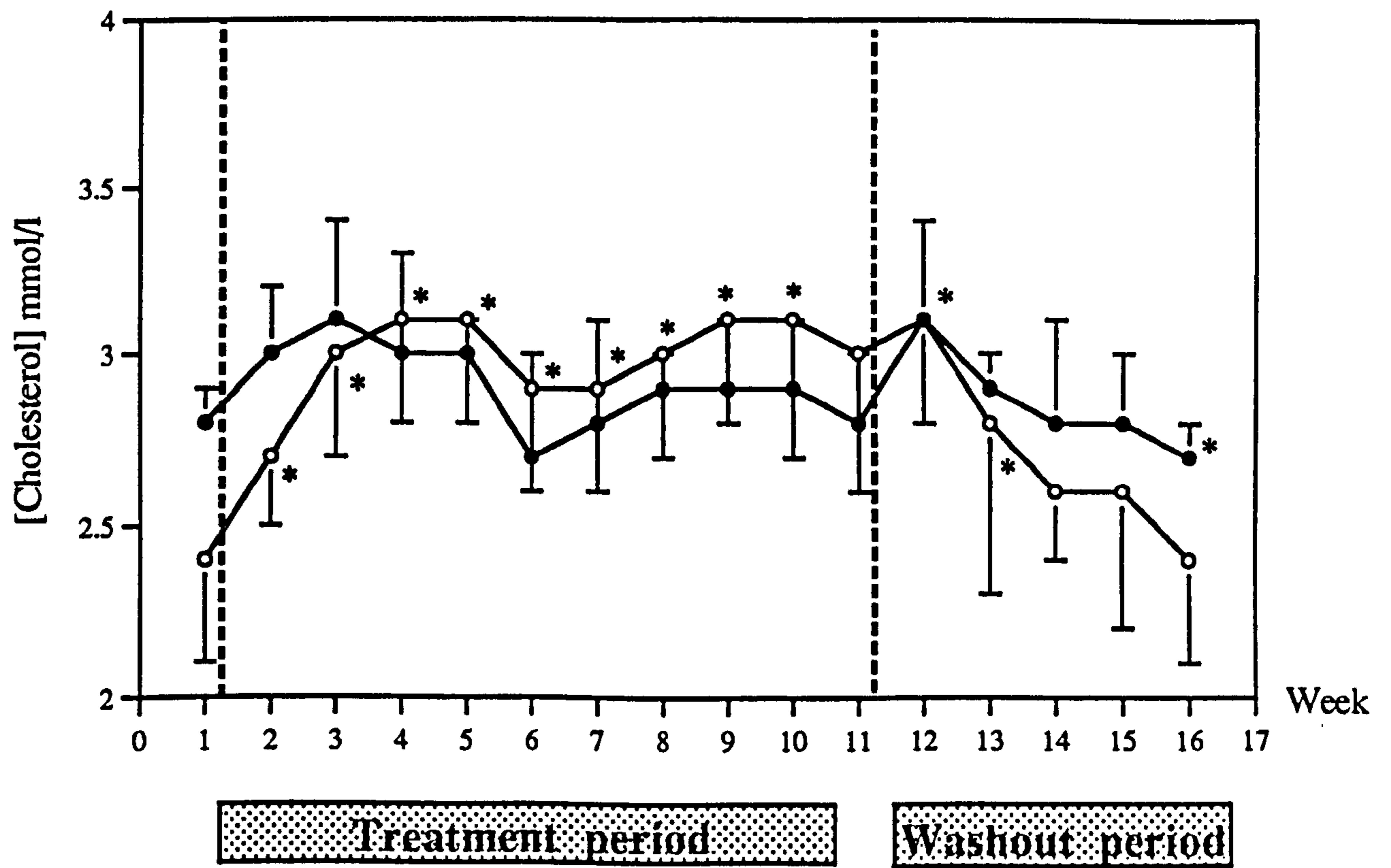
Fig 6.2 Resting plasma cholesterol concentration (mean \pm SD) at the end of the baseline period and during the feeding and washout periods.

Week 1 end of baseline period

Weeks 2-11 feeding period

Weeks 12-16 washout period.

* Significant difference from week 1 within group F ($p < 0.05$).



—●— Group C

—○— Group F

Fig 6.3 Resting plasma triglyceride concentration (mean \pm SD) at the end of the baseline period and during the feeding and washout periods.

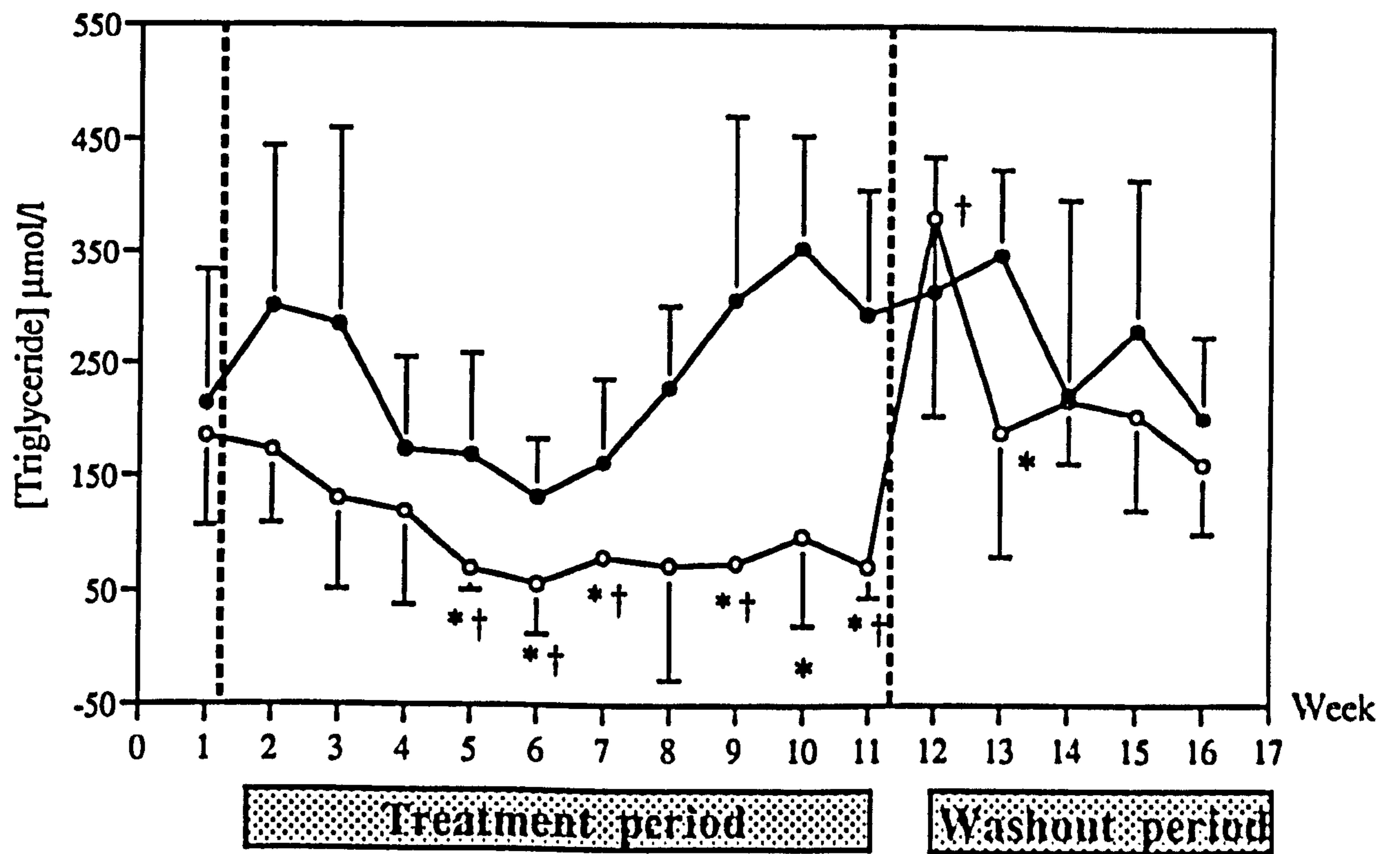
Week 1 end of baseline period

Weeks 2-11 feeding period

Weeks 12-16 washout period

* Significant difference from week 1 within group F ($p < 0.05$).

† Significant difference from corresponding week between groups ($p < 0.05$).



—●— Group C

—○— Group F

Plasma post pentosan polysulphate total lipase activity

Plasma T. Lip activity measured before the injection of PP was negligible and was unchanged in either dietary group during the sampling period. Horses in group F, however, exhibited a significant increase in post PP T. Lip activity during fat supplementation ($p < 0.05$, Fig 6.4). Post PP T. Lip activity was significantly elevated above the baseline activity after 3 weeks of fat supplementation. Peak enzyme activity was reached after either 6 or 10 weeks of fat supplementation in individual horses (Fig 6.5). Mean post PP T. Lip activity was no longer significantly different from the baseline activity 5 weeks after the withdrawal of the fat supplemented diet.

Muscle metabolites and enzyme activities

There was no significant interaction between sampling week and dietary group with respect to muscle glycogen concentration ($p > 0.05$, Table 6.5). Similarly, there was no significant difference in muscle triglyceride concentration prior to and after the period of fat supplementation, either within or between dietary groups ($p > 0.05$, Table 6.6). Prior to the adjustment of muscle enzyme activity for the fibre composition of biopsy samples there was no significant change in either muscle CS or β -HAD activity, as a result of fat supplementation. However, there was a significant increase in the adjusted muscle CS activity after 10 weeks of fat supplementation in dietary group F ($p < 0.05$, Table 6.6). There was no corresponding change in adjusted CS activity in dietary group C. There was a trend towards an increase in adjusted muscle β -HAD activity after 10 weeks of fat supplementation in dietary group F, however, this increase was not significant ($p > 0.05$, Table 6.6)

Fig 6.4 Post PP plasma T. Lip activity (mean \pm SD) at the end of the baseline period and during the feeding and washout periods.

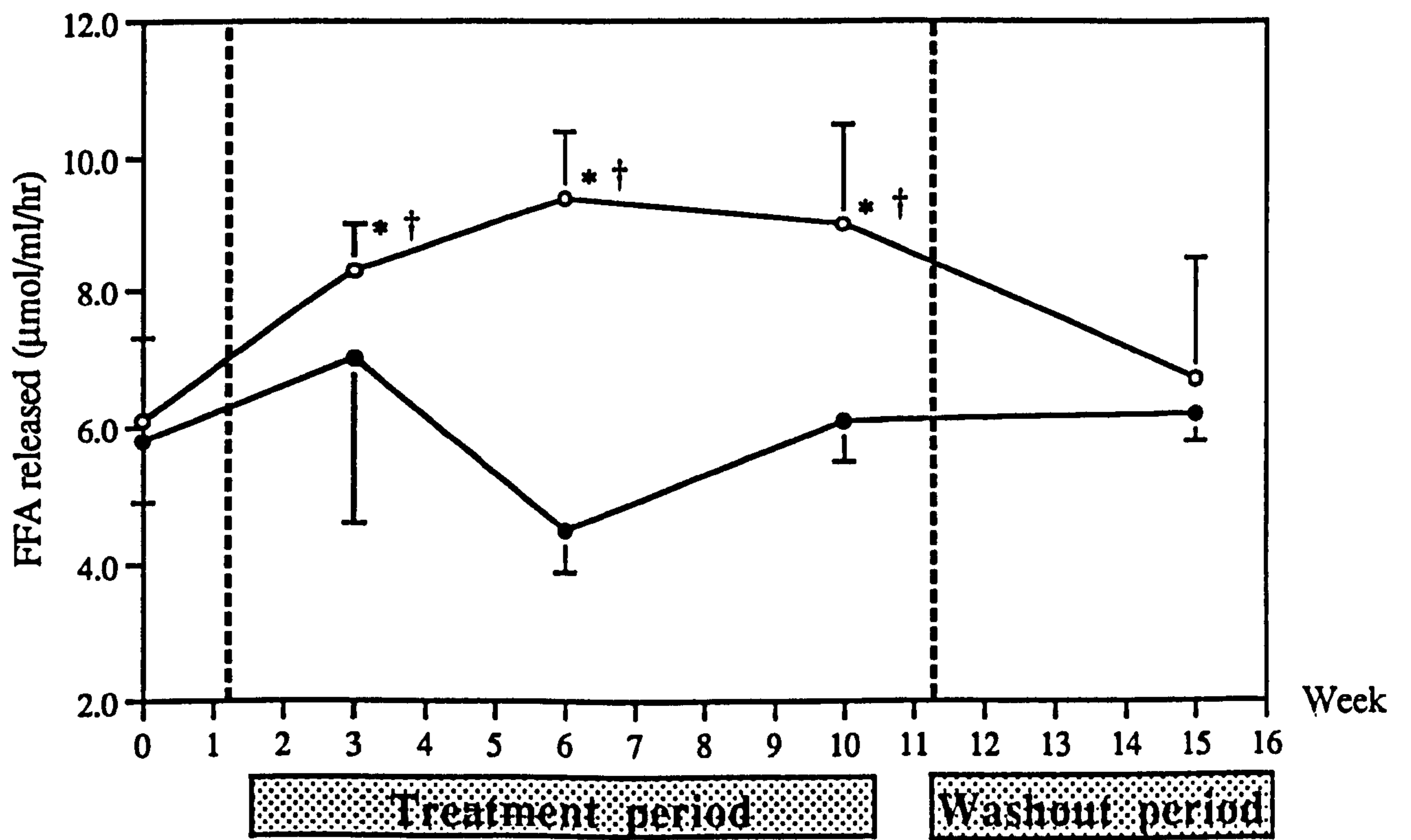
Week 1 end of baseline period

Weeks 2-11 feeding period

Weeks 12-16 washout period

* Significant difference from week 1 within group F ($p < 0.05$).

† Significant difference from corresponding week between groups ($p < 0.05$).



—●— Group C

—○— Group F

Fig 6.5 Post PP plasma T. Lip activity in individual horses (group C) during the baseline period, feeding and washout periods.

Week 0 baseline period

Weeks 2 - 11 feeding period

Weeks 12-16 washout period

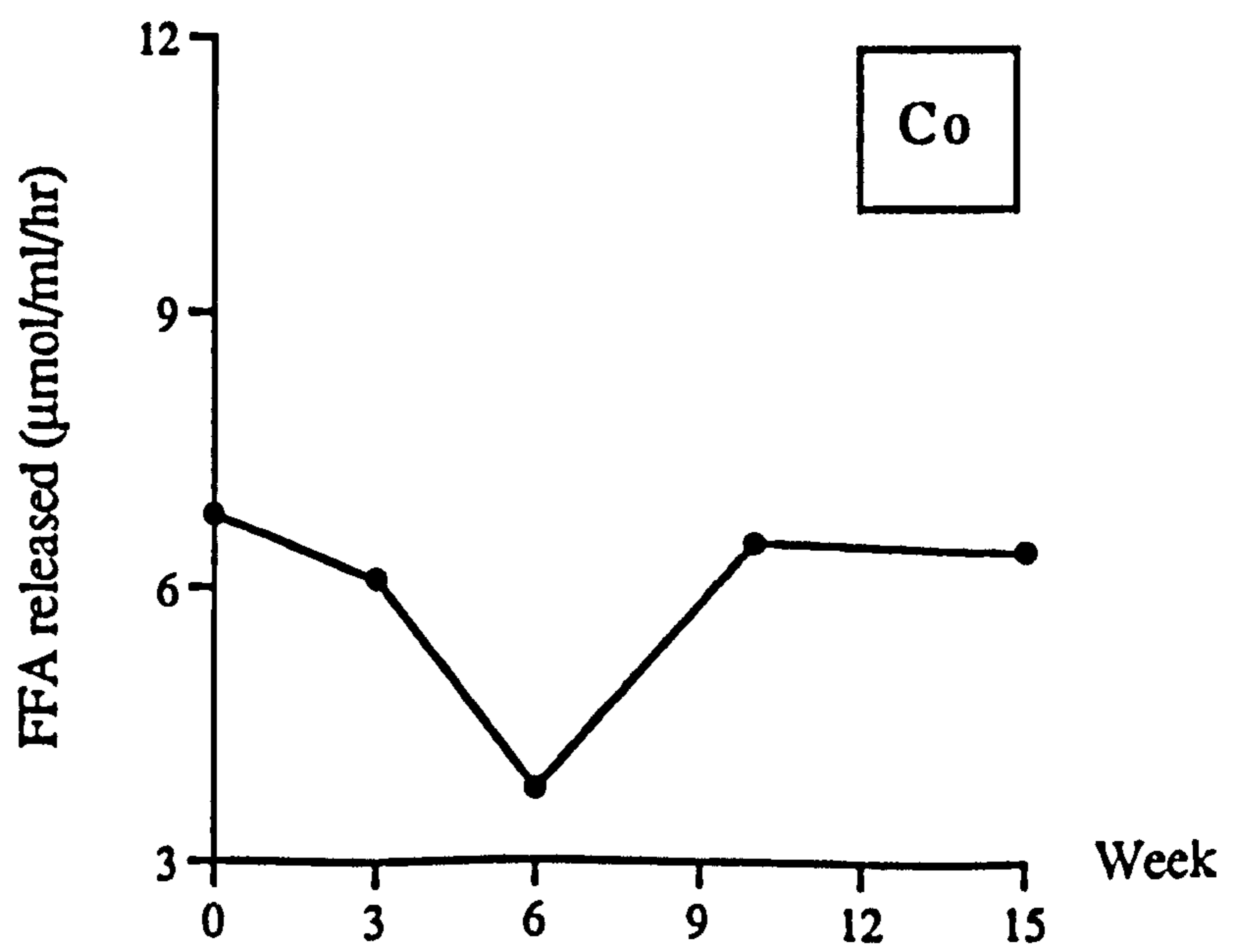
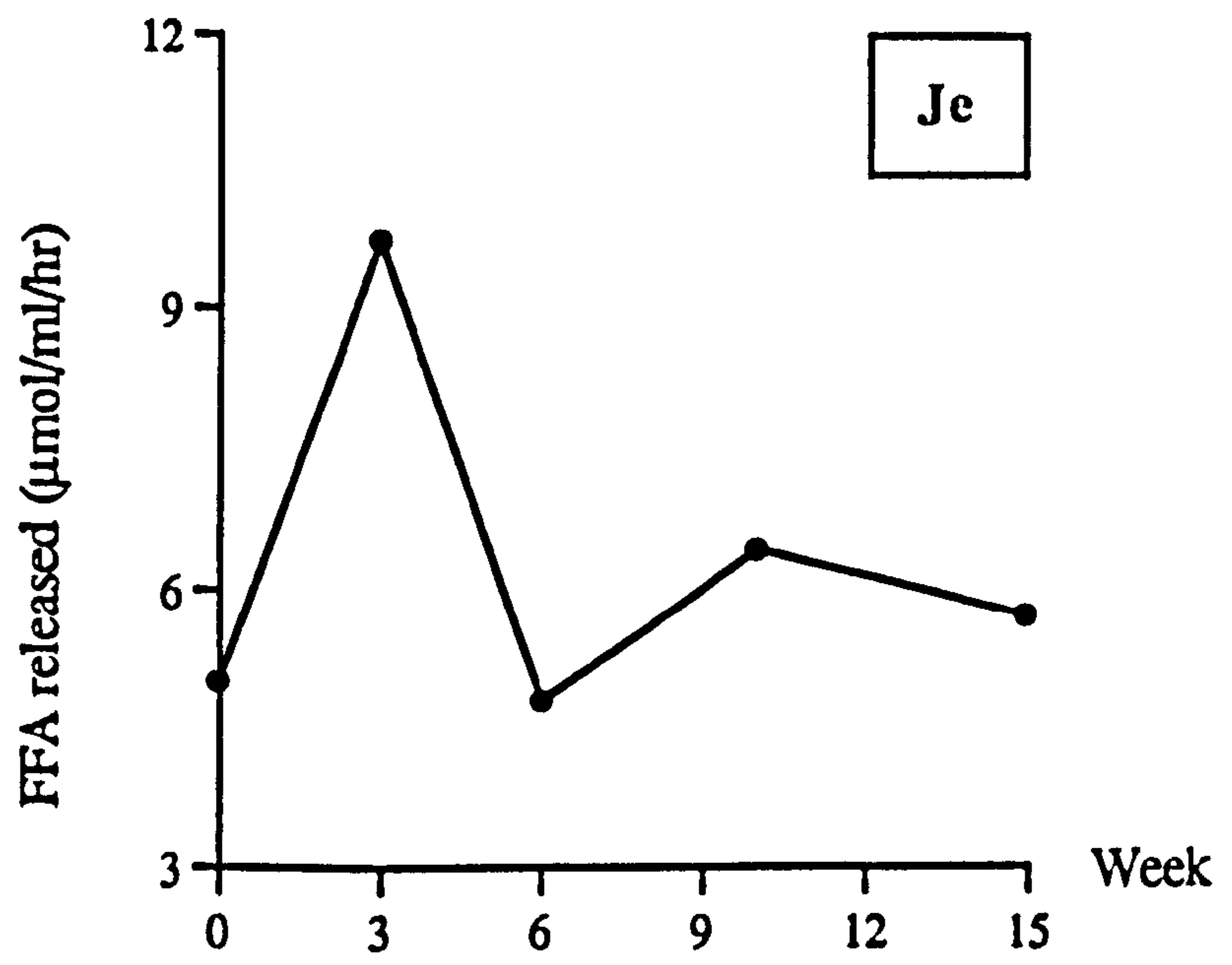
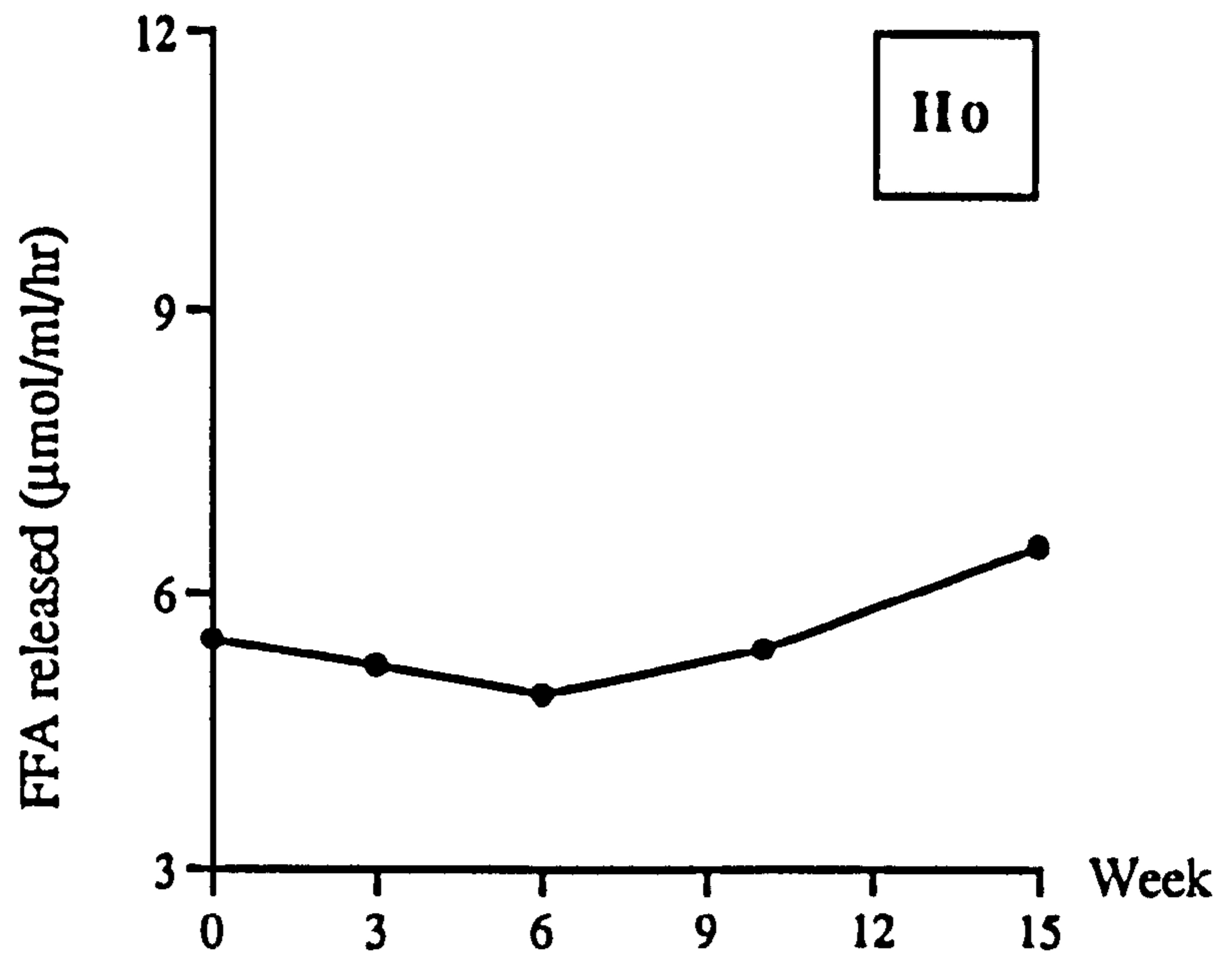


Fig 6.6 Post PP plasma T. Lip activity in individual horses (group F) during the baseline period, feeding and washout periods.

