Aspects of Growth and Development of the Pasture-fed Thoroughbred Foal in New Zealand

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Abstract

In each of 2 years, pregnant mares (n = 10 and n = 23) kept at pasture were divided into copper supplemented or unsupplemented groups, and injected with calcium copper edetate or saline. Their foals were examined, weighed, measured and clinically scored at 2 weekly intervals from birth to 160 days of age to assess growth and development, and evidence of developmental orthopaedic disease (DOD). Foal liver biopsies were harvested by Tru-cut biopsy needle at different ages for determination of copper concentration. At 160 days of age, articular surfaces were examined for cartilage irregularities, which were sampled for histology and histochemistry. Cartilage samples were harvested from irregularities and defined sites for histology. Distal third metacarpal and metatarsal bones (Mc3 and Mt3) were sawn frontally, radiographed and processed for histology.

The parenteral copper supplementation had no effect on mare or foal liver copper concentration, and was not associated with reduced evidence of DOD in foals. The prevalence and severity of DOD lesions was very low. Two different patterns of decline in foal liver copper concentration were observed. Enlargements of the distal Mc3 and Mt3 physeal region were present in all foals, but were not associated with pain, lameness, or abnormalities in the metaphyseal growth plate.

In vivo techniques to assess body composition could be used to predict chemical body composition, particularly ultrasonographic rump fat thickness measurements. Fillies were significantly fatter than colts at 160 days of age, despite no differences in mean birth weight and weight gain. The only growth parameters associated with the prevalence of DOD lesions was rapid growth rate between 5 and 6 months of age, which was associated with more lesions in the tibiotarsal joint.

The New Zealand Thoroughbred industry should weigh and condition score foals at monthly intervals, keeping careful records. Foals can be successfully

raised at pasture, with good growth, and a low incidence of DOD lesions at 160 days of age, without being excessively fat. Copper injections should not be given to horses, but oral supplementation with copper should be considered for pregnant mares in late gestation.

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Chapter 1

Introduction, literature review and objectives

Introduction

The basis of any animal industry is the successful breeding of suitable animals, to produce and raise young stock in sufficient numbers, and to use and sell at a cost-effective price. In the New Zealand Thoroughbred industry the emphasis for production has been on an extensive rather than intensive basis, with breeding and growing young animals at pasture. Grass is a relatively inexpensive feed source in New Zealand, and the temperate climate allows grass growth throughout the year, and animals can be kept outdoors through every season.

However, there are few data on the basic aspects of growth in the horse, which is surprising in an industry that concentrates on growing quality animals on an individual basis. The size and shape of an animal at the time of sale affects the potential price purchasers will pay; those perceived as being small, poorly developed, or likely to have defects which will impede function, are not favoured.

This chapter summarises the scientific literature available on aspects of growth and development that are investigated in this thesis. Attention is focused on assessment of growth and composition, aberrations of skeletal growth in the young horse known collectively as 'developmental orthopaedic disease' and their aetiopathogenesis, and the influence of copper on skeletal growth, with particular reference to the horse. The topic of growth and development is vast, as it is a fundamental feature of every living organism; hence the review is limited to aspects that relate to the thesis objectives (page 104).

New information on growth and development in the young horse raised at pasture would reduce the existing deficits in knowledge, and could improve the equine industry's capabilities to produce quality stock of higher value. This chapter thus deals with the pertinent literature, and outlines hypotheses and aims of the experimental work to be determined in later chapters.

Literature Review

1.1 The horse

The early horses (Equus caballus) were the last of the common livestock animals to be domesticated, at around 5000 BC. Economic progress has probably depended more on the horse than any other species (Clutton-Brock, 1989). Horses are considered genetically less variable than other livestock species such as sheep, cattle and pigs, as there has been little selection for increased milk or meat production. As fast-moving ungulates, the potential for speed and strength has been exploited and developed by man. Horses were selectively bred to produce either animals that could run faster or heavier types that could pull loads for longer.

The first domestic horses were probably the size of large ponies, of medium build, with upstanding manes (Bökönyi, 1983). In general, mammals that developed in the cold northern latitudes tended to be large and heavy bodied with relatively short legs, while mammals in hotter climates had longer legs and finer limb proportions (Clutton-Brock, 1989). There is wide variation in size and conformation of modern domestic horses, from small stocky ponies from North Europe such as the Exmoor pony, heavy horses from North and Central Europe such as the Percheron, and slender-limbed horses from the south such as the Arab. However, it is now generally accepted that all domestic horses descended from the same progenitor (Willoughby, 1981). The breeds and types of horses that are known today developed through artificial selection by man, and natural selection for adaptation to local climatic and environmental conditions (Clutton-Brock, 1989).

1.1.1 The Thoroughbred

The history of the Thoroughbred horse began in England. Development of the breed is in part due to the Tudor and Stuart kings in the 17th and 18th centuries, who were keenly associated with horse racing. They encouraged selective

breeding to improve the local running horses by importation of Eastern horses (Willett, 1975). The local horses were mares that had been crossed with horses brought to England by the Romans and French Normans in the 11th century (Evans, 2000). In the late 1600's significant numbers of horses with Arabian, Turk and Barb ancestry were imported to try to improve the speed of the mares. Three predominant male Thoroughbred lineages are recognised from the mid-1800's, namely the Darley Arabian, Byerley Turk and the Goldolphin Arabian. The lineage of all Thoroughbreds around the world can be traced to these 3 sires. In the modern population 95% of paternal linkages are attributable to the Darley Arabian Line (Cunningham et al. 2001). Pedigree analyses estimate that 30 founder mares contribute to 94% of modern maternal lineages (Cunningham et al. 2001). However, variation in mitochondrial DNA suggests the number of founding mares may be low as 12 (Hill et al. 2002).

There are more than 300 000 Thoroughbred horses worldwide (Cunningham et al. 2001). The Thoroughbred population is essentially closed, and concerns have been expressed about loss of genetic variation, and an increased heritability of genetic disease (Holden, 1991). Wastage in the Thoroughbred industry is considered to be high, with overall wastage figures as high as 72.8% (Jeffcott et al. 1982). However, few studies have carefully evaluated wastage in young horses, between birth and entering race training, where the level of wastage may be high. A survey of Canadian Thoroughbreds revealed that up to 27% of foals were reported to have a health problem between 2 weeks and 1 year of age, with 6% of foals dying in this period (Morley and Townsend, 1996). Bourke (1995) suggested that only 30% of live Thoroughbred foals bred for racing could be expected to start in a race. A survey of Thoroughbreds in the United Kingdom estimated that 10% of foals born were not named (a requirement to enter training) and 14% of named animals were not trained (Jeffcott et al. 1982).

1.1.1.1 The Thoroughbred in New Zealand

During the 19th century horseracing was the most popular organised pastime in New Zealand, and by 1875 it was estimated that one in four New Zealanders attended a race meeting regularly (Grant, 2001). Today racing struggles in comparative terms, but it still employs more than 30 000 people (Grant, 2001).

Around 5000 foals are registered each year in the New Zealand Thoroughbred Studbook. Around 7700 to 8000 mares are bred from September 1st, so foaling generally starts from mid-August onwards. Commonly, foals are kept with their dams at pasture until weaning at 4 to 6 months of age.

The major sales for yearling horses are in January and February, and intensive preparation of yearlings begins about 4 months earlier. Preparation includes boxing (stabling), grain feeding and some form of controlled exercise, such as using a horse walker. Most yearlings presented for sale in New Zealand are 17 months of age or less, in comparison to yearlings at the premiere Australian sales being some 3 months older, and therefore larger.

The cost of breeding a foal and raising it until it is sold as a yearling has been estimated at around $51 000, including a $20 000 service fee (Archer, 2001). The New Zealand yearling sales aggregate was $55 million in 2001/2002, compared to $72 million in 1999/2000, with the estimated value of horse exports around $115 million in 2001/2002 (source: New Zealand Thoroughbred Racing).

In order to achieve good prices at the yearling sales, there is a drive for vendors to present 'well-grown', 'mature' individuals that appear to be ready to enter race training in the next spring and race as two-year-olds. Several of the larger studs weigh their young horses at frequent intervals during preparation for yearling sales to monitor weight gains, and most use visual assessment to monitor the condition of the horse, and adjust each horse's ration of grain feed appropriately.

1.2 Growth and development

'Growth and development' is a term whose sense is appreciated, but the exact meaning is often not understood (Batt, 1980). Hammond (1971) defined growth as the increase in weight until mature size is reached, and development as the changes in conformation and shape. Growth involves the differentiation of organs, changes of size and body proportions, and ageing changes in function (Hammond, 1971).

Growth is a complicated process of cell replication, tissue differentiation, matrix formation, cell death and many other interrelated mechanisms (Gluckman, 1986). It is not a uniform process, but rather a series of adaptations to the current and future needs of the animal (Lawrence and Fowler, 2002). Growth occurs by cell multiplication (hyperplasia), cell enlargement (hypertrophy) or by incorporation of extra-cellular material (Davies, 1989).

There are many definitions used for the term growth. Broadly, growth can be defined as any temporal change in any parameter that can be measured (Moss-Salentijn, 1982). Some authors confine the use of the term growth to describe increases in living substance, as opposed to accumulation of excess fat or water. Growth usually refers to an increase in weight or size of an individual (Warriss, 2000), and this definition will be used for the purposes of this thesis.

1.2.2 Describing growth and development

The dynamic changes occurring in the size, shape and proportion of an animal are complex (Lawrence and Fowler, 2002) and can be difficult to assess and interpret. Growth can be assessed as an increase in mass per unit time, or as changes in form or composition resulting from differential growth of the component parts of the body.

1.2.2.1 Development and maturity

While growth is an increase in size or mass, maturation or development is the attainment of a specialised and mature state. Maturity is often used to describe the attainment of adult weight or the asymptote to the sigmoidal growth curve, or in relation to the ability to reproduce. The process of maturation is based upon six considerations: the relationship of maturity to chronological time, the identification of indicators of maturity, the independence of different processes of maturity within the individual, the appreciation of uneven maturation, sexual dimorphism, and the relationship of maturity to size (Cameron, 1997).

Many consider the papers of Taylor (Taylor, 1965; Taylor and Fitzhugh, 1971; Taylor, 1980a; Taylor, 1980b; Taylor, 1980c; Taylor, 1985) to be definitive in mathematically describing growth and maturity. However, mature weight can be difficult to determine, especially for meat-producing animals, with few published reports on expected mature size for different breeds and sexes (Kirton and Morris, 1989). Humans reach their mature size at around 18 and 20 years of age for females and males respectively. However, adult weights tend to fluctuate, mainly in respect to changing fat content. The size of the adipose tissue mass is not under a strict set-point control (Jequier and Tappy, 1999), and under conditions of excess dietary energy intake fat mass will continue to increase throughout life.

Genetic selection for growth rate in production animals has tended to increase mature size (Lawrence and Fowler, 2002). The larger the mature size of an animal, the longer it tends to take to mature. For example, mice normally attain full mature size by 20 to 25 weeks of age, while cattle take 5 to 6 years to attain mature size. The degree of maturity of a growing individual is expressed as the ratio of the bodyweight to its mature weight. The time (in days) a species takes to reach any particular degree of maturity tends to be directly proportional to its mature weight raised to the power of 0.27 (Taylor, 1965).

Maturity can also be defined in terms of the body components bone and muscle, and when they first reach a steady state (Davies, 1989). A mature animal has a

higher muscle to bone ratio than during its development. The percentage of muscle decreases as animals enter the 'fattening' stage, where the proportion of fat in the body starts to increase. Meat producing animals are usually slaughtered near the end of their pubertal growth spurt, at one-third and two-thirds of mature body size (Berg and Butterfield, 1976), when the ratio of muscle to bone has increased, but fat is also starting to increase. The most appropriate age to slaughter an animal is often a compromise between attaining a good ratio of muscle to bone, without excessive fat that is required to be trimmed off. The concept of maturity can also be used to describe fat growth. For example, early maturing animals have more fat at a given weight than late maturing animals.

The major chemical components of the bodies of vertebrates are water, fat, protein and ash. Moulton (1923) first described the concept of chemical maturity, a state where the relative fatness of an animal does not influence the chemical composition of the fat-free body (protein, water and ash in body). This implies that the concentration of water, protein and ash in a body are fairly constant for a species. However, others have described wide variation in the chemical composition within and between species (Reid et al. 1968), although results included animals at different stages of maturity.

Growth and maturity of body tissues can be described by the allometric growth coefficient ($y= ax^b$ where y = size of the organ or part, a = a constant, x = size of the rest of the body, and b = the growth coefficient of the organ or part). For example, protein and water increase with a coefficient of around 1.0, bone < 0.7 and fat > 1.6. (i.e. if liveweight increases 100%, protein weight increases 100%) (Huxley, 1932; Berg and Butterfield, 1976).

Maturity in horses

All breeds of horses probably achieve their mature weight and height by 5 years of age (Lewis, 1995), although there are relatively few growth data for growing and mature animals. The relative proportion of mature weight of the Thoroughbred foal at birth and two years of age, compared other grazing species, is given in Table 1.1. Between-breed differences in rate of attainment

of mature weight are greater than the differences in rate of attainment of mature height (Frape, 1998). For instance, by 12 months of age the Shetland pony has attained 73% and 94% of mature weight and height respectively, while the Percheron horse at the same age has attained 59% and 89% of mature weight and height respectively (Hintz, 1980 cited by Frape, 1998).

Table 1.1 Relative proportion of mature weight at birth and 2 years of age in 4 species

	Thoroughbred Horses[1]	Friesian Cattle[2]	Corriedale Sheep[3]	Red Deer[4]
Birthweight	8 - 11%	7 - 9%	9.5 - 10.5%	9 - 10%
Weight at 2 years	87 - 92%	84 - 90%	95 - 100%	65 - 85%

Sources

1. Hintz et al. 1979; Lewis, 1995
2. Holmes et al. 1984
3. Pitchford, 1993
4. Asher and Adam, 1985; Moore et al. 1988

1.2.2.2 Sexual dimorphism

Sexual dimorphism is any morphological variation between sexually mature males and females. Sexual size dimorphism is common in animals, including sheep, cattle, deer and swine, and humans, with males being larger than females. The difference in size is largely the consequence of testicular steroid secretions (Ford and Klint, 1989). After neonatal castration of bulls and rams, growth rate is reduced relative to intact males, but continues at a faster rate than that of females of the respective species (Ford and Klint, 1989). In sexually dimorphic species the main factors influencing size are age, sex and genetic potential (Fennessy et al. 1981;Frampton and Nugent, 1992).

The horse shows relatively little sexual dimorphism in size, when determined by weight or height at the withers. Mature Thoroughbred mares and stallions weigh around 500 and 545 kg respectively, and are 160 and 162 cm in height respectively (Lewis, 1995). Pagan et al. (1996) weighed 172 Thoroughbred mares and 25 Thoroughbred stallions in Kentucky, with average weights of

570 kg and 580 kg respectively. Colts tend to be bigger than fillies at birth, and this difference increases with age (Hintz et al. 1979). However, compared to fillies of the same age, colts are not bigger as a percentage of their mature size, and they tend to reach their mature size more slowly than fillies (Hintz et al. 1979; Lewis 1995; Frape 1998). There are no reports on the size of castrated horses (geldings) compared to stallions and mares, although it is likely they are intermediate in size, as observed in most other mammalian species.

1.2.2.3 Efficiency of growth

When daily feed intake exceeds that required for maintenance (energy required to maintain an animal at a constant weight) then there is energy available for growth. The efficiency of animal growth is the ratio of energy in the feed consumed to the energy in live weight gained. The animal body has evolved to grow in such a manner that it is functionally efficient (Berg and Butterfield, 1976), although the efficiency of growth varies widely between species (Black, 1988). In terms of converting dietary energy to edible product, hens (producing eggs) and dairy cattle (producing milk) are considered more efficient than swine, broiler chickens and beef cattle (Beitz, 1985).