Week 0 baseline period

Weeks 2-11 feeding period

Weeks 12-16 washout period

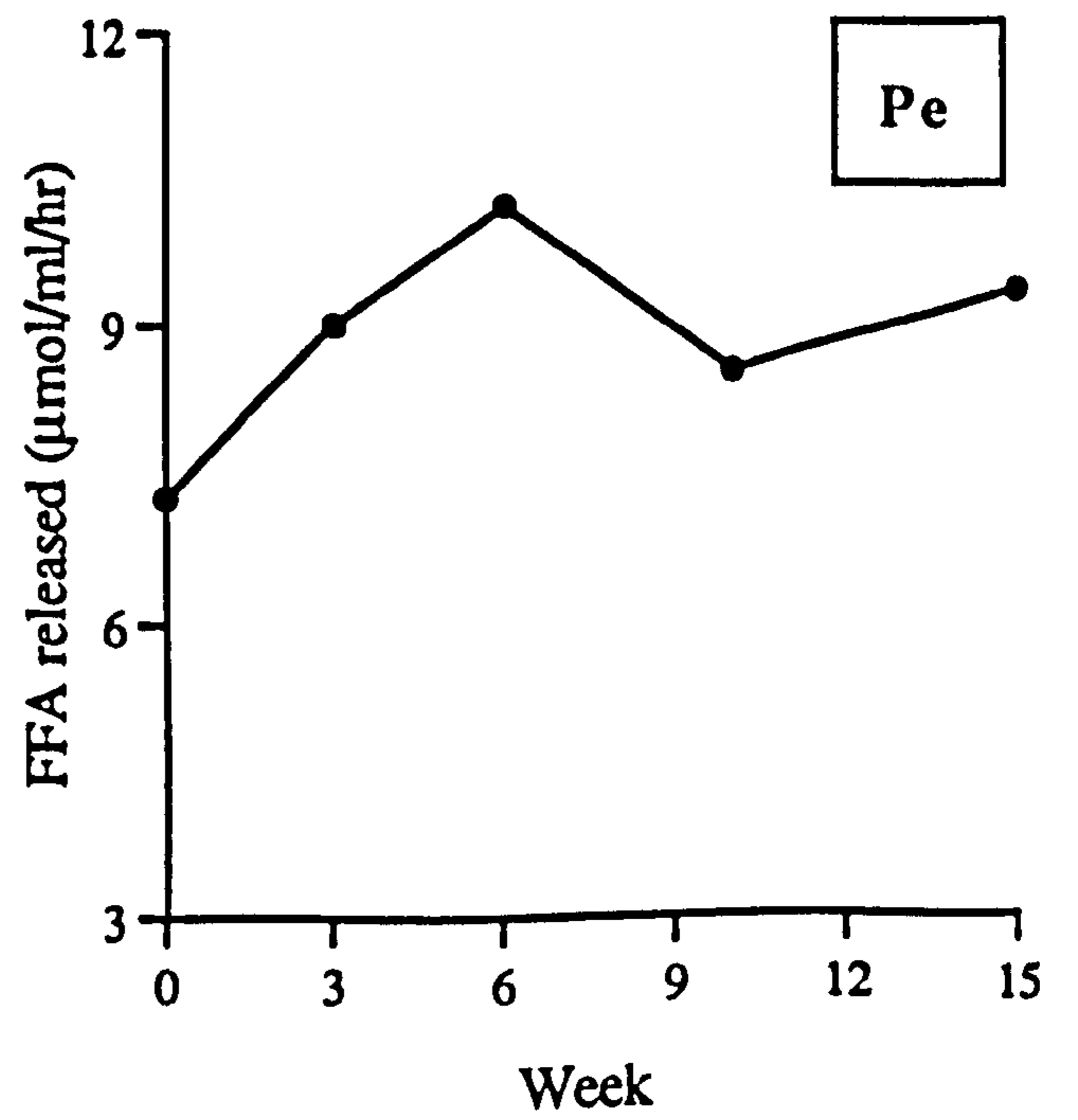
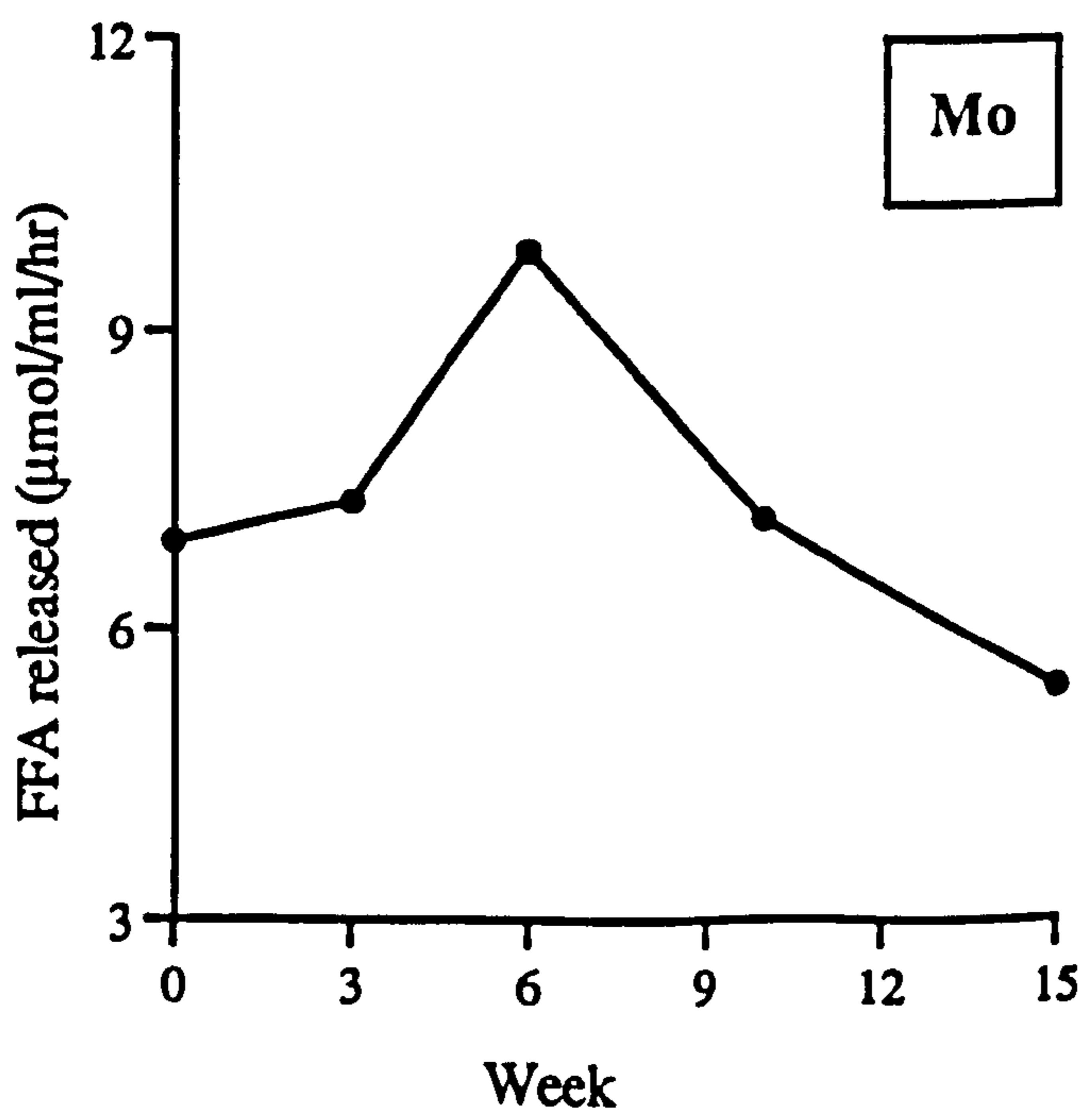
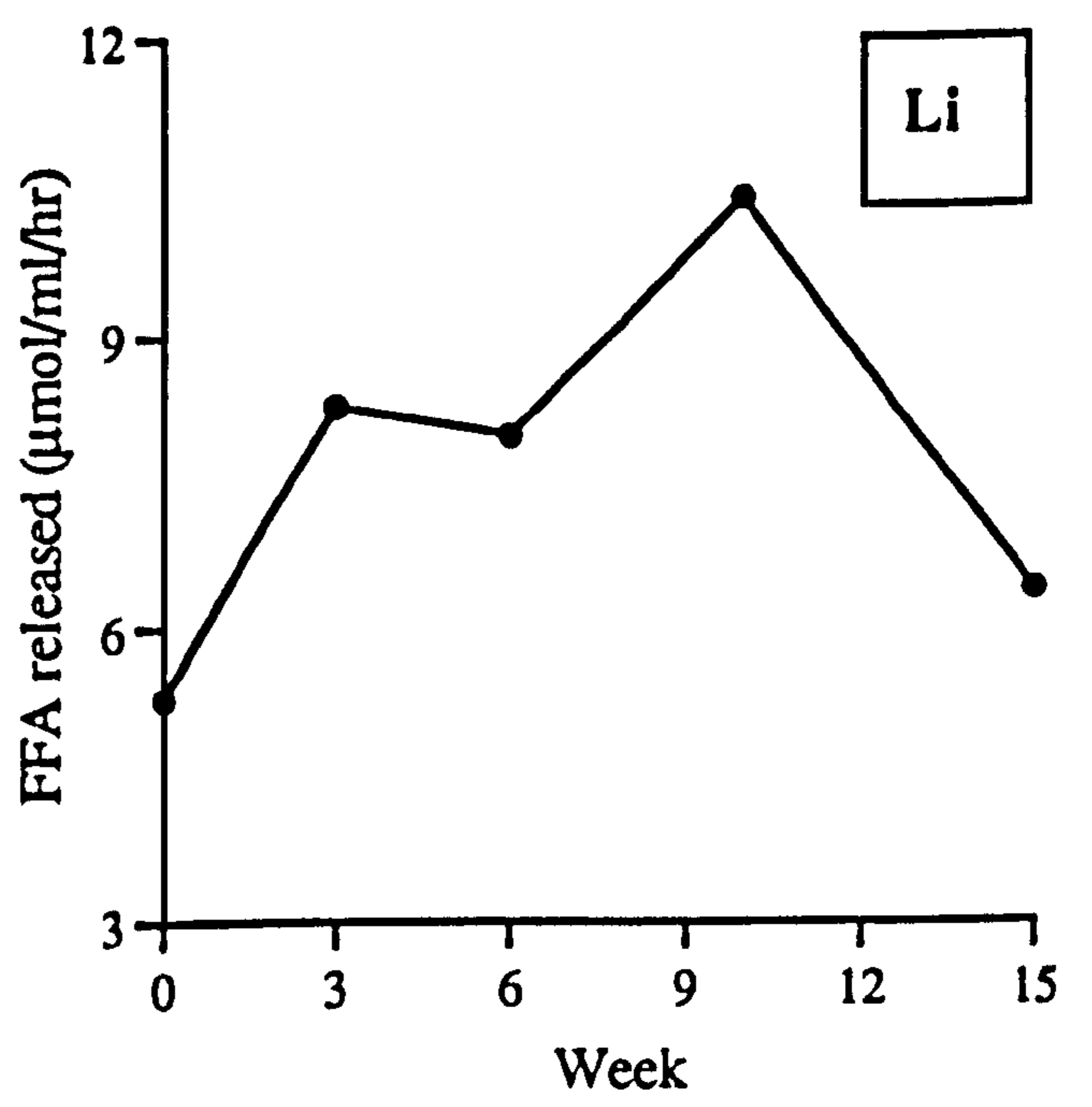
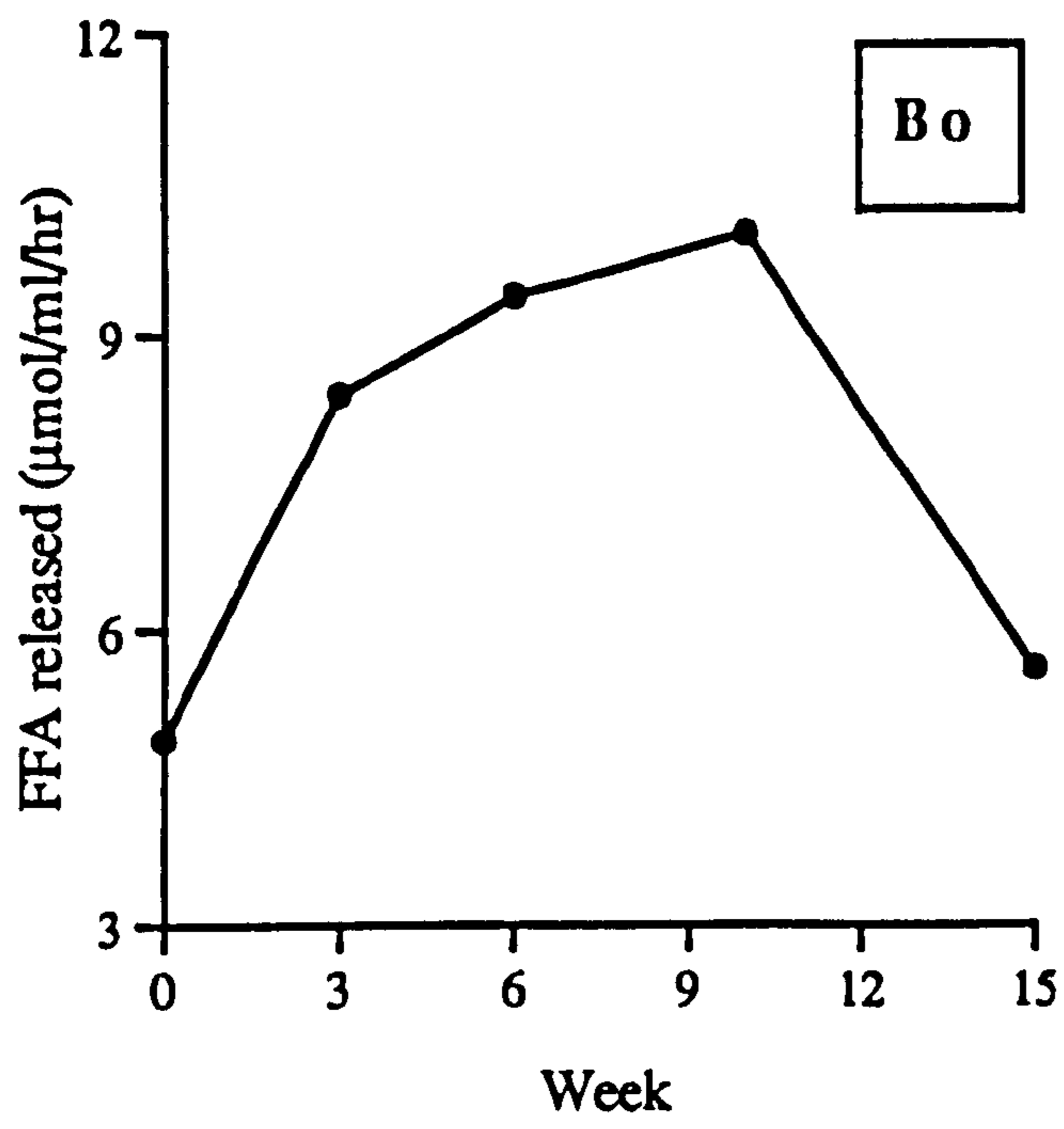


Table 6.5 Muscle glycogen concentration (mean \pm SD) during the baseline, feeding and washout periods in horses in dietary groups C and F.

Pre	Prior to fat supplementation
3 Weeks	Following 3 weeks of fat supplementation of horses in dietary group F
6 Weeks	Following 6 weeks of fat supplementation of horses in dietary group F
10 Weeks	Following 10 weeks of fat supplementation of horses in dietary group F
Washout	Following 5 weeks of withdrawal of the fat supplemented diet of horses in group F

	Muscle Glycogen (mmol/kg dw)	
	Dietary Group	
	Control	Fat Supplemented
Pre	727 ± 80	596 ± 26
3 Weeks	600 ± 66	575 ± 46
6 Weeks	679 ± 29	610 ± 53
10 Weeks	672 ± 49	620 ± 25
Washout	653 ± 26	647 ± 29

Table 6.6 Muscle triglyceride concentration (mmol/kg dw), CS and β -HAD activity (IU/kg dw) and fibre composition of muscle biopsy samples (fractional area occupied) from individual horses (groups C and F) prior to and following 10 weeks of fat supplementation of horses in dietary group F.

CS, β -HAD and triglyceride ratio - ratio of CS, β -HAD or triglyceride to the combined fractional area occupied by type I and IIa fibres in the biopsy sample.

* Significantly different from the corresponding Pre concentration or activity ($p < 0.05$)

Horse/Dietary group	Fractional Area Occupied							Ratio		
	CS	β-HAD	Triglyceride	I	Ila	I Ib	I + Ila	CS	β-HAD	Triglyceride
Pre										
Bo F	119.6	17.9	6.6	0.18	0.61	0.21	0.79	152	22.7	8.4
Li F	120.8	27.5	3.9	0.16	0.55	0.29	0.71	170.6	38.8	5.5
Mo F	83.6	18.4	3.3	0.09	0.41	0.50	0.50	165.9	36.5	6.5
Pe F	150.1	27.1	14	0.13	0.46	0.42	0.58	258.8	46.7	24.1
Co C	105.5	9.9	38.8	0.19	0.38	0.43	0.57	184.4	17.3	67.8
Ho C	169.7	23.9	2.7	0.23	0.41	0.36	0.64	266.4	37.5	4.2
Je C	93.2	22.9	12.5	0.05	0.30	0.65	0.35	263.3	64.7	35.3
Mean C	122.8 ± 41.1	18.9 ± 7.8	18.0 ± 18.7					238.0 ± 46.5	39.8 ± 23.8	35.8 ± 31.8
Mean F	118.5 ± 27.2	22.7 ± 5.3	7.0 ± 4.9					186.8 ± 48.6	36.2 ± 10.0	11.1 ± 8.7
10 Weeks Supplementation										
Bo F	116.3	24	4.3	0.13	0.46	0.42	0.58	199.5	41.2	7.4
Li F	107.4	16	1.8	0.03	0.55	0.42	0.58	186.8	27.8	3.1
Mo F	95.9	16.3	5.2	0.07	0.30	0.63	0.37	260.6	44.3	14.1
Pe F	114.9	18.3	10.3	0.10	0.28	0.62	0.39	298.4	47.5	26.8
Co C	145.1	20.1	14	0.12	0.54	0.34	0.66	220.2	30.5	21.2
Ho C	113.3	22.4	3.7	0.13	0.54	0.33	0.67	168.1	33.2	5.5
Je C	86.3	20.6	4.7	0.07	0.33	0.60	0.40	215.8	51.5	11.7
Mean C	114.9 ± 29.4	21.0 ± 1.2	7.5 ± 5.7					201.4 ± 28.9	38.4 ± 11.4	12.8 ± 7.9
Mean F	108.6 ± 9.3	18.7 ± 3.7	5.4 ± 3.6					236.3 ± 52.4 *	40.4 ± 8.7	12.9 ± 10.3

Discussion

Alterations in muscle metabolism, following a period of exposure to a fat supplemented diet have been widely reported during exercise in the horse. The mechanism involved in the reported shift in metabolism during exercise has not as yet been elucidated. A direct substrate effect due to a change in the diet, or an adaptational response that affects the ability to utilise fat during exercise may explain the observed response. Alternately, an alteration in exercise metabolism may occur as a consequence of a change in the hormonal milieu, as a result of the variation of the ratio of fat : carbohydrate in the diet.

The digestibility of ether extract was increased with fat supplementation, as has been previously described (McCann *et al* 1987; Rich *et al* 1981). The increased digestibility of ether extract probably reflects the increased oil content of the fat supplemented diet. Furthermore the fat present in the control diet was integral fat, i.e. contained in cereal grains and this may have reduced its digestibility. The increased fibre digestibility is probably a reflection of the increased fibre content of the fat supplemented diet.

Plasma FFA concentration at rest was variable throughout the feeding study in both groups of horses. There was no significant interaction between dietary group and sampling time. An increase in the pre-exercise plasma FFA concentration was suggested to increase the utilisation of fat during subsequent low intensity exercise as described in Chapter 5. However, a dietary induced elevation in resting FFA concentration was not demonstrated in this study and therefore probably would not contribute to the increase in fat utilisation previously reported by other authors. Plasma glucose concentration was also not significantly influenced by dietary treatment. No significant differences were identified in muscle glycogen concentration, either within or between dietary groups. The concentration of muscle glycogen was similar to that previously reported (Essen-Gustavsson *et al* 1984; Snow *et al* 1985; Snow and Harris 1991). Random variation in muscle glycogen concentration probably reflects differences in biopsy site, which may be partly attributed to variation in the fibre composition of biopsy samples. Type I fibres may quantitatively

contain less muscle glycogen than type II fibres (White and Snow 1987). Further variation may have been introduced due to serial biopsies being taken alternately from the right and left middle gluteal muscle. Snow and Harris (1991) estimated that the variance between sampling sites, within horses undertaking race type training, was 57 mmol glycosyl units/kg dm. As was discussed earlier, previous studies have reported an increase in resting muscle glycogen concentration in response to a period of fat supplementation. However, the concentration of muscle glycogen in these former studies was much lower in comparison to those of this study and to that previously reported (Essen-Gustavsson *et al* 1984; Snow *et al* 1985; Snow and Harris 1991). The observed increases in resting muscle glycogen concentration may have occurred as the result of differences in the gross or net energy content of the control and fat supplemented diets. Furthermore the training and nutritional status of the horses prior to the studies cannot be overlooked as a precipitant of the increase in muscle glycogen concentration. In agreement with the studies in rats of Miller *et al* (1984) and Simi *et al* (1991) fat supplementation was associated with an increase in muscle CS and β -HAD activity. Statistical significance was, however, only achieved with respect to the increase in muscle CS activity. Nevertheless the results suggests that an increase in the capacity of muscle to oxidise FFA may have occurred as the result of fat supplementation. However, although an attempt was made to correct for differences in the fibre composition of biopsy samples, confirmation of the increase in oxidative capacity could only be achieved using measurements of CS and β -HAD in single muscle fibres. Unlike the study of Conlee *et al* (1990) no significant change in muscle triglyceride concentration was observed as a result of fat supplementation.

Plasma cholesterol concentration was significantly increased during the fat supplementation period in horses in group F. The increase in cholesterol concentration was, however, small and remained within the normal concentration range. Furthermore, the concentration was not significantly different from that of group C. An increase in the plasma cholesterol concentration evoked by a high fat or fat supplemented diet has been previously reported in both horses and rats (Hill *et al* 1960; Hambleton *et al* 1980; Rudney and Sexton 1986). The

increase in cholesterol concentration may have arisen as a consequence of either increased dietary intake or as a result of increased cholesterol-genesis. The cholesterol content of both diets C and F is likely to be minimal. The main ingredients of both diets; cereal grains, forages, grain by-products and soya oil have an extremely low cholesterol content (Lentner 1981). It is therefore likely that the increase in cholesterol concentration was due to endogenous synthesis, possibly as the result of increased production of acetyl CoA due to an increased flux through β -oxidation. It has previously been suggested that cholesterol-genesis may represent an alternative pathway for acetyl CoA when lipogenesis is suppressed (Hill *et al* 1960). Alternatively, an increase in the triglyceride : protein ratio of VLDL, intermediate density lipoproteins and LDL may have led to a stimulation of endogenous cholesterol synthesis. As triglycerides are removed from circulating lipoproteins they are replaced by cholesterol ester in order to maintain lipoprotein stability which may in turn stimulate endogenous cholesterol synthesis.

Plasma triglyceride concentration and plasma post PP T. Lip activity were unchanged in the control group of horses. The corresponding enzyme activity was increased in the horses in group F by on average about 50% as a result of fat supplementation. Furthermore, the increase in enzyme activity was associated with a significant reduction in plasma triglyceride concentration. Similarly, the fasting activity of post heparin plasma LPL is higher in suckling foals compared to adult ponies (Watson *et al* 1993a). This may reflect the relatively higher fat content of the diet of the former. An increase in muscle heparin releasable LPL activity has been reported to occur as the result of feeding diets containing between 65-78% of the energy as fat, in Man and in rats (Delorme and Harris 1975; Jacobs *et al* 1982). Delorme and Harris (1975) also reported a simultaneous decrease in the activity of adipose tissue LPL. Plasma post PP T. Lip activity represents the sum of heparin releasable LPL and HL. The functional location of heparin releasable LPL is the endothelial cell surface of extra-hepatic tissue capillaries, mainly adipose tissue and skeletal muscle (Robinson 1987). Hence, the observed increase in plasma post PP T. Lip activity may have resulted from an increase in either or both adipose or muscle tissue LPL, or an increase in the

(Robinson 1987). Hence, the observed increase in plasma post PP T. Lip activity may have resulted from an increase in either or both adipose or muscle tissue LPL, or an increase in the activity of HL. An increase in the activity of heparin releasable muscle LPL, as a result of dietary manipulation (Delorme and Harris 1975), fasting (Delorme and Harris 1975; Lithell *et al* 1978) or exercise (Borensztajn *et al* 1975; Taskinen *et al* 1980) is associated with a reciprocal decrease in the activity in adipose tissue. In other words as the capacity for uptake of FFA by skeletal muscle, from lipoprotein associated triglycerides is increased, there is a reciprocal decrease in their uptake by adipose tissue. HL is responsible for remodelling circulating lipoprotein and is involved in the removal of cholesterol and phospholipids from high and low density lipoproteins (Jansen *et al* 1980; Watson *et al* 1993b). Summerfield *et al* (1984) reported that HL activity was lower in rats fed a high fat diet in comparison to those fed a high carbohydrate diet. The reduced HL activity, however, was associated with a lower plasma cholesterol concentration (Summerfield *et al* 1984). HL acts on lipoprotein particles with a much lower triglyceride content than LPL and hence the reduction in plasma triglyceride observed is probably the result of an increase in LPL activity. The decrease in plasma triglyceride concentration, associated with the fat supplemented diet, indicates a better management of the fat load and has been previously reported in the horse (Duren *et al* 1987). Harris and Felts (1973) reported an increase in the rate of removal of labelled triglyceride from the plasma in rats fed a high fat diet. The increase in the activity of post PP T. Lip was probably the result of an increase in muscle LPL that was larger than a reciprocal decrease in adipose tissue LPL activity.

The stimulus for the increase in post PP T. Lip activity is unknown. The changes in LPL activity may be have been induced by hormonal changes that occurred as the result of the change in diet. Insulin has previously been implicated in the regulation of adipose tissue and muscle LPL (Jacobs *et al* 1982; Kiens *et al* 1989). The effect of insulin on LPL activity, however, appears to be tissue dependant, and may be the result of an insulin mediated change in tissue glucose metabolism (Kiens *et al* 1989). Pagan *et al* (1994) reported that plasma

insulin concentration was lower both at rest and during subsequent exercise, after a period of adaptation to a fat supplemented diet in comparison to a high carbohydrate control diet.

In summary, fat supplementation was associated with a better management of the fat load as was illustrated by the decrease in resting plasma triglyceride concentration . The reduction in plasma triglyceride concentration may have occurred as the result of an increased ability of muscle to utilise lipoprotein associated plasma triglyceride, as a result of an increase in the activity of muscle LPL. Although the fat supplemented diet was associated with an increase in total cholesterol concentration this remained within the normal range at all times. Additionally fat supplementation may have resulted in an increase in muscle oxidative capacity as indicated by the increase in muscle β -HAD and CS activity in the horses in dietary group F.

Chapter 7

Metabolic Response to Low, Moderate and Moderate/High Intensity Exercise in Horses Fed a Low Fat or Fat Supplemented Diet.

Introduction

Many studies have previously been carried out to investigate the effect of fat supplementation on the metabolic response to exercise in the equine. The results of these studies have often been contradictory and the effect of fat supplementation on exercise metabolism remains unresolved. It has been widely suggested that an increase in the level of dietary fat, fed chronically, may alter substrate utilisation during subsequent exercise (Hambleton *et al* 1980; Duren *et al* 1987; Pagan *et al* 1987; Hintz *et al* 1987; Moser *et al* 1991; Harkins *et al* 1992; Scott *et al* 1992; Hodgson *et al* 1994). This may in turn lead to alterations in exercise performance (Webb *et al* 1987; Harkins *et al* 1992). Many of the suggested shifts in substrate metabolism have been based on indirect measurements of fuel utilisation, such as changes in plasma glucose, lactate, FFA, and muscle glycogen concentration.

At the end of both prolonged low intensity exercise (Hambleton *et al* 1980; Hintz *et al* 1987) and moderate to high intensity exercise of short duration (Duren *et al* 1987; Harkins *et al* 1992; Pagan *et al* 1994), plasma glucose concentration has consistently been reported to be higher, in association with a fat supplemented diet in the horse. It is suggested that this may be the result of a glucose sparing effect. Plasma lactate concentration in response to exercise, following a period of adaptation to a fat supplemented diet, has been reported to be lower (Pagan *et al* 1993), higher (Webb *et al* 1987) or no different (Duren *et al* 1987; Meyers *et al* 1989; Harkins *et al* 1992) relative to that measured in horses fed a high carbohydrate control diet. Glycogen utilisation is reported to increase during moderate to high intensity exercise, in response to feeding a fat supplemented diet in the horse (Oldham *et al* 1990; Jones *et al* 1991; Scott *et al* 1992). These findings, however, may reflect an observed increase in resting muscle glycogen concentration seen in the latter studies. Muscle glycogen utilisation during exercise was unchanged when resting muscle glycogen concentration was unaffected by fat supplementation (Pagan *et al* 1987; Hintz *et al* 1987; Hodgson *et al* 1994)

Measurement of RER represents a more direct method of assessing substrate utilisation during exercise. However, its use in equine studies has been limited (Pagan *et al* 1987; Meyers *et al* 1989). Pagan *et al* (1987) reported a lower RER during low and moderate intensity exercise following fat supplementation when compared to a control group of horses receiving a low fat high carbohydrate diet. However, RER was similarly lower in a group receiving a high protein diet in the same study. This may suggest that the differences in RER measured were due to differences in the carbohydrate content of the 3 diets. Meyers *et al* (1989) failed to report any significant effect of diet on RER at a similar low intensity exercise, although, the trend towards a lower RER with the fat supplemented diet was apparent.

Previous studies investigating the effect of fat supplementation on exercise metabolism in the horse have varied in terms of :-

- a) The intensity and duration of the exercise.
- b) The nature and amount of fat included in the diet.
- c) The breed, age and training status of the horses .
- d) The experimental design of the feeding regimens, i.e. crossover designs or discrete subject groups.
- e) The feeding status of the horses prior to commencement of exercise.

Some of these differences may explain the inconclusive and often inconsistent results that have been previously reported. The aim of the present study was to assess the metabolic response to exercise, of 3 grades of intensity, in response to feeding a fat supplemented diet. The horses used were at a stable level of fitness and energy balance prior to the start of the period of supplementation. The study design used incorporated two discrete subject groups. This was done in order that both within horse and between horse comparisons could be made. A short crossover design, such as a 4 X 4 Latin square, was considered inappropriate since shifts in substrate utilisation may occur as the result of an adaptational response to the diet, of unknown period of induction and washout.

Materials and Methods

As part of the feeding study described in the previous chapter a series of exercise tests were additionally performed during the different feeding phases of the study. Horses in both dietary groups (F and C) underwent exercise tests of low (T), moderate (SC) and moderate/high intensities (FC), on 4 separate occasions. The low intensity test was a trotting exercise test (T), the moderate intensity test incorporated a period of slow canter (SC) and the moderate/high intensity test incorporated a period of fast canter (FC). The exercise tests were undertaken at the end of the baseline period, between weeks 4-5 (F5) and weeks 9-10 (F10) of the feeding period and at the end of the washout period. Dietary treatment during these periods was as described in Chapter 6. It was difficult to standardise the time of day at which exercise tests were performed due to the large number of tests carried out within 1 week. The time at which each of the 3 grades of exercise test was undertaken was therefore standardised within horses but randomised between horses. Each horse undertook a single exercise test in any 1 day and this was followed by at least 1 day, in which light exercise (walking and trotting) was undertaken, before the next exercise test was performed. The horses performed the 3 different exercise tests in the same order on each occasion; i.e. the SC exercise test was followed by the T exercise test and then FC exercise test, respectively. All horses were well accustomed to the treadmill and all associated equipment and had completed each exercise test with ease on several occasions before the baseline exercise tests were performed. All exercise tests were carried out at a room temperature of 20⁰C and at 60% relative humidity. The Exercise test protocols were as follows :

T Exercise Test

1' Walk	1.6 m/s	0 ⁰ incline
40' Trot	3.2 m/s	0 ⁰ incline
10' Walk	1.6 m/s	0 ⁰ incline

SC Exercise Test

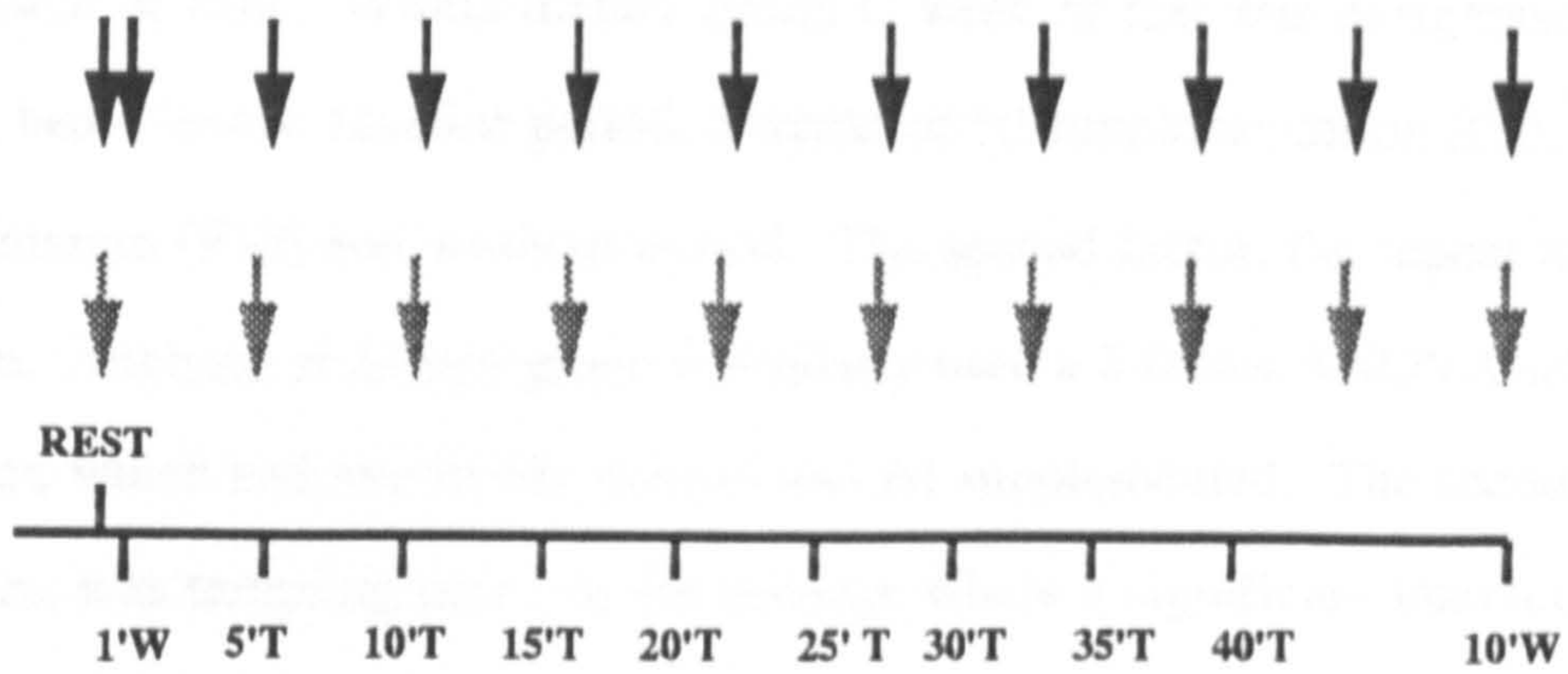
10' Walk	1.6 m/s	0° incline
5' Trot	3.2 m/s	0° incline
5' Walk	1.6 m/s	0° incline
10' Canter	7.0 m/s	0° incline
10' Walk	1.6 m/s	0° incline

FC Exercise Test

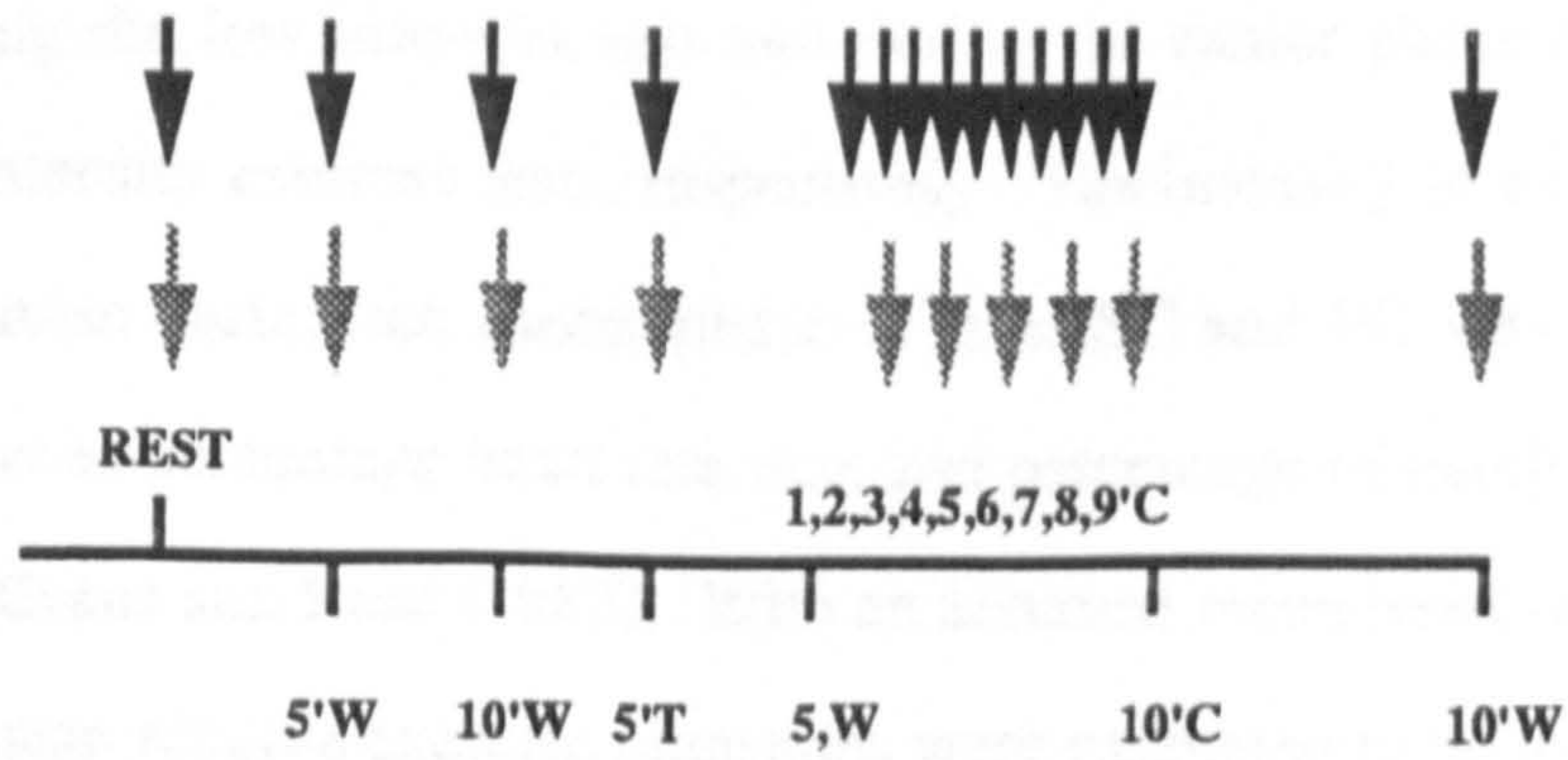
10' Walk	1.6 m/s	0° incline
5' Trot	3.2 m/s	0° incline
5' Walk	1.6 m/s	0° incline
10' Canter	10.0 m/s	0° incline
10' Walk	1.6 m/s	0° incline

Horses were catheterised as previously described in Chapter 2 and blood samples taken throughout exercise as shown over-leaf. Blood samples were immediately centrifuged and treated, as described previously, for the analysis of plasma FFA, lactate and glucose concentration. Respiratory measurements for calculation of RER were made as overleaf, using an open flow respiratory mask system as described in Chapter 2. Heart rate was measured throughout exercise as previously described, Chapter 2.

T Exercise Test



SC and FC Exercise Tests



Respiratory Data Aquisition

Blood Sample

- W** walk (1.6 m/s)
- T** trot (3.2 m/s)
- C** canter (7 or 10 m/s)

Statistical analysis

A 2-factor ANOVA for repeated measures was used to identify any significant effect of dietary treatment or time. Within dietary group C week of test was designated as the first factor, which had 4 levels: baseline period, 5 weeks of fat supplementation (F5), 10 weeks of fat supplementation (F10) and washout period. The second factor, the repeat measure, was sampling time. Analysis of dietary group F similarly used a 2-factor ANOVA where the first factor was diet, which had two levels: control and fat supplemented. The second factor, the repeat measure, was sampling time. In the instance where a significant interaction between week of test and time or dietary treatment and time was identified, further analysis was carried out. A paired Student's t test was applied to identify differences within dietary group and an unpaired Student's t test to identify differences between dietary groups.

Results

Mean heart rates during the baseline exercise tests were 94 ± 12 , 128 ± 16 and 157 ± 16 beats per minute during the low intensity test and during the canter phase of the moderate and moderate/high intensity exercise tests, respectively. The intensity of exercise test T and the intensity of exercise during the canter phase of tests SC and FC was estimated using the relationship between percentage heart rate max and percentage of maximum oxygen uptake, as described by Evans and Rose (1987). With an assumed mean heart rate max of 210 beats per minute the mean relative exercise intensities were estimated to be 7.5, 34 and 56% of $VO_2 \text{ max}$ for exercise tests T, SC and FC respectively. There was no significant difference in heart rate either within or between dietary groups with respect to all exercise intensities ($p > 0.05$).

Baseline exercise tests - respiratory exchange ratio

There was a large inter-horse variation in RER measured during exercise test T ($p < 0.0001$), as illustrated by the large standard deviation in Fig 7.1. RER initially increased during the first 5 minutes of trotting and then declined slowly. Following 25 minutes of trotting and

thereafter, RER was significantly lower than that measured after the first five minutes of trotting ($p < 0.0001$).

RER steadily increased during the first 3-5 minutes of the canter phase, of both the SC and FC exercise tests, before reaching a plateau or steady-state (Fig 7.1). Following 4 minutes of canter RER was no longer significantly different from that measured during the previous minute during exercise test SC. During exercise test FC the RER measured after 3 minutes of canter was not significantly different from that measured after 2 minutes. The inter-horse variation in RER during the canter phase of both exercise tests SC and FC was markedly less than that during exercise test T (Fig 7.1).

Low Intensity exercise tests (T)

There was no significant difference in the RER measured between weeks during exercise test T, in the control group of horses (Figs 7.2). ANOVA revealed a significant interaction between week of test and measurement time, in the horses in group F. Further statistical analysis of the horses in group F indicated a significant difference in RER, measured between the serial exercise tests, that was related to the period of fat supplementation. RER was significantly lower, in the latter stages of the exercise tests performed after 5 and 10 weeks of fat supplementation, relative to the baseline exercise tests (Fig 7.2). RER was also significantly increased, relative to exercise test (F10), once the horses in group F were reverted to the control diet. There was no significant difference in RER at any time between the two dietary groups ($p > 0.05$). There was a trend, however, for the RER of horses in group F to be lower than that of group C during exercise tests F5 and F10 (Fig 7.3). The degree of change in RER measured during the serial exercise tests was different between horses. Horses Bo, Mo and Pe exhibited a clear drop in RER during exercise tests F5 and F10 relative to the baseline test, whereas horse Li showed minimal variation in RER between exercise tests (Fig 7.4).

Fig 7.1 RER (mean \pm SD) during the low intensity exercise test (T) and during the canter phases of the moderate (SC) and moderate/fast (FC) intensity exercise tests in horses in both dietary groups C and F during the baseline period.

***** RER significantly different ($p < 0.05$) from that measured after 5 minutes of exercise.

† RER significantly different ($p < 0.05$) from that measured after 1 minute of canter exercise during exercise test SC

‡ RER significantly different ($p < 0.05$) from that measured after 1 minute of canter exercise during exercise test FC

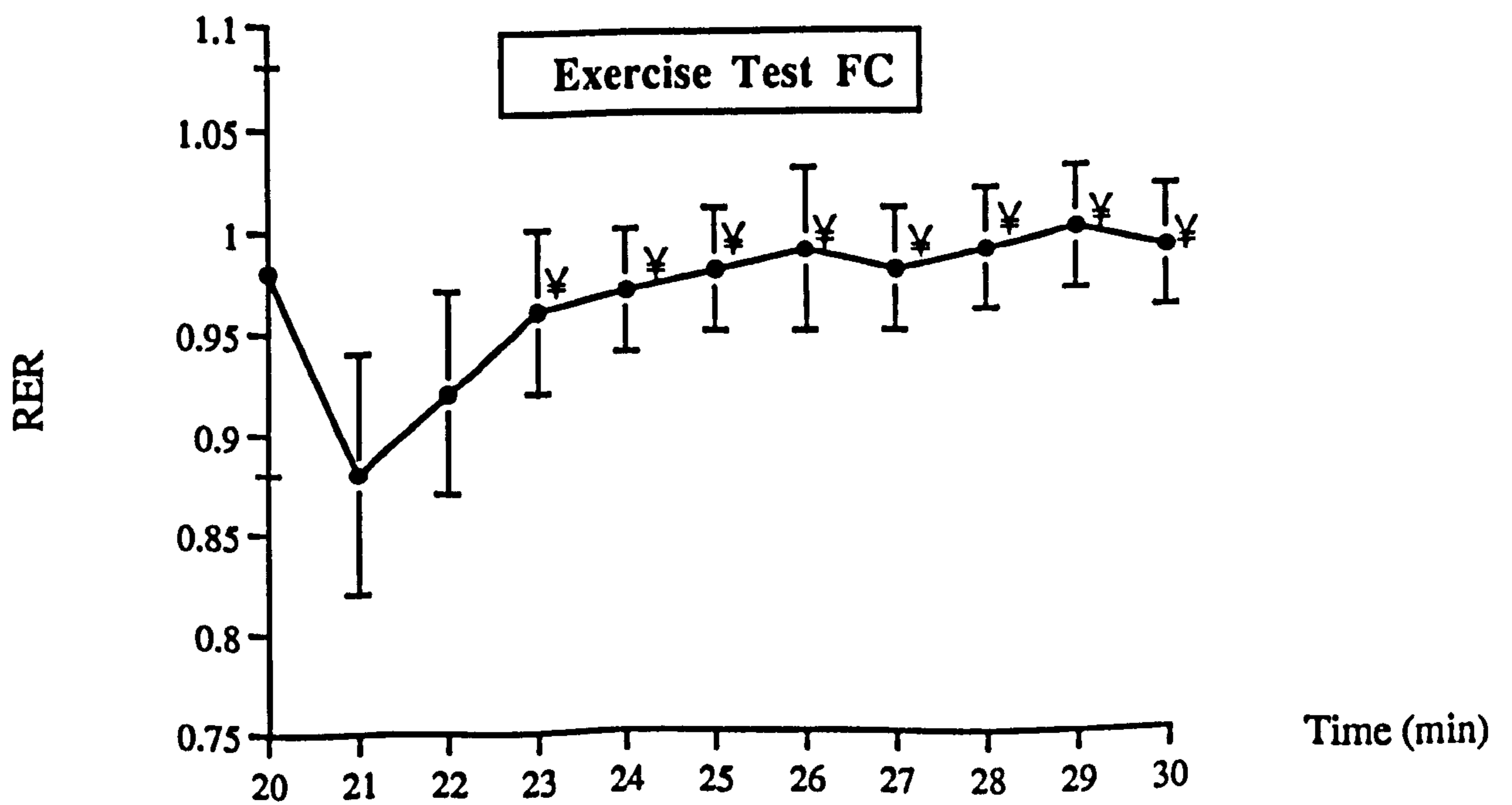
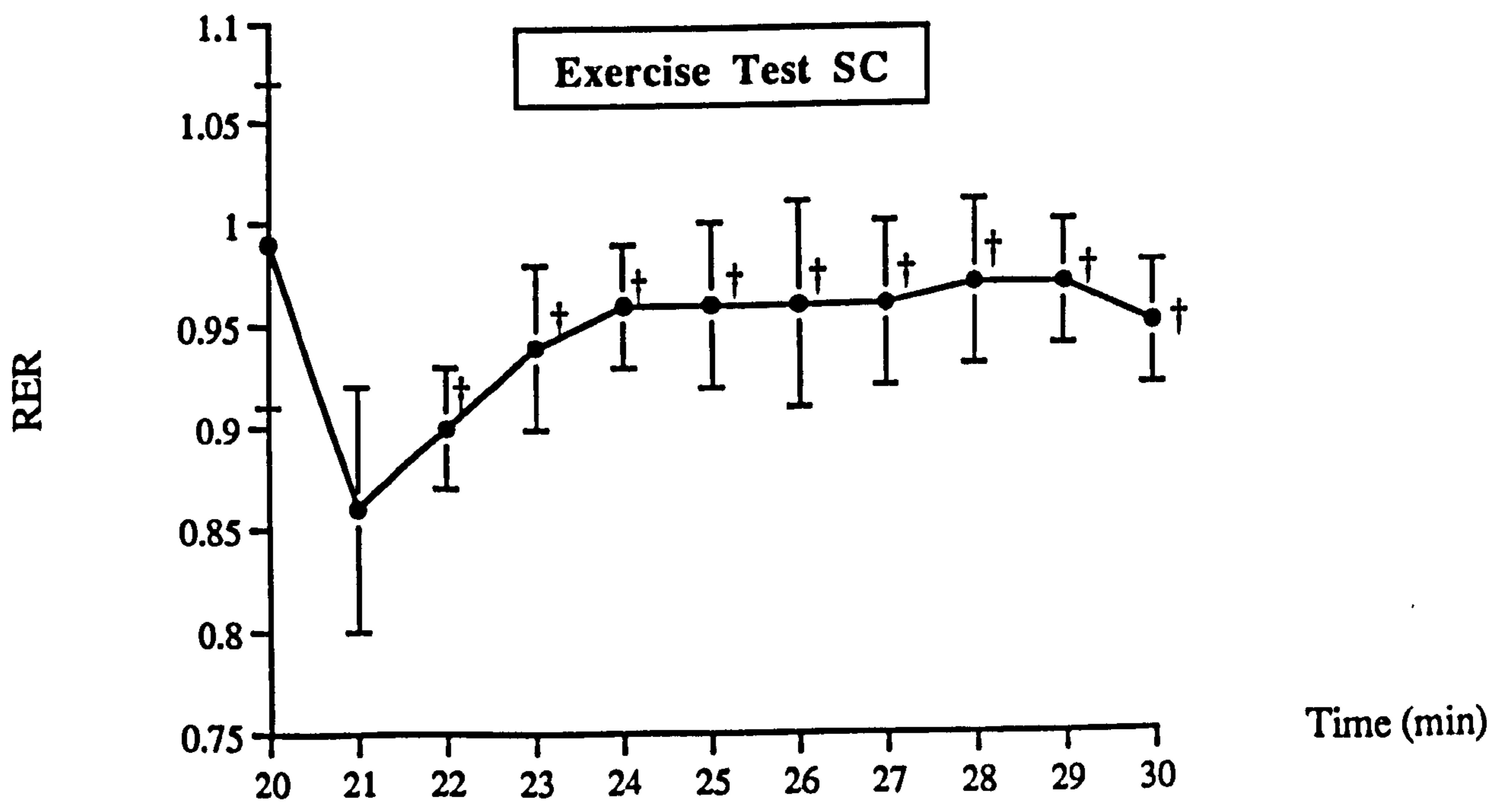
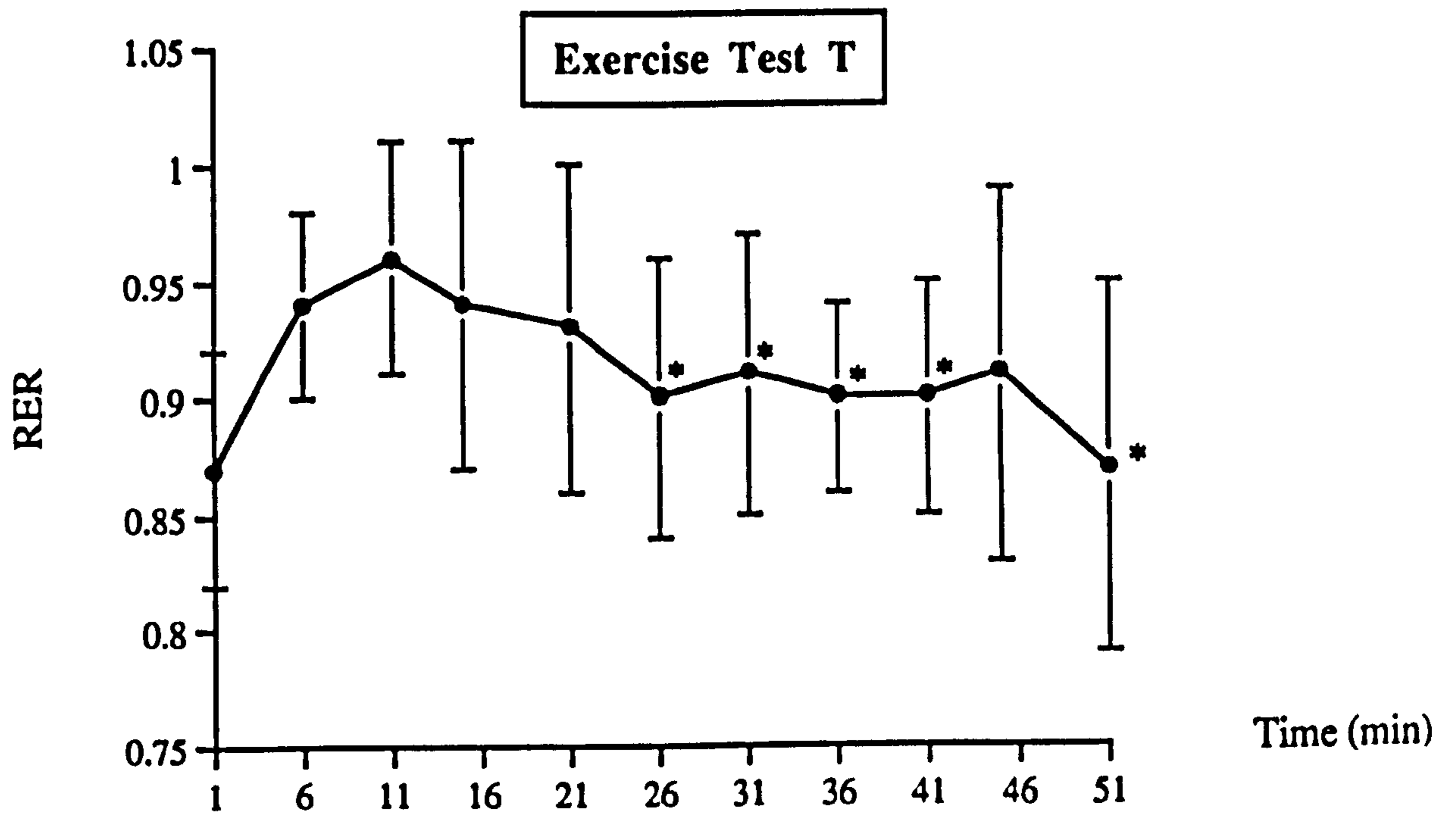


Fig 7.2 Mean RER during exercise test T during the baseline period the feeding period (F5 and F10) and the washout period in horses in dietary groups F and C respectively.

* / * RER significantly different ($p < 0.05$) from that measured at the corresponding time during the baseline exercise test.

† / † RER significantly different ($p < 0.05$) from that measured at the corresponding time during the washout exercise test.

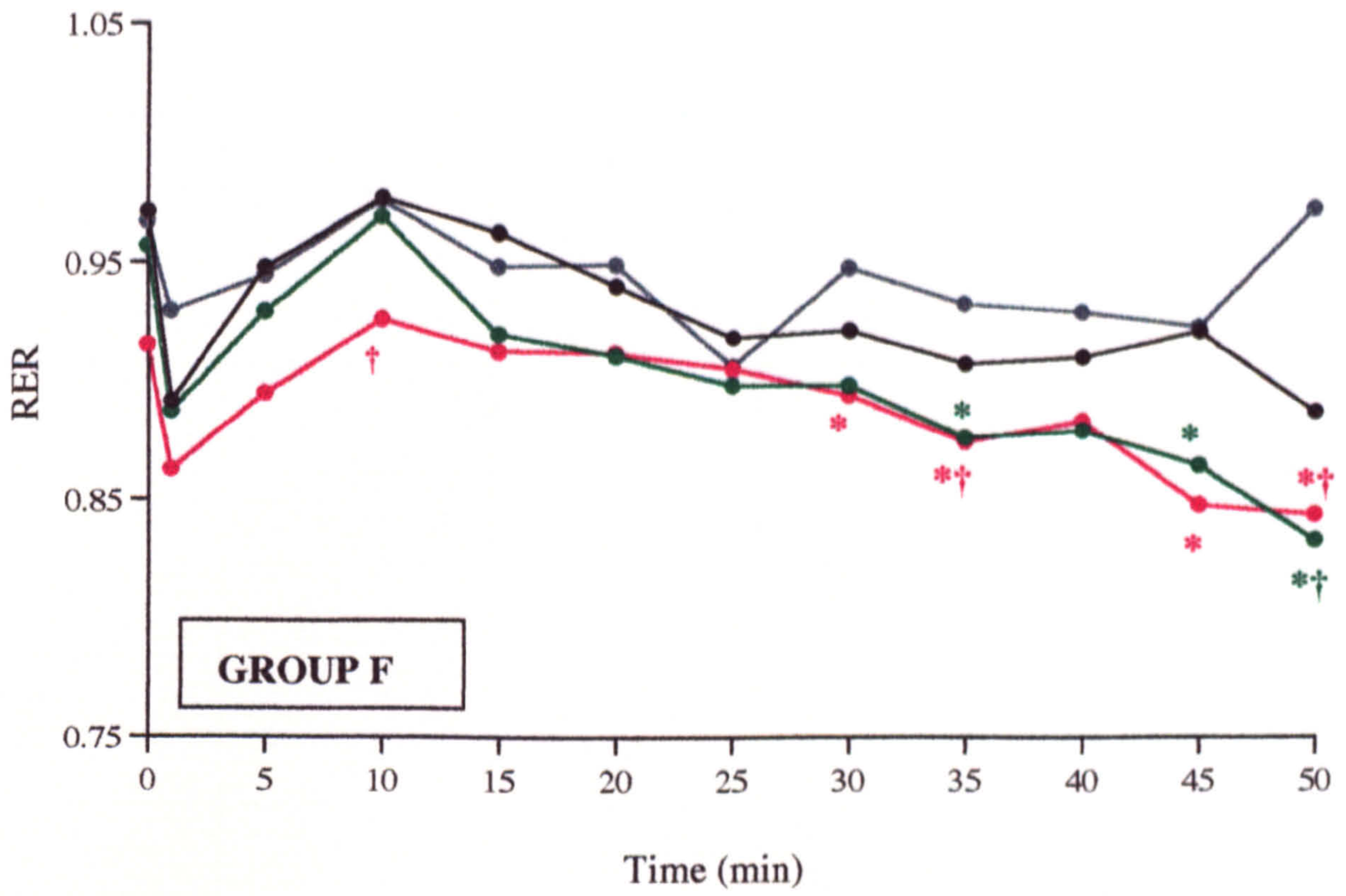


Fig 7.3

A comparison of RER (mean \pm SD) during a 50 min period in 10 subjects in obesity group C and F for the exercise (F5 and F10) and washout periods. The results, showing (F5) and (F10) and washout periods.