1.2.2.4 Temporal growth

The general phenomenon of growth in relation to time is referred to as temporal growth. Simplistically, growth can be considered as consisting of a self-accelerating phase, followed by a linear phase, then a decelerating phase that fades out with maturity. For animals that have been fed to meet requirements for maintenance and growth throughout postnatal life, liveweight plotted against time produces a characteristic sigmoid growth phase (Figure 1.1).

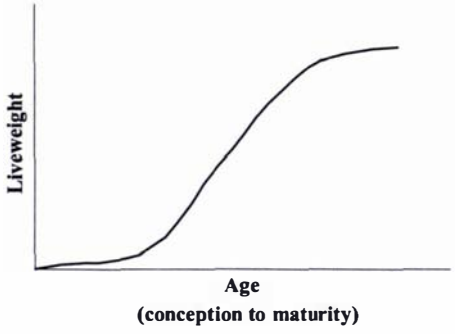


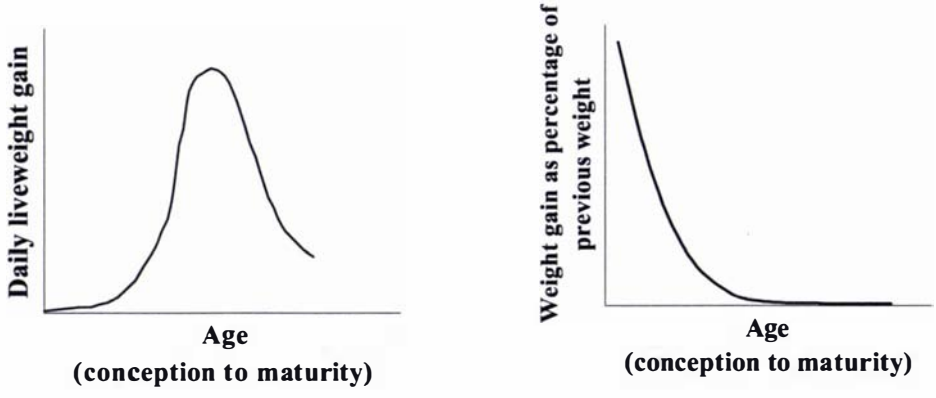
Figure 1.1 The typical relationship between age and weight of an animal.

The growth curve is phasic, with weight increasing slowly at first, then the weight increase reaches a maximum, then decreases so liveweight increases in older individuals is small. The point of inflection on the curve occurs when growth rate is maximal. Growth is most efficient at this time because the proportion of total energy available to the individual that is used for maintenance is relatively lower than at any other time or phase of the growth curve (Warriss, 2000). The decelerating phase occurs as the animal approaches its mature weight. It is associated with a relative constancy of feed intake, and a gradually diminishing increase in body weight, until intake is equivalent to maintenance requirements (Lawrence and Fowler, 2002). This is regarded as mature body weight, but this weight is not stable, and varies within individuals depending on feed supply and variables such as exercise level, reproductive status and environmental factors.

Growth curves for most animals are similar in shape, although in humans the growth curve is distorted by the disproportionately long period as infants and juveniles (Warriss, 2000). Standardised growth curves constructed for 9 different domestic species using data from early embryonic to late postnatal growth, plotting metabolic age (age raised to the power of 0.27) against the degree of maturity, were very similar in shape (Taylor, 1980c). These data imply that mean growth curves for different species show great similarity, despite large difference in mature size between species.

Growth over time can be described as average growth rate (also known as absolute growth rate), which is calculated by dividing the increase in weight over a particular time by the length of this period (Figure 1.2a). In this way growth can be described in terms of kg day^{-1}, for example. There is large variation in average growth rate between species. For example, although humans and sheep have similar birth and mature weights, maturity in humans is achieved in 15 to 20 years, while in sheep it is achieved in 1.5 to 2 years (Black, 1988).

Relative growth rate is calculated as the increase in weight over a period of time, divided by the initial weight. It is usually expressed as percentage, so growth rate is described as a proportional increase over a period of time, allowing comparisons of growth between animals (or tissues) with different sizes. Relative growth rate progressively decreases throughout life (Figure 1.2b).



a) b)

Figure 1.2 The relationships between age and a) average daily growth rate and b) relative growth rate.

1.2.2.5 Genetic influences on growth

When nutrition is adequate, variations in growth are largely genetic in nature, due to the actions of many genes. Genetic differences can be observed between

breeds, and between strains within breeds. The most obvious difference between different genotypes is in mature size. For example, the mature weight of Percheron horses is 725 to 1000 kg, while the mature weight of Arab horses is 400 to 450 kg (Evans, 2000).

Genetic effects may also be due to mutations in single genes. A range of mutations in the myostatin gene result in hereditary muscle hypertrophy (culard or dopplender or 'double muscling'), perhaps the most dramatic genotype effect on growth and body composition, and occurs in several breeds of cattle such as the Belgian Blue (Picard et al. 1995). In contrast, a mutation in a regulatory RNA in the callipyge gene discovered in sheep results in extreme hypertrophy of muscle in specific areas, but has no effect on birthweight or growth rate (Carpenter et al. 1996; Freking et al. 1998).

Genetic influences on growth in horses

Mares have a diffuse epitheliochorial placenta that attaches to the entire endometrium. Uterine size is directly related to size of the mare, so size of the mare determines the area for placentation and foetal growth (Allen et al. 2002); parity may also have an effect. Varying maternal size can enhance or restrict the normal genetic potential for a breed (Walton and Hammond, 1938; Tischner, 1985; Tischner, 1987). Using artificial insemination to make reciprocal matings between Shire horses and Shetland ponies, Walton and Hammond (1938) demonstrated that foals born to larger mares were heavier at birth, and differences were still considerable at 4 years of age. Embryo transfer of pony embryos into draft-type mares resulted in larger foals at birth with higher growth rates prior to weaning, compared to sibling pony foals out of pony mares (Tischner, 1985; Tischner, 1987). After weaning differences in size and weight gradually became less evident, although differences in height were still evident at 53 months of age (Tischner and Klimczak, 1989). Allen et al. (2002) reported similar influences of dam size on foal birthweight. Pregnancies were established by embryo transfer of pony embryos in Thoroughbred mares, or Thoroughbred embryos in pony mares, or by artificial insemination of pony in pony mares and Thoroughbred in Thoroughbred mares. Pony foals from

Thoroughbred dams were larger at birth than pony foals from pony dams, and than TB foals from pony dams.

Pregnancies established by embryo transfer on full-siblings in multiparous and nulliparous recipient mares had no effect the size and weight of foals by four months of age, although foals from multiparous mares weighed more at birth, and during early lactation (Pool-Anderson et al. 1994).

The estimates of heritability of growth measurements in Thoroughbred horses at one year of age are relatively high (0.60 for weight and 0.72 for height), and indicate that selecting for height and weight can result in genetic change in these measurements (Hintz et al. 1978). Heritability for average mature weight in Hereford cattle was 0.47 (Meyer, 1995).

1.2.2.6 Photoperiodic control of growth

In species that are seasonal breeders there is evidence for photoperiodic control of growth. Growth rates are slower, and food intake is less during winter months compared to summer months in sheep and deer under ad-libitum feeding (Blaxter and Gill, 1979; Fennessy, 1982). Growth rates of foals in Central Kentucky were reduced during winter months, regardless of when foals were born (Pagan et al. 1996), but the effects of feed supply could not be eliminated.

1.2.2.7 Hormonal control of growth

A large number of hormones and growth factors have been implicated in the control of aspects of growth. Generally they act by promoting or inhibiting the proliferation or differentiation of cells. Growth hormone is produced by the pituitary gland, and promotes growth through peptide hormones known as somatomedins, such as insulin-like growth factors. Insulin inhibits protein degradation during fasting (Warriss, 2000). Sex hormones, oestrogen and testosterone, promote growth in the female and male, resulting in size and fatness differences between sexes (Warriss, 2000). Insulin-like growth factor

may influence muscle growth systemically, while local factors such as mechanical growth factor act locally in muscles (Goldspink, 2002).

1.2.3 Compensatory growth

Compensatory growth following a period of undernutrition is a very constant feature of animal growth (Wilson and Osbourn, 1960). After a period of undernutrition and growth below the genetic potential, animals, in particular herbivores, show increased growth rates when food supply is abundant again. This phenomenon is also called 'catch-up growth' or 'rebound growth'. Growth in this period is rapid, and more efficient than animals of the same age that have been fed adequately. Efficiency of growth in the compensatory period may be even higher when animals have previously recovered from another prior severe body weight loss (Williams and Sheedy, 1987).

Cattle wintered on low planes of nutrition during winter gain weight more rapidly when turned out on spring grass than animals that received an adequate or generous plane of nutrition over winter (Wilson and Osbourn, 1960). Similar observations have been made in children offered an ample balanced diet after a prolonged period of malnutrition (Stearns and Moore, 1931). In ruminants, compensatory growth is greater when the diet quality and quantity is improved (i.e. a greater nutrient density) in addition to offering more food (Hornick et al. 2000).

1.2.3.1 Compensatory growth in horses

Feed restriction of young ponies to maintain a constant weight over a 147 to 180 day period resulted in decreased heart rate, interpreted as consistent with decreased metabolic rate in response to undernutrition. When ponies that had been energy restricted were allowed to graze pasture ad libitum, heart rates increased immediately. The dry matter (DM) intake on metabolic size basis ($liveweight^{0.73}$) was higher in ponies that had been feed restricted, compared to ponies allowed to grow normally (Ellis and Lawrence, 1978c). Compensatory

growth in young horses has been associated with flexural deformities (Hintz, 1996).

1.2.4 Growth in the horse

1.2.4.1 Prenatal growth

Prenatal growth is strongly influenced by the size of the dam, and in particular the size of the uterus (Allen et al. 2002). Pregnancies of Thoroughbred foals established in recipient pony mares resulted in evidence of muscle underdevelopment in foals at birth, and some foals were dysmature (normal gestational length but small or immature) or premature (gestation length less than 320 days). It was suggested that intrauterine growth restriction resulted in reduced muscle and fat deposition (Allen et al. 2002). Twin pregnancies in the horse usually result in foetal death or undersize, due to the restricted placental area for each foetus resulting in intrauterine growth retardation (Ginther and Douglas, 1982).

During the last 3 months of gestation the size of the equine foetus enlarges dramatically, with energy requirements of the dam increasing up to 20 % above maintenance requirements National Research Council (NRC) (1989). Energy restriction of dams in the last three months of gestation (50% of NRC recommendation for dietary energy) resulted in an average loss of 26 kg liveweight, but had no effect on foal birth weight (Banach and Evans, 1981). Jordan (1982) also reported that feeding mares to lose 10 or 20% of maintenance weight had no effect on birthweight, but did not specify the period of time for which mares were underfed. However, the equine foetus has been shown to be highly dependant on maternal glucose supplies in late gestation as the equine foetus has limited glucogenic capacity (Fowden, 1997), and the gravid uterus accounts for 70% of the maternal rate of glucose metabolism in late gestation (Evans, 1971). Thus, Fowden et al. (1994) considers that the equine foetus is vulnerable to nutritional challenge during gestation. The foal gains 23% of its birthweight in each of the last two months of gestation (Meyer and Ahlswede, 1978).

When maternal nutrition is adequate, foetal growth is tightly regulated so birthweight and size of the offspring are appropriate to the maternal skeletal size (Loveridge and Noble, 1994). Dystocia is uncommon in mares, with 4% incidence reported in Thoroughbreds (Vandeplassche, 1992; McGladdery, 2001). Dystocia in mares is usually due to foetal malpositioning, and not foeto-pelvic size disproportion (Roberts, 1986), in contrast to cattle. Higher incidences of dystocia (about 10%) in Belgian draft horses may reportedly be due to muscular hypertrophy of the foetus (Vandeplassche, 1992).

Gestational length is highly variable in the horse compared to other farm livestock (Davies Morel et al. 2002), with a normal range of 320 to 360 days (Rossdale, 1976), and a mean of 343 days (Marteniuk et al. 1998) in Thoroughbreds. Platt (1978) considered the most important influences on gestation length were the time of year when conception occurred, and the influence of the individual dam. There is a trend for mares to have similar gestation lengths in successive years, and a tendency for mares bred early in the season to have longer gestation lengths than mares bred later (Ropiha et al. 1969; Marteniuk et al. 1998). Male offspring have longer gestation lengths than female offspring (Ropiha et al. 1969; Marteniuk et al. 1998; Davies Morel et al. 2002), which is not uncommon in other species, although the reasons for the differing gestation lengths are unclear. There is limited evidence of a quadratic relationship between mare age or parity with foal birthweight in Thoroughbreds (Ryan et al. 2000).

1.2.4.2 Postnatal growth in the horse

After birth the offspring is able to reach its true potential size, without the constraints of maternal uterine size. There are considerable differences between species in the size of the offspring relative to the mother, although the differences in proportion of mature weight between domestic grazing species are small (Table 1.1, page 9). The newborn foal is born in an advanced state of maturity and size, at about 10% of the dam's weight in light-breed horses (Platt, 1978). The average birth weight of Thoroughbred foals is around 55 kg

(Hintz et al. 1979; Jelan et al. 1996). Colts are heavier at birth than fillies (Hintz et al. 1979), which may be related to gestation length. Thoroughbred foals born early in the season were lighter at birth compared to foals born later in the season in Canada (Hintz et al. 1979), although effects of birth month on birthweight were not apparent in Quarter Horses in Florida (Heusner, 1991).

The maximum adult weight of horses is related to birthweight to a large extent, partly because the mature numbers of cells in many tissues of the adult have been achieved by birth or soon after (Frape, 1998). In general, differences in growth rate after the neonatal period have little influence on mature size (Frape, 1998). Platt (1978) suggests that twin foals, and very small singleton foals, will develop into small adults, and are unlikely to perform well. However, in lambs, singletons were heavier at birth and matured 15% faster but were not heavier at maturity than multiples (Pitchford, 1993). For most species a positive correlation exists between birthweight and weight at maturity (Bridges et al. 2000). Birthweight of Percheron horses is strongly related to mature body size (Bridges et al. 2000). When human twins were considered as individuals, twins who were heavier at birth were taller and heavier as adults than were lighter twins (Loos et al. 2002). In cattle, birthweight accounts for 36 to 60% of variation in absolute weight gain, and birthweight in combination with relative growth rate accounted for all the variation in absolute weight gain (Bailey and Mears, 1990).

It is recommended that the nutrient requirements of the young horse must be met to achieve optimum growth and development and structural soundness at maturity (NRC, 1989). However, the concept of optimum growth and development has not been defined. The NRC (1989) recommends that growth of the foal should produce a smooth curve that is steepest in the first few months of life, and gradually levels after 2 years of age. However, there is little experimental evidence to support this recommendation (Fennessy, 1997), especially in regard to preventing, or reducing the incidence of developmental skeletal abnormalities. The potential for maximum weight gain persists to until around 9 months of age in the horse, at which time it gradually declines

(Frape, 1998). During this period of rapid growth, articular cartilage lesions may occur (Stromberg, 1979), but many less severe lesions seem to resolve before 11 months of age (van Weeren and Barneveld, 1999).

Smooth growth curves are relatively simply to model mathematically. It has been suggested that growth is not continuous, and instead proceeds by growth bursts followed by periods of no growth (saltation and stasis hypothesis) (Lampl et al. 1992; Lampl and Johnson, 1997; Lampl et al. 1998; Lampl et al. 2001), although most evidence supports the continuous growth model (Klein et al. 1994; Heinrichs et al. 1995).