- Baseline
- F5
- F10
- Washout

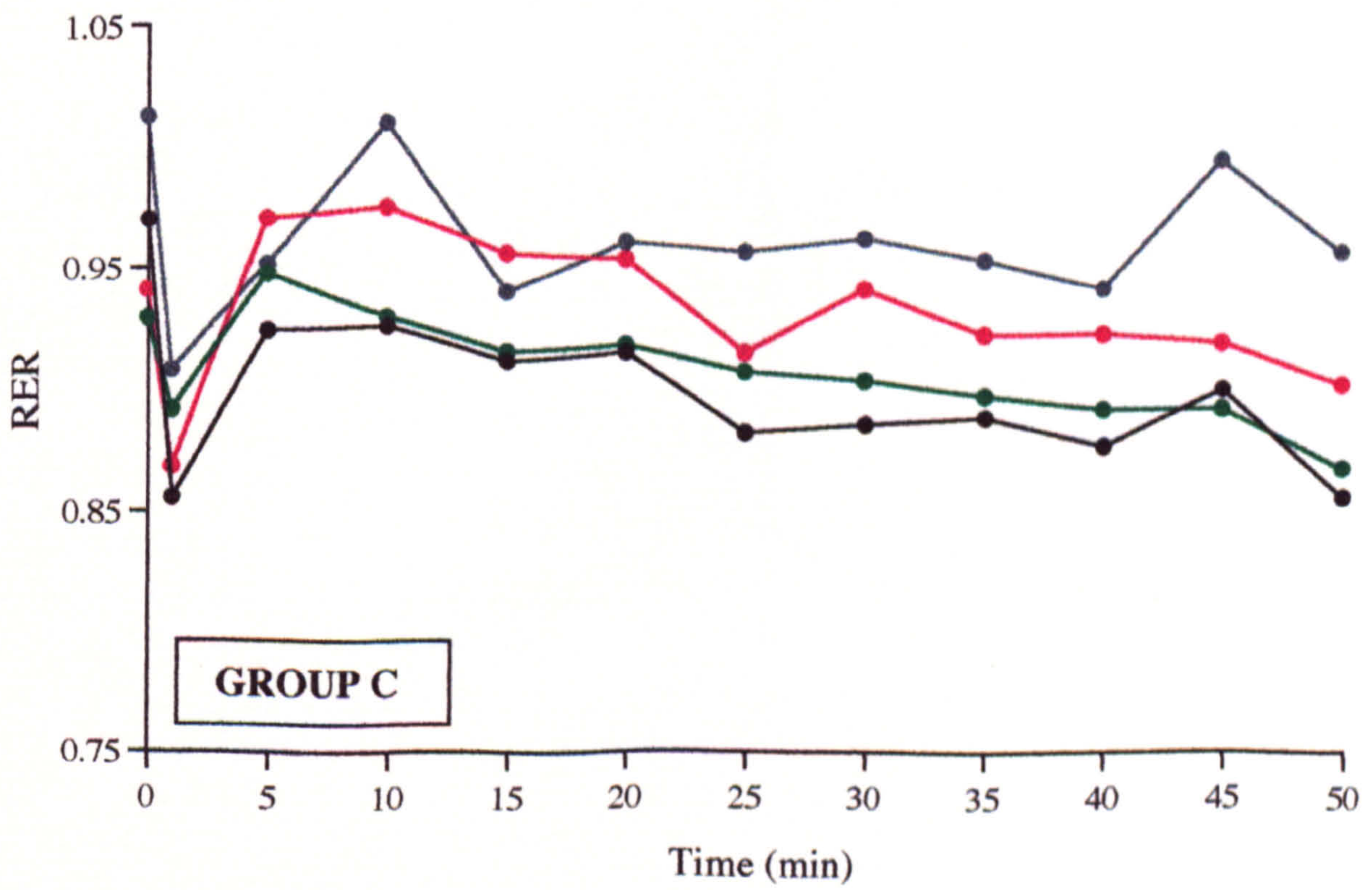
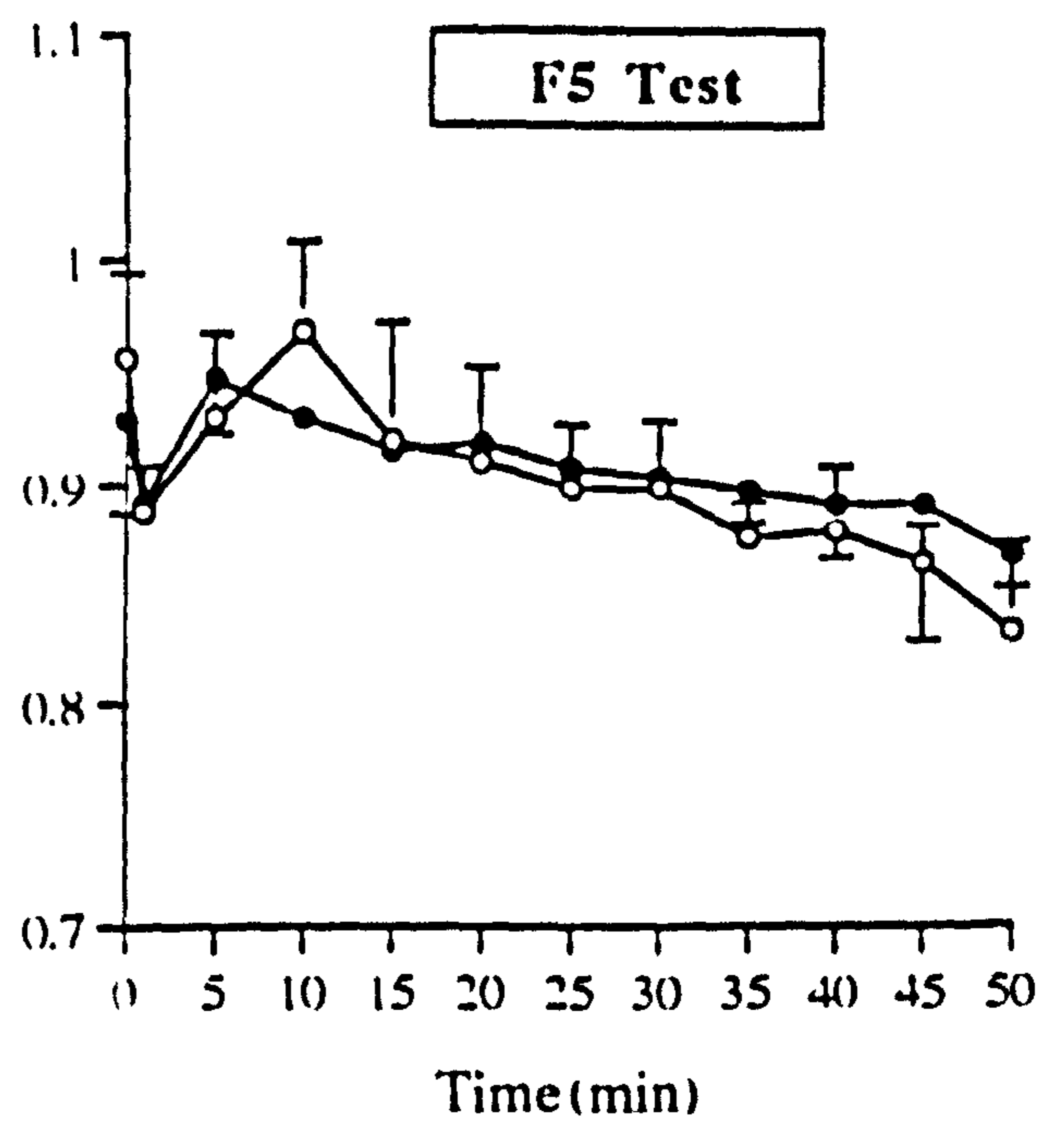
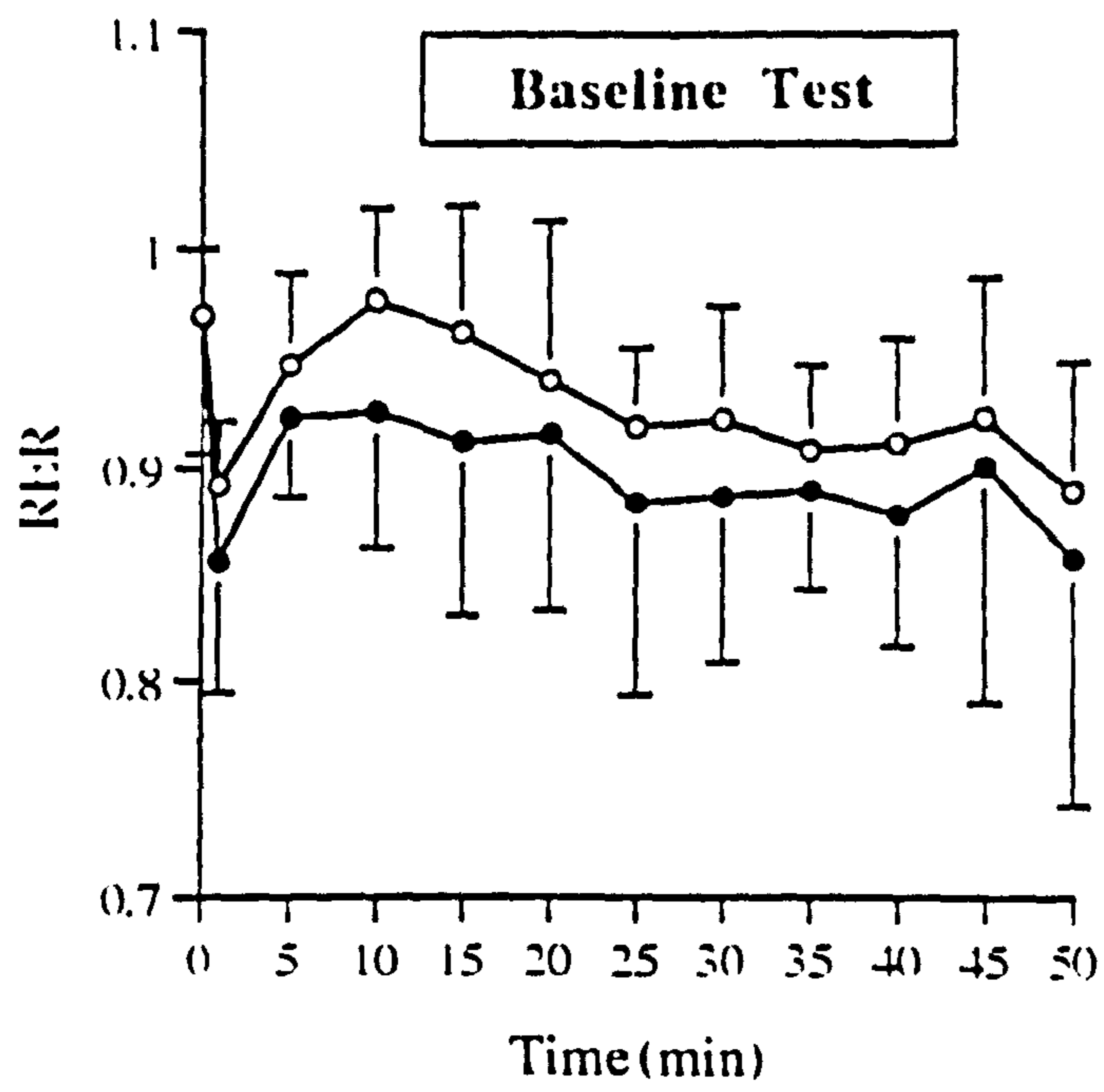


Fig 7.3 **A comparison of RER (mean \pm SD) during exercise test T in horses in dietary groups C and F for the exercise tests performed during the baseline, feeding (F5 and F10) and washout periods respectively.**



—○— Group F
 —●— Group C

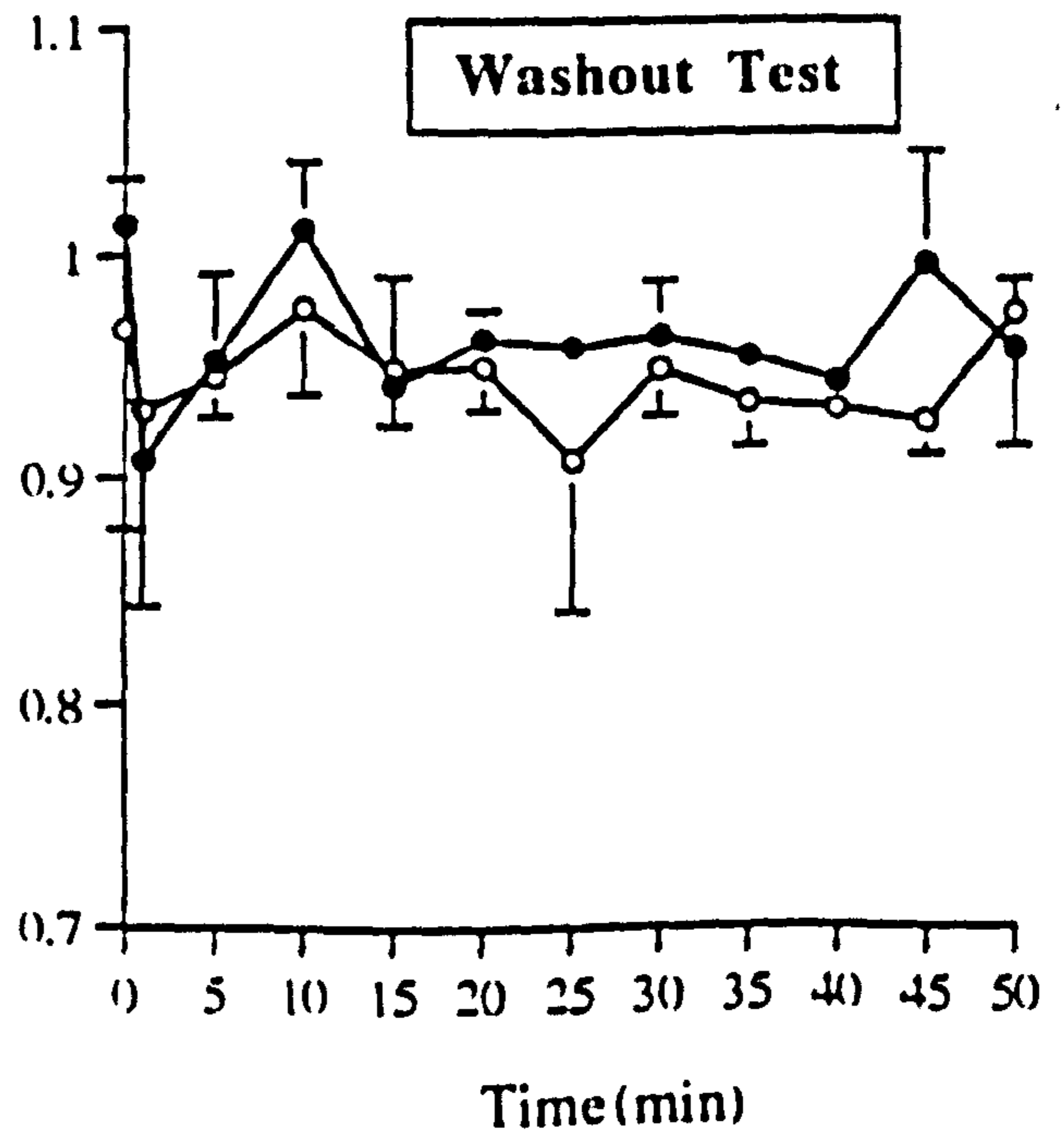
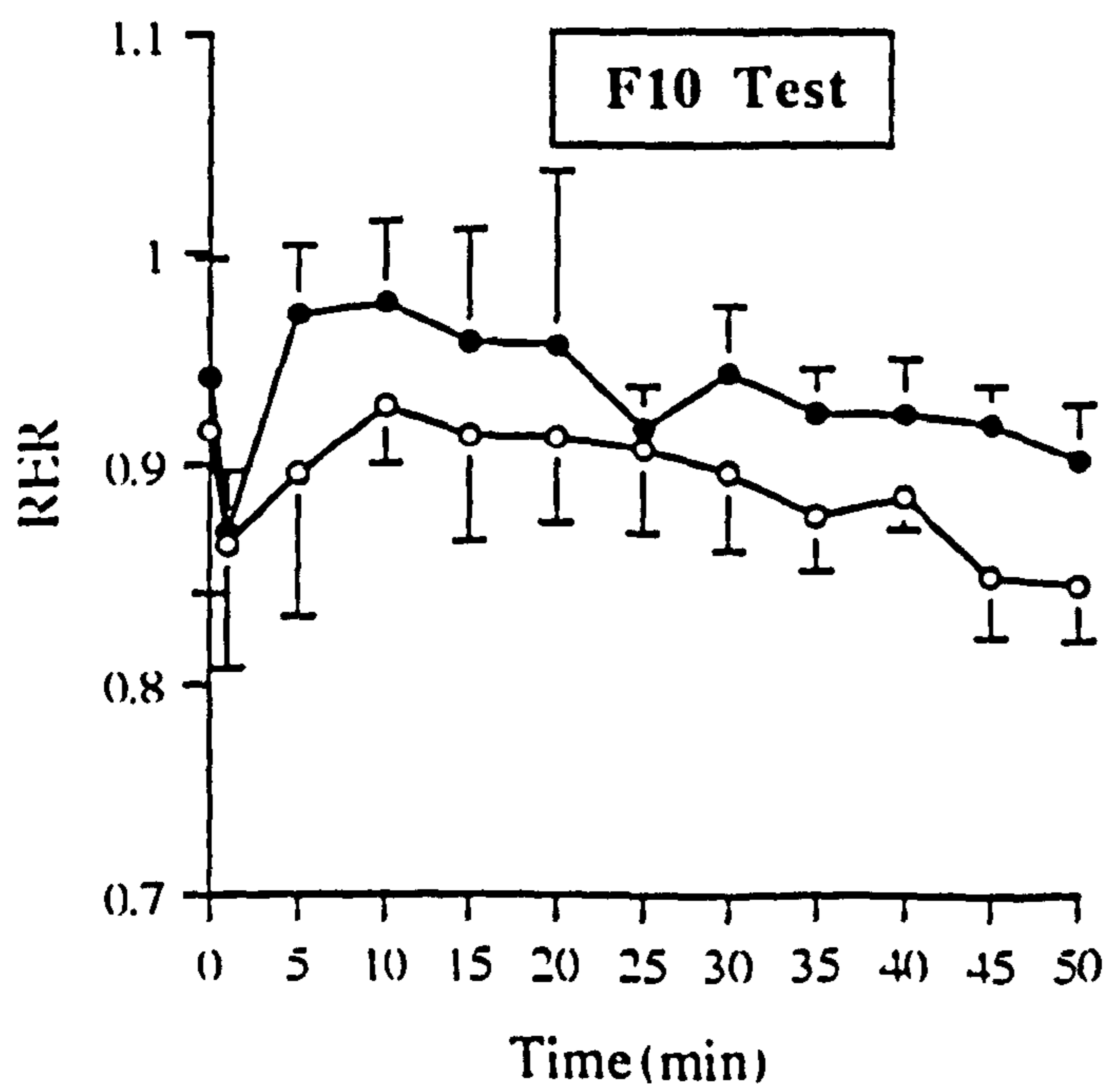
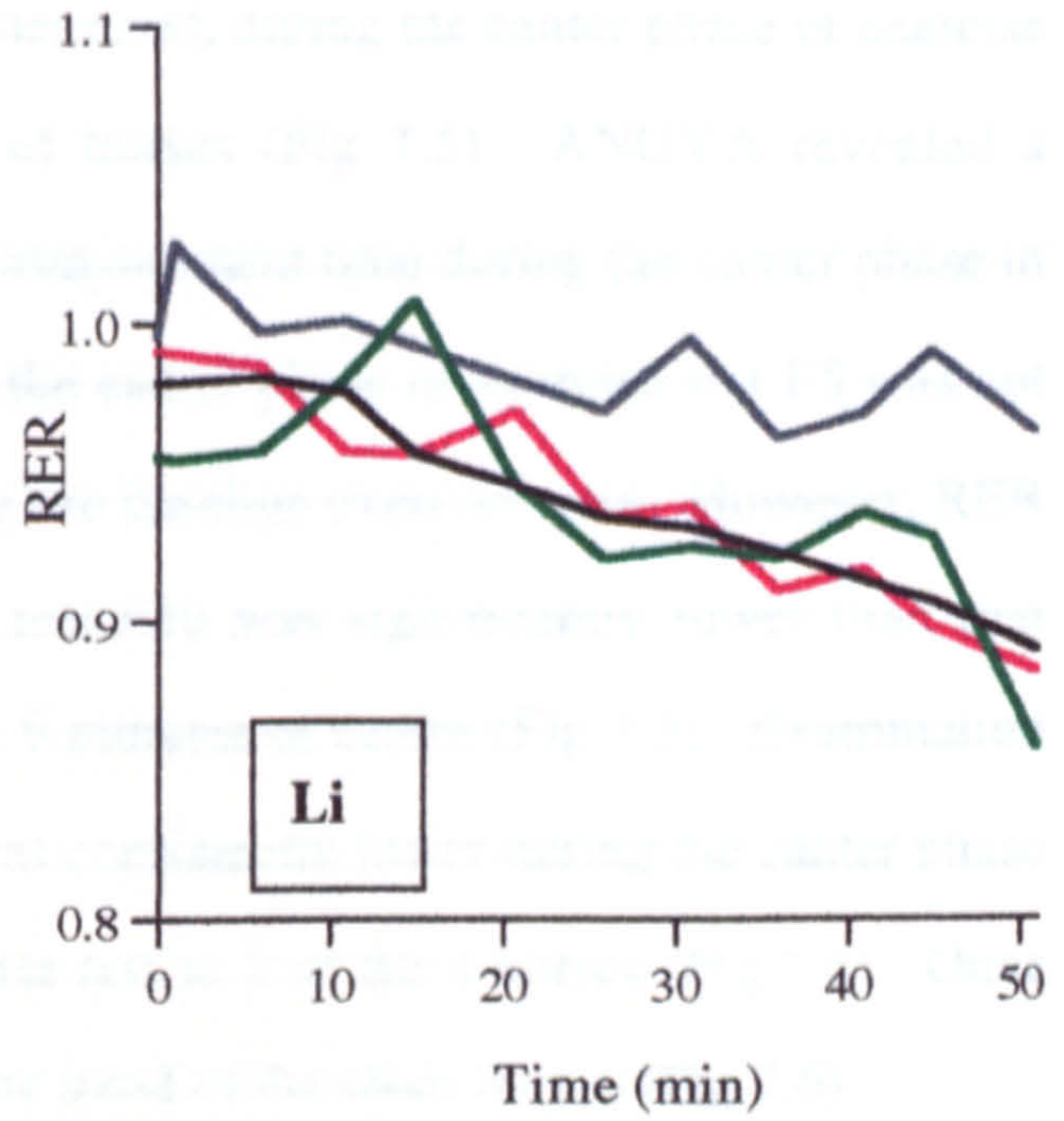
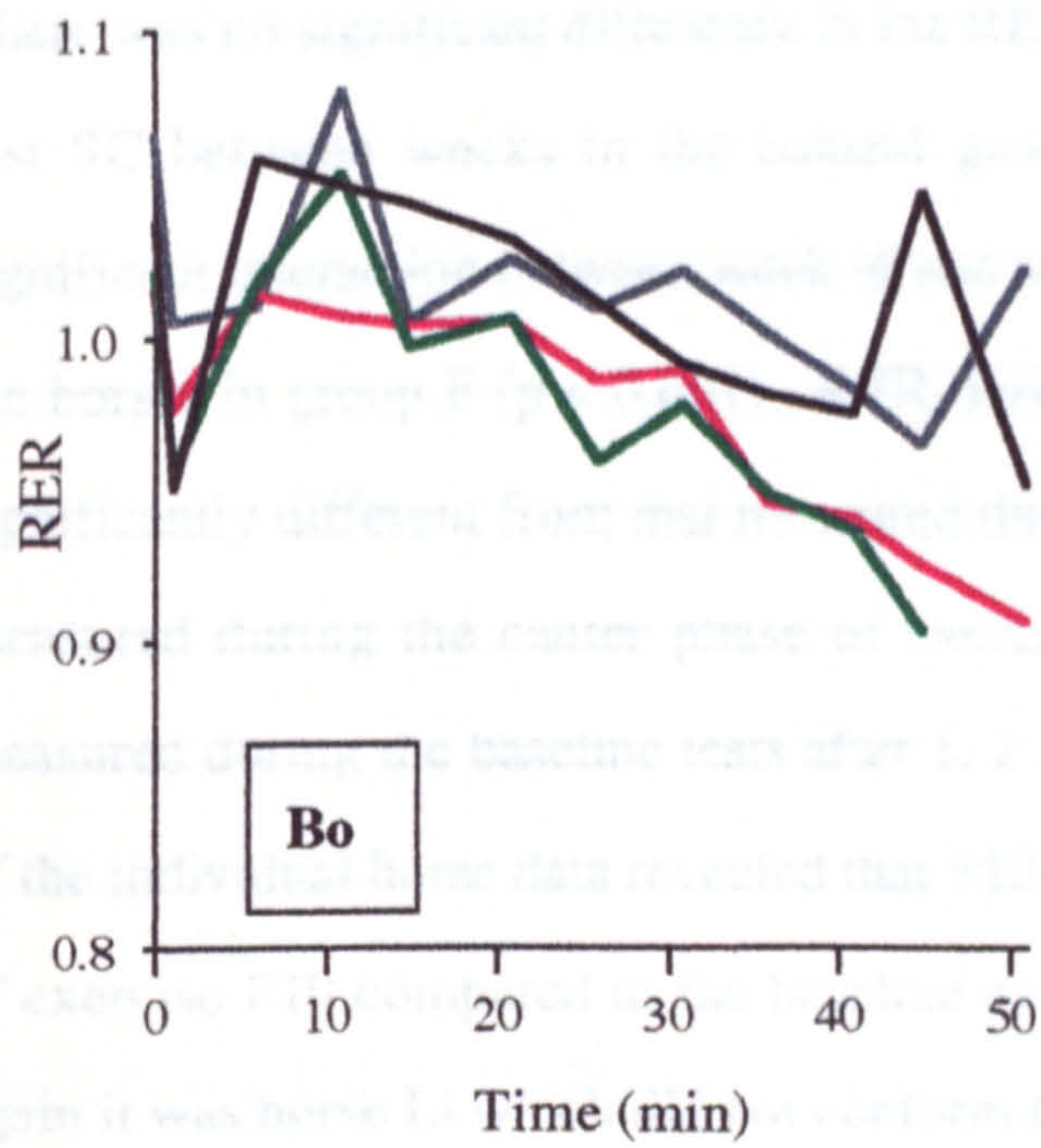
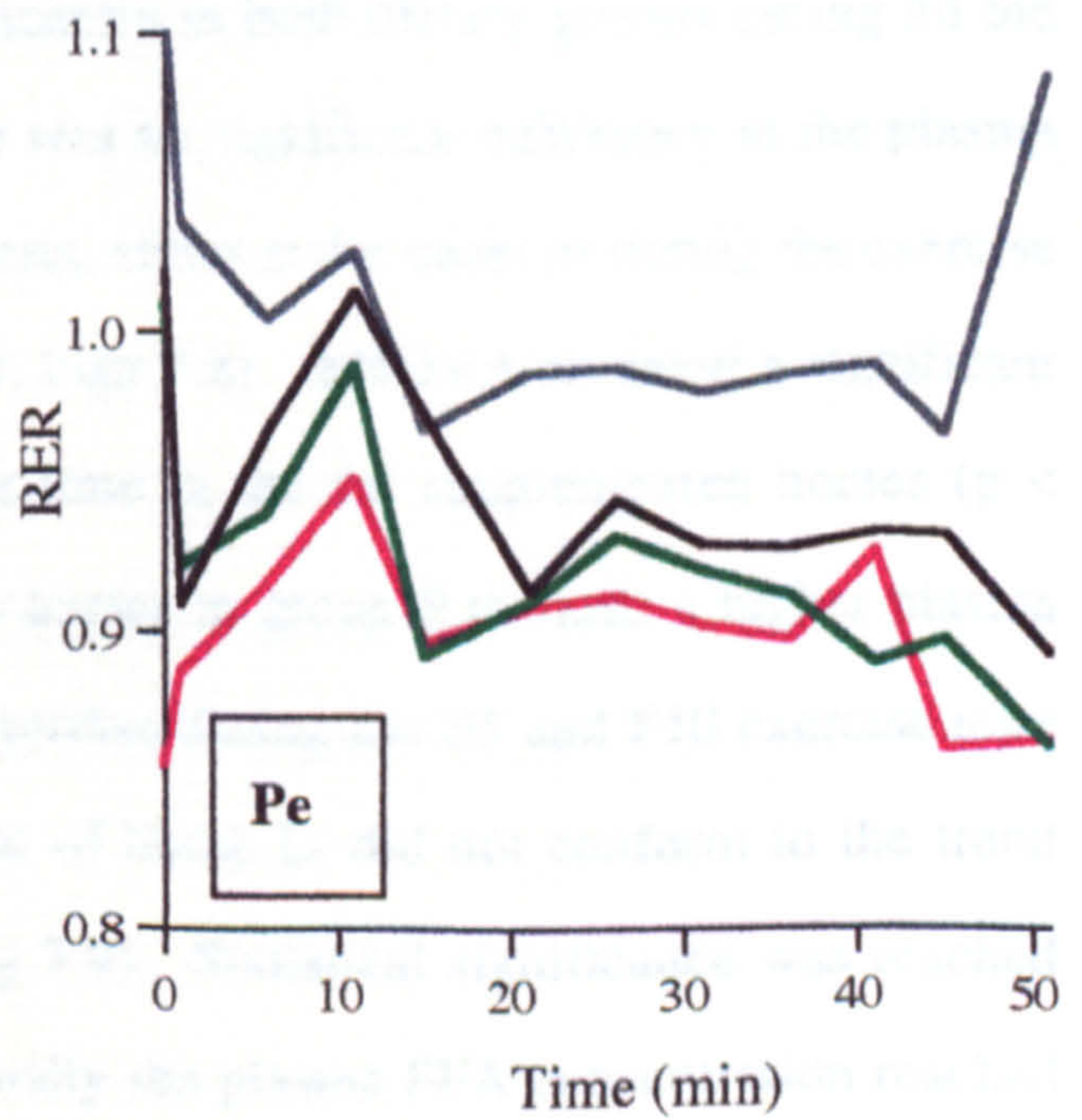
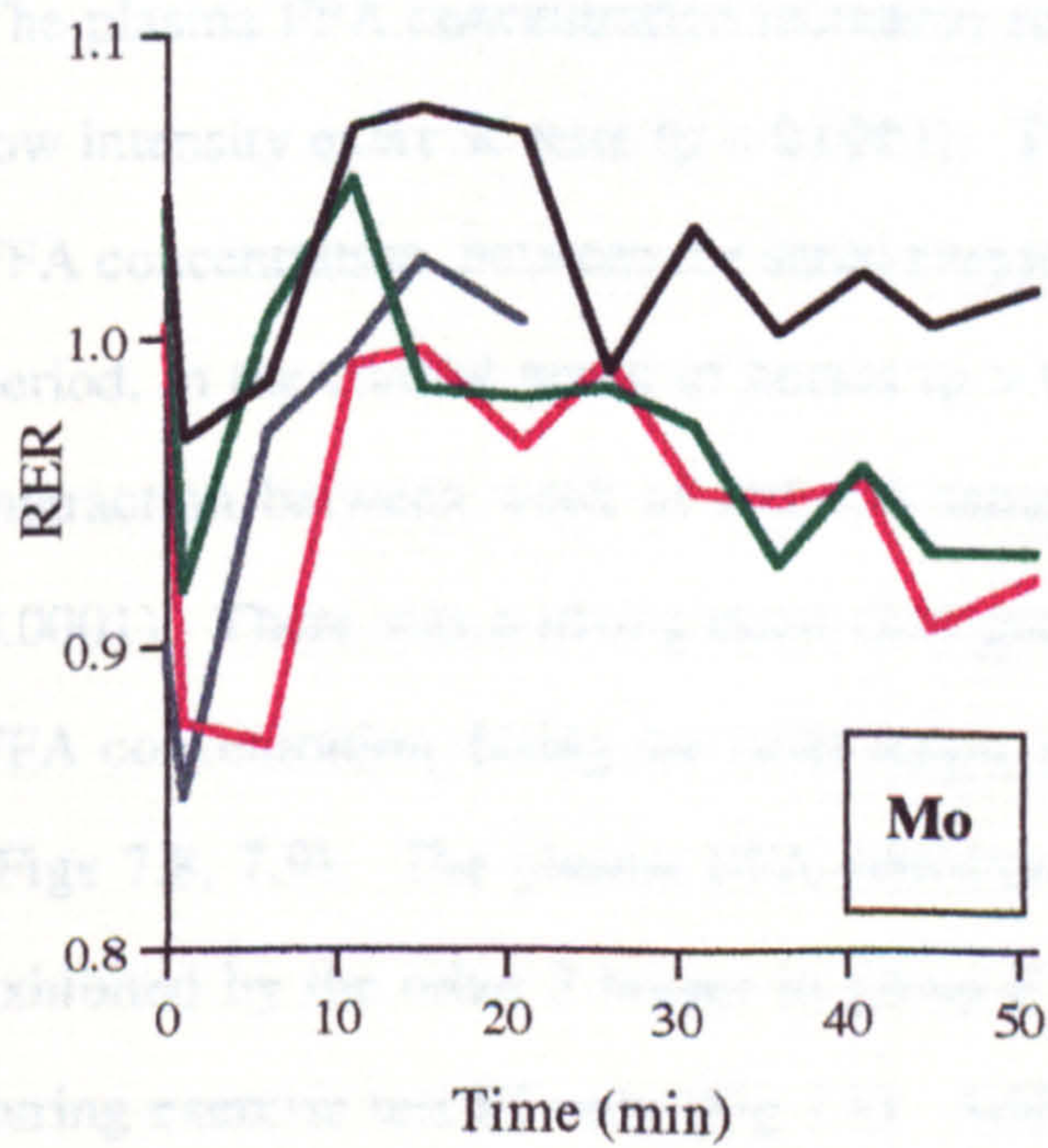


Fig 7.4 RER measured in individual horses (Bo, Li, Mo, Pe) in dietary group F in response to exercise test T during the baseline, feeding (F5 and F10) and washout periods.



- Baseline
- F5
- F10
- Washout



Moderate exercise tests (SC)

There was no significant difference in the RER measured, during the canter phase of exercise test SC between weeks in the control group of horses (Fig 7.5). ANOVA revealed a significant interaction between week of test and measurement time during the canter phase in the horses in group F ($p < 0.001$). RER during the canter phase of exercise test F5 was not significantly different from that measured during the baseline exercise tests. However, RER measured during the canter phase of exercise test F10 was significantly lower than that measured during the baseline tests after 1, 2 and 9 minutes of canter (Fig 7.5). Examination of the individual horse data revealed that RER was consistently lower during the canter phase of exercise F10 compared to the baseline exercise test in 3 of the 4 horses (Fig 7.6). Once again it was horse Li which did not conform to the trend of the other horses (Fig 7.6).

Moderate/fast exercise tests (FC)

There was no significant difference in the RER measured during the canter phase of exercise tests FC in either dietary groups and there was no significant difference in RER measured between dietary groups ($p > 0.05$, Fig 7.7).

Plasma free fatty acid concentration

The plasma FFA concentration increased significantly in both dietary groups during all the low intensity exercise tests ($p < 0.0001$). There was no significant difference in the plasma FFA concentration between the serial exercise tests, either at the onset or during the exercise period, in the control group of horses ($p > 0.05$, Figs 7.8). ANOVA revealed a significant interaction between week of test and sampling time in the fat supplemented horses ($p < 0.0001$). There was a strong trend amongst the horses in group F towards a higher plasma FFA concentration during the latter stages of exercise during the F5 and F10 exercise tests (Figs 7.8, 7.9). The plasma FFA concentration of horse Li did not conform to the trend exhibited by the other 3 horses in group F (Fig 7.9). Statistical significance was reached during exercise test F5 only (Fig 7.8). Additionally the plasma FFA concentration reached

during the washout test was significantly lower in the latter stages of exercise, relative to exercise test F5 ($p < 0.05$, Fig 7.8).

Plasma FFA concentration increased slowly during the canter phase of exercise tests SC and FC and continued to rise rapidly during the walking recovery phase (Figs 7.10 and 7.11). There was no significant difference in plasma FFA concentration between any of the serial SC or FC exercise tests, either within or between dietary group. During exercise test FC the walking recovery period was associated with a sharp increase in FFA concentration in both dietary groups (Fig 7.11).

Plasma lactate concentration

Plasma lactate concentration declined in the early stages of exercise test T before increasing during the latter stages, although the concentration remained below 2 mmol/l throughout exercise. Plasma lactate concentration increased during the first two minutes of the canter phase of exercise test SC and then either declined or remained at a constant level (< 4.5 mmol/l) for the remainder of the period of canter. During the canter phase of exercise test FC the lactate concentration was typically lower than 9 mmol/l. However, the plasma lactate concentration of horse Mo reached 18 mmol/l during the exercise test performed after 10 weeks of fat supplementation. There was no significant effect of either week of test or dietary group on plasma lactate concentration during either the slow, moderate or moderate/fast exercise tests ($p > 0.05$, Table 7.1).

Plasma glucose concentration

There was no significant interaction between week of test and diet with respect to plasma glucose concentration either within or between dietary groups for any of the exercise tests (Table 7.2).

Fig 7.5 Mean RER during the canter phase of exercise test SC completed during the baseline period, the feeding period (F5 and F10) and the washout period in horses in dietary groups F and C respectively.

* RER significantly different ($p < 0.05$) from that measured at the corresponding time during the baseline exercise test.

† RER significantly different ($p < 0.05$) from that measured at the corresponding time during the washout exercise test.

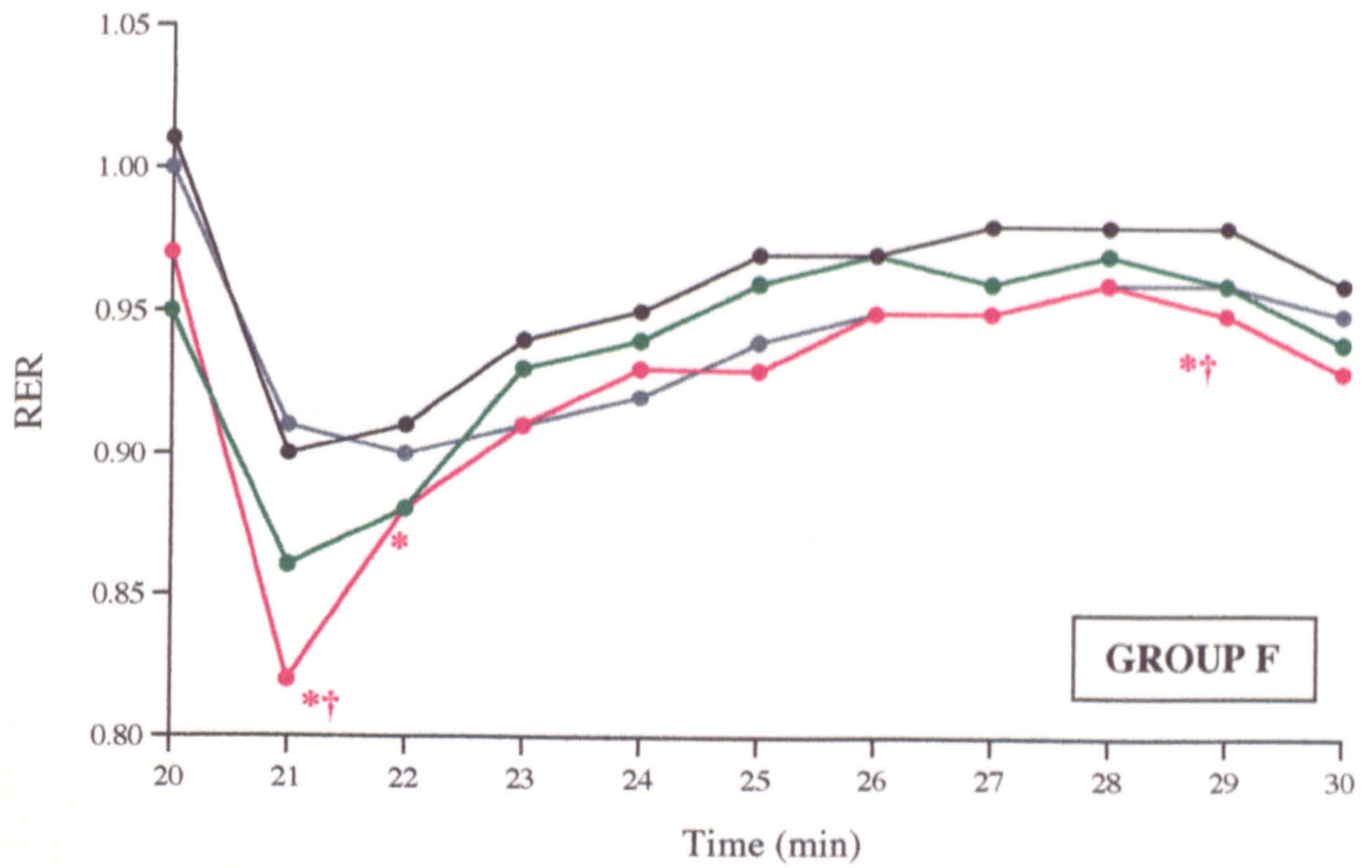


Fig 7.6

RER increased in response to the higher phase of exercise at 20 min. During the recovery period (21-24 min) and washout period.

- Baseline
- F5
- F10
- Washout

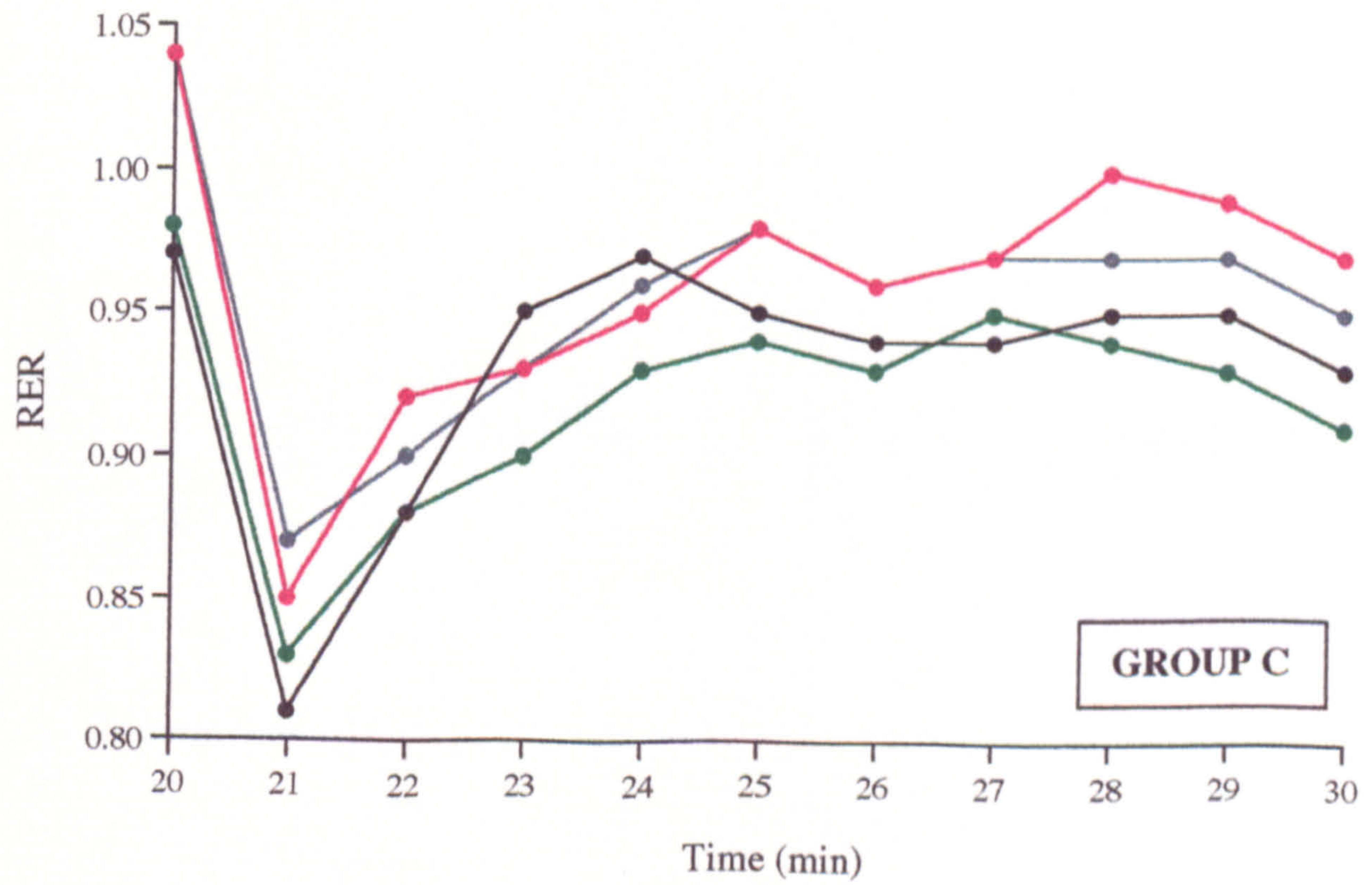


Fig 7.6 RER measured in individual horses (Bo, Li, Mo, Pe) in dietary group F, in response to the canter phase of exercise test SC during the baseline, feeding (F5 and F10) and washout periods.

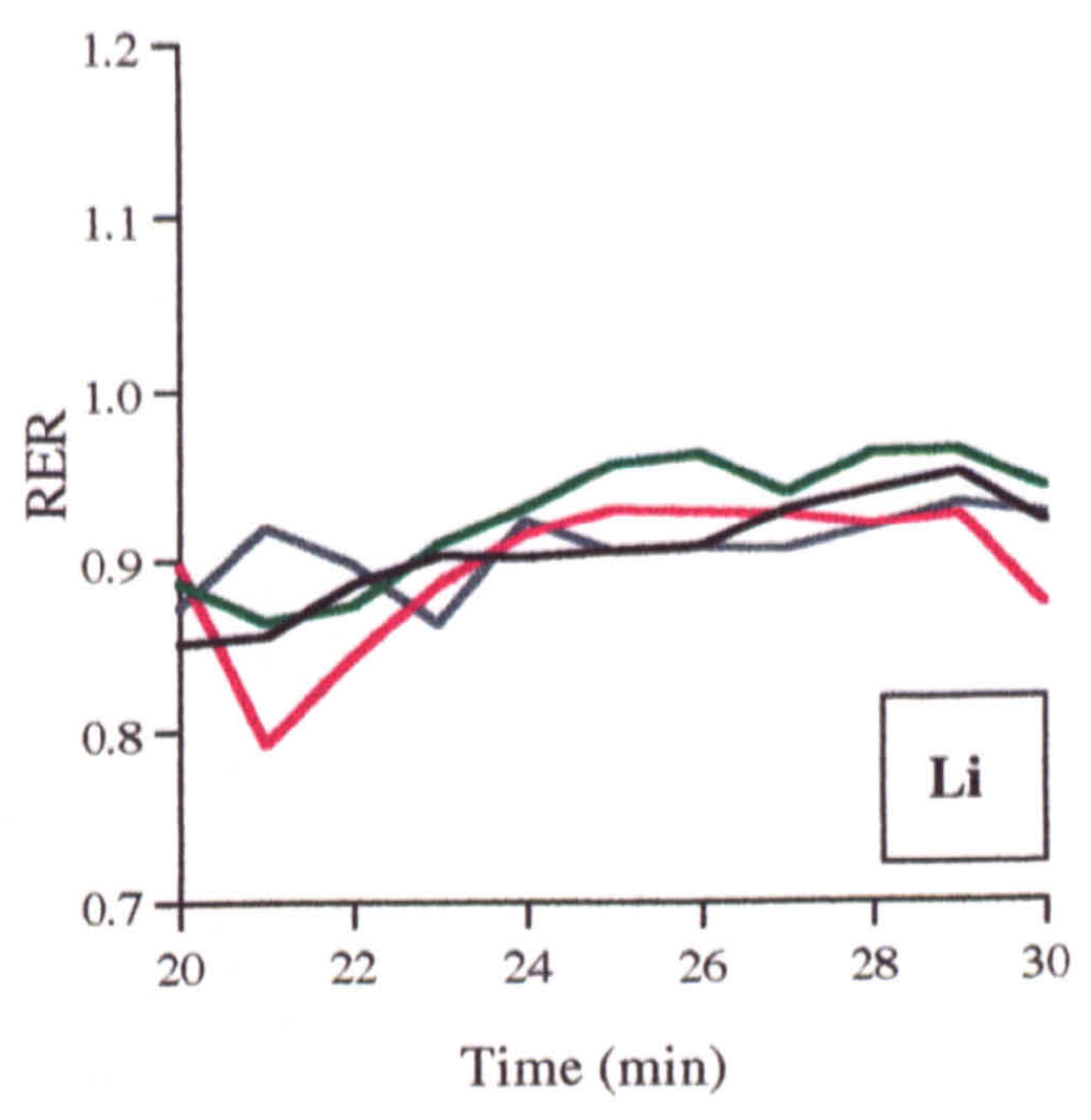
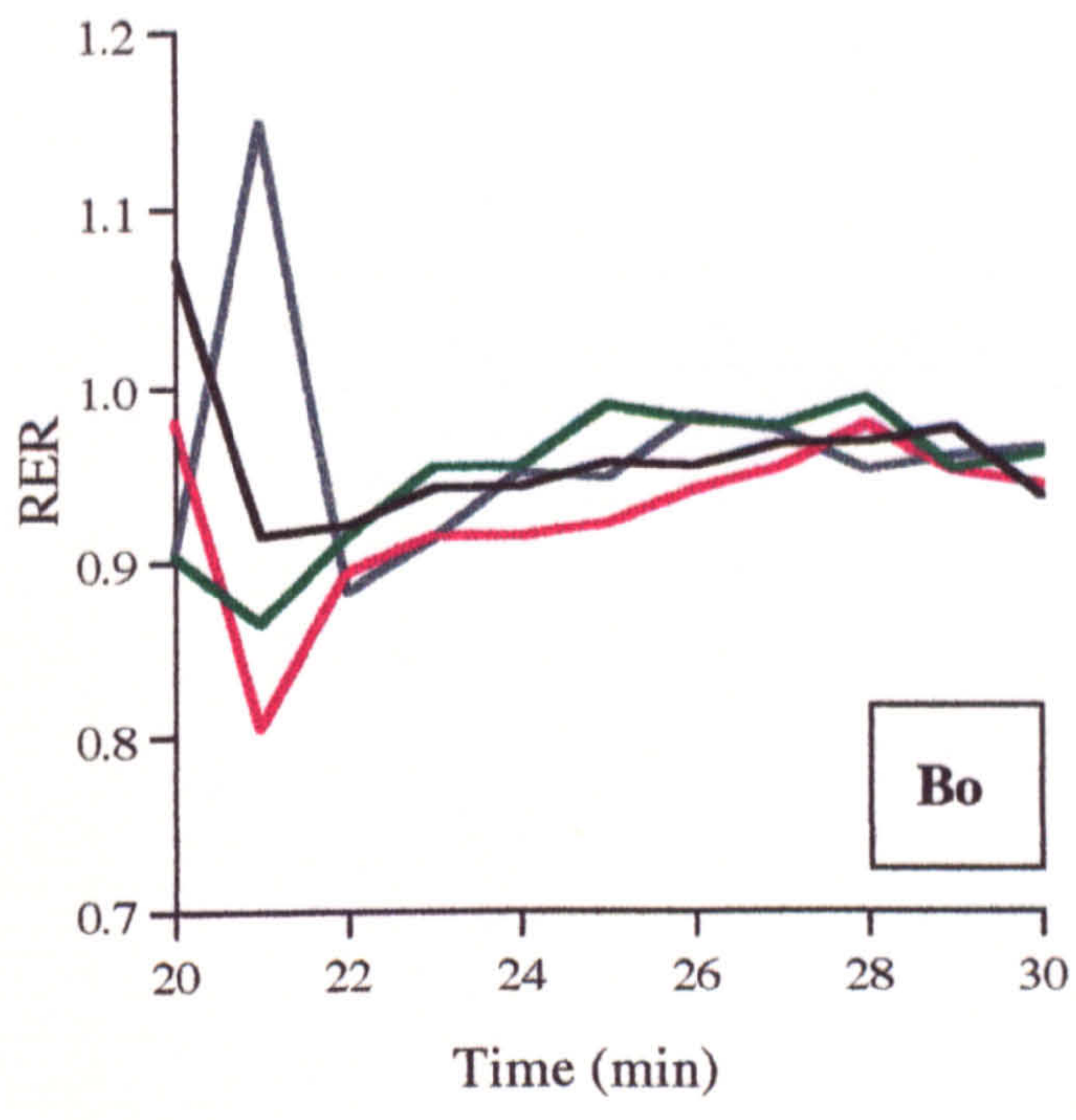


Fig 7.7

RER (green & red lines) during the baseline period, the food period (F5 and F10) and the washout period in subjects Bo (left) and Li (right).

- Baseline
- F5
- F10
- Washout

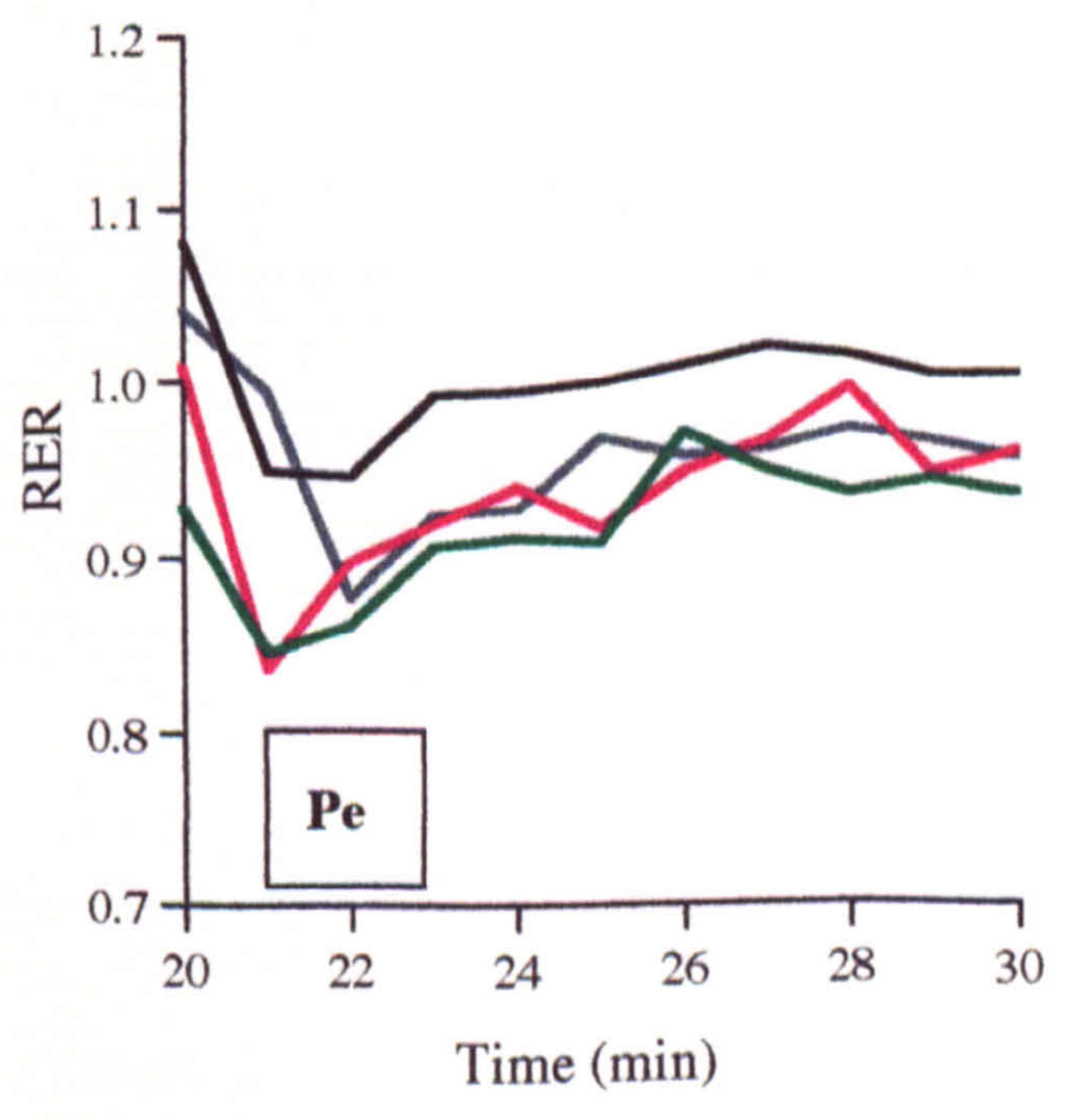
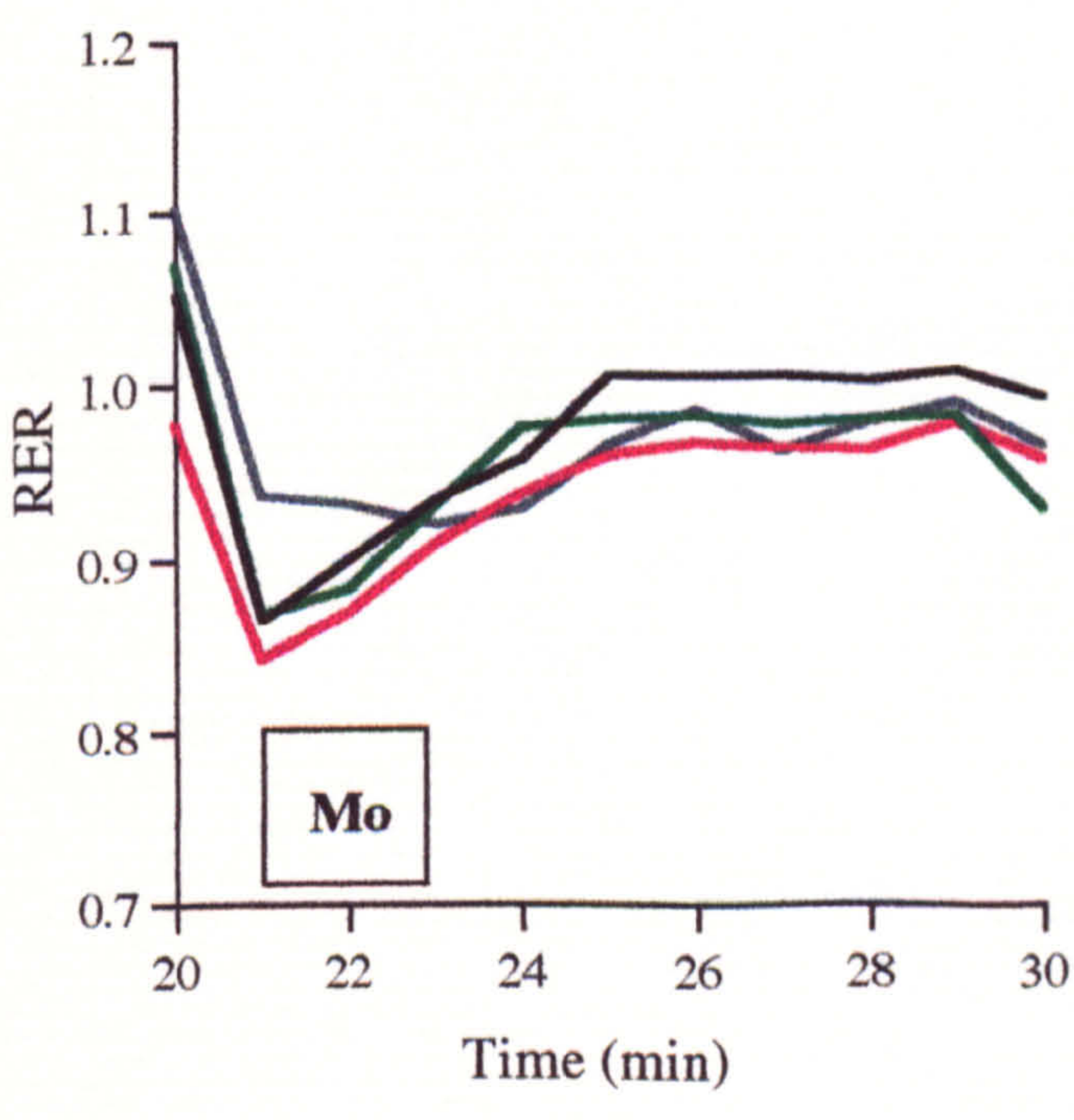


Fig 7.7 RER (mean \pm SD) during the canter phase of exercise test FC completed during the baseline period, the feeding period (F5 and F10) and the washout period in horses in dietary groups F and C respectively.

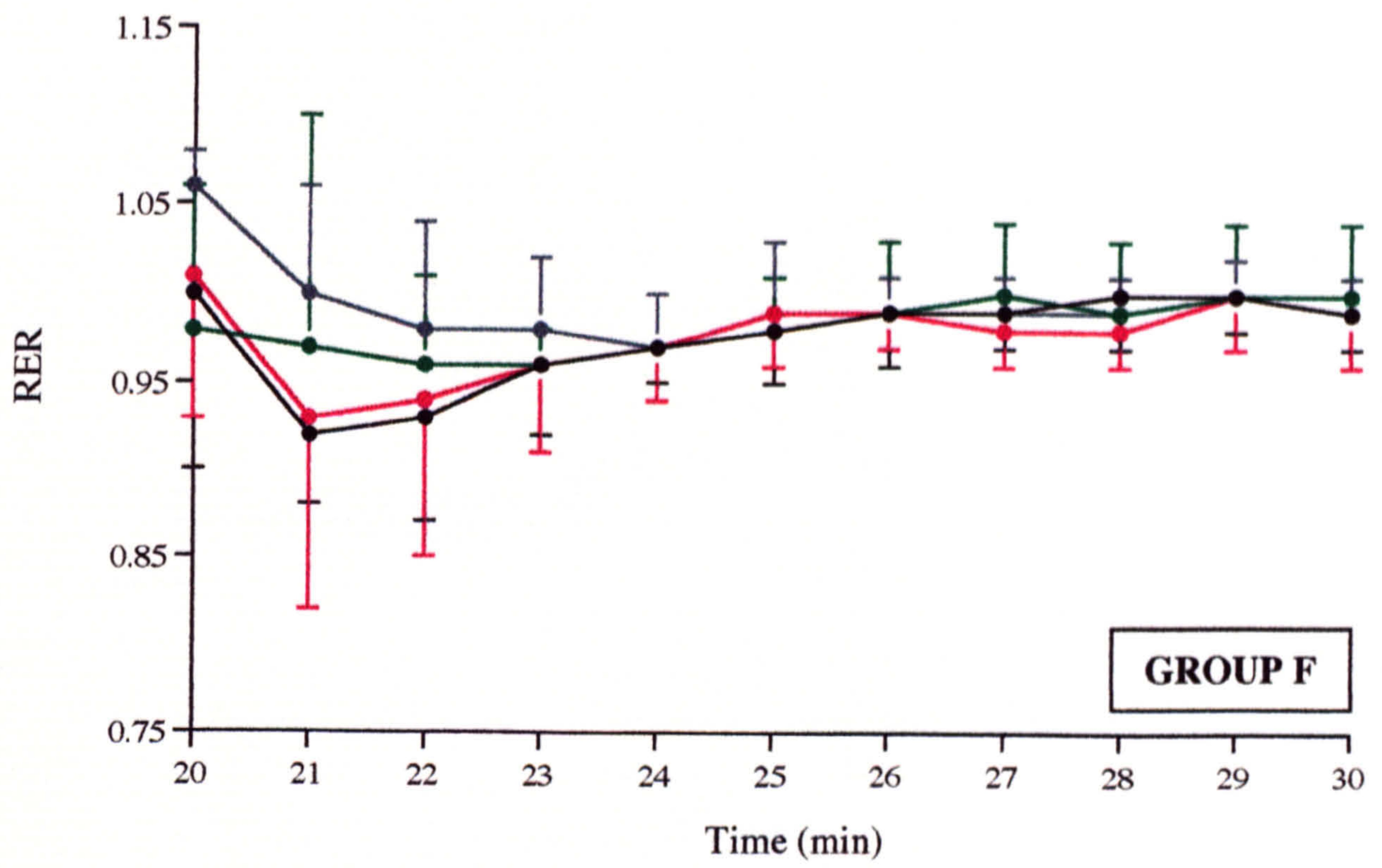


Fig 7X

Mean plasma ITA concentration during the baseline, feeding (F5 and F10) and washout periods in the control (group 1) and C (group 2) respectively.