There are relatively little published growth data for horses, particularly growth from birth to maturity. Most studies focus on the Thoroughbred, so there are limited comparisons of growth between breeds. The largest study of growth rate in horses included data on more than 1990 Thoroughbred horses foaled from 1958 to 1975 at Windfields Farms, Ontario, Canada (Hintz et al. 1979). In this study mature weight was estimated to be 500 and 545 kg for mares and stallions respectively, with mature height at the withers of 160 and 162 cm. At 6, 12 and 18 months of age, horses achieved 46, 67 and 80% of their mature weight respectively and 83, 90 and 95% of their mature height. Growth data collected in the early 1970s from a small number of studs in England (Green, 1976), were very similar to the Canadian data. Data from Thoroughbred horses in Kentucky showed a higher weight gain of horses in the autumn compared to the Canadian data (Pagan et al. 1996). Data were collected from nearly 800 Thoroughbred horses in Ireland, and growth rates were similar to the Canadian data, with higher growth rates of Canadian foals in the first 4 months of life.

Hintz (1985) noted that much more was expected of young horse growth than in previous times, and this is likely to be even more pertinent today. Hintz (1983) noted that the percentage of mature weight achieved at 6, 12 and 18 months of age was 40, 60 and 73% respectively, prior to 1950, while in more recent reports was 46, 66 and 81% respectively. Rapid growth rates have been implicated in the development of osteochondrosis in pigs, poultry and cattle, as well as in horses (Olsson and Reiland, 1978; Stromberg and Rejno, 1978;

Reiland et al. 1978; Pagan and Jackson, 1996). The potential for maximum weight gain persists to until around 9 months of age in the horse, at which time it gradually declines (Frape, 1998). Rapid growth rates in heifers during the prepubertal period can cause severe retardation of milk production potential (Sejrsen and Purup, 1997), which could potentially also be a problem in mares, leading to decreased growth of the suckling foal.

There are numerous published papers examining the short-term effect of different diets on growth rates of young horses (for example Ott and Asquith, 1986; Thompson et al. 1988; Webb et al. 1989; Scott et al. 1989; King and Nequin, 1989; Gibbs et al. 1989; Ott and Asquith, 1989; Thompson, 1995; Breuer et al. 1996; Ott and Kivipelto, 1998; Coleman et al. 1999; Ott and Kivipelto, 1999; Ott and Johnson, 2001; Ott and Kivipelto, 2002). However, the numbers of horses studied are usually low, and frequently dietary treatment effects are not significant between groups, so few conclusions can be made about optimal nutrition and growth rate of the group. Several longer-term feeding trials (6 months or longer) have shown that high weight gains occurring as a result of nutritional manipulation contribute minimally (Topliff et al. 1988; Szczurek et al. 1988); or transiently (Cymbaluk and Christison, 1989a; Cymbaluk et al. 1990) to musculoskeletal abnormalities in young horses.

Growth of horses in New Zealand

Thoroughbred foals in New Zealand are grown at pasture, with comparatively low grain inputs compared to many overseas operations, where horses may be housed and fed large quantities of grain. In New Zealand there are little published data on growth rates of Thoroughbreds. Birthweights and liveweight gains vary between Thoroughbred studs (Goold, 1991; Fennessy, 1997). Comparison of growth rates of small numbers of horses in New Zealand (Goold, 1991; Fennessy, 1997; Grace et al. 2002) with those of Pagan et al. (1996) and Hintz et al. (1979) are similar.

1.3 Body composition

As an animal grows from conception to maturity, its body proportions and composition change because growth rates of different organs and tissues of the body vary as the whole animal grows (Beitz, 1985). The order of growth is similar for all mammalian species, and seems based on the relative importance of the functions for the body parts. For example, fat is the latest maturing tissue.

In most species, muscle grows by hyperplasia to increase fibre numbers during the embryonic and foetal stages, with fibre numbers fixed at birth (Wigmore and Stickland, 198). Postnatally muscle growth is mainly due to increase in fibre hypertrophy, through increases in length and cross-sectional area. However, there are species differences, such as in the rat, where muscle fibre hyperplasia continues for a period after birth (Lawrence and Fowler, 2002).

Fat is a common term to describe the adipose or fatty tissue of the body. For this thesis the terms fat and adipose tissue will be used interchangeably. Fat cells filled with lipid are known as adipocytes; when the tissue consists almost entirely of adipocytes organised into lobules it is known as adipose tissue. Within adipose tissue there are also connective tissue, blood vessels and nerves, so when fat depots are chemically analysed they also contain protein and water. The major fat depots are subcutaneous, perirenal, omental and associated with muscles (intra- and intermuscular) (Warriss, 2000). The proportion of total body fat at each depot varies between species. There are also large differences in adiposity between breeds of sheep, cattle and pigs. As well as being present in adipose depots, adipocytes are also present in muscle, bone and organs.

When excess dietary energy is supplied for maintenance and growth of non-adipose tissues, lipid can be deposited in adipose tissue. When insufficient dietary energy is available for growth and maintenance, fatty acids can be released from adipose tissue and used for energy. Fat depots increase in size by hyperplasia, and increase in adipocyte volume. New adipocytes can differentiate from fibroblast-like pre-adipocytes at any time in life (Jequier and

Tappy, 1999). The degree of hyperplasia and cell volume enlargement varies between sites and breeds. For example, there are large species differences in the development of subcutaneous fat. In ruminants, subcutaneous fat is the fastest growing depot relative to total fat, while in pigs it is the largest proportion of total body fat throughout growth (Davies, 1989).

Bone growth will be discussed in detail in Section 1.4, page 46.

1.3.1 Carcass composition

In production animals, growth is frequently described with reference to the weight of the carcass rather than liveweight. The definition of carcass varies between authors and species, but usually relates to the liveweight of the body less the weight of the head, skin, distal limbs, and most of the viscera. Kidneys and perirenal fat may be left on the carcass or removed. The carcass of the pig often includes the skin and head.

The carcass of any species can be physically dissected into bone, muscle and fat/adipose tissue. As an animal grows, the carcass weight as a proportion of liveweight increases, and the muscle to bone ratio increases, while the percentage of bone in the carcass decreases. Relative to carcass weight, fat mass increases steadily at a faster rate than the whole carcass, so fat content (as a percentage of liveweight or carcass weight) increases dramatically as animals advance in maturity. Fat is the most variable tissue in the body, and much effort is directed in meat producing animals to controlling fatness through nutritional or genetic manipulation.

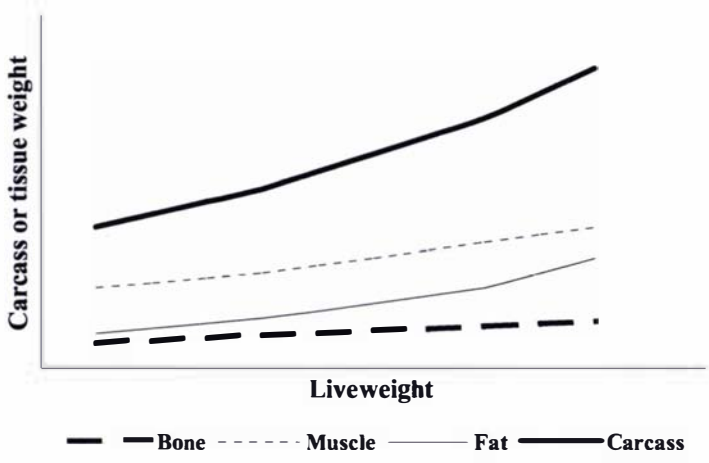


Figure 1.3 Growth of carcass and carcass tissues relative to liveweight

There are gender influences on carcass composition, with females of most species maturing at lighter weights than males, and entering the rapid fattening stage sooner. Also, different breeds commence the rapid fattening at difference ages, with breeds of smaller mature weight tending to fatten at an earlier age than breeds with a higher mature weight.

1.3.2 Chemical composition

Protein, water and ash percentages decrease with age and fattening, while fat percentage increases.

The fat-free body is the portion of the body for which the composition is determined, less the fat in the part of the body. For example, if a carcass is chemically analysed, the fat-free body is the carcass weight less the weight of chemically analysed fat. Some authors use the terms fat-free and lean body mass interchangeably, but lean more commonly refers to the edible portion of a carcass or muscle tissue without fat. To avoid confusion the term fat-free mass will be used in this thesis to describe the weight of tissue less the fat mass in that tissue.

As animals grow the concentration of protein and ash in the fat-free body increase, as the water concentration decreases.

1.3.3 Assessment of body composition

Accurate assessment of body composition is of great importance to humans, especially in health management, and assessment of athletes, and overweight or obese patients. Assessment of body composition is also important in production animals. There is particular interest in the body composition with respect to fat. In humans the fat content of the body may influence morbidity, mortality and effect of medical treatment. In meat-producing animals, accurate assessment of fatness allows animals to be slaughtered at the most appropriate degree of maturity for profitability.

Body composition has been shown to be a critical determinant for successful performance in human athletes in a variety of sports (Barr et al. 1994). There is limited evidence of an inverse relationship between fatness and performance of elite Standardbred athletes (Kearns et al. 2002b), possibly due to a reduced fat-free mass in fatter horses, resulting in a decreased power-to-weight ratio for the horse (Hodgson, 2002). However, the study used only a small number of horses, and the statistical power of the results has been questioned (Hodgson, 2002). While it is clear that differences in masses and proportions of muscles, fat and water can have implications for performance in horses, there is a great need for research in this area (Evans, 2002).

An ideal technique to assess body composition should be accurate, easy to perform, inexpensive, applicable to a wide range of ages and compositions, and suitable for application in the live animal with minimal perturbation of subsequent performance (Ferrell and Cornelius, 1984). Techniques to estimate body composition must be both valid (measure what they intend to measure) and reliable (yield the same information with repeated applications) (Barr et al. 1994).

There are many techniques to describe body composition in humans and animals, which are reviewed in this section, but not all of them are suitable for predicting body composition in the horse. There is a paucity of published data on horse body composition, primarily due to the difficulties of techniques used to assess body composition in the horse (Kearns et al. 2002a). Many techniques are considered unsuitable because of the size of the horse, or the technical problems associated with the techniques.

Firstly, a brief summary follows on the different compartment models that underlie the techniques to assess body composition.

1.3.3.1 Compartment models for assessment of body composition

All body composition techniques rely on the division of the body into various compartments. The most simplistic model is a single compartment composed of weight only, which gives no indication of body composition. Any weight in excess of a maximum number is considered to be due to fat, independent of height, muscularity or hydration status.

In the two-compartment model the body is divided into fat and fat-free masses. The two-compartment model is the basis for many body composition methods and prediction equations that are used in evaluation of body composition of humans (Lukaski, 1987; Jebb and Elia, 1995). At its simplest, a weight measurement is used with another variable such as height, by reference to tables of ideal body weight, to give an indication of body composition. This assumes that excess weight is due to excess fat. Other two compartment models include hydrodensitometry and dilution techniques to assess total body water (Sections 1.3.3.5 page 30 and 1.3.3.6 page 31 respectively).

The three-compartment model divides the body into fat, water, and combined protein and mineral. This is usually achieved by combining body composition techniques, such as hydrodensitometry to assess fat and fat-free mass, and isotopic dilution to assess total body water. Assumptions for techniques using the three-compartment model may include the density of fat being 0.9 kg L^{-1},

and the ratio of protein to mineral being fixed, with a density of 1.52 kg L^{-1} (Jebb and Elia, 1995).

The four-compartment model divides the body into fat, water, protein and mineral. Techniques include total body chemical analysis (Section 1.3.3.14, page 40), or a combination of in-vivo techniques.

Further compartments can include the division of total body water into extracellular and intracellular water, or elemental analysis. Most body composition techniques used in humans are based on two compartment models, but with the development of new techniques more complex models may be considered.

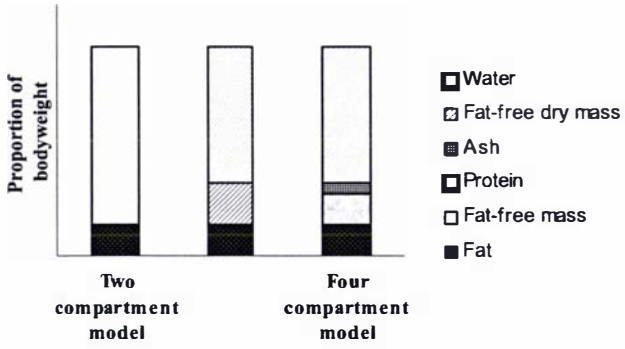


Figure 1.4 Examples of 2-, 3- and 4- compartment body composition models

1.3.3.2 Condition scoring

Body condition scores are commonly used in many species, including cattle (Edmonson et al. 1989; Ferguson et al. 1994), sheep (Russel, 1984), horses (Henneke et al. 1983; Carroll and Huntington, 1988), cats (Laflamme, 1997), and dogs (Donoghue et al. 1991). Condition scoring is a visual and/or tactile evaluation of the amount of subcutaneous fat in an animal, and overcomes individual and breed differences in body size and weight (Russel, 1984), but is subjective due to possible assessor bias and inconsistency (Evans, 1978).

In meat-producing animals, condition scores are not useful in predicting composition in animals where muscle accretion is of greatest importance (Lawrence and Fowler, 2002). However, they are useful as indicators of other types of productivity. In several species, positive relationships between higher body condition score and improved reproductive and lactation performance have been demonstrated, including cattle (Waltner et al. 1993; Pedron et al. 1993; Domecq et al. 1997a; Domecq et al. 1997b), sheep (Russel, 1984) and horses (Henneke et al. 1984; Gentry et al. 2002a; Gentry et al. 2002b). In cattle, subcutaneous fat is highly correlated with internal (visceral) fat (Wright and Russel, 1984), the latter being an important indicator of lactation capability (Taylor and Murray, 1991).

Two condition scoring techniques have been described for horses using scales of 0 to 5 (Carroll and Huntington, 1988) and 1 to 9 (Henneke et al. 1983), with low scores assigned to emaciated individuals, and high scores to obese individuals. Both techniques rely on visual and palpable assessment of subcutaneous fat depth at a number of sites. The relationships between condition scores and actual fat masses or fat percentages determined by dissection or chemical composition in horses have not been investigated. Kearns et al. (2002a) considers condition scores are not useful for appraising total fat mass or percentage in horses. However, in sheep and cattle, condition scores are highly correlated with percentage body fat determined by chemical composition (Russel et al. 1969; Wright and Russel, 1984).

Studies relating body condition score to athletic performance in the horse have been extremely limited. In endurance horses, condition score was positively correlated with distance successfully completed in 160 km endurance races (Garlinghouse and Burrill, 1999), although Lawrence et al. (1992) found no significant difference in condition scores of finishers and non-finishers in a 160 km endurance race. Condition scores of endurance horses entered in 160 km races, mostly of Arab breeding, had average condition scores of 4.5 (Garlinghouse and Burrill, 1999) and 4.7 (Lawrence et al. 1992), compared to 5.0 and 5.5 for Thoroughbreds and Standardbreds in training (Gallagher et al. 1992) respectively, using the scoring system of Henneke et al. (1983).

Studies relating condition scores and growth of young horses are very limited. Pagan et al. (1996) weighed and condition scored Thoroughbred foals at monthly intervals from birth to 18 months of age, and found that fillies tended to be fatter at each age interval. The greatest difference in condition score occurred at four months of age, with fillies mean condition score of 6.5 compared to 6 for colts, using the scoring system of Henneke et al. (1983).

1.3.3.3 Anthropometry and linear measurements

In humans, measurements of the body (anthropometry) with callipers and tape measures are widely used to predict body composition, as they are cheap and easy to perform (Forbes, 1999). Anthropometric measurements include skinfold thickness at various sites, circumferences and lengths at various body parts or regions, and a number of weight-for-stature indices.

The most commonly used indirect method for assessing body fatness in humans is based on measurements of subcutaneous fat (Fanelli and Kuczmarski, 1984), assessed by multiple skinfold thickness measurements, most commonly taken with a calliper as a fold of skin and subcutaneous fat tissue. Although the technique is simple and quick there are several limitations. Inter- and intra-subject variation in compressibility cannot be controlled, it is not possible to palpate the fat-muscle interface, and interpretable skinfold measurements are difficult to obtain from obese patients (Fanelli and Kuczmarski, 1984).

Values from skinfold thickness measurements at various sites, and measurements of bone dimensions and limb circumferences have been used in multiple regression equations to predict body density and to calculate body fatness and fat-free mass. Most prediction equations using skinfold thickness measurements have been validated against hydrodensitometric data (Barr et al. 1994), from Caucasians mostly, so prediction equations are not applicable to other ethnic groups because of ethnic differences in fat distribution and composition of the fat-free mass (Heyward, 1996).

Skinfold thickness measurements are not useful in most animal species to predict body fat, either because they are not repeatable or related to body fat mass, or are difficult to obtain. However, in cattle, anal skin fold thickness is related to total body fat (Charles, 1974; Johnson and Davis, 1983). The repeatability of the technique from day to day is not high (Simm et al. 1983; Somervaille et al. 1986). Prediction equations for body composition using anal skinfold thickness measurements may be limited to defined populations of animals (Stanford et al. 1998).

In production animals, the use of linear body measurements such as length, width, depth, height and circumference of various body parts to predict body composition have generally given poor results. Kempster (1986) and Topel and Kauffman (1988) examined published linear measurement data of animals and concluded they were not accurate predictors of body composition.

There are very limited data on the relationships between linear measurements in the live horse and body composition. Length and height measurements of 8 horses were highly correlated with total body water, determined by deuterium dilution (reviewed in Section 1.3.3.6, page 31) (Forro et al. 2000).

1.3.3.4 Weight

Weight represents the sum of the somatic chemical constituents, and is probably the least valid estimate of body composition (Barr et al. 1994) as it gives no indication of the size or proportions of the fat and fat-free masses. Infrequently weight is used alone, in a single compartment model, for assessment of fatness in humans and animals. Any weight in excess of a maximum number for a given species, breed, sex and age is considered to be due to excess fat. Due to the obvious limitations of this single compartment model it should not be used alone in assessment of fatness in any species (Jebb and Elia, 1995).

In humans a commonly used derivative of bodyweight is body mass index (BMI), where weight in kilograms is divided by the square of height in metres. A high index (>25) is assumed to indicate the patient is overweight, while a low

index (<18.5) is assumed to indicate the patient is underweight (Hamilton et al. 2000). Neither weight nor BMI can distinguish between relative contributions of fat and fat-free body mass (Garn et al. 1986), and BMI is poorly correlated to percentage body fat (Clarys et al. 1999). For example, a weightlifter is likely to have a high BMI, but the bodyweight is associated with the fat-free mass (mainly skeletal muscle) and not fat. However, because of the ease of use, and repeatability of measurements, BMI is very widely used, although its applicability in assessing body composition is controversial.

1.3.3.5 Hydrodensitometry

The earliest, and probably most frequently used, two-compartment model in humans is hydrodensitometry, or underwater weighing. The fat and fat-free masses have different densities; if these are assumed to be constant between individuals, the fat and fat-free mass can be calculated from the whole body density. Body volume is calculated by weighing the subject in air and in water, using Archimedes principal that the volume of an object submerged in water is equal to the volume of water displaced. It is also assumed that there is a constant level of hydration (73.2%), and a constant proportion of bone mineral to muscle in the fat-free body (Lukaski, 1987).

The technique requires the co-operation of the individual, including exhaling before underwater weighing. The total body volume underwater must be corrected for residual lung volume (Wilmore, 1969) to enable accurate assessment of body fatness. Smaller errors can be due to time of day, exercise, stage of menstrual cycle, illness and medications in humans (Ellis, 2000). The difficulties in using this technique preclude its use in live animals. Historically hydrodensitometry was considered the 'gold standard' for human body composition assessment, but this is no longer considered true (Heyward, 1996), due to the errors associated with the technique.

Many field techniques used to assess body composition in humans, such as bioelectrical impedance analysis (Section 1.3.3.8 page 33) have been validated against hydrodensitometric data, so the assumptions for hydrodensitometry are

an inherent part of many newer body composition techniques, including the associated errors (Barr et al. 1994). Results of techniques that rely on hydrodensitometrically derived equations to estimate body fat must be interpreted with considerable caution (Barr et al. 1994). In contrast to humans, many in-vivo techniques to assess body composition in animals have been validated against dissection or chemical composition data, which are considered to have smaller measurement errors, and rely on fewer assumptions (Sections 1.3.3.13, page 39, and 1.3.3.14, page 40 respectively).

1.3.3.6 Dilution techniques and total body water

Hydrometry, the measurement of total body water; can be used to assess body composition, because there is an inverse relationship between the proportions of water and fat in animals and humans. Dilution techniques measure water, and so indirectly by difference, give an estimate of adipose tissue. An inherent assumption in this calculation is that the fat-free mass contains 73.2% water. The techniques require the administration of specific tracers (chemicals that are easily analysed in samples of blood, saliva or urine) that are assumed to distribute evenly in body water. It is also assumed that no tracer is lost before equilibrium is reached, and the sampled component represents all the body water pools (Sainz and Tulloh, 1990). The tracer is given orally or intravenously, and time is allowed for it to distribute, before sampling blood, saliva or urine to determine the tracer concentration and thence calculate the tracer dilution. Fat-free mass is then calculated from the total body water, assuming the hydration fraction of the fat-free mass is fixed. However, hydration of the fat-free mass is known to decrease with age (Berg and Butterfield, 1976) and is also affected by malnutrition and disease states. Fat is calculated as the difference between the fat-free mass and body weight, and is assumed to contain no water.

Tracer doses of labelled water (tritium, deuterium and oxygen-18) have been extensively used in humans and animals to assess total body water (Panaretto, 1968; Ellis, 2000). Total body water in humans is most commonly measured using the deuterium oxide isotopic dilution technique (Jebb and Elia, 1995).

The use of tritiated water exposes subjects to β-radiation, whereas deuterium oxide is a stable non-radioactive isotope. The concentration and distribution of the hydrogen isotopes must be corrected for exchange with non-aqueous hydrogen; failing to do this can lead to an overestimation of total body water by 1 to 5% in humans (Sheng and Huggins, 1979) and 4% in rats (Foy and Schnieden, 1960).

Dilution techniques have been used by researchers in production animals to predict total body water, and indirectly by difference give an estimate of adipose tissue (Robelin, 1973; Sheng and Huggins, 1979). They are considered to be accurate and relatively low cost research tools for prediction of body composition in live animals (Robelin, 1984). However, others have reported no reported benefit of dilution techniques over using body weight or empty body weight (liveweight less gastrointestinal contents) to predict body composition in animals (Velazco et al. 1997). The variability of body water in the gastrointestinal tract of ruminants may limit the precision of dilution techniques to predict total body water (Robelin, 1973), which may also be true in the horse. Various researchers have used two tracers, one for body water and the other for empty body water or gastrointestinal water, in an attempt to overcome this problem (Arnold and Trenkle, 1986).

Several dilution techniques have been used to calculate total body water in the horse including antipyrine (Spurlock et al. 1985), deuterium oxide (Andrews et al. 1997; Forro et al, 2000), ethanol (Elser et al. 1983), urea (Lawrence et al. 1986) and tritiated water (Julian et al. 1956; Deavers et al. 1973; Dunkle et al. 1985). Lawrence et al. (1986) reported a high correlation between total body water determined by chemical composition of the organ free body with total body water determined by urea dilution ($r = 0.71$, $p < 0.03$). Total body water estimated by ethanol dilution was not significantly different to total body water of the empty body determined by chemical analysis (Elser et al. 1983), however, there were relatively high standard errors associated with estimation of body water by ethanol dilution. Antipyrine and tritiated water techniques are considered cumbersome (Andrews et al. 1997), and antipyrine is probably not

accurate enough to distinguish between animals of similar weight (Sainz and Tulloh, 1990). The use of deuterium oxide in dilution techniques to predict body composition of horses appears promising (Andrews et al. 1997).

1.3.3.7 Total body electrical conductivity

Electrical conductivity is a rapid, safe, non-invasive and non-contact method for assessing body composition in humans. Originally the technique was developed in the 1970's for rapid assessment of the fat and lean proportions of meat packages, carcasses and livestock, and in the early 1980's was adapted for human use (Sutcliffe and Smith, 1995). Total body electrical conductivity (TOBEC) operates on the principal that impedance of a radiofrequency coil is changed when a body (or meat cut) is inserted, and the change of the impedance is related to the volume of body electrolytes (Sutcliffe and Smith, 1995). Effectively the TOBEC instrument is calibrated with a measurement of total body water (Ellis, 2000). Cost may be a limiting factor for the technique in humans and animals as the TOBEC machine is very expensive (Lukaski, 1987). A disadvantage of the technique is that the geometrical shape of the sample or animal determines the strength of the response (Robin et al. 2002). Small changes in temperature of the sample or animal result in up to 10% variation in the response (Robin et al. 2002). There are no published reported of TOBEC use in horses, probably due to the size of the horse.

1.3.3.8 Bioelectrical impedance analysis

Bioelectrical impedance analysis (BIA) has been widely used in humans, in both clinical and research settings (Foster and Lukaski, 1996). It is generally considered to have acceptable reliability and accuracy, and is widely used due to the low cost, portability, safety and convenience (Ellis, 2000).

The method for determining body impedance is based on the conduction of an applied electrical current in an organism (Lukaski, 1987). Generally a tetrapolar method is used, to minimise contact impedance or skin-electrical interactions (Hoffer et al. 1969), with 2 electrodes placed on the wrist and 2 on the

contralateral ankle in humans. A small alternating current is introduced via an electrode at the wrist and at the ankle, and the corresponding voltage is measured in the other electrodes. The impedance is the ratio of the voltage to the current applied (Foster and Lukaski, 1996). In the single frequency machine a constant current of 50 Hz, 800 mA is passed through the body, which is imperceptible to the patient. Multifrequency BIA machines are available, which allow resistance and reactance measurements to be made over a wide range of frequencies. Human patients are generally required to lie supine while BIA measurements are taken, with measurements taken at least 2 hours after eating, and 30 minutes after voiding (Lukaski et al. 1985).

The theoretical basis for the procedure is unclear (Yanovski et al. 1996). The hypothesis that BIA can be used to assess fat-free mass (and therefore fat) is based on the principle that the impedance of a geometrical system is related to conductor length and configuration, cross-sectional area and signal frequency (Lukaski, 1987). Each biological tissue acts as either an insulator or conductor to the current. The fat-free mass contains nearly all the body water and electrolytes, and so conducts electricity much better than fat, which contains little water and acts as an insulator. The resistance values obtained are considered as indirect body parameters and must be calibrated with a more direct body composition method (Ellis, 2000). There are large numbers of regression equations for using BIA measurements to predict body composition in humans (Forbes, 1999), and most include a linear measurement such as height, and weight. Most regression equations for humans were derived using hydrodensitometry and reference values from the two-compartment model, and are specific to age-defined and race-defined populations (Heyward, 1996).

The validity of BIA depends heavily on assumptions of a constant relationship between water, electrolytes, fat-free body mass and fat mass in the body (Stewart et al. 1993). In humans, menstrual status, hydration status, activity levels and skin temperature affect BIA results. Standardisation of the measurements is also necessary due to issues with the instrumentation itself (Deurenberg et al. 1988).

BIA has been used in animals to predict body composition in live and dead animals, including cats (Elliott et al. 2002), dogs (Scheltinga et al. 1991), cattle (Marchello et al. 1999), pigs (Swantek et al. 1992), and sheep (Slanger et al. 1994; Berg and Marchello, 1994; Berg et al. 1996; Hegarty et al. 1998). Forro et al. (2000) recorded multifrequency BIA measurements in 8 horses, and concluded the technique was useful technique to assess total body water. However, including impedance data in regression analyses for predicting total body water did not improve the predictive power of the equations over those using length and height. The large volume and variability of hindgut water in the horse may influence the accuracy of BIA results (Kearns et al. 2002a).

1.3.3.9 Ultrasound

Ultrasonographic imaging is used for assessment of body composition in animals and humans. In ultrasonographic imaging, electrical activity is converted in a probe to high-frequency ultrasonic energy, which is then transmitted to the body in short pulses. The ultrasonic waves impinge perpendicularly on interfaces between tissues that differ in acoustical properties, and some of the energy is reflected to the receiver in the probe, and is transformed to electrical energy (Lukaski, 1987). Real-time ultrasound equipment (B-mode) emits many sound waves simultaneously along a linear path; reflected sound waves are presented on the monitor as a two-dimensional image or cross-section. Ultrasound machines are relatively inexpensive, portable and easily used, but the sound signals do not travel deep enough into the body to scan the whole body in cross-section. The depth and resolution of the image are affected by the frequency of the ultrasound pulses.

In humans, ultrasonographic fat measurements are considered to be a good predictor of body fat (Fanelli and Kuczmarski, 1984), but predictions are made by hydrodensitometrically derived regression equations, so care must be taken in interpretation of results (Barr et al. 1994). The pressure at which the probe is applied to the skin can distort the apparent thickness of adipose tissue, so uniform and constant pressure needs to be applied (Lukaski, 1987). Methodological problems can also arise when the muscle-adipose tissue

interface is irregular (Barr et al. 1994) and when there is fat layering, which reportedly is common in women (Hayes et al. 1988). Many of the machine-associated problems have been overcome with newer ultrasonographic technology.

Ultrasonographic technology is used in a wide variety of animals to assess the depth and area of subcutaneous fat stores and muscle. However, the accuracy of ultrasonographic measurements in prediction of body composition is variable, and is dependant on species, the ultrasound machine, and the skill of the technician (McLaren et al. 1991; Houghton and Turlington, 1992). The techniques have commercial application in pigs with accurate prediction of the percentage of lean cuts before slaughter, but similar results have not been obtained in lambs, perhaps due to the small variation in fat depth and muscle area, or simply due to the low absolute values. However, in the last 20 years there have been marked improvements in the imaging capabilities of ultrasound machines. Recent work indicated ultrasound measurements were a better predictor of saleable meat yield than liveweight in sheep (Stanford et al. 1995). Stanford et al. (1998) noted that regardless of the precision of ultrasound measurements, sheep body composition analysis has been significantly improved using indices based on ultrasonographic measurements.

Ultrasound in horses

In horses, ultrasonographic fat thickness measurements are reportedly highly repeatable, precise, accurate and easy to perform (Westervelt et al. 1976; Kane et al. 1987; Kearns et al. 2001; Kearns et al. 2002a). Equine researchers use prediction equations developed by Westervelt et al. (1976) and Kane et al. (1987) to calculate body fat from ultrasonographic rump fat thickness measurements.

Westervelt et al. (1976) assessed fat thickness in ponies and horses "directly posterior to the scapula, 5 cm lateral from the spinous processes between the 12^{th} and 13^{th} ribs, and over the rump 5 cm lateral from the midline at the centre of the pelvic bone". The thickest area of fat detected ultrasonographically was measured. In 15 ponies, rump ultrasonographic and actual fat thickness

measurements at post-mortem were highly correlated ($r = 0.85$, $p < 0.01$). The chemical composition of a further 11 ponies and 8 horses were determined; the percentage ether-extractable fat was highly correlated with rump fat thickness measurements. Prediction equations were developed for percentage ether extractable fat in the empty body of horses and ponies from rump fat thickness measurements. ($R^2 = 0.86$, coefficient of variation (CV) 2.4%, $R^2 = 0.64$, CV 3.8% for horses and ponies respectively).

Kane et al. (1987) measured ultrasonographic and actual rump fat thickness (at post-mortem) at 5 sites over the right side of croup after the area was clipped, the first site 6 cm anterior to the tailhead, and approximately 10 cm off midline; each subsequent site was 5 cm anterior. A figure indicates the ultrasound probe was placed perpendicular to the vertebral column. The mean ultrasonographic rump fat thickness measurements at the 5 sites varied from 0.6 to 2.0 cm, and at each site were highly correlated with actual fat thickness measured at post-mortem, but correlations decreased as fat thickness decreased. The carcass composition (total body less head, blood and viscera) was determined for each animal, and was highly correlated with ultrasonographic and actual fat thickness measurements. Prediction equations for percentage empty body fat in the carcass from ultrasonographic and actual fat thickness measurements were given, but the authors cautioned that a standardised sampling technique must be used for ultrasound measurements.