RER significantly different ($p < 0.05$) from the value recorded at the corresponding time during the baseline exercise test.

ITC significantly different ($p < 0.05$) from the value recorded at the corresponding time during the washout exercise test.

- Baseline
- F5
- F10
- Washout

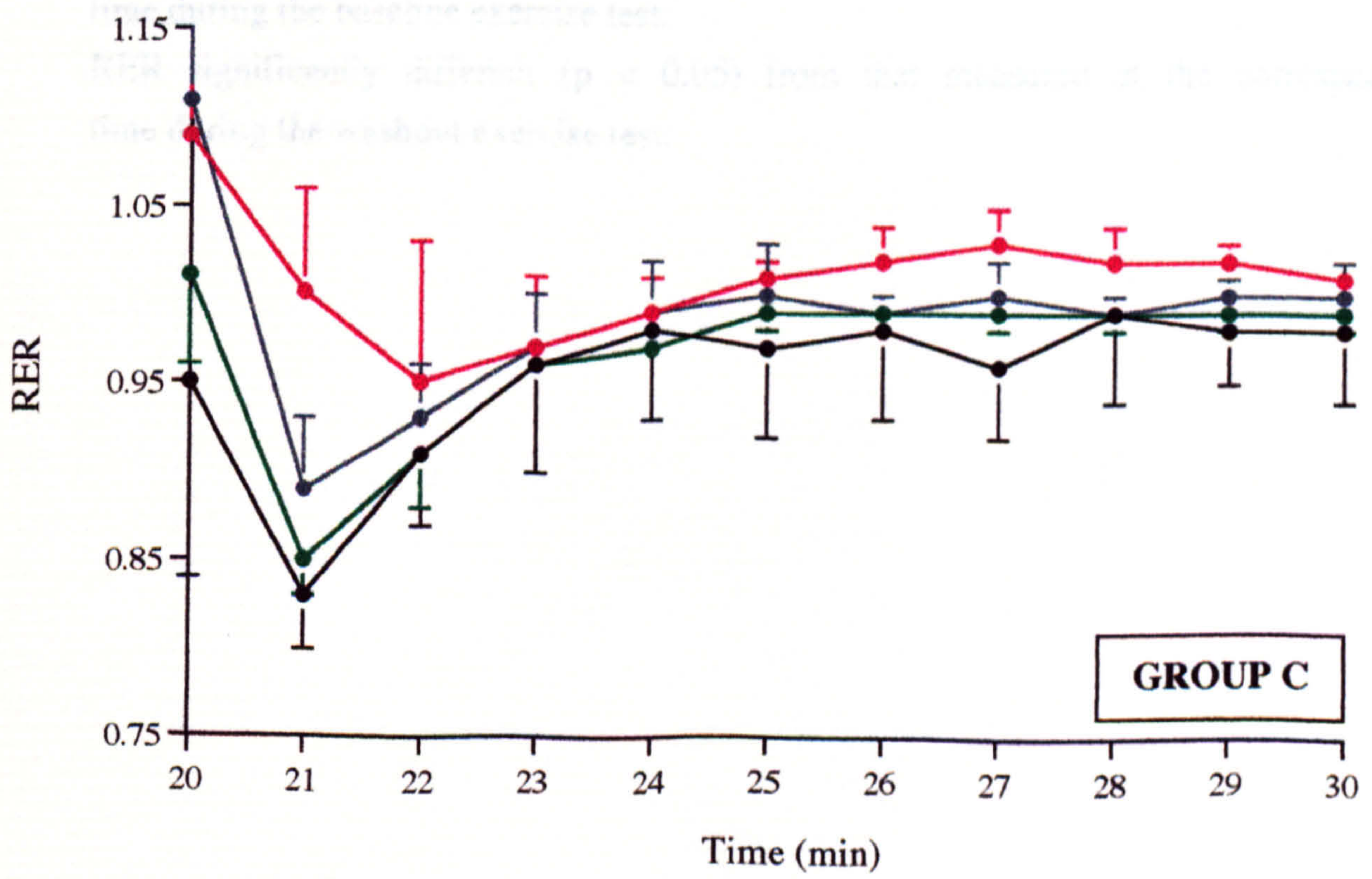


Fig 7.8 Mean plasma FFA concentration during exercise test T, performed during the baseline, feeding (F5 and F10) and washout periods by the horses in dietary groups F and C respectively

* RER significantly different ($p < 0.05$) from that measured at the corresponding time during the baseline exercise test.

† RER significantly different ($p < 0.05$) from that measured at the corresponding time during the washout exercise test.

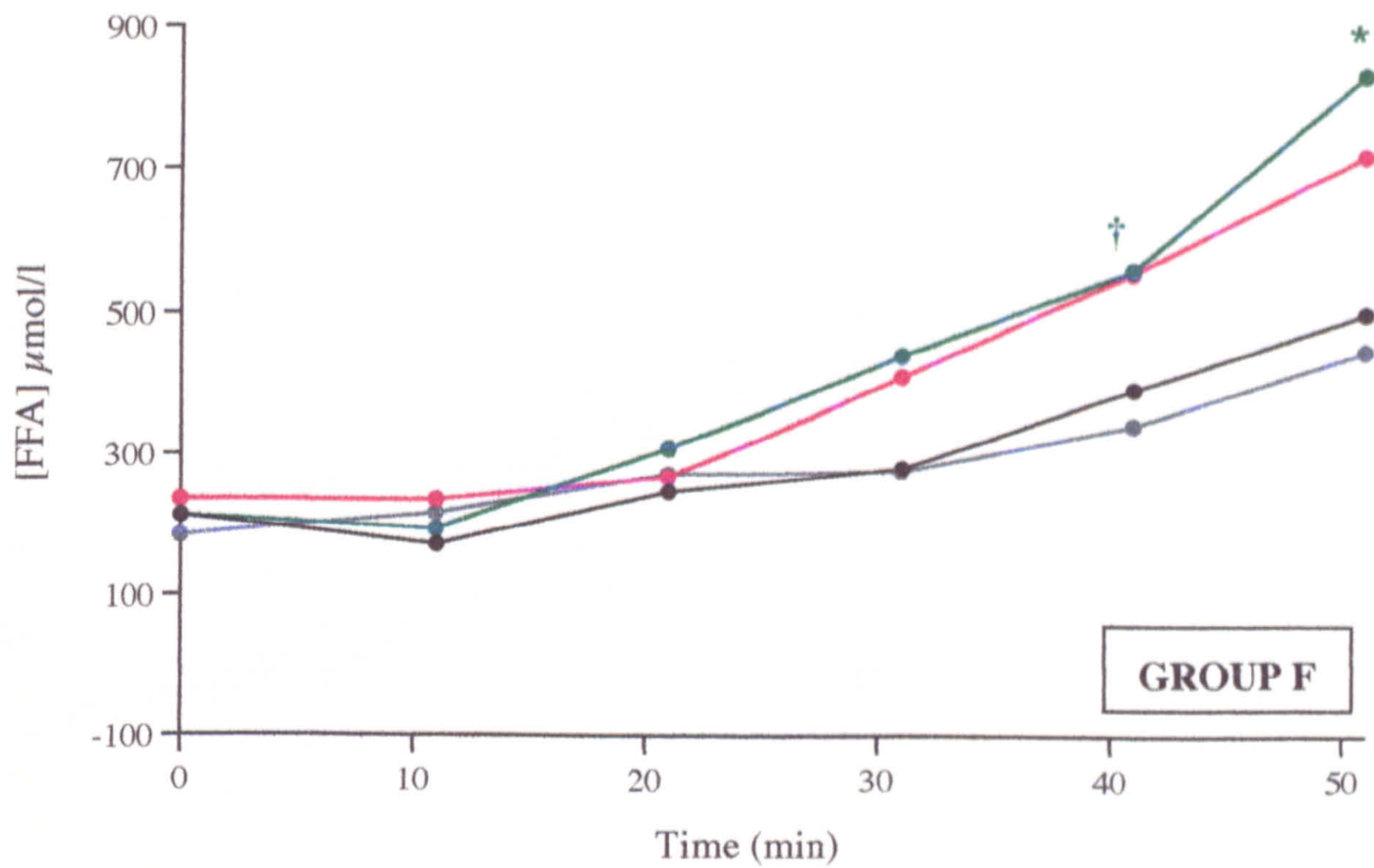


Fig 7.9

Plasma FFA concentrations in individual horses (Pret, Eq, 1984) were in control group F during exercise and T performed during the treatment involving F5 and F10 and without paraffin.

- Baseline
- F5
- F10
- Washout

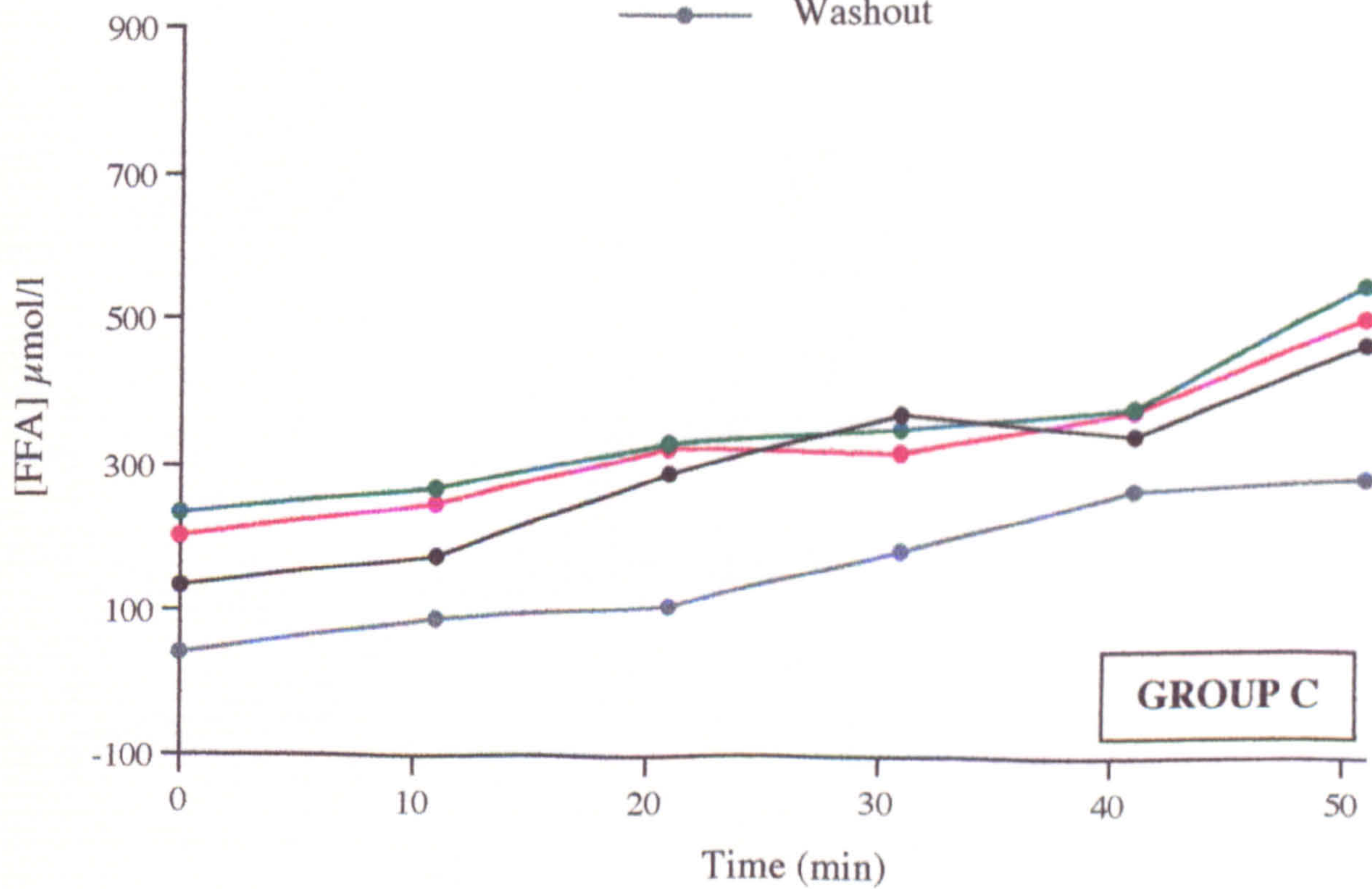


Fig 7.9

Plasma FFA concentration in individual horses (Bo, Li, Mo, Pe) in dietary group F during exercise test T, performed during the baseline, feeding (F5 and F10) and washout periods.

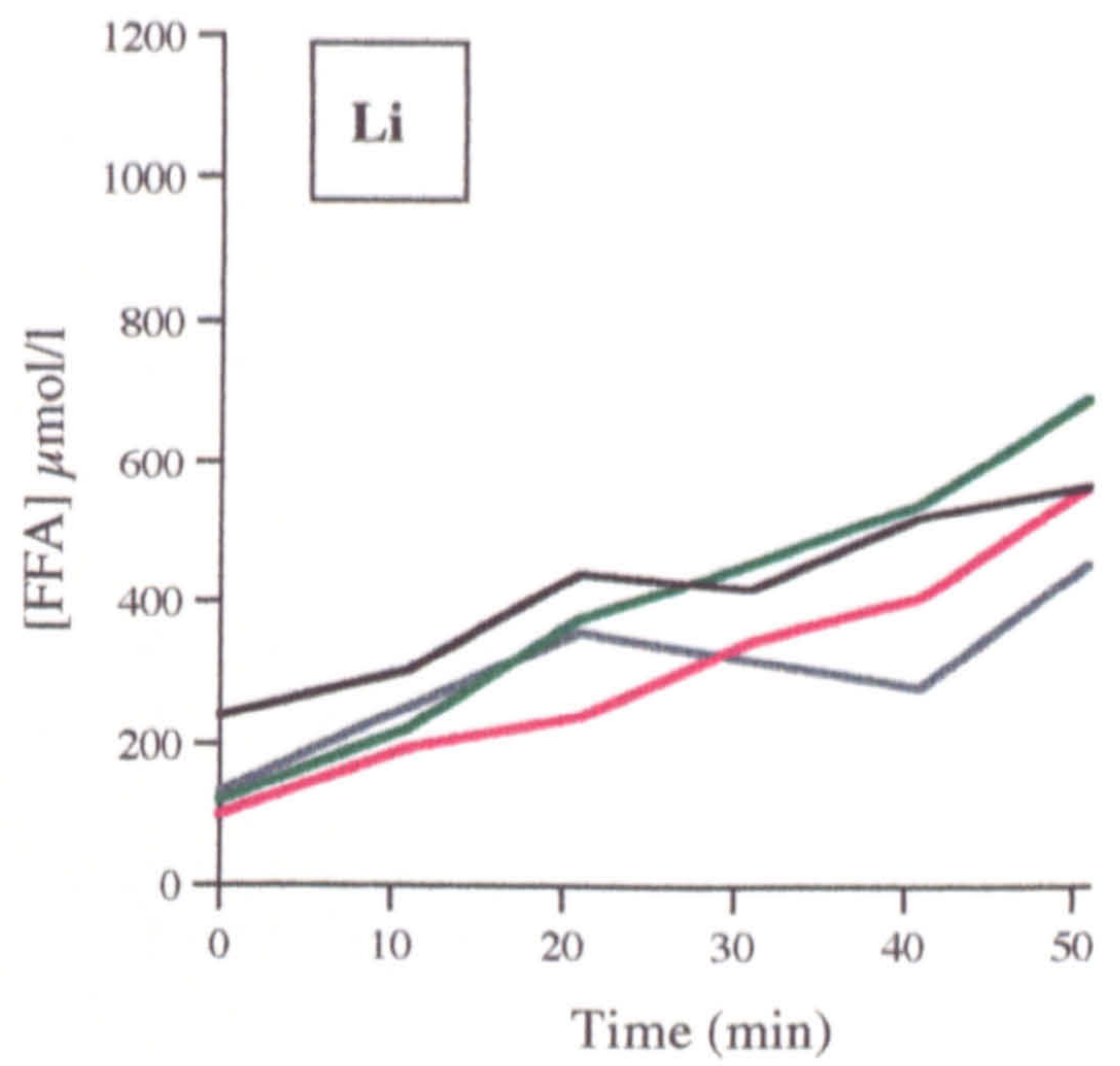
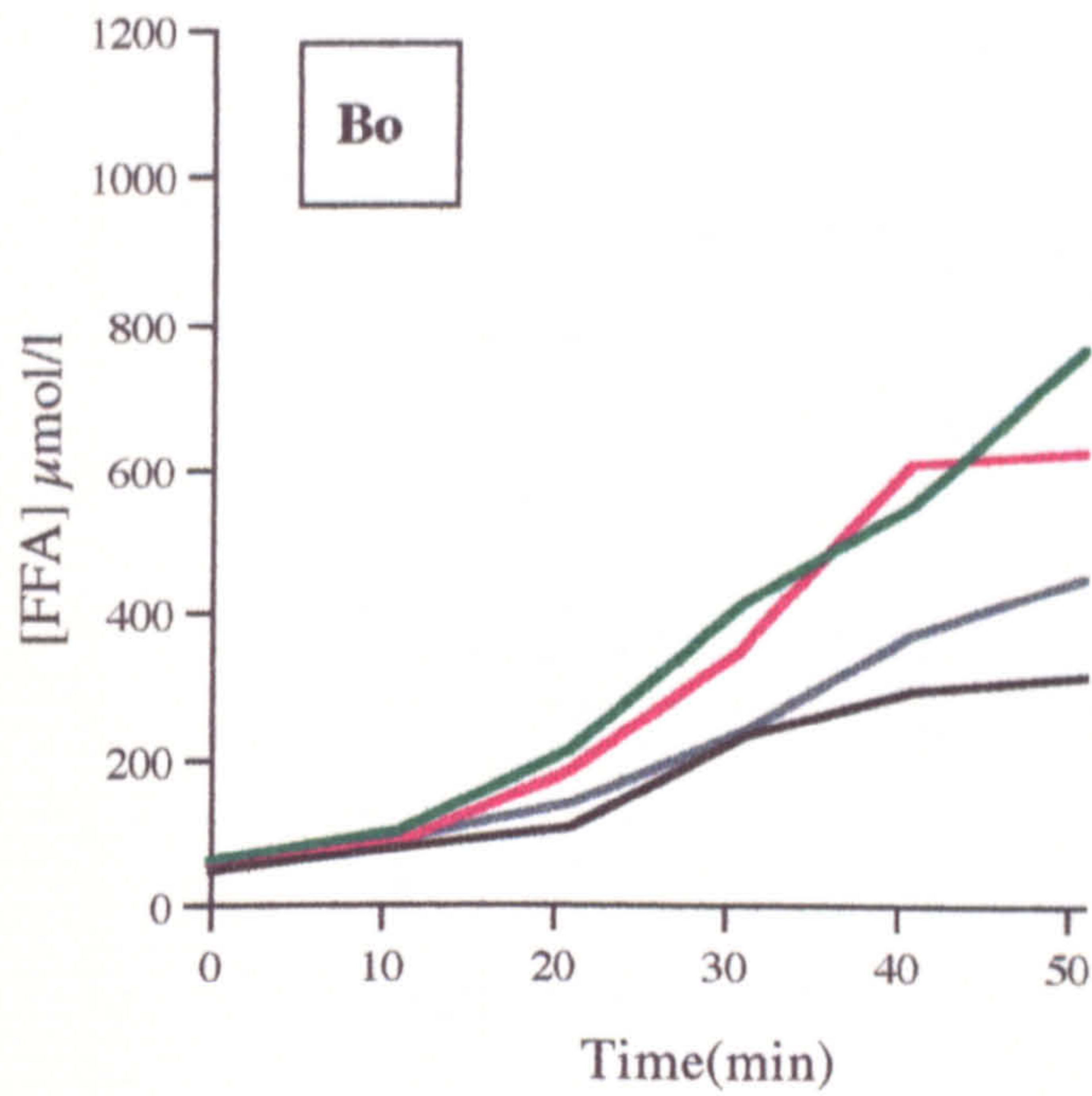


Fig. 7.10 Effects of β -adrenergic blockade on the increase in plasma free fatty acid concentration during the last 50 min of exercise in 10 subjects with normal ($n = 5$) and obese ($n = 5$) subjects. The subjects received either placebo (Bo) or propranolol (Li) during the last 50 min of exercise. The values are the mean \pm SEM. The lines represent the following conditions: Baseline (black), F5 (green), F10 (red), and Washout (blue).

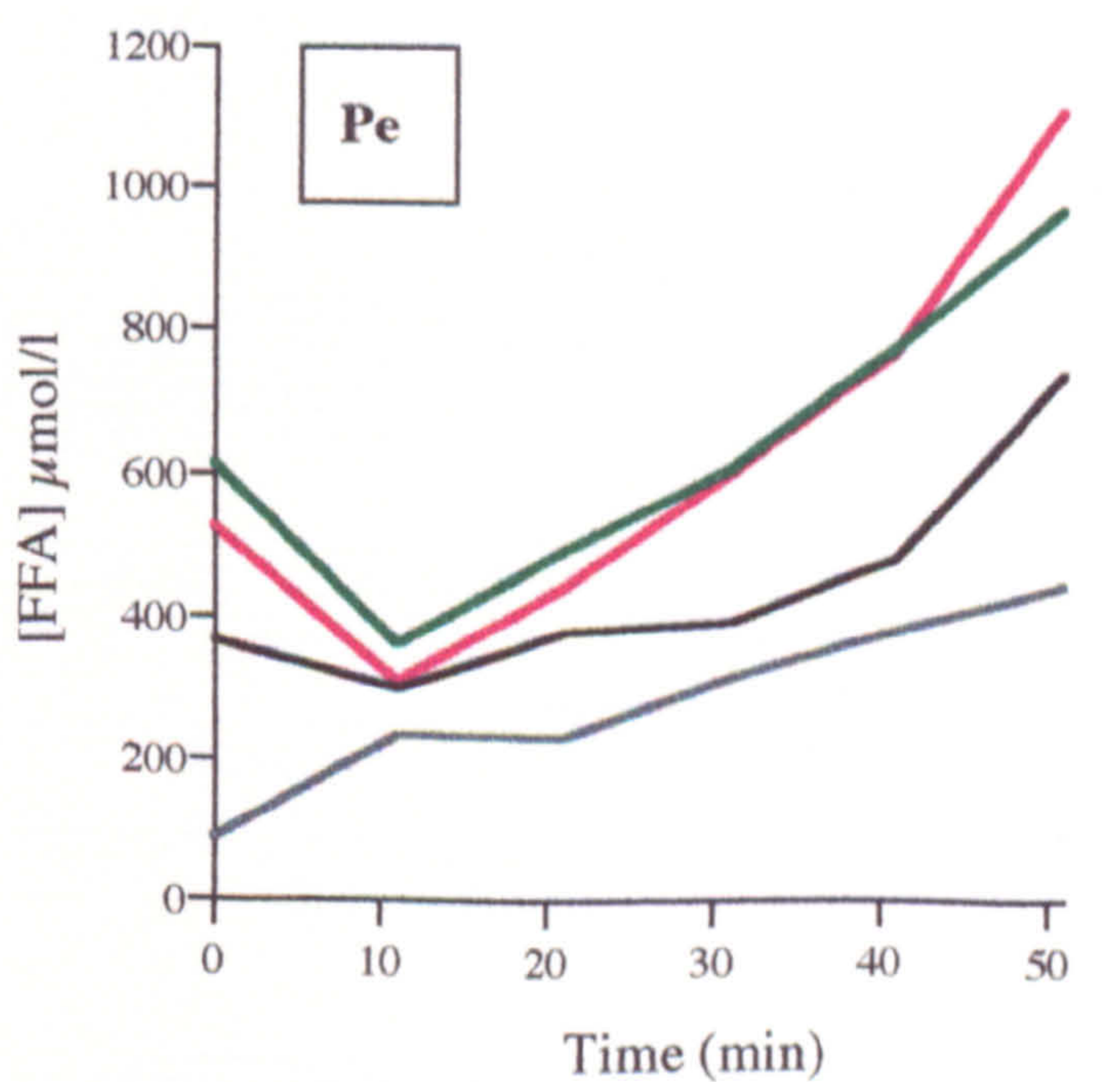
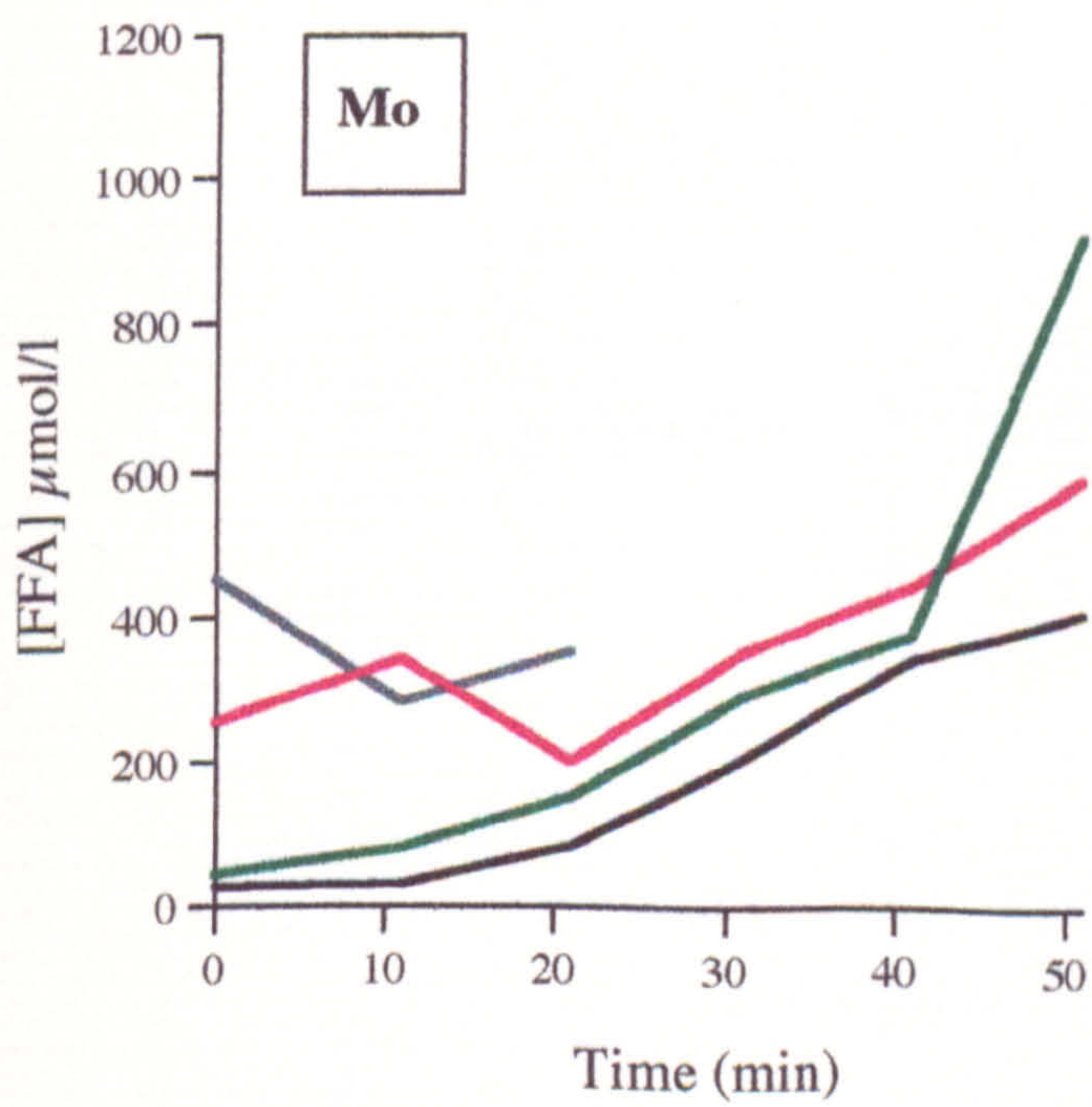


Fig 7.10 Plasma FFA concentration (mean \pm SD) during the canter and recovery phases of exercise test SC in horses in dietary groups F and C respectively during the baseline, feeding (F5 and F10) and washout periods.