There are differences in methodology and site choice to ultrasonographically assess rump fat thickness between researchers (Westervelt et al. 1976; Kane et al. 1987). Based on these data it would seem advisable for those planning to use ultrasound to develop their own methods for taking into account the principles outlined by Westervelt et al. (1976); and Kane et al. (1987). Researchers need to clearly define the location of the most suitable site at which to assess fat thickness ultrasonographically, the orientation of the ultrasound probe, and the repeatability of measurements.

1.3.3.10 Computed tomography

Computed tomography (CT) relates small difference in x-ray attenuation to differences in physical density of tissues to construct a 2-D image in any plane (Waite et al. 2000). The image is of a 'slice' (10 to 13 mm thick), usually in cross section of the body or body part (Lukaski, 1987). The volume of fat-free and fat tissue in each slice can be calculated. The number of slices depends on the purpose of the CT scan. Multislice CT for measurement of tissue mass is very accurate (Mitsiopoulos et al. 1998). However, it is an expensive apparatus, and subjects are exposed to ionising radiation (Lukaski, 1987).

Application in other species has been limited by the size of the animal, so its use for body composition has been largely restricted to animals smaller than humans, including pigs (reviewed by Szabo et al. 1999), goats (Sorensen, 1992), sheep (Young et al. 1996; Jopson et al. 2002) deer (Jopson et al. 1997), and rabbits (Romvari et al. 1998). In horses, CT use has been restricted to estimation of bone mineral content and density rather than body composition (Firth et al. 1999; Waite et al. 2000), and for clinical diagnostic purposes.

1.3.3.11 Magnetic resonance imaging

Magnetic resonance imaging (MRI) is a safe, non-invasive technique for direct body composition analysis in humans (Lukaski, 1987). Cross-sectional images of the body are generated that are similar to CT images, with high contrast between soft tissues. During MRI examinations the atoms of the body, positioned in a strong magnetic field, take up energy from the external energy source and re-emit that energy as a function of time (Szabo et al. 1999).

It is a useful technique to assess visceral and subcutaneous abdominal fat separately, which has growing importance in human medicine, with high correlations between visceral fat and coronary heart disease (reviewed by Alexander, 2001). MRI studies in live animals have shown high accuracy for prediction of fat and fat-free mass (reviewed by Baulain, 1997). However, the

size and current high cost of the MRI machine limits its usefulness to animals smaller than humans (Szabo et al. 1999).

1.3.3.12 Dual-energy x-ray absorpitometry

Dual-energy x-ray absorpitometry (DXA) is a rapid, non-invasive technique that is widely used in humans to assess bone density or mineral content, or body composition (Grier et al. 1996). DXA is based on the three compartment model of body composition. DXA scans the sample at two x-ray levels, to provide a two-dimensional image and measurements of bone mineral, fat and lean content (muscles, organs and water), and total tissue mass (Mitchell et al. 1998). The hydration status of the body has only small effects on the soft-tissue estimates (Heyward, 1996), compared to other methods of body composition assessment. DXA has been validated against hydrodensitometry, skinfold thickness and BIA in humans (Johansson et al. 1993), but not against total body chemical composition or dissection.

DXA has been used to assess body composition in dogs and cats (Toll et al. 1994; Munday et al. 1994; Lauten et al. 2001) and carcass and live composition in piglets and pigs (Ellis et al. 1994; Mitchell et al. 1996; Picaud et al. 1996). It is unlikely to be used for body composition assessment in whole horse unless a larger densitometer, or one that is vertically oriented, is designed (Grier et al. 1996).

1.3.3.13 Dissection

Dissection of cadavers generally divides the body into fat, muscle and bone, a three compartment model of body composition. The abdominal and thoracic organs, and digesta may be considered as another compartment. It is a labour-intensive and time-consuming technique, the actual time depending on the degree of accuracy required. The obvious major limitation of the technique is that it cannot be used in longitudinal studies where beginning and end time points must be measured (Kearns et al. 2002a). A review of adult human dissection data revealed published results for only 51 cadavers including

weights of skin, adipose, muscle and bone tissue, with the majority of reports published in the 19th century (Clarys et al. 1999).

Dissection studies have been used extensively in many species, particularly for production animals. Changes in tissue proportions during normal growth of production animals are well documented in dissection studies (Berg and Butterfield, 1976). Dissection data allow validation of new body composition techniques, such as ultrasonographic fat thickness measurements and BIA used in animals. However, in humans none of the techniques to estimate body fat have been validated against dissection data (Clarys et al. 1999). Dissection of carcasses or bodies allows fat to be subdivided into different locations, such as perirenal or retroperitoneal. When physical dissection is followed by analysis of chemical composition it allows calculation of fat within muscle and bone (Berg and Butterfield, 1976).

Dissection studies in the horse

A limited number of studies have used physical dissection of the horse to determine body composition, including those of Gunn (1987) and Webb and Weaver (1979) and Robelin et al. (1984). Thoroughbred horses had higher muscle to bone ratios compared to other non-Thoroughbred horses (Gunn, 1987), although there was little difference in muscle to bone ratio among 5 different French breeds of horses (Robelin et al. 1984). The only dissection data for young horses are those of Robelin et al. (1984) in horses bred for meat production, between 12 and 30 months of age.

1.3.3.14 Chemical composition

Chemical body composition is the classical approach to investigate hydration of the fat-free mass, which is among the best known and most widely used constants of body composition techniques (Wang et al. 1999). The technique is expensive, time-consuming and tedious (Berg and Butterfield, 1976). It is important to ensure the sample for chemical composition is homogenous, which is done by mincing, grinding and mixing of the tissue.

Chemical composition in animals has been carried out on the whole body, the empty body or the carcass, so considerable care must be taken when comparing results from different studies. Visceral organs have a high hydration status, so values for hydration of the fat-free mass of the whole body are considerably higher than that of carcass alone (Wang et al. 1999). Concern has been expressed that the assumed hydration constant of the fat-free mass of 0.732 used in many in vivo human body composition techniques is based on chemical composition data of 50 eviscerated guinea pigs (Pace and Rathbun, 1945), combined with limited data on the chemical composition of the whole bodies of rats, rabbits, cats, dogs and monkeys (Wang et al. 1999). However, this 'value' is confirmed by numerous studies in production animals (Reid et al. 1968).

Body fat determined by chemical composition is the total of all lipid in the body, which gives no indication of where fat was stored. An additional problem is that the choice of lipid extraction solvent and the time allowed for extraction has an effect on the amount of material extracted (Dobush et al. 1985; Wang et al. 1999), so again comparing studies must be done cautiously.

Analysis of chemical composition has been carried out in a wide range of animals, including dogs (Sheng and Huggins, 1971), pigs, sheep and cattle (Reid et al. 1968). The fat-free composition varies between and within species, and fat percentage ranges from 1 to 65% of the empty body weight (Reid et al. 1968). Chemical composition has not been widely used in human research, with a review of the literature by Wang et al. (1999) summarising the fat-free hydration status of only 9 human cadavers.

Chemical composition of horses

There are limited data on the chemical body composition of horses. Various authors reporting on the proportions of the basic 4 components (Robb et al. 1972; Kane et al. 1987; McCann et al. 1988) in horses from 12 months of age to adults. Others have described the mineral content of foetuses and young horses in detail (Schryver et al. 1974; Meyer and Ahlswede, 1978; Grace et al. 1999b). More research on the chemical body composition of young horses is required for a definitive description of growth, beyond that based solely on changes in

weight and height. Chemical body composition data for horse can also be used to validate in vivo techniques to estimate body composition.

1.3.4 The importance of fat assessment in vivo

Fat is an essential component for both health and metabolism (Kearns et al. 2002a). However, excessive fatness is a major health issue in the human population. It is estimated that over 30% of Americans are overweight by at least 20% (Houseknecht et al. 1998). In general, obesity (greater than 15% above normal weight for a given age, height and sex) is not a problem in agricultural animals, but excess fatness is an issue for meat producing animals. In cats and dogs, the prevalence of excessive fatness and obesity is increasing (Edney and Smith, 1986; Butterwick, 2000), and is associated with diabetes mellitus and many other diseases.

In the horse population, excessive fatness and obesity is a growing problem (Buff et al. 2002). Overweight horses and ponies are predisposed to developing laminitis (Sloet van Oldruitenborgh-Oosterbaan, 1999), a painful condition resulting from failure of the lamellae of the inner surface of the hoof capsule that normally suspend the pedal bone (Pollitt, 1999). Obesity is a risk factor for the development of hyperlipaemia, a disorder of lipid metabolism that primarily affects ponies, donkeys and miniature horse and donkey breeds (Watson, 1998). Kronfeld (1998) claims that fatness impairs reproductive performance in horses, although there is little evidence to support this (Henneke et al. 1984; Gentry et al. 2002b). Excess fatness may impair athletic performance (Kronfeld, 1998), probably through a decreased power to load ratio (Hodgson, 2002), and exacerbation of musculoskeletal conditions such as degenerative joint disease. Overweight (and presumably excessively fat), rapidly growing young horses may be predisposed to developmental orthopaedic diseases (Section 1.5.1, page 57) (Stromberg, 1979). Excess fatness in young horses may reduce future lactation potential, as it does in growing heifers (Sejrsen and Purup, 1997).

The distribution of body fat has important implications for disease in humans, with overall fatness being an insensitive indicator of the risk of metabolic

complications of obesity (Kissebah et al. 1982; Krotkiewski et al. 1983; Lapidus et al. 1984). Increased visceral fat stores are significant risk factors for the development of cardiovascular disease and type II diabetes in humans (Katzmarzyk et al. 1999). There are less health risks associated with fat stores at other sites, such as subcutaneous abdominal fat, so much research effort is being focused on techniques to assess visceral fatness. Currently, visceral fat stores can be only directly quantified in living patients with imaging techniques such as CT and MRI. There is evidence that visceral adiposity begins early in life (Goran and Gower, 1999; Goran and Malina, 1999), which may also be true in animals. The distribution of fat in meat-producing species at slaughter has been widely studied (for example, Taylor and Murray, 1991; Afonso and Thompson, 1996; Kolstad, 2001). However, the relationship between fat distribution and disease received little attention in animals. In the horse, altered distribution of fat may be associated with hyperplasia of the pituitary pars intermedia (van der Kolk, 1998).

A major constraint in the quantification of total body fatness and its regional distribution, and determining the relationships to fatness to health, disease and performance, is the lack of appropriate in vivo body composition techniques. The need for simple, repeatable techniques to assess body composition in live horses is great (Westervelt et al. 1976). Published data on fatness in horses assessed by various body composition techniques are presented in Table 1.2. The data are very limited, but there may be breed-related differences in fat and distribution (Kearns et al. 2002a).

Table 1.2 Review of body fat percentages reported in the horse

Methodology to calculate body fat	Author	Subjects	n	Age	Condition, sex and treatment (where specified)	Body fat percentage (range or mean ± standard error) of empty body weight*, unless stated
Chemical composition	Kane et al. 1987	Horses	6	1 - 26 years	Good body condition	10.1 – 24% of carcass weight
	Robb et al. 1972	Ponies	11	8 months-18 years		6.6 - 18.9
	Westervelt et al. 1976	Horses	8			15.88 ± 1.99
		Ponies	6		Not exercised	15.03 ± 0.56
			5		Exercised	8.96 ± 0.80
	Schryver et al. 1974	Mixed-breed light horses	4	4 months	Geldings	5.3
			8	1 year	Mixed sex	16.4
			12	2 years	Mixed sex	40.2
	Elser et al. 1983	Ponies	10	Mature	Geldings	8.06 ± 1.31
	Lawrence et al. 1986		10			15.4 ± 4.1% of carcass weight
	McCann et al. 1988	Predominately Quarter horse breeding	12	Aged		9.41 ± 2.42% of carcass weight (perirenal fat removed)
Dissection	Gunn, 1987	Thoroughbreds	9	Prenatal to mature	Mixed	1.12% of liveweight[a]
		Non-Thoroughbreds	5			2.11% of liveweight
	Webb and Weaver, 1979	Thoroughbreds and ponies	12	1.5 – 14 years	Mixed sex, some with chronic disease, emaciated to good condition	5.06
	Robelin et al. 1984	French breeds		12 months		10.9 % of carcass weight
				18 months		9.4 % of carcass weight
				24 months		12.9 % of carcass weight
				30 months		14.2 % of carcass weight-

[a] Some Thoroughbreds reported to have no dissectable fat

*Empty body weight = liveweight less gastrointestinal contents

Methodology to calculate body fat	Author	Subjects	n	Age	Condition, sex and treatment (where specified)	Body fat percentage (range or mean ± standard error) of empty body weight*, unless stated
Dilution techniques to estimate total body water	Julian et al. 1956	Light breeds	6	1 – 11 years		2.1 – 24.7% of liveweight
		Percherons	4	3 – 10 years		15 – 33% of liveweight
	Deavers et al. 1973	Ponies	11	10 months to 4 years	Mixed sex	0 – 18.8% of liveweight
Ultrasono-graphic rump fat thickness	Lawrence et al. 1992	Mostly Arabians or part-Arabians	38	7 – 19 years	Mixed sex endurance horses. Condition score 3.6 – 5.2 (scale 1-9)	7.8[a] (Top finishers 6.5; non-finishers 11)
	Kearns et al. 2001	Standardbreds	23	10 ± 3 years	Mares	22.3[b]
	Kearns et al. 2002b	Standardbreds	6	3.5 ± 0.6 years	Lean male elite athletes	7.4 ± 0.9 [b]
			8	3.1 ± 0.4 years	Lean female elite athletes	9.9 ± 0.5 [b]

*Empty body weight = liveweight less gastrointestinal contents

[a] Using rump sites and prediction equations of Kane et al. (1987)

[b] Using rump sites of Westerveld et al. (1976) and prediction equations of Kane et al. (1987)

1.4 Skeletal growth

The skeletal morphology of the adult animal represents an elegant compromise between structural obligation and metabolic responsibility (Bain and Watkins, 1993). The skeleton functions to support the body, to provide the system of levers used in locomotion, and to protect the soft internal organs. Bone also has a key role in mineral homeostasis, supplying a reserve of calcium, phosphate and other ions.

Skeletal morphology, the shape and form of adult bones, is determined by mechanical stress and bone adaptation, which is dynamically controlled by the mechanical environment through growth, modelling and remodelling (Done and Goody, 1996). Modelling is responsible for altering bone shape, while remodelling is responsible for the maintenance of tissue mass and architecture in the adult skeleton (Bain and Watkins, 1993).

1.4.1 Equine skeletal anatomy

The mature horse skeleton consists of 205 bones, although numerical variations occur in the carpal, tarsal and coccygeal bones (Getty, 1975a). The appendicular skeleton includes 40 bones in the two forelimbs and 40 bones in the two hindlimbs.

1.4.2 Classification of bones

Bones can be divided into 4 main types, based on their shape and function.

The long bones are in the limbs, and have an elongated, cylindrical form, with enlarged extremities. Long bones act as supporting columns, and as levers (Getty, 1975b). In general, long bones develop from at least three centres of ossification: the primary ossification centre in the shaft (diaphysis) and the secondary ossification centres at each extremity (epiphysis).

Cuboidal bones include the small carpal and tarsal bones. When cuboidal bones are grouped together, such as in the carpus and tarsus, the many articulations allow complex movements, and may also diminish concussive forces (Dyce, Sack and Wensing, 1987).

The flat bones, such as the skull, rib, pelvis and scapula, provide attachments for muscles, and protect the organs they cover. Flat bones consist of two layers of compact bone, with intervening spongy bone and marrow.

Irregular bones are those of irregular shape, unpaired and in a median plane, such as the vertebrae.

Histologically there are three types of bone. Compact or cortical bone is found in the shafts of long bones and the outer surfaces of flat bones. Cancellous or trabecular bone occupies the metaphyses, epiphyses and apophyses, and consists of a three-dimensional meshwork of trabeculae and interconnecting struts. Woven bone is an immature form of bone, which is also present at fracture sites.

1.4.3 Bone growth

Bone development is known as ossification or osteogenesis. All bones are derived from mesenchyme, but develop by two different processes, known as intramembranous ossification and endochondral ossification respectively.

1.4.3.1 Intramembranous ossification

Intramembranous ossification proceeds without a cartilage model, and the bone tissue forms directly in areas of vascularised mesenchyme. Examples of bones that develop by intramembranous ossification are flat bones of the skull. Ossification begins at a spherical centre of ossification. The process extends from the centre to the periphery of the future bone, so a network of bony trabeculae is produced, which rapidly thicken. The trabeculae coalesce to form a bony plate, separated from adjacent bones by fibrous tissue, which becomes the periosteum when the bone attains its mature size.

1.4.3.2 Endochondral ossification

Long bones develop indirectly from mesenchyme through a process called endochondral ossification, which results in cartilage being changed into bone. A temporary cartilage model or anlage precedes each long bone. Endochondral ossification occurs at the primary ossification centre of the diaphysis and the secondary ossification centre of the epiphysis in long bones and in the cuboidal bones in all mammals (Hurtig and Pool, 1996). Endochondral ossification occurs through a series of well-defined steps, involving chondrocyte differentiation, matrix calcification, vascular invasion and ossification (Anderson, 1989).

1.4.4 Development of the epiphysis, metaphysis and diaphysis

Nomenclature to describe the growth plates of long bones is inconsistent in the literature. Frequently the metaphyseal growth plate is referred to as the epiphyseal growth plate, epiphyseal disc or epiphyseal cartilage, which is confusing, as it implies that endochondral ossification at this site results in epiphyseal growth, when it actually results in metaphyseal growth. Often the nomenclature does not distinguish the metaphyseal growth plate from cartilage of the primary ossification centre, or true epiphyseal growth cartilage. For the purposes of this thesis the term metaphyseal growth plate or physis will be used to describe the transverse disk of hyaline cartilage between the epiphysis and the diaphysis of long bones.

In the foetus, bones are first present as cartilage templates or anlages. Aggregation of undifferentiated mesenchyme is followed by differentiation of cells in the core of the aggregation into chondrocytes. Spindle shaped cells at the periphery form the perichondrium, a sheath around the cartilage (Vortkamp et al. 1996). As the central chondrocytes cease proliferating and become hypertrophic, the altered extracellular matrix allows blood vessels to invade from the perichondrium. As vascularisation proceeds, osteoblasts and bone marrow cells replace the cartilage with bone.

1.4.4.1 Primary centre of ossification

Periosteal capillaries grow into the calcified cartilage in the middle region of the anlage, and then supply its interior. The presence of capillaries initiates the development of the primary centre of ossification. Osteogenic cells give rise to osteoblasts that lay down bone matrix on the calcified cartilage, eventually forming cancellous bone. This process replaces much of the cartilage anlage. As the ossification front reaches towards the ends of the bone template the cartilage derived from mesenchyme is replaced by cartilage produced by newly proliferated chondrocytes (Horton, 1993). This cartilage becomes organised into a growth plate, which occupies the narrow space between the epiphyseal cartilage and the expanding bone. The innermost layers of cancellous bone in the mid-shaft region are resorbed, leaving the medullary cavity, which becomes populated with myeloid cells surrounded by bone cortex. At this stage the bony diaphysis still has cartilaginous epiphyses.

1.4.4.2 Secondary centres of ossification

Most secondary centres of ossification develop postnatally. In the centre of the epiphysis chondrocytes hypertrophy, and the surrounding matrix calcifies and begin to break down. Osteogenic cells and capillaries grow into the spaces, and osteoblasts deposit bone matrix on the cartilage matrix. The centre of ossification in the epiphysis develops radially by endochondral ossification, enlarging the dimensions of the bone, contributing only slightly to the bone length. The centre of the epiphysis ossifies first, so the ends of the long bone have a covering of epiphyseal growth cartilage that is continuous with articular cartilage, also known as the articular-epiphyseal complex. The articular cartilage is devoid of blood vessels, while the epiphyseal cartilage contains cartilage canals and blood vessels.

At the metaphysis of the long bone epiphyseal cartilage is continuous with the metaphyseal growth plate. Eventually endochondral ossification of epiphyseal cartilage is complete, and a subchondral bone plate forms adjacent to both articular and metaphyseal cartilage.

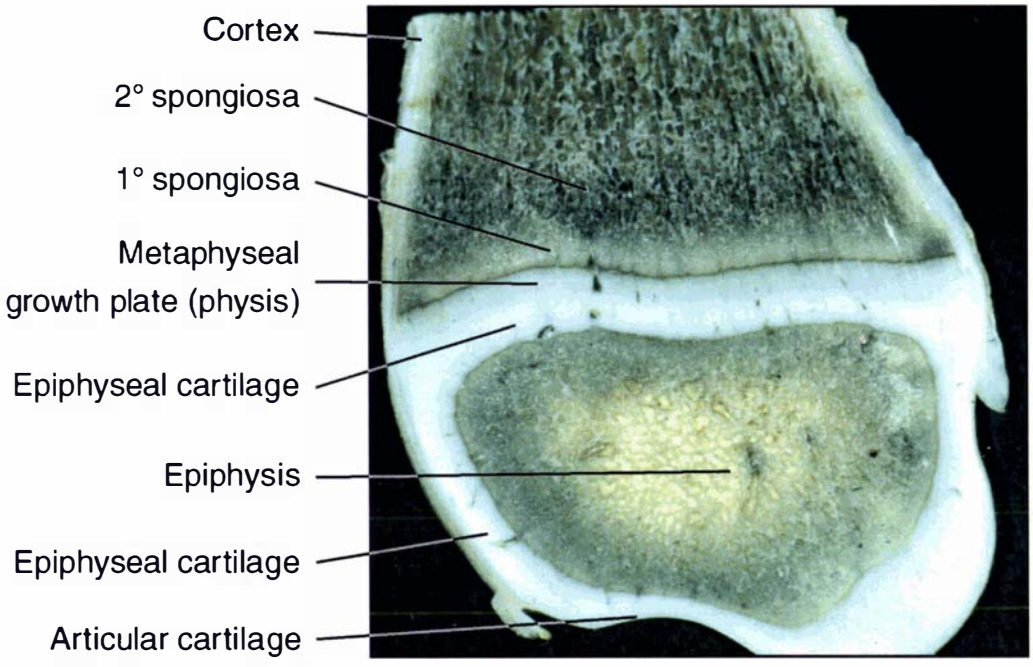


Figure 1.5 En face view of a sagittal section through the medial styloid process of the distal radius of a foal born 2 months prematurely (image courtesy of Professor Elwyn Firth).

1.4.5 Growth cartilage

The growing cartilage of the epiphysis and metaphysis consists of histologically and biochemically distinguishable layers or zones. While the zones exhibit abrupt transitional regions, they are physically, metabolically and developmentally interdependent (Brighton, 1978). The division of growth cartilage into resting, proliferating and hypertrophic zones, as described by Brighton, (1978), will be used in this thesis. The sizes of the zones vary between species, with humans and large animals having relatively large resting zones compared to laboratory animals (Little, 1973). The degree of organisation of chondrocytes in growth cartilage also varies between species, with avian growth cartilage less well organised than mammalian cartilage (Leach and Gay, 1987).

1.4.5.1 The resting zone

In the resting zone (or reserve zone) chondrocytes are small and oriented randomly. They divide at infrequent intervals to become the precursors of cells in the proliferative zone.

1.4.5.2 The proliferative zone

In the proliferative zone chondrocytes are stacked into columns or clusters, oriented parallel to the long axis of the bone (Horton, 1993). The cells become flattened, with synchronous cell division in each column. When this cell division stops the column 'grows out' (Little, 1973). The cells enlarge, and the column is temporarily replaced by intercellular matrix until a new column is formed from cells in the resting zone.

1.4.5.3 The hypertrophic zone

In the hypertrophic zone cells are larger, and have a major role in mineralisation, through production of matrix vesicles, rich in alkaline phosphatase (reviewed by Anderson, 1989). Matrix vesicles are the putative sites of initial mineralisation within cartilage (Boyan et al. 1990). There is no

intercellular substance in this zone, so it is a structurally weak region (Turner, 1987). In the zone of calcification the matrix between cells gradually becomes calcified, starting with the formation of matrix vesicles. Metaphyseal blood vessels penetrate the transverse septa and lacunae as the terminal hypertrophic chondrocytes degenerate (Horton, 1993). The blood vessels also provide osteoblasts and chondroclasts for bone production and break down of remaining cartilage respectively. Osteoid matrix is formed on the columns of calcified cartilage, resulting in primary spongiosa. This is eventually replaced by secondary spongiosa, which lacks remnants of the cartilage core.

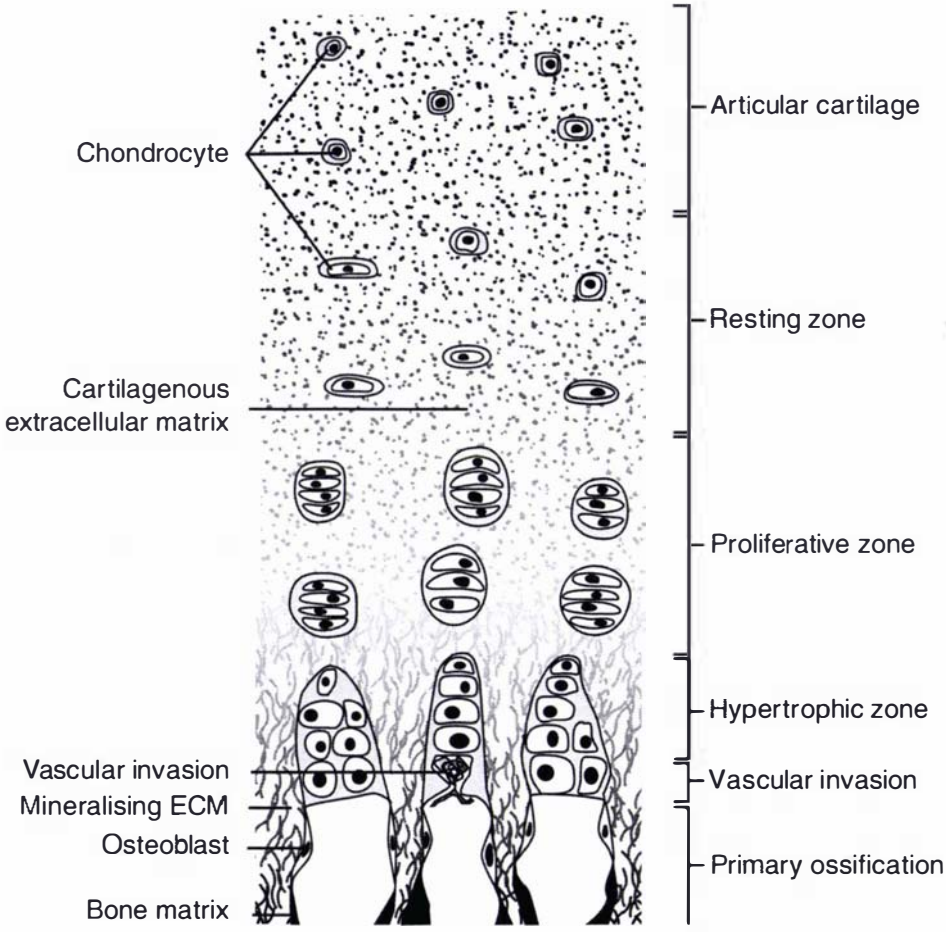


Figure 1.6 Schematic of epiphyseal growth cartilage. After Horton (1993), Figure 6, page 78.

1.4.6 Growth plate closure

The characteristic cellular organisation of the physeal growth plate remains until the growth plate closes, which happens in an orderly temporal pattern. In general, the most distal growth plates close before more proximal growth plates. In humans the last growth plates of long bones close after puberty (Little, 1973). The rat is unique in that the physeal growth plates remain open indefinitely (Little, 1973).

By 18 months of age, 95% of the final adult height is reached in the horse (Hintz et al. 1979) but the growth plates of the proximal scapula, ulna, femur and tibia do not close until three years of age or older in the horse (Getty, 1975a). Longitudinal bone growth has been demonstrated by the placement of stainless steel pins transversely in the extremities of long bones, and bone growth assessed by serial radiographs in pony foals from birth to around 2 years of age (Heinze and Lewis, 1968; Campbell and Lee, 1981); and horses from birth up to 2 years of age (Fretz et al. 1984; Smith et al. 1991). In-vivo methods of bone labelling have also been described in the foals to determine the rate of bone growth between birth and 104 days of age (Goyal et al. 1981).

1.4.7 Regulation of skeletal growth

The regulation of endochondral ossification occurs by the integration of a complex array of both local and systemic growth factors (Loveridge and Noble, 1994). Intrinsic or genetic factors principally play a role in the ability of skeletogenic cells to differentiate, undergo mitosis, and produce specific extracellular matrix components (Moss-Salentijn, 1982). Extrinsic or extracellular factors affect the timing and rates of such events (Moss-Salentijn, 1982).

1.4.7.1 Systemic factors

Growth hormone (GH) or somatotropin is produced by the anterior pituitary gland, and secreted in a pulsatile manner. It is a key regulator of somatic

growth, and is mediated via somatomedins. Acting through both systemic and local insulin-like growth factor (IGF) production, GH can stimulate bone formation and resorption. Growth hormone receptors have been identified in growth cartilage and bone of many species. GH acts directly to stimulate chondrocyte differentiation, and indirectly through stimulation of production of IGFs in the liver.

The majority of intrauterine and postnatal skeletal growth is regulated by the endocrine actions of IGF-1 (Spagnoli and Rosenfeld, 1996), which is secreted from the liver primarily, and other non-hepatic tissues. IGF-1 is actively transported, and exerts its endocrine actions at the growth plate (Loveridge and Noble, 1994). GH may augment IGF-1 action by inducing differentiation of chondrocytes and stimulating local production of IGF at the growth plate (Spagnoli and Rosenfeld, 1996).

Plasma IGF-1 concentrations are positively correlated with body size in the dog (Eigenmann et al. 1984), but not in other species (Zangger et al. 1987). High levels of IGF-1 are present in the blood of young horses compared to adults, concurrent with rapid growth (Malinowski et al. 1996).

1.4.7.2 Local factors

Local factors may include GH, IGF-I and IGF-II, transforming growth factor-β B (TGF-β), basic fibroblastic growth factor (bFGF) and bone morphogenic proteins (BMP) (Reddi, 1994), but there are likely to be many other contributing local factors (Vortkamp et al. 1996). Extrinsic local factors that are involved in the control of long bone growth include static compression, which leads to decreased longitudinal bone growth. Application of a static compressive load on a growth plate by stapling can retard, and ultimately stop, long bone growth (Bylander et al. 1981; Bylander et al. 1983). Stapling one side of a growth plate will retard growth on that side, but the other side of the growth plate will continue to grow. Unilateral temporary stapling or compression wiring across a growth plate may be used in the horse to correct angular limb deformities (Vaughan, 1976; Campbell, 1977). A lack of dynamic compression due to

immobilisation or paralysis will result in decreased long bone growth, but is preceded by a transient increase in growth rate (Moss-Salentijn, 1982).