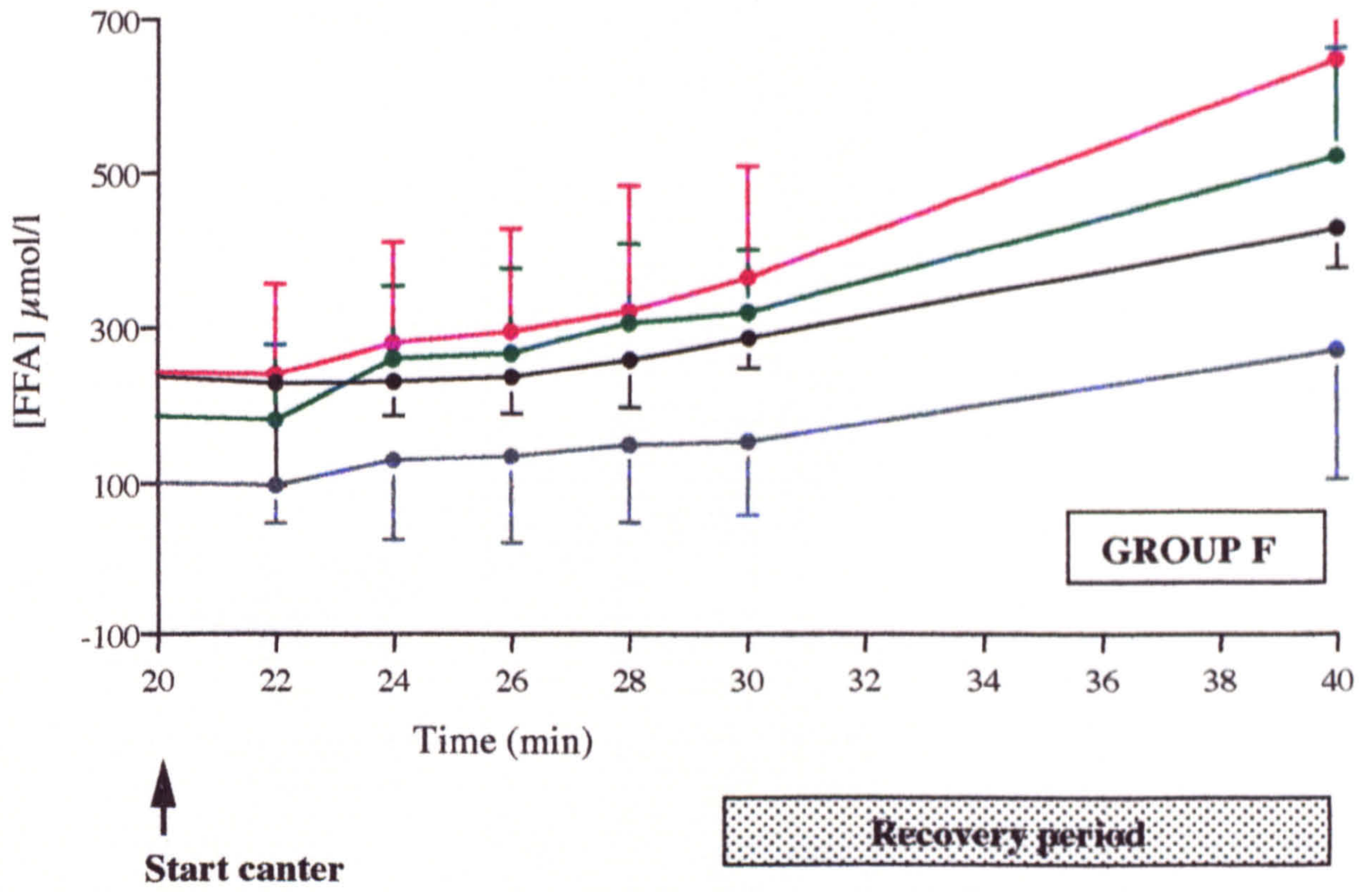


Fig 7.11
 Plasma FFA concentrations (mean \pm SD) during the baseline and recovery phases of constant rate PC in horses in dietary groups F and U, respectively during the baseline, loading and recovery periods.

- Baseline
- F5
- F10
- Washout

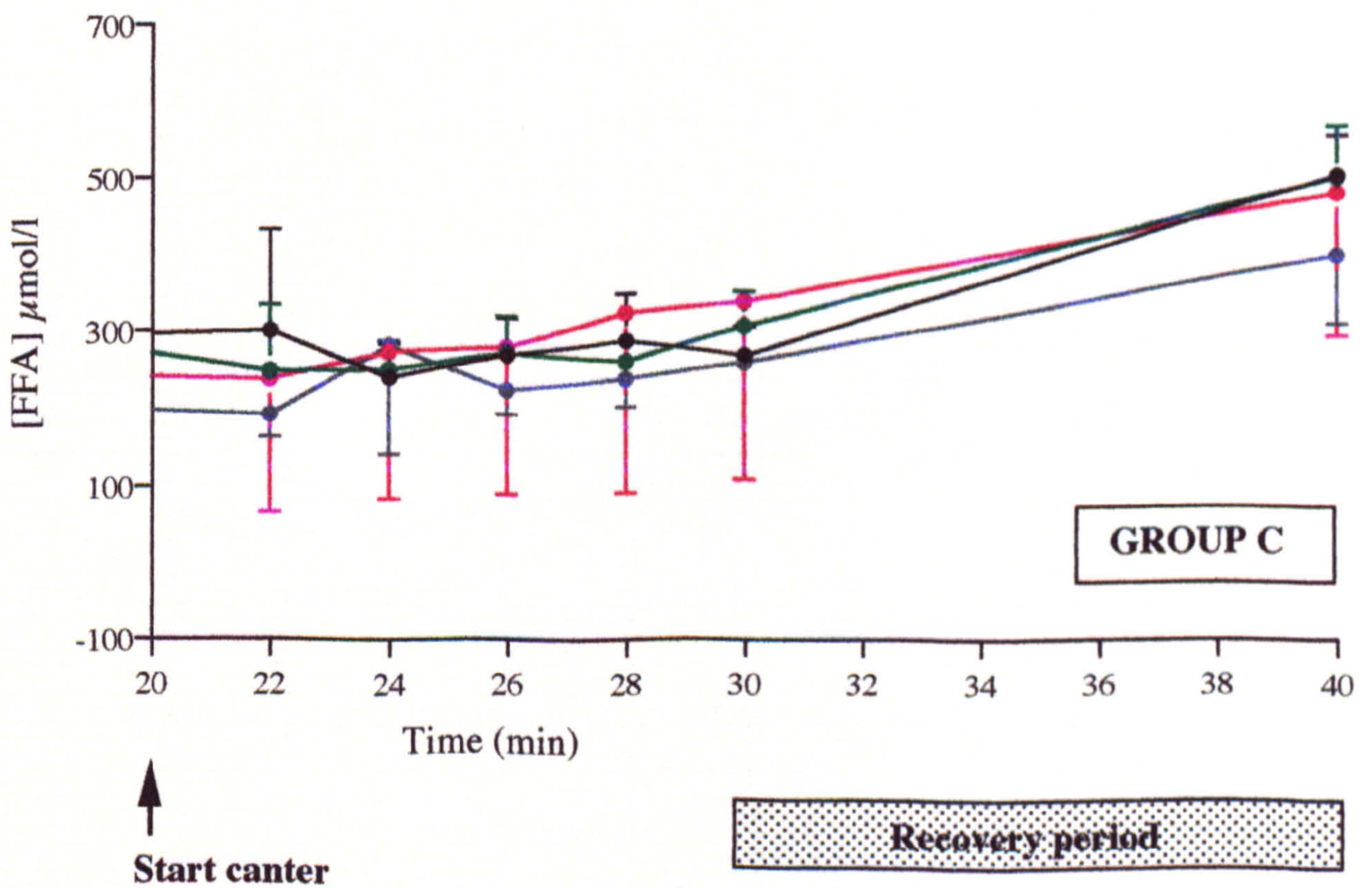


Fig 7.11 Plasma FFA concentration (mean \pm SD) during the canter and recovery phases of exercise test FC in horses in dietary groups F and C respectively during the baseline, feeding (F5 and F10) and washout periods.

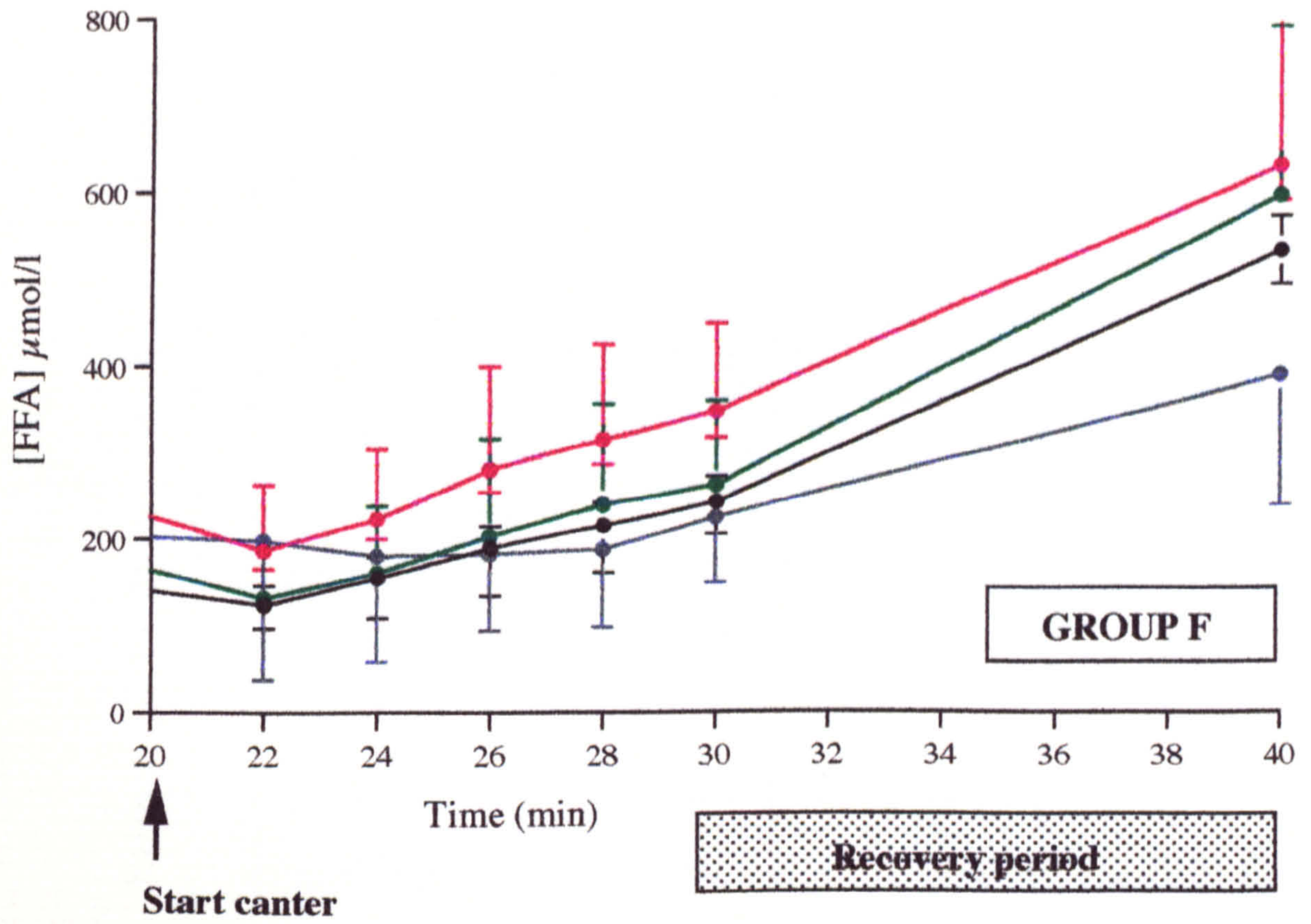


Table 7.1

Plasma glucose, lactate, SC and FC during the recovery period in the recovery groups C and F.

- Baseline
- F5
- F10
- Washout

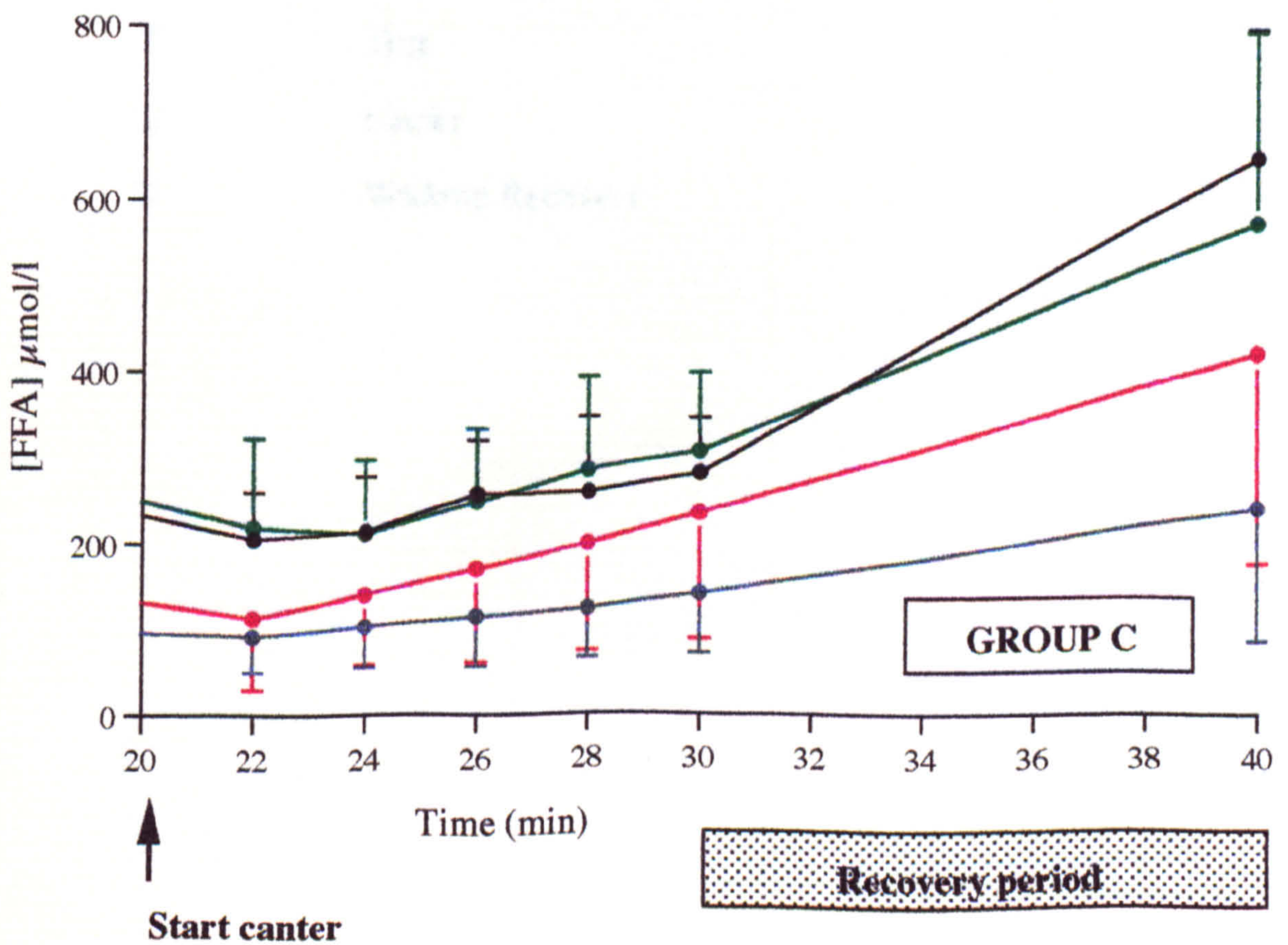


Table 7.1 Plasma glucose concentration (mmol/l, mean \pm SD) during exercise tests T, SC and FC during the baseline, feeding and washout periods in horses in dietary groups C and F.

Pre E	Pre Exercise
T	Trot
C	Canter
R	Walking Recovery

GROUP F

GROUP C

Sample Time	Baseline	Feeding 5	Feeding 10	Washout	Baseline	Feeding 5	Feeding 10	Washout
T								
PREE	5.9 ± 0.5	5.7 ± 0.4	5.6 ± 0.5	5.8 ± 0.6	5.5 ± 0.7	5.5 ± 0.4	5.6 ± 0.5	5.9 ± 0.4
10T	4.9 ± 0.9	4.6 ± 0.6	5.3 ± 0.4	5.2 ± 0.3	5.1 ± 0.8	5.0 ± 0.5	5.2 ± 0.1	4.8 ± 0.9
20T	4.4 ± 1.2	4.4 ± 1.1	5.2 ± 0.7	5.3 ± 0.6	4.9 ± 0.6	5.3 ± 0.0	5.1 ± 0.4	4.0 ± 1.5
30T	4.7 ± 0.9	4.6 ± 1.0	5.3 ± 0.7	5.2 ± 0.6	5.2 ± 0.4	5.3 ± 0.2	5.1 ± 0.3	4.3 ± 1.8
40T	5.2 ± 0.5	5.0 ± 0.6	5.7 ± 0.4	5.5 ± 0.2	5.5 ± 0.2	5.5 ± 0.1	5.2 ± 0.1	4.9 ± 1.1
10R	5.6 ± 0.2	5.6 ± 0.2	5.8 ± 0.3	5.5 ± 0.4	5.8 ± 0.7	5.7 ± 0.4	5.4 ± 0.2	5.4 ± 0.4
SC								
PREE	5.4 ± 0.6	6.2 ± 0.4	5.5 ± 0.3	5.7 ± 0.7	5.3 ± 0.1	6.2 ± 0.6	5.9 ± 0.4	5.3 ± 0.4
10W	5.0 ± 0.6	5.5 ± 0.5	5.3 ± 0.3	5.3 ± 0.5	5.1 ± 0.3	5.5 ± 0.4	5.6 ± 0.5	5.3 ± 0.3
5T	5.0 ± 0.8	5.3 ± 0.5	5.4 ± 0.4	5.0 ± 0.4	5.2 ± 0.7	5.6 ± 0.2	5.6 ± 0.4	5.5 ± 0.5
2C	5.0 ± 0.9	5.1 ± 0.6	5.3 ± 0.7	5.2 ± 0.5	5.3 ± 0.6	5.7 ± 0.2	5.6 ± 0.7	5.4 ± 0.3
4C	4.9 ± 0.6	5.2 ± 0.4	5.3 ± 0.7	5.4 ± 0.6	5.4 ± 0.2	5.7 ± 0.4	5.5 ± 0.7	5.6 ± 0.5
6C	4.9 ± 0.7	5.0 ± 0.5	5.3 ± 0.6	5.2 ± 0.2	5.5 ± 0.4	5.8 ± 0.5	5.3 ± 0.9	5.7 ± 0.6
8C	4.9 ± 0.6	5.0 ± 0.6	5.4 ± 0.6	5.2 ± 0.3	5.5 ± 0.7	5.7 ± 0.6	5.2 ± 0.9	5.8 ± 0.7
10C	5.0 ± 0.6	5.3 ± 0.7	5.5 ± 0.7	5.0 ± 0.3	5.5 ± 0.8	5.6 ± 0.5	5.4 ± 1.2	5.9 ± 0.8
10R	5.2 ± 0.7	5.7 ± 0.4	5.7 ± 0.7	5.2 ± 0.3	5.6 ± 0.4	5.9 ± 0.3	5.9 ± 0.8	5.7 ± 0.3
FC								
PREE	5.3 ± 0.2	5.6 ± 0.3	5.7 ± 0.4	5.7 ± 0.3	5.3 ± 0.2	5.7 ± 0.1	5.9 ± 0.3	5.8 ± 0.0
10W	5.1 ± 0.2	5.2 ± 0.7	5.6 ± 0.3	5.3 ± 0.4	5.2 ± 0.3	5.1 ± 0.1	5.4 ± 0.3	5.0 ± 0.6
5T	4.9 ± 0.3	5.0 ± 0.8	5.2 ± 0.4	5.5 ± 0.5	5.3 ± 0.4	5.4 ± 0.2	5.1 ± 0.9	5.1 ± 0.7
2C	5.2 ± 0.4	5.2 ± 0.9	5.4 ± 1.1	5.5 ± 1.2	5.7 ± 0.7	5.3 ± 0.1	4.9 ± 1.1	5.2 ± 1.2
4C	5.2 ± 0.4	5.5 ± 1.0	5.7 ± 1.2	5.8 ± 1.4	5.6 ± 0.4	5.4 ± 0.5	5.2 ± 1.1	5.3 ± 1.6
6C	5.2 ± 0.5	5.7 ± 1.4	6.1 ± 1.5	5.5 ± 1.6	5.6 ± 0.5	5.6 ± 0.1	5.3 ± 1.5	5.0 ± 1.4
8C	5.3 ± 0.4	5.8 ± 1.3	6.1 ± 1.6	5.7 ± 1.5	5.5 ± 0.4	5.9 ± 0.2	5.6 ± 1.3	5.0 ± 1.3
10C	5.3 ± 0.5	5.9 ± 1.4	6.0 ± 2.3	5.8 ± 1.4	5.8 ± 0.7	6.4 ± 0.0	5.5 ± 1.4	5.0 ± 1.6
10R	5.8 ± 0.4	6.5 ± 1.4	6.6 ± 1.7	6.2 ± 1.2	6.0 ± 0.4	6.6 ± 0.3	5.9 ± 1.3	5.4 ± 1.2

Table 7.2 Plasma lactate concentration (mmol/l, mean \pm SD) during exercise tests T, SC and FC during the baseline, feeding and washout periods in horses in dietary groups C and F.

Pre E	Pre Exercise
T	Trot
C	Canter
R	Walking Recovery

Sample	Baseline		Feeding 5		Feeding 10		Washout	
	Group F	Group C	Group F	Group C	Group F	Group C	Group F	Group C
T								
Pre E	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.4	1.2 ± 0.1
10T	0.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	0.5 ± 0.2	0.8 ± 0.1
20T	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.6 ± 0.1
30T	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.7 ± 0.1
40T	0.3 ± 0.1	0.6 ± 0.3	0.6 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.7 ± 0.1
10R	0.4 ± 0.0	0.7 ± 0.2	0.6 ± 0.4	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.1
SC								
Pre E	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.9 ± 0.4	0.8 ± 0.4	0.7 ± 0.1
2'C	1.0 ± 0.2	1.4 ± 0.8	1.2 ± 1.0	1.1 ± 0.7	1.0 ± 0.4	1.2 ± 0.5	2.5 ± 1.4	1.2 ± 0.4
4'C	0.8 ± 0.1	1.1 ± 0.5	0.9 ± 0.8	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	1.5 ± 0.8	0.9 ± 0.3
6'C	0.8 ± 0.2	1.0 ± 0.3	0.7 ± 0.5	0.7 ± 0.3	0.6 ± 0.2	0.9 ± 0.3	1.0 ± 0.4	0.9 ± 0.2
8'C	0.7 ± 0.2	1.0 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.3
10'C	0.8 ± 0.3	1.2 ± 0.3	0.6 ± 0.1	0.8 ± 0.3	0.7 ± 0.2	1.0 ± 0.2	0.8 ± 0.3	0.9 ± 0.3
10R	0.5 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	0.5 ± 0.2	0.6 ± 0.1
FC								
Pre E	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	1.0 ± 0.2
2'C	3.4 ± 2.0	2.3 ± 0.7	4.7 ± 2.8	2.3 ± 1.1	5.5 ± 8.1	2.5 ± 1.2	3.6 ± 1.7	3.2 ± 1.7
4'C	2.1 ± 1.0	1.7 ± 0.7	2.9 ± 1.2	1.8 ± 1.0	4.7 ± 7.0	1.9 ± 0.9	3.1 ± 1.0	2.4 ± 1.2
6'C	2.0 ± 0.6	1.7 ± 0.5	2.6 ± 1.1	1.8 ± 0.9	3.8 ± 4.9	1.8 ± 0.7	4.0 ± 2.7	2.1 ± 0.9
8'C	2.3 ± 0.3	1.9 ± 0.7	2.8 ± 1.1	2.0 ± 1.1	3.3 ± 3.0	2.0 ± 0.7	3.1 ± 1.4	2.6 ± 1.0
10'C	2.4 ± 0.6	2.3 ± 0.8	3.0 ± 1.1	2.3 ± 1.3	3.1 ± 2.5	2.2 ± 0.9	3.1 ± 0.3	2.7 ± 1.0
10R	0.9 ± 0.2	1.0 ± 0.3	1.1 ± 0.4	0.9 ± 0.4	1.3 ± 0.8	0.9 ± 0.3	0.9 ± 0.2	1.2 ± 0.3

	Baseline		Feeding 5		Feeding 10		Washout	
Sample	Group F	Group C	Group F	Group C	Group F	Group C	Group F	Group C
T								
Pre E	0.7 ± 0.2	0.8 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.5 ± 0.1	0.8 ± 0.2	0.9 ± 0.4	1.2 ± 0.1
10T	0.4 ± 0.1	0.7 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.3	0.5 ± 0.2	0.8 ± 0.1
20T	0.4 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.6 ± 0.2	0.4 ± 0.2	0.6 ± 0.1
30T	0.3 ± 0.1	0.6 ± 0.2	0.3 ± 0.1	0.5 ± 0.1	0.3 ± 0.2	0.5 ± 0.2	0.5 ± 0.2	0.7 ± 0.1
40T	0.3 ± 0.1	0.6 ± 0.3	0.6 ± 0.4	0.4 ± 0.1	0.4 ± 0.1	0.5 ± 0.2	0.4 ± 0.1	0.7 ± 0.1
10R	0.4 ± 0.0	0.7 ± 0.2	0.6 ± 0.4	0.5 ± 0.0	0.4 ± 0.1	0.6 ± 0.2	0.5 ± 0.1	0.7 ± 0.1
SC								
Pre E	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.7 ± 0.1	0.6 ± 0.2	0.9 ± 0.4	0.8 ± 0.4	0.7 ± 0.1
2C	1.0 ± 0.2	1.4 ± 0.8	1.2 ± 1.0	1.1 ± 0.7	1.0 ± 0.4	1.2 ± 0.5	2.5 ± 1.4	1.2 ± 0.4
4C	0.8 ± 0.1	1.1 ± 0.5	0.9 ± 0.8	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.3	1.5 ± 0.8	0.9 ± 0.3
6C	0.8 ± 0.2	1.0 ± 0.3	0.7 ± 0.5	0.7 ± 0.3	0.6 ± 0.2	0.9 ± 0.3	1.0 ± 0.4	0.9 ± 0.2
8C	0.7 ± 0.2	1.0 ± 0.3	0.6 ± 0.2	0.7 ± 0.3	0.7 ± 0.2	0.9 ± 0.2	0.9 ± 0.3	0.9 ± 0.3
10C	0.8 ± 0.3	1.2 ± 0.3	0.6 ± 0.1	0.8 ± 0.3	0.7 ± 0.2	1.0 ± 0.2	0.8 ± 0.3	0.9 ± 0.3
10R	0.5 ± 0.2	0.8 ± 0.2	0.5 ± 0.1	0.6 ± 0.1	0.5 ± 0.2	0.7 ± 0.1	0.5 ± 0.2	0.6 ± 0.1
FC								
Pre E	0.6 ± 0.2	0.7 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.6 ± 0.2	0.8 ± 0.2	0.8 ± 0.3	1.0 ± 0.2
2C	3.4 ± 2.0	2.3 ± 0.7	4.7 ± 2.8	2.3 ± 1.1	5.5 ± 8.1	2.5 ± 1.2	3.6 ± 1.7	3.2 ± 1.7
4C	2.1 ± 1.0	1.7 ± 0.7	2.9 ± 1.2	1.8 ± 1.0	4.7 ± 7.0	1.9 ± 0.9	3.1 ± 1.0	2.4 ± 1.2
6C	2.0 ± 0.6	1.7 ± 0.5	2.6 ± 1.1	1.8 ± 0.9	3.8 ± 4.9	1.8 ± 0.7	4.0 ± 2.7	2.1 ± 0.9
8C	2.3 ± 0.3	1.9 ± 0.7	2.8 ± 1.1	2.0 ± 1.1	3.3 ± 3.0	2.0 ± 0.7	3.1 ± 1.4	2.6 ± 1.0
10C	2.4 ± 0.6	2.3 ± 0.8	3.0 ± 1.1	2.3 ± 1.3	3.1 ± 2.5	2.2 ± 0.9	3.1 ± 0.3	2.7 ± 1.0
10R	0.9 ± 0.2	1.0 ± 0.3	1.1 ± 0.4	0.9 ± 0.4	1.3 ± 0.8	0.9 ± 0.3	0.9 ± 0.2	1.2 ± 0.3

Discussion

Examination of the RER data, measured during the baseline exercise tests, revealed a large inter-horse variation in the response to low intensity exercise. This suggests that there is a large difference in the use of fat and carbohydrate as fuel sources between horses during this type of exercise. This may reflect differences in the individual horse's inherent capacity to utilise fat and carbohydrate. The relative contribution of fat and carbohydrate as fuel sources during low intensity exercise may be largely influenced by genetic factors, as a function of differences in the fibre composition of the skeletal muscle mass and thus associated variation in mitochondrial density, enzymes of fat and carbohydrate catabolism and associated differences in the storage of endogenous muscle fuel stores. The relative contribution of fat and carbohydrate to energy production is also affected by a number of factors including: diet, state of training and nutritional status. Their relative contribution during exercise therefore in an individual depends on the interaction between exercise intensity and the above factors.

The time period between consumption of the pelleted feed and the commencement of exercise tests was not standardised between horse. This may have contributed to the variation in RER observed during exercise test T. However, the horses had almost constant access to forages which, as reported in Chapter 3, serves to prevent decreases in plasma glucose concentration, characteristic of the post-absorptive period in Man (Hoffer 1988). In addition, horses were not exercised for at least 2 hours following the consumption of pelleted feeds. Furthermore, the time at which an individual horse undertook any of the 3 exercise tests on the four separate occasions was standardised to eliminate this as a cause of variation between serial exercise tests.

The inter-horse variation in RER became lower as the intensity of exercise increased and was associated with a corresponding increase in mean RER. The increase in carbohydrate utilisation and associated rise in RER with increase in exercise intensity is well documented in other species. These results suggest that the increased utilisation of carbohydrate is

uniform or obligatory and is less affected by the factors discussed with respect to low intensity exercise.