1.4.8 Nutritional influences on skeletal growth in the horse

1.4.8.1 Dietary energy and protein

Growth, when measured by body weight and size, is a sensitive parameter for nutritional adequacy (Nap and Hazewinkel, 1994). Most malnutrition is due to both energy and protein deficits (Lewis, 1995), and results in decreased weight gain or weight loss, with less dramatic influence on increases in body height and length. In dogs, dietary energy restriction resulted in a 10 to 20% reduction in longitudinal growth, while the ratio of body length to body weight nearly doubled (Dammrich, 1991). In studies by Ellis and Lawrence (1978b), weanling filly foals were maintained at a constant weight for 6 months through dietary restriction, followed by 6 months of ad libitum pasture. There was a marked delay in closure of the metaphyseal growth plates of the proximal and middle phalanx and distal metacarpus of restricted fillies that had been feed restricted compared to those that had been allowed to grow normally. Delayed growth plate closure may be a mechanism to allow the animal to attain a normal or near-normal skeletal size when dietary energy intake improves (Ellis and Lawrence, 1978b).

Severe restriction of dietary protein results in the disease kwashiorkor in children, characterised by growth failure and muscle wasting. Dietary protein deficiency results in reduced growth rates in young horses (Lewis, 1995) and reduced feed intake. Young horses fed low protein diets, or those lacking in lysine, grew less rapidly in height and weight than those with adequate dietary protein (Hintz et al. 1971).

1.4.8.2 Calcium and phosphorus

In most species only severe calcium deficiency results in disease (Loveridge and Noble, 1994). Children with chronic malabsorption have reduced calcium

absorption and poor growth, but their bones appear normally mineralised (Loveridge and Noble, 1994). A severe deficiency of dietary calcium or excess dietary phosphorus causes hypersecretion of parathyroid hormone (PTH), resulting in fibrous osteodystrophy, or nutritional secondary hyperparathyoidism (NSH) (David et al. 1997). This disease was commonly associated with feeding high levels of bran to horses at the turn of the century (Harris, 1998), and in dogs fed home-made diets high in meat, potatoes and bread (Kronfeld, 1984). Bran is high in phosphorus and phytate (phytates impair absorption of dietary calcium), while the homemade diets of dogs are low in calcium. In horses the disease is now more likely to occur when grazing tropical grasses such as kikuyu, with high levels of oxalates that bind dietary calcium so it is not available to the horse. Clinical signs of NSH depend on the magnitude of the calcium-phosphorus imbalance, dietary oxalates, and age (Ramirez and Seahorn, 1997), and are most pronounced in horses with high calcium requirements (growing horses, pregnant and lactating mares). In well-established cases of NSH there may be enlargement of facial, mandibular and maxillary bones due replacement of resorbed bone with excess fibrous tissue, lameness due to microfractures and loss of subchondral bone, and weight loss due to painful mastication as periodontal bone is absorbed (Ronen et al. 1992; Ramirez and Seahorn, 1997; David et al. 1997).

Phosphorus deficiency leads to increased plasma calcium, through vitamin D active metabolites, resulting in bone demineralisation and osteomalacia (Greiwe-Crandall et al. 1993). Feed intake may decrease, resulting in reduced growth rate (Lewis, 1995). Phosphorus deficiency is uncommon in most species, including horses (Lewis, 1995), and dogs, but is an endemic problem for humans and animals in many Third World countries (Nap and Hazewinkel, 1994).

High dietary calcium intake results in excess calcitonin secretion, inhibiting the conversion of cartilage to bone and resorption of calcium from bone (Lewis, 1995). In the horse calcium supplementation at more than 5 times NRC (1989) requirements had no deleterious effects (Jordan et al. 1975). Similarly, there were no effects of excess dietary calcium or phosphorus (342% and 388% of

NRC recommendations respectively) on growth rate and height in horses (Savage et al. 1993b). In giant breeds of dogs, excess calcium may lead to decreased body weight and growth in size (Hedhammer et al. 1974), and cartilage and bone abnormalities (Goedegebuure and Hazewinkel, 1986).

1.5 Aberrations of skeletal growth in the horse

Skeletal growth, characterised by increasing skeletal dimensions, is a primary determinant of overall body growth. Longitudinal bone growth in the appendicular skeleton is achieved through the highly organised, sequential process of endochondral ossification. This relies on temporal and spatial organisation of macromolecules, and can be influenced by many factors, including nutrition and the availability of minerals, physical activity, hormones and toxins (Palmer, 1993). Disruption of endochondral ossification results in cartilage retention in metaphyseal cartilage and articular-epiphyseal cartilage, and has been reported in many species. In growing foals disturbance of endochondral ossification is a feature of the group of diseases collectively called developmental orthopaedic disease (DOD).

Disturbances in skeletal growth may also result from injuries, such as fractures, and possibly infections (Martens and Auer, 1980), but will not be considered in this literature review.

1.5.1 Developmental orthopaedic disease

A panel sponsored by the American Quarter Horse Association in 1986 coined the term 'developmental orthopaedic disease' to describe diseases where there was a failure of conversion of cartilage to bone (McIlwraith, 1986). Originally DOD was defined to include the diseases of osteochondrosis, acquired angular limb deformities, subchondral bone cysts, physitis, flexural deformities and cuboidal bone malformation. Cervical vertebral malformation may also be

included in the syndrome (Jeffcott, 1993), and is has been suggested that juvenile arthritis should also be included, when it arises from joint deformities (Watrous et al. 1991; McIlwraith, 1993b).

There is confusion in the literature in defining horses with DOD. Pool (1987) included any inherited or acquired disease that affects the structure and function of the bone and joint, as well as attachments of the skeleton, thus including defects in endochondral ossification, intramembranous ossification, growth, and bone modelling and remodelling. Gabel (1988) proposed the term 'metabolic bone disease' be used in preference to DOD as it implies a common pathogenesis in the diseases. However, in the human literature, metabolic bone disease is not related to musculoskeletal development, but instead describes the loss of mineral from the skeleton leading to osteoporosis (Hurtig and Pool, 1996). An identical pathogenesis for all the DOD diseases is also considered most unlikely (Jeffcott, 1997). Hurtig and Pool (1996) consider the term DOD is useful to describe all skeletal conditions associated with growth, but warn that the broadly defined syndrome with diverse causes may cause confusion. The DOD syndrome also includes diseases that may not result from abnormal endochondral ossification (McIlwraith, 1993a), such as flexural limb deformities.

In this thesis the term DOD will be used to describe the group of diseases outlined in the following sections, many of which may be associated with disrupted normal progression of endochondral ossification.

1.5.1.1 Osteochondrosis

Osteochondrosis is the result of failure of endochondral ossification, occurring in articular-epiphyseal cartilage, and metaphyseal cartilage (Jeffcott and Henson, 1998). Cartilage growth is rapid, but chondrocyte differentiation is disturbed, with subsequent loss of the prerequisites for bone formation, provisional calcification and vascular invasion (Olsson, 1982). This results in cartilage retention (Poulos et al. 1978; Reiland, 1978c), with necrosis in the basal layers of the retention. In the articular-epiphyseal cartilage the necrotic

area may subsequently fissure under biomechanical stress (McIlwraith, 1993b). If the fissure reaches the articular surface a flap may form, known as osteochondrosis dissecans. It has been suggested that when inflammation is present in conjunction with articular damage the term osteochondritis dissecans is used (Jeffcott, 1993). Osteochondrosis may result in clinically evident joint effusion and lameness, and joint lesions may result in secondary degenerative joint disease.

Several classification systems have been proposed to differentiate osteochondrosis lesions in horses. Pool (1993) proposed that osteochondrosis be classified as primary (idiopathic or genetic) or secondary (acquired). Lesions in the idiopathic form of osteochondrosis occur at predilection sites, and it is proposed they form as a result or excessive biomechanical forces on normal structure, or normal forces on weakened structure. Lesions are generalised, but not symmetrical in the acquired form of osteochondrosis (Pool, 1993). Rooney (1975) proposed that osteochondrosis is classified into articular and non-articular (or marginal) forms, with the former being mostly osteochondrosis dissecans lesions, and the latter associated with cartilage infolding. Stromberg, (1979) classified osteochondrosis as articular or physeal, and further classified articular osteochondrosis lesions into peripheral and central, with both resulting in cartilage flaps, although only peripheral flaps were capable of ossification, and marginal flaps could form into subchondral cystic lesions. However, not all cystic lesions are marginal, for example those found in the medial femoral condyle and the proximal interphalangeal joint (McIlwraith, 1982; Dowling et al. 1998). Hurtig and Pool (1996) categorised osteochondrosis lesion in the horse as typical or atypical. The typical pattern manifested as one or two lesions in a characteristic, possibly bilaterally symmetrical, location, although the lesion could be clinically silent. In contrast, the atypical pattern manifested as multiple articular, and sometime physeal, lesions in characteristic sites and other random locations in the skeleton.

It has been suggested the term dyschondroplasia is preferable to describe primary lesions of osteochondrosis in growth cartilage (Olsson, 1978; Jeffcott,

1991), although Jeffcott (1993) conceded that the term osteochondrosis is so ingrained in current thinking that it would be difficult to effect a change. However, not all osteochondrosis lesions are associated with dyschondroplastic cartilage (McIlwraith, 1993a). In clinical cases dissection between cartilage and subchondral bone has been observed, without thickening of the cartilage, or alteration in the subchondral bone (McIlwraith, 1993a). Similarly radiographic bone lucency was reported to develop after endochondral ossification had ceased (McIntosh and McIlwraith, 1993).

In humans, it is hypothesised that necrosis of bone adjacent to articular cartilage is the underlying defect that later presents as osteochondrosis dissecans (Pappas, 1981). However this hypothesis is based on examination of specimens obtained at surgery, years after the onset of joint pain (Carlson et al. 1991), so the primary defect may have occurred in growth cartilage many years earlier. Early lesions in humans have not been examined, and recent descriptions of certain osteochondroses indicate ischaemic necrosis is not a primary event but appears to follow a fracture or other traumatic insult (Resnick, 1995). In many cases the clinical presentation and patho-morphologic features of osteochondroses in horses and humans are different, and the common name confuses.

Pool (1993) suggested that typical equine osteochondrosis is primary, and is characterised by multiple random cartilage lesions in physes and articular cartilage in multiple skeletal sites, but not bilaterally symmetrical.

Originally, the histological hallmarks for osteochondrosis were considered to be thickening of cartilage, disturbance of endochondral ossification, cartilage degeneration and cartilage necrosis (Rejno and Stromberg, 1978). Henson et al. (1997b) stated that despite the range of histological changes described it remains difficult to accurately identify lesions accurately. Henson et al. (1997b) described the disrupted normal sequential transition of chondrocytes, resulting in accumulations of large numbers of small, rounded chondrocytes, apparently arrested at the pre-hypertropic stage. Others have also noted cluster formation, increased spatial separation between chondrocyte columns and haemorrhage (Savage et al. 1993a), microfractures and degenerative foamy matrix (Hurtig et

al. 1993) and the presence of chondonecrosis, myelofibrosis and clefts between cartilage and bone (Carlson et al. 1995). Pearce et al. (1998a) described the presence of chondrocyte clusters, subchondral fibrosis and subchondral sclerosis surrounding fibrous material associated with cartilage invaginations, and obvious thickening of the hypertrophic layer in retained cartilage cores.

Tibial dyschondroplasia in broilers and turkeys is also classified as osteochondrosis, with retention of chondrocytes in the proximal metaphysis (Poulos et al. 1978). Proliferating chondrocytes multiply and develop into transitional chondrocytes, but fail to differentiate any further (Poulos et al. 1978). The retained cartilage is not calcified, and vessels cannot penetrate. In pigs, turkeys and broilers osteochondrosis/tibial dyschondroplasia is always a generalised disease, histologically and often also macroscopically (Olsson, 1982).

1.5.1.2 Physitis

Physitis is one of the terms used to describe enlargements of metaphyseal growth plates in young animals, particular in horses and cattle. It is also called physeal dysplasia (Jeffcott, 1996), epiphysitis (Rooney, 1963), nutritional epiphysitis and physosis (Firth, 1990). The term epiphysitis, although commonly used by horse owners, is an erroneous one, as the disease affects the metaphysis and physis primarily. The term also infers that the primary lesion is inflammation of the physis, which is unlikely in many clinical cases (Firth, 1990). The confused terminology, and the meanings implied, is not yet resolved. In the literature review of this thesis the term physitis will be used, as it is the popularly used term in scientific literature. Later in this thesis the inappropriateness of this term will be addressed.

Physitis commonly occurs at the distal radius and distal third metacarpus/metatarsus of horses, at 1 to 2 years of age and 4 to 8 months of age respectively (Turner, 1987). Physitis results in altered contours of the bone, and possible disturbance in longitudinal growth (McIlwraith, 1993b). Clinically affected horses may be lame, but not all have radiographic evidence of defective

ossification (McIlwraith, 1993b). Some affected horses have radiographic evidence of retained cartilage cores, irregularly thickened growth plates, metaphyseal sclerosis and abnormal trabecular patterns (Hurtig and Pool, 1996). There is only one clinical report on the histologic changes associated with physitis (Rooney, 1963), probably because the disease is self-limiting and thus early lesions have not been described. Hurtig and Pool (1996) describe crushing of the hypertrophic chondrocyte layer, fracture of the calcified cartilage layer or newly formed bone, crushing of the growth plate or protrusion of growth plate margins into the perichondrium. Some authors consider physitis to be a physeal form of osteochondrosis in horses (Rejno and Stromberg, 1978; Jeffcott, 1991), while others consider it is not a form of osteochondrosis, but should be considered one of the diseases in the DOD syndrome (Hurtig and Pool, 1996).

1.5.1.3 Acquired angular limb deformity

Angular limb deformities (ALD) are axial deviations of a limb in a frontal plane, and may be perinatal or developmental (acquired). Developmental factors include excessive dietary energy, trauma, excessive exercise and conformational defects (Auer, 1983). ALD may occur in association with osteochondrosis and cuboidal bone malformation (Fretz, 1980; McLaughlin et al. 1981). However, as only a few ALD can be definitively associated with abnormal endochondral ossification, they should be considered as one of the DOD diseases, but not a manifestation of osteochondrosis (Hurtig and Pool, 1996).

1.5.1.4 Flexural limb deformity

Flexural limb deformities result in joint restriction in a flexed position or the inability to completely extend a joint (Adams and Santschi, 2000). Flexural deformity may be incited from painful musculoskeletal conditions (Wagner and Waltrous, 1990), including osteochondrosis (Moore and McIlwraith, 1977) and physitis (Cymbaluk and Christison, 1989b), or may occur in association with rapid growth (Hintz, 1996), or malpositioning in utero (Rooney, 1966). Despite the findings of Hintz (1996), acquired flexural deformity could not be

experimentally induced in a group of weanling horses rapid weight gains and limited exercise, and there was no effect of a low hoof angle (Szczurek et al. 1988).

Flexural limb deformities are commonly referred to as contracted tendons Wagner, however, the implied pathogenesis may be incorrect (Barr et al. 1994) as a pathological process within a tendon or muscle has not been demonstrated (Gerring, 1989).

1.5.1.5 Subchondral bone cysts

Subchondral bone cysts are also described as subchondral cystic lesions and cyst-like lesions to avoid the implication the lesions are true cysts, although McIlwraith (1998) considers they can be called a cyst, as they have a lining. Subchondral bone cysts can affect nearly every appendicular joint in the horse (Textor et al. 2001), and may be the result of subchondral bone trauma (Verschooten and De Moor, 1982; Kold et al. 1986) or osteochondrosis (Rooney, 1975; Stromberg, 1979; Trotter and McIlwraith, 1981; McIlwraith, 1982; McIlwraith, 1993a). It is postulated that necrosis of retained cartilage in the horse may lead to fragmentation or to necrosis and bone resorption, resulting in cyst development and expansion (Trotter and McIlwraith, 1981; Stowater et al. 1986).

Experimentally, trauma applied to articular cartilage and subchondral bone resulted in subchondral cystic lesions (Kold et al. 1986; Ray et al. 1996). Subchondral bone cysts are also encountered in advanced osteoarthritis in humans (Hurtig and Pool, 1996), although a common pathology between subchondral cystic lesion in horses and cystic bone lesions in humans could not be established (Von Rechenberg et al. 1998).