There was a strong trend towards a lower RER throughout the 50 minutes of exercise test T, during the period of fat supplementation of horses in group F, with respect to the baseline exercise test. The trend was apparent in only 3 of the 4 horses, although the difference was significant in the latter stages of exercise. The results suggest an increase in the utilisation of fat during the exercise tests following fat supplementation. RER was also significantly higher during the washout test, i.e. once the horses in group F had been reverted to the control diet. The increase in fat oxidation may have been due to either an increase in plasma FFA or triglyceride utilisation or an increase in the oxidation of endogenous muscle triglyceride. In agreement with previous studies in rats, using much higher levels of fat supplementation (Miller *et al* 1984; Simi *et al* 1991), a significant increase in muscle CS activity and a trend towards an increase in muscle β -HAD activity was observed in response to fat supplementation in Chapter 6. The suggested increase in oxidative capacity may have contributed to the observed increase in fat utilisation during exercise. A significant increase in muscle triglyceride concentration following fat supplementation has been reported, again in rats (Abumrad *et al* 1978; Conlee *et al* 1990). A similar increase in the concentration of muscle triglyceride was not apparent in this study, as was discussed in the previous chapter. However, a significant increase in the plasma post PP T. Lip activity measured at rest, which may have resulted from an increase in muscle heparin releasable LPL, was reported during the period of fat supplementation (Chapter 6). An increase in the activity of the latter would enhance the capacity for uptake of plasma triglyceride derived FFA by muscle. An increase in plasma triglyceride utilisation may therefore have contributed to the observed decrease in RER.

Examination of the individual horse data revealed that after completion of 20 minutes of exercise the horses in group F exhibited a trend towards an increased plasma FFA concentration during tests F5 and F10, which reached significance after 40 minutes of exercise during exercise test F5. The increased plasma FFA concentration may have

reflected either increased FFA mobilisation or reduced extraction by exercising muscle; since plasma FFA concentration represents the net difference between FFA mobilisation and oxidation or re-esterification. An increase in the exercise induced elevation of FFA concentration has been previously described in human subjects fed a high fat, low carbohydrate diet (Galbo *et al* 1979; Jansson *et al* 1982; Jansson and Kaijser 1982; Lambert *et al* 1994) and in horses fed a fat supplemented diet (Essen-Gustavsson *et al* 1991). Jansson *et al* (1982) suggested that an increased rate of lipolysis during exercise, as a result of feeding a carbohydrate poor or fat rich diet, may occur due to an accentuation of the sympatho-adrenal response to exercise, as indicated by a greater exercise induced increase in nor-adrenaline. A similar enhancement in the exercise induced increase in adrenaline has also been reported in response to a high fat diet (Galbo *et al* 1979; Jansson *et al* 1982).

The concentration of insulin, a potent anti-lipolytic hormone, decreases during exercise (Galbo *et al* 1977). Following feeding of a fat supplemented diet insulin has been reported to be lower during exercise both in man (Galbo *et al* 1979; Jansson *et al* 1982) and in the horse (Pagan *et al* 1994) when compared to a high carbohydrate fed control group. Increased FFA mobilisation may not necessarily reflect an increase in the rate of lipolysis but may occur simply as an increase in FFA release as a result of either a reduction in FFA recycling or a reduction in the retention of FFA within adipocytes.

RER was consistently lower during the canter phase of exercise test SC in 2 of the 4 horses, following 5 weeks of fat supplementation. Following 10 weeks of fat supplementation RER was consistently lower, during the canter phase of exercise, in 3 of the 4 horses. In all these instances the lower RER was associated with an enhanced exercise induced increase in plasma FFA concentration. Horse Li failed to show a similar trend in RER and similarly the increase in FFA concentration during exercise tests F5 and F10 was lower than that during the baseline test. There were no significant differences in RER measured either within or between dietary groups with respect to the moderate/fast exercise tests. The recovery period was associated with a sharp rise in plasma FFA during exercise tests FC in both dietary

groups. This may be the result of a continued high rate of lipolysis or may simply reflect release of FFA's retained within adipocytes during the canter phase of the exercise. Romijn *et al* (1993) reported that the rate of lipolysis immediately following high intensity exercise was rapidly decreased in man, but similarly was associated with an increased rate of FFA release into plasma. As was discussed by these authors, the decreased appearance of FFA in plasma during high intensity exercise may have resulted from the entrapment of FFA within adipocytes. The FFA then being released during the low intensity recovery period. The entrapment of FFA may be the result of a reduction in adipose tissue blood flow and hence albumin available for FFA transport, mediated through alpha-adrenergic activity or a high FFA-albumin ratio in adipose tissue (Bulow *et al* 1985; Romijn *et al* 1993)

Horse Li failed to show the changes in plasma FFA concentration and associated difference in RER as exhibited by the other horses in group F as a result of fat supplementation. During the baseline tests horse Li exhibited the lowest RER of all horses in group F, over all exercise intensities. This horse may have an inherent high capacity for fat oxidation relative to the other horses in group F and was therefore unaffected by the introduction of fat into the diet.

RER increased with increasing exercise intensity and the variation in RER both between dietary groups and between exercise sessions was low during the moderate/high intensity exercise tests. This suggests that the increase in carbohydrate utilisation in response to the increase in exercise intensity, as indicated by a higher RER, was uniform or obligatory between horses. The failure to observe any change in RER as a result of fat supplementation may reflect the high dependence on carbohydrate metabolism at this intensity of exercise.

In Summary, fat supplementation was associated with a reduction in RER during low and moderate intensity exercise which was accompanied by an increase in the exercise induced elevation of plasma FFA. An increased delivery of plasma FFA to the working muscle may have resulted in an increased rate of FFA oxidation, and hence a reduction in RER. It was suggested in Chapter 5 that the thoroughbred horse has the capacity to increase the

contribution of fat to energy production, during prolonged low intensity exercise, in the presence of an elevation in plasma FFA concentration. Furthermore it was suggested that the oxidative capacity of muscle may be increased as a result of fat supplementation (Chapter 6). Additionally an increased activity of muscle LPL, reflected by an increase in the plasma post PP T. Lip activity may have increased the capacity to utilise plasma triglycerides. The failure to observe any significant change in RER during the moderate/high intensity exercise tests suggests that carbohydrate was the predominant fuel source and that further utilisation of fat was inhibited.

Chapter 8

General Discussion

The thoroughbred horse has been selectively bred for speed of running and has the capacity for a high rate of carbohydrate utilisation. This can be inferred from the reported proportion of type II fibres in biopsy samples of the middle gluteal muscle, in comparison to other breeds as reviewed by Snow (1983). The thoroughbred horse also exhibits increased muscle glycogen storage (Snow *et al* 1985) and higher activities of phosphofructokinase (Cutmore *et al* 1985), phosphorylase, CS (Sewell *et al* 1994) and LDH (Roneus and Lindholm 1991), in comparison to Man (Simoneay *et al* 1983; Harris *et al* 1974; Essen *et al* 1975). Conversely, the thoroughbred horse has lower activities of heparin releasable LPL, as described in Chapters 4 and 6, and β -HAD (Lindholm *et al* 1983; Simoneay *et al* 1983; Chan 1984) which are key enzymes of fat metabolism. These differences may simply reflect the higher proportion of type II fibres in mixed muscle biopsy samples but suggest that the thoroughbred has developed as a carbohydrate dependant athlete.

Horses maintained on a commercial low fat diet exhibited a low concentration of plasma long chain FFA, as measured by HPLC, during the major part of a single 24 hour period. Furthermore, the plasma FFA concentration was unaffected by feed intake. The early hours of the morning (2-9am) prior to feeding were characterised as the period of greatest variability in plasma FFA concentration. A proportion of the horses studied showed a large increase in plasma FFA concentration localised at around 7am, as described in Chapter 3. Plasma FFA have been reported to exhibit a diurnal rhythm, which was related to food intake in human subjects (Hollister and Wright 1956). The diurnal rhythm in FFA concentration in Man, however, is likely to have resulted from changes in circulating glucose and insulin concentration, the plasma FFA concentration decreasing sharply after food intake and rising during the post-absorptive period. The horse, a semi-ruminant herbivore, does not undergo a true post-absorptive period as it has the ability to ferment structural carbohydrates producing VFA which are metabolised (Bergman 1990). As a result of the ability to metabolise VFA the consumption of forages, which are rich in fibre, ensures that glucose homeostasis is not compromised in between feeding periods. In order to account for the reported circadian

rhythm in plasma FFA described in Chapter 3 all subsequent exercise studies were carried out during the period of least variability in plasma long chain FFA concentration.

A comparison of the lipolytic and anticoagulative effects of HEP and PP indicated that a higher dose (x3) of PP was required to elicit a comparable lipolytic effect to HEP in the horse, as in other species (Cururachi *et al* 1967; Scully *et al* 1983). Injection of both HEP and PP in isolation resulted in a significant increase in plasma T. Lip activity. Whilst co-administration of HEP or PP with a triglyceride emulsion produced a substantial increase in plasma FFA. The effect of PP injection on clotting function as measured by APTT was approximately 9 times less than that of HEP, in agreement with Cururachi *et al* (1967) and Scully *et al* (1983). Due to the risks of prolongation of clotting time associated with HEP administration (Smith 1990) PP was used in conjunction with a triglyceride emulsion to produce the pre-exercise elevation in plasma FFA described in Chapter 5.

Measurements of RER during low, moderate and moderate/high intensity exercise indicated that the use of fat as a fuel source was dependant on both the intensity and duration of exercise, as described in Chapters 5 and 7. The contribution of carbohydrate to energy production increased with increasing exercise intensity as indicated by an increase in RER. An increase in the contribution of fat to energy production occurred with duration of low intensity exercise, as indicated by a decrease in RER. During low intensity exercise RER, however, increased during the first 5-10 minutes of exercise, indicating an increased reliance on carbohydrate metabolism during this period. As reported in Chapter 5, this initial increase in RER occurred even in the presence of a pre-exercise elevation of plasma FFA. This suggests that during the early period of exercise plasma FFA concentration is not limiting to its oxidation. An initial rise in RER was also apparent following acceleration to canter during the moderate and moderate/high intensity exercise tests in Chapter 7. A plateau or steady state in RER was achieved only after 4 or 3 minutes of canter during the moderate or moderate/high intensity exercise tests respectively. As was discussed in Chapter 5, FFA utilisation may be inhibited at the onset of exercise as a result of an increase in the rate of

glycolysis and associated decrease in the pH of the cell or through direct inhibition by glycolytic intermediates.

An increase in the ratio of acetyl carnitine : free carnitine may reduce the availability of the latter for transport of long chain FFA across the mitochondrial membrane. An increase in the concentration of acetyl carnitine in skeletal muscle in the rat has been reported to occur in response to high intensity exercise of short duration (Carter *et al* 1981). Furthermore a simultaneous and stoichiometric decrease in the concentration of free carnitine has been reported in equine muscle (Foster and Harris 1987a; Foster and Harris 1987b). Foster (1989) demonstrated that the major accumulation of acetyl carnitine can occur after only 2 minutes of moderate intensity exercise, in the absence of a significant accumulation of lactate in plasma. Following the pre-exercise elevation of plasma FFA concentration (Chapter 5), the increase in RER observed during the first 5-10 minutes of exercise was accompanied by a sharp decrease in the plasma FFA concentration. An increase in the extraction of plasma FFA by skeletal muscle as a result of an increase in muscle blood flow at the onset of exercise, together with a delay in adipose tissue lipolysis may explain the rapid fall in plasma FFA concentration. The corresponding increase in RER, however, suggests that the uptake of FFA into muscle occurred without a corresponding increase in FFA utilisation. This suggests that FFA may have been retained in some form in the cell cytoplasm, possible in the form of fatty acyl carnitine.

In the presence of a pre-exercise elevation of plasma FFA concentration the thoroughbred horse appeared to have the capacity to increase the contribution of fat to energy production during prolonged low intensity exercise, as was discussed in Chapter 5. The increase in fat utilisation, however, was not uniform and varied between horses; it was suggested that this may be related to the inherent ability of an individual horse to utilise fat. The variability in the utilisation of fat at low exercise intensities may be due to individual variability in: the rate of lipolysis and release of FFA, the transport capacity into muscle and then into the mitochondrion and finally the FFA oxidising capacity of the muscle cells; all of which may

be influenced by genetic factors and state of training. The variability in RER measured between horses for a standard exercise was high at low exercise intensity decreasing as the intensity of exercise increased. Individual horses were exercised at the same absolute intensity in the studies of Chapters 5 and 7. Differences in individual VO_{2max} would therefore influence the relative work intensity of exercise performed, which may effect substrate utilisation and account for some of the variability in RER observed between horses .

Brooks and Mercier (1994) described the cross-over concept as a method of explaining the pattern of substrate utilisation in an individual at any point in time during exercise. These authors suggest that substrate utilisation at a particular time is function of exercise intensity responses (which increase carbohydrate utilisation) and endurance-training responses (which promote fat oxidation). Furthermore, they propose that a theoretical point "the crossover point" exists during exercise which represents the exercise intensity at which carbohydrate utilisation predominates over fat utilisation. At exercise intensities above the cross-over point a further increment in carbohydrate oxidation and decrement in fat oxidation occurs. The intensity of exercise at which the crossover point occurs may vary between individuals.

An increase in RER above that observed during prolonged low intensity exercise was measured during moderate and moderate/high intensity exercise in the thoroughbred horse. Additionally, the increase in exercise intensity was characterised by a lower inter-horse variability in RER and thus substrate utilisation. This suggests that the increase in carbohydrate utilisation with increasing exercise intensity, as indicated by RER, is more uniform or obligatory and is less influenced by the factors discussed with respect to low intensity exercise. The moderate and moderate/high intensity exercise were approximately equivalent to 34 and 56% VO_{2max} respectively. In Man the literature suggests that at exercise intensities of $\leq 50\%$ VO_{2max} fat oxidation predominates (Edwards 1934; Gollnick and Saltin 1988; Bulow 1988). However, during high intensity exercise $\geq 70\%$ VO_{2max} carbohydrate is the predominant fuel source, with the crossover point occurring somewhere between the two exercise intensities. During moderate intensity exercise without fat

supplementation RER was reported to be generally around 0.95, suggesting a large contribution of carbohydrate to energy production. It was estimated that the moderate intensity exercise performed in Chapter 7 was equivalent to a relative exercise intensity of only 34% $\text{VO}_{2\text{max}}$. It is possible therefore that the crossover point, at which carbohydrate metabolism predominates during exercise, may occur at a lower relative exercise intensity in the thoroughbred horse in comparison to Man. Although, the effect of a prolonged period of exercise at the same relative intensity on substrate selection would require further investigation.

In recent years the use of supplemental fat in equine diets has attracted much interest. This is highlighted by the emergence of a number of commercially produced 'high fat' diets. The impetus for the production of these diets has been two-fold. Firstly fat represents a very energy dense foodstuff containing over twice the amount of energy of either carbohydrate or protein. The increasing popularity of equine events such as endurance racing and 3-day eventing, which require a high level of fitness and training, have made horse owners and trainers more aware of the need for high energy diets. Traditional performance horse feed has been high in carbohydrate with a minimal content of fat. Previous association of carbohydrate based diets with musculo-skeletal problems such as rhabdomyolysis, osteochondrosis and developmental orthopaedic disease in horses, however, have encouraged the use of fat in equine diets. Additionally, feed refusals and associated weight loss often pose a problem in horses undergoing hard training. The inclusion of fat in the diet enables the energy content of the horses feed to be increased whilst the feed bulk may be reduced. This often ensures an adequate energy intake and thus maintenance of weight.

Secondly, there have been many reports of shifts in substrate utilisation during exercise of varying intensities as the result of fat supplementation in the rat, the horse and in Man (see Chapters 6 and 7). Furthermore increases in exercise performance have also been described. Little information, however, is available as to the proposed mechanism for the shift in substrate utilisation as a result of fat supplementation in the horse. The aim of the study

described in Chapter 6 and 7 was to report any changes in resting blood and muscle parameters, occurring in response to feeding a fat supplemented diet, which may partially explain corresponding changes in exercise metabolism.

In agreement with the studies in rats of Miller *et al* (1984) and Simi *et al* (1991) fat supplementation was associated with an increase in muscle CS and β -HAD activity. Statistical significance was, however, only achieved with respect to the increase in muscle CS activity. Nevertheless, the results suggests that an increase in the oxidative capacity of muscle may have occurred as the result of fat supplementation. Although an attempt was made to correct for differences in the fibre composition of serial biopsy samples confirmation of the increase in oxidative capacity could only be achieved using measurements of CS and β -HAD in single muscle fibres. Unlike the studies of Abumrad *et al* (1978) and Conlee *et al* (1990) no significant increase in muscle triglyceride concentration was observed as the result of fat supplementation. The level of fat fed in the former studies, expressed as a percentage of the total energy content of the diet, was however much greater (78%) than that present in the diets used in Chapter 6 and 7. Generally in previous equine studies the amount of fat contained in fat supplemented diets is lower than that used in studies in other species. This is partially due to the physical problems of feed manufacture but also because of the need to maintain a minimum roughage content of the diet for normal gut function in the equine.

Fat supplementation was not associated with any significant change in resting muscle glycogen concentration in agreement with Pagan *et al* (1987); Greiw *et al* (1989) and Hodgson *et al* (1994) and in contrast to the studies of Meyers *et al* (1989) Olham *et al* (1990); Harkins *et al* (1992) and Scott *et al* (1992). The elevation in muscle glycogen concentration, observed in the latter studies, may have been influenced by the relatively low concentration of muscle glycogen reported both before and after the period of fat supplementation. Furthermore, differences in the gross energy content of the control and fat supplemented diets together with the training and nutritional status of the horses prior to fat

supplementation cannot be overlooked as a precipitant of the observed increase in resting muscle glycogen concentration.

The consumption of a fat supplemented diet over a period of 10 weeks resulted in a mean 50% increase in post PP T. Lip activity, which was associated with a significant reduction in plasma triglyceride concentration. This suggests that the plasma triglyceride clearing capacity of the horse was increased in response to fat supplementation. Similarly Harris and Felts (1973) reported an increase in the rate of removal of labelled triglyceride from the plasma of rats fed a high fat diet. The post HEP plasma LPL activity is also elevated in suckling foals consuming mares milk which would be expected to have an elevated fat content (Watson *et al* 1993a). As was discussed in Chapter 6 the increase in plasma post PP T. Lip activity may reflect an increase in muscle LPL activity. Muscle HEP releasable LPL has been reported to increase in Man and in the rats in response to a high fat diet (DeLorme and Harris 1975; Jacobs *et al* 1982). The reduction in plasma triglyceride concentration, which probably reflects an increase in the triglyceride clearing capacity of this plasma, has been previously reported in the horse in response to fat supplementation (Duren *et al* 1987). The period of fat supplementation was also characterised by an increase in plasma cholesterol concentration possibly due to increased production of acetyl CoA as a result of an increased flux through β -oxidation (Hill *et al* 1960). The total cholesterol concentration, however, remained within the normal range at all times.

The control diet, as described in Chapter 6, provided a typical 500 kg horse with approximately 110g of fat, whereas, the fat supplemented diet provided 500 g of fat to a horse of similar weight. Energetically the fat content of the fat supplemented diet was approximately equivalent to about 20% of the total energy content (DE) of the diet. The absolute content of the fat supplemented diet was relatively low in comparison to that used in comparable studies in Man. However, fat supplementation in this study represented a 5 fold increase in the relative fat content of the diet. In comparison, studies in Man which have used diets containing about 78% of the total energy content as fat, may have only represented

a 2 fold increase in the fat content. A typical human athlete may consume approximately 38% of the energy intake as fat under normal conditions (Wilmore and Costill 1994). The relative increase in the fat content of the diet may therefore be an important factor in the adaptational response to fat supplementation in the horse.

An increase in the utilisation of fat during prolonged low intensity exercise was apparent as the result of fat supplementation. RER was significantly lower during the latter stages of exercise, following 5 and 10 weeks of fat supplementation, when compared to both the baseline and washout periods respectively. On examination of the individual horse data RER was consistently lower than both the baseline and washout tests in 3 of the 4 horses studied. The lower RER in these 3 horses was associated with an increased exercise induced elevation in plasma FFA concentration, as has been previously reported in Man (Galbo *et al* 1979; Jansson *et al* 1982; Lambert *et al* 1994). As was discussed in Chapter 7 the elevated plasma FFA concentration may have arisen due to increased FFA mobilisation, either as the result of enhanced lipolysis or possibly as a result of increased FFA release.

A similar increase in fat utilisation was suggested to occur, as a result of fat supplementation, during moderate intensity exercise. This was also associated with an increased plasma FFA concentration during the latter stages of exercise. Examination of the individual horse data, however, indicated that the effect was not as clear as that observed during low intensity exercise. The difference in RER during the 4 phases of the feeding study were small. In contrast to that observed during low intensity exercise, the RER was not consistently lower during the period of fat supplementation. The effect of a more prolonged exercise period of similar intensity is unknown and may increase the utilisation of fat further, which would be particularly relevant to horses competing in endurance type events. No significant difference was observed in RER between weeks during the moderate/high intensity exercise tests and carbohydrate represented the major energy source.

The proposed increase in fat utilisation during low and moderate intensity exercise may have occurred as the result of an increased ability to utilise plasma triglyceride, as the result of an increase in the activity of muscle LPL, together with an increase in the availability of plasma FFA. The results in Chapter 5 suggest that the horse has the capacity to increase the contribution of fat to energy production, during prolonged low intensity exercise, in the situation where the availability of plasma FFA is increased. Additionally the trend towards an increase in muscle β -HAD activity and significant increase in CS activity, as a result of fat supplementation, suggests an increase in the muscle oxidative capacity which may have contributed to the observed increase in utilisation of fat during exercise in Chapter 7. Many of the adaptations described in the response to fat supplementation could be mediated through differences in the hormonal response to feeding. The circulating level of insulin, a potent anti-lipolytic hormone, has been reported to be lower both at rest and during exercise following a prolonged period of fat supplementation in the horse (Pagan *et al* 1994). Similarly a reduced concentration of circulating plasma insulin has been reported in response to a high fat diet in Man (Galbo *et al* 1979; Jansson *et al* 1982). Insulin is implicated in the regulation of adipose tissue lipolysis and FFA mobilisation both at rest and during exercise (Hales *et al* 1978; Galbo *et al* 1979 & 1983). Furthermore, insulin may also be involved in the reciprocal regulation of muscle and adipose tissue LPL activity (Jacobs *et al* 1982; Kiens *et al* 1989). The lower circulating level of insulin observed following fat supplementation may merely reflect a decrease in the soluble carbohydrate content of the diet rather than the fat content per se. In support of this notion Pagan *et al* (1987) reported a lower RER during moderate intensity exercise in response to isocaloric diets containing elevated levels of either fat or protein, compared to a high carbohydrate diet.

The time course of changes which occurred in response to the fat supplemented diet were as follows: A significant increase in plasma cholesterol concentration occurred after only 1 week of fat supplementation. The increase in post PP T.Lip activity was apparent after 3 weeks of fat supplementation and was associated with a significant reduction in plasma triglyceride concentration after 4 weeks of fat supplementation. All the above changes were

reversed following 5 weeks withdrawal of the fat supplemented diet. Increased fat utilisation during low intensity prolonged exercise may have beneficial effects on endurance capacity, although this question was not addressed in the present series of studies. It was observed that not all horses responded in the same manner to either an acute increase in the availability of plasma FFA or to chronic fat supplementation. However, to the owners and trainers of horses it is of little importance whether the equine population as a whole respond positively to fat supplementation. An adaptational response in a significant proportion of horses, which may increase endurance capacity, is more relevant.

Problems encountered and future strategies

The large volume of samples produced during the studies in Chapters 4, 5, 6 and 7 necessitated the use of a rapid automated method for the analysis of plasma FFA. The plasma concentration of FFA_t calculated by the addition of FFA_i concentration, as measured by HPLC, was relatively lower than resting concentrations reported using the automated colourimetric method. They were, however, in agreement with those measured by Rose (1982) using gas chromatography. The FFA_t concentration measured by HPLC showed little variation during the most part of the day and night cycle. The difference observed in resting plasma FFA concentration may simply reflect methodological differences. Alternately the higher FFA concentration observed in Chapters 4, 5, 6 and 7 may be the result of FFA release as part of the stress response. The horses used in the study in Chapter 3 were catheterised an hour before the 1st sample was withdrawn and remained in their stables throughout the 24 hour sampling period. Blood sampling during the preceding chapters was either carried out by venepuncture or followed catheterisation in the treadmill room. This may have been associated with stress as an anticipatory response to the forthcoming procedures, exercise etc. Furthermore, the HPLC method described in Chapter 2 quantified FFA with a carbon chain length greater than and including C:14. The higher FFA concentrations measured using the colourimetric kit may have been as a result of simultaneous measurement of FFA with a carbon chain length < C:14. This is especially relevant to the horse which produces short chain VFA mainly acetic, butyric and propionic acids as a result of microbial fermentation in

the hind gut (Bergman 1990). At rest acetate represents a significant energy source in the horse (Pethick *et al* 1993). The role of acetate and other VFA as a fuel source during exercise is, however, unknown and requires further investigation.

In order to ensure that the fat supplemented diet used in Chapters 6 and 7 was isocaloric to the control diet it was necessary to reduce the bulk of ingredients with a high carbohydrate content and to increase those with a high fibre content. The fibre content of the fat supplemented diet was therefore higher than that of the control diet. The effect of this difference in fibre composition of the two diets is unknown and requires further investigation. Furthermore, the effect of the difference in the carbohydrate content on the hormonal response to feeding and subsequent exercise is an area to be investigated. Lower plasma insulin concentration observed as a result of fat supplementation (Pagan *et al* 1994) may have arisen as the result of a reduction in the soluble carbohydrate content of the diet.

Ideally all of the exercise tests carried out in Chapter 7 would have been performed at the same time of day or after a standardised period of time following feeding. It was, however, impractical to carry out the exercise tests in this manner due to the large number of horses used in this study and the volume of exercise tests performed within a single week. In order to minimise the influence of feeding status, the horses were not exercised for at least 2 hour following the consumption of the pelleted portion of the diet and had almost constant access to forages. Furthermore, the time at which the exercise tests were carried out was standardised within horses but was also randomised between dietary groups, as described in Chapter 7.

In summary the areas of interest for future investigation include: the role of VFA during exercise and the effect of fibre content of the diet. Further investigation of the hormonal response to high carbohydrate versus fat supplemented diets and the effect on subsequent exercise metabolism. Confirmation of the observed increase in muscle LPL activity in response to fat supplementation through direct measurements of muscle samples.

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Appendix 1

Muscle Section Staining Technique

Reagents

- 1 0.2 M sodium acetate (27.22 g sodium acetate trihydrate in 1000 ml distilled de-ionised water).
- 2 50% acetic acid (50 ml glacial acetic acid in 50 ml distilled de-ionised water).
- 3 Glycine / calcium chloride (3.00 g glycine + 2.94 g calcium chloride dihydrate in 1000 ml distilled de-ionised water).
- 4 0.1 M potassium hydroxide (5.60 g in 1000 ml distilled de-ionised water).
- 5 2% calcium chloride (10.00 g calcium chloride dihydrate in 500 ml distilled de-ionised water).
- 6 1% cobalt chloride (5.00 g cobalt chloride in 500 ml distilled de-ionised water).
- 7 1% ammonium polysulphide (1 ml 10% ammonium polysulphide in 10 ml distilled de-ionised water).

Preparation **NB. Prepare the reagents in this order**

- 8 Calibrate pH meter between 7 and 10 using a two point calibration.
- 9 Add approximately 45 ml of glycine / calcium chloride solution to each of two coplin jars. Incubate @ 20°C. One jar adjust to pH 9.8 and the other to pH 9.6 using 0.1m potassium hydroxide.
- 10 Add 75 mg of ATP disodium salt (1.5 mg / ml, 3 mmol). to the coplin jar containing about 45 ml glycine / calcium chloride buffer at pH 9.8 (the ATP will bring the pH back down to around 9.6) then correct the pH to 9.6 again.
- 11 Calibrate pH meter between 4 and 7 using a two point calibration.
- 12 Incubate, in a coplin jar, approximately 45 ml sodium acetate at 20 °C. Once it is at the right temperature slowly add the acetic acid until pH 4.500 (Great accuracy is

needed). As this reagent is by far the most important, the pH must be achieved as close to the time of staining as possible to decrease the chance of inaccuracies.

- 13 Incubate 2 other coplin jars, one containing 2% calcium chloride the other 1% cobalt chloride at 20 °C. Leave until solutions are at the correct temperature.

Method

- 14 Incubate a slide, which has four muscle sections mounted on it, in the sodium acetate/acetic acid buffer for 5 mins. (The timing is critical).
- 15 Rinse the slide at least four times in separate water containers with distilled water. Change the water after each rinse. (A couple of seconds for each rinse)
- 16 Rinse the slide in the glycine / calcium chloride solution (pH 9.6) for 30 seconds. NB. Do not rinse between the glycine buffers.
- 17 Incubate the slide in the ATP / glycine / calcium chloride solution (pH 9.6) for 30 minutes.
- 18 Rinse the slide four times.
- 19 Incubate the slide in 2% calcium chloride solution for 90 seconds.
- 20 Rinse the slide four times.
- 21 Incubate the slide in 1% cobalt chloride for 3 minutes.
- 22 Rinse the slide four times.
- 23 Flood the slide with ammonium polysulphide for 2 minutes. (Carry out in fume cupboard).
- 24 Rinse the slide with distilled water.
- 25 Mount a coverslip on the slide using UV free aqueous mountant and seal the edges of coverslip using clear nail varnish.

Results

- 27 If the stain has worked correctly type I fibres will be black, type IIA fibres white / very light brown and type IIB fibres will be dark brown.

Chemicals

ATP disodium salt: From equine muscle.	Order Sigma: A 5394
Calcium chloride 2-hydrate AnalR	Order BDH: Prod 10070
Sodium Acetate Trihydrate	Order Sigma: S 8625
Glycine	Order Sigma G 7126

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