1.5.1.6 Cervical vertebral malformation

Cervical vertebral malformation, or the 'wobbler syndrome' is a disease of horses characterised by spinal cord compression and neurologic dysfunction,

due to stenosis of the vertebral canal (Reed et al. 1986). Overfeeding and vigorous exercise may contribute to the development of cervical vertebral malformation, and is suggested that restriction of dietary energy, with provision of adequate minerals and vitamins, may halt the progression of the disease (Donawick et al. 1989).

Cervical vertebral malformation may occur in association with osteochondrosis of the facets of the intervertebral joints (Powers et al. 1986; Stewart et al. 1991), although not all horses with cervical osteochondrosis have cervical vertebral malformation (Stewart et al. 1991; Beck et al. 2002). Stewart et al. (1991) found that horses with cervical vertebral malformation had more severe, but not more numerous osteochondrosis lesions than unaffected horses. Breeding mares and stallions affected with cervical vertebral malformation did not increase the incidence of the disease in their offspring, but they did have a higher incidence of other DOD entities (Wagner et al. 1985).

1.5.2 Proposed aetiologies for developmental orthopaedic disease

DOD is considered to be multifactorial, with proposed aetiologies including genetic, traumatic, endocrine, and various nutritional factors including overfeeding, or imbalances of minerals or vitamins (Kronfeld et al. 1990; Williams and Pugh, 1993; Hurtig and Pool, 1996; Jeffcott, 1996).

1.5.2.1 Genetics

Familial predisposition and heritability may have a role in the development of osteochondrosis in humans (Duthie and Houghton, 1981), horses (Stromberg, 1979) and dogs (Paatsama et al. 1971). A familial predisposition was reported in horses with cervical vertebral malformation (Dimock, 1939), although the latter study was performed before radiography and myelography were available, so apparent 'wobblers' may have had other diseases (Wagner et al. 1985).

Radiographic surveys have shown a correlation between the incidence of osteochondrosis in the progeny and their sires (Jeffcott, 1997). Stromberg and

Rejno (1978) reported an overrepresentation of osteochondrosis in the progeny of two stallions. Philipsson et al. (1993) reported a heritability coefficient of 0.24 to 0.27 for osteochondrosis in Standardbred trotters in Sweden. Similarly, Schougaard et al. (1990) and Grondahl and Dolvik, (1993) determined high heritability coefficients for osteochondrosis. Heritability estimates of osteochondrosis in Maremmano horses were lower, and it was estimated that with selection against osteochondrosis the frequency of the disease could reduce from 16 to 2 % in 5 generations (Pieramati et al. 2003). However, care must be taken when comparing heritability values from different studies as the method of estimating heritability affects results, with non-linear methods giving lower heritabilities than linear methods (Pieramati et al. 2003). The mode of inheritance is suggested to be polygenic, rather than due to a single gene pair (Philipsson et al. 1993). The presence of a genetic marker for osteochondrosis has been reported in the pig (Andersson-Eklund et al. 2000); selection for such a marker has the potential to improve the reduction in frequency of osteochondrosis (Pieramati et al. 2003). Divergent selection has been successful in producing lines of poultry that differ in incidence of tibial dyschondroplasia (Wong-Valle et al. 1993a; Wong-Valle et al. 1993b).

1.5.2.2 Trauma and exercise

Biomechanical trauma may contribute to the pathogenesis of osteochondrosis, and is the most widely proposed factor for its development in all species, although it is unlikely it is the sole cause (Ekman and Carlson, 1998). Direct mechanical trauma may result in osteochondral necrosis and fragmentation of cartilage. However, it is difficult to differentiate lesions resulting from excessive mechanical trauma on normal cartilage from those resulting from normal mechanical trauma on structurally weakened cartilage (Pool, 1993). Repeated microtrauma may be an important factor in lesion prevalence at predilection sites (Olsson, 1987). Cartilage is thicker in direct weight-bearing surfaces, where mechanical forces are high, and may be more predisposed to shear forces (Pool, 1993).

In immature cartilage, endothelium lined cartilage canals permit blood flow from the metaphyseal and epiphyseal circulation to epiphyseal and articular cartilage (Hurtig and Pool, 1996). It is proposed that in pigs and horses mechanical shearing of cartilage canals at sites where biomechanical forces are high, such as the lateral trochlear ridge of the femur, may result in osteochondrosis (Hurtig and Pool, 1996) and subsequently local ischaemia. Cartilage canals are absent from the articular epiphyseal cartilage of the distal tibia and the femoral condyles by 3 and 5 months of age respectively (Carlson et al. 1995), indicating osteochondrosis could be initiated before chondrification but not after.

Housing animals on hard flooring increases the prevalence and severity of osteochondrosis in boars and cattle (Murphy et al. 1975; Perrin et al. 1978; White et al. 1984). Loading and unloading pigs, including dropping them from a height of less than 1 metre, resulted in a high incidence of osteochondrosis, compared to pigs that had not been loaded and unloaded (Nakano and Aherne, 1988). Many early osteochondrosis lesions heal (Hill et al. 1984; Carlson et al. 1988), so avoidance of trauma during the vulnerable timeframe could reduce the likelihood of clinically evident disease (Ekman and Carlson, 1998).

There is very limited evidence that enforced exercise may have a protective effect on the development of osteochondrosis in young horses fed high energy diets, but exercise is detrimental if lesions were already present (Bruin and Creemers, 1994). A large study by van Weeren and Barneveld (1999) found that enforced exercise did not have an aetiological role in the development of osteochondrosis in genetically predisposed Warmblood foals, but may have influenced the appearance and distribution of osteochondrosis lesions.

It is speculated that obesity may increase the probability of rats developing cartilage disorders, probably as a result on increased mechanical stress on growth cartilage (Kember and Walker, 1971), but studies in other species have not found a statistical relationship between fat content or body mass and cartilage disorders. Studies in pigs fed repartitioning agents have shown no difference in the frequency or severity of cartilage abnormalities between

animals fed repartitioning agents, resulting in decreased fat mass and increased lean mass, compared to control animals with greater fat mass (Hill and Dalrymple, 1987; He et al. 1993).

1.5.2.3 Ischaemia

Ischaemia of cartilage or subchondral bone results in osteochondral lesions in pigs (Carlson et al. 1989). Experimental occlusion of cartilage canals resulted in osteochondrosis of the epiphyseal cartilage (Carlson et al. 1991). Similarly, surgical interruption of the metaphyseal blood supply resulted in osteochondrosis lesions in the growth plate (Trueta and Amato, 1960; Riddell, 1975).

Normally cartilage canals disappear through chondrification, a process whereby the lumen narrows and become filled with cartilage (Lufti, 1970), or constricted by cartilage. Histologically evident osteochondrosis lesions have been reported in association with necrotic cartilage canals, and it is hypothesised that abnormal cartilage canals result in ischaemia and chondronecrosis in pigs and horses (Kincaid et al. 1985; Woodard et al. 1987a; Carlson et al. 1995). Ekman and Carlson (1998) propose an unknown vascular aetiology of osteochondrosis could explain why lesions occur at multiple predilection sites in pigs, and why they are often bilaterally symmetrical. They further hypothesise that an unidentified factor related to rapid growth may initiate vascular necrosis. However, in horses there is reported to be little association between rapid growth and osteochondrosis (Glade et al. 1981; Hoppe, 1984; Glade and Belling, 1984), although recently associations have been reported between rapid growth rate and osteochondrosis in Warmblood foals (van Weeren et al. 1999; Firth et al. 1999). Also, although osteochondrosis is often bilateral in horses, involvement at multiple sites is uncommon (Pool, 1993; Hurtig and Pool, 1996).

Cartilage canals persist as arcades or short vessel complexes until epiphyseal bone growth occupies almost all of the epiphyseal cartilage (Firth and Poulos, 1993). The vessels remain for different times in different bone ends, and within sites in each bone end. The temporary presence of the vessels has been

suggested to be the reason why subchondral epiphyseal osteomyelitis (E-type infection, Firth et al. 1980) has a specific site and age localisation. Vessels in the thickest cartilage remain open the longest (Firth and Goedegebuure, 1988). The presence of open vessels in epiphyseal cartilage may account for 'windows of susceptibility' to development of osteochondrosis, if vascular damage or pathology is responsible for cartilage retention, typical of articular osteochondrosis.

1.5.2.4 Endocrine influences

Nutritionally induced effects on cartilage growth may be mediated by the endocrine system (Glade and Belling, 1986). It is hypothesised that postprandial hyperglycaemia and hyperinsulinaemia induced by high grain intake may cause changes in T_4 and growth hormone release, and result in osteochondrosis (Glade and Belling, 1986; Glade, 1986). Ralston (1996) demonstrated in a small number of horses that postprandial hyperglycaemia and hyperinsulinaemia after a grain meal were higher in horses with osteochondrosis, compared to horses without osteochondrosis, supporting the hypotheses of Glade and Belling (1986) and Glade (1986). In Warmblood foals, IGF-1 activity was significantly lower for osteochondrosis positive foals compared to unaffected foals (van Weeren et al. 1999). IGF-1 promotes chondrocyte differentiation in culture (Henson et al. 1997a), and it was proposed that reduced IGF-1 concentrations might contribute to the development of osteochondrosis (Sloet van Oldruitenborgh-Oosterbaan et al. 1999).

Prolonged treatment with glucocorticoids negatively affects cartilage and bone, may induce parathyroid hormone resistance (Glade et al. 1982), and causes a systemic shift to catabolism through reduced efficiency of nutrient utilisation, resulting in decreased weight gain and bone growth (Glade et al. 1981). Dexamethasone given to pony foals for 3 months or longer resulted in osteochondrosis, and degenerative joint disease (Glade et al. 1980; Glade et al. 1983). Such a chronic administration of exogenous corticosteroids indicated wide difference from the pathogenesis of naturally occurring osteochondrosis.

1.5.2.5 Nutrition

Dietary energy and protein

Rapid growth rates have been correlated with the frequency and severity of osteochondrosis (Olsson and Reiland, 1978). The rapid growth achieved is a combination of the genetic ability of the animal to grow, and the nutritional supplementation for growth. Breeds that have not been selected for rapid growth, such as the wild European hog, show a low incidence of osteochondrosis (Reiland, 1978a).

Strong associations exist between rapid growth rates, high energy feed intake and osteochondrosis in broiler chickens and pigs (Olsson and Reiland, 1978; Savage et al. 1993a; Hurtig and Pool, 1996). Reducing dietary energy in pigs reduced the severity and frequency of osteochondrosis (Reiland, 1978a; Carlson et al. 1988). However, other studies have indicated no difference in severity or frequency of osteochondrosis when growth rate was restricted (Grondalen, 1974a; Grondalen, 1974b; Nakano et al. 1984).

The role of dietary energy as a predisposing factor for osteochondrosis in cattle is not well understood (Trostle et al. 1998). Swedish bulls fed high energy and protein diets from 100 kg liveweight to maturity had a higher frequency and severity of osteochondrosis lesions at slaughter than bulls fed low energy and protein diets (Reiland et al. 1978). However, the growth rates achieved in that study were low in comparison to cattle in commercial environments in America, which reportedly have a low incidence of osteochondrosis (Trostle et al. 1998).

Excess dietary energy (129 percent above NRC, 1989 recommendations) in the young horse appears to be a reliable model to induce skeletal aberrations such as osteochondrosis at post-mortem examination and histologically, but curiously was not associated with rapid weight gain (Savage et al. 1993a). Similarly Glade and Belling (1984) were able to induce osteochondrosis, assessed histologically in distal radial physeal biopsies, by providing a high energy and protein diets for 8 months (130% above NRC, 1989 recommendations), but there was no association between osteochondrosis and growth rate. Excess

dietary energy has also been associated with clinically evident physitis and flexural deformities in young horses (Thompson et al. 1988; Cymbaluk et al. 1990). Overall, there is little evidence to support an association between rapid growth rates and DOD in the horse (Sandgren et al. 1993; Pagan and Jackson, 1996). However, rapid growth rates and larger sized animals are often cited as contributing factors to development of DOD (Stromberg, 1979; Fischer and Barclay, 1984; Lewis, 1995; Hurtig and Pool, 1996; Jeffcott, 1997). Recent studies in Warmblood foals, genetically predisposed to osteochondrosis, have reported associations between osteochondrosis and rapid growth rate in the third month of life (Firth et al. 1999) or from birth to 11 months of age (van Weeren et al. 1999).

Overnutrition of Great Dane pups with respect to energy, protein, calcium and phosphorus resulted in increased frequencies of skeletal abnormalities such as osteochondrosis and hip dysplasia (Hedhammer et al. 1974; Kasstrom, 1975). Excess dietary protein did not result in skeletal abnormalities in horses (Schryver et al. 1987; Savage et al. 1993a), or pigs (Reiland, 1978b; Woodard et al. 1987b).

The source of excess dietary energy may be important in the pathogenesis of osteochondrosis. Ralston (1996) reported that young horses fed diets high in soluble carbohydrate had postprandial hyperinsulinaemia/hyperglycaemia and a higher than normal incidence of osteochondrosis. The association between postprandial hyperinsulinaemia/hyperglycaemia of feeds with different glycaemic indices and osteochondrosis in the horse has not been investigated further.

Calcium, phosphorus and vitamin D

High levels of dietary calcium fed to pregnant ewes resulted in retardation of cartilage differentiation in their foetuses, evidenced by retained cartilage in the proximal humeral epiphysis and metaphysis (Corbellini et al. 1991). It has been proposed that calcium oversupplementation is the main causative factor in the development of osteochondrosis in the dog (Hazewinkel et al. 1985;

Hazewinkel et al. 1991), although the calcium to phosphorus ratio is of little importance (Hazewinkel et al. 1991).

There is little evidence of deleterious effects of excessive dietary calcium in the horse. There were no reported detrimental effects of feeding calcium at five times the requirement when adequate phosphorus was provided (Jordan et al. 1975). Feeding calcium at approximately three times NRC (1989) recommendations had no effect on the frequency or severity of dyschondroplasia/osteochondrosis lesions in weanling foals (Savage et al. 1993b). However, Krook and Maylin (1988) considered that high levels of dietary calcium were a risk factor for osteochondrosis lesions associated with catastrophic racetrack fractures.

An epidemiological study on farms in Ohio and Kentucky identified that the lowest dietary calcium was recorded on farms with highest incidence and severity of developmental orthopaedic disease, based on clinical examination (Knight et al. 1985). Ration evaluation after dietary correction revealed an increase of dietary calcium to nearly twice the recommended level, and a decrease in the incidence of DOD in the following year (Gabel et al. 1987).

Excessive dietary phosphorus (388% above NRC, 1989 requirements) resulted in a greater frequency and severity of osteochondrosis lesions in foals compared to those fed normal or high calcium diets (Savage et al. 1993b), but foals receiving high phosphorus diets did not develop nutritional secondary hyperparathyroidism.

The fat-soluble D vitamins originate from plant or animal sources, and act to maintain plasma calcium concentration (Lewis, 1995). Deficiency can result in rickets in the young, and osteomalacia in the adult, but naturally occurring cases or experimentally induced disease has not been conclusively reported in the horse (Lewis, 1995). Vitamin D is synthesised in the skin in response to exposure to daylight, so deficiency is unlikely to occur in the horse.

Copper, zinc and molybdenum

Associations between primary and secondary copper deficiency and osteochondrosis have been reported in sheep (Suttle et al. 1972), cattle (Smith et al. 1975a; Suttle and Angus, 1978; Woodbury et al. 1999), deer (Thompson et al. 1994; Audige et al. 1995) and pigs (Pond et al. 1990). Primary copper deficiency occurs when dietary intake of copper is low, while secondary copper deficiency occurs when dietary copper intake is adequate, but elevated dietary levels of other mineral antagonists impede copper uptake or utilisation. In ruminants, elevated dietary levels of molybdenum, and/or sulphur, may induce secondary copper deficiency. Other divalent heavy metal cations such as cadmium can also compete with copper at the site of intestinal absorption (Freking et al. 1998). Primary and secondary copper deficiency has been implicated as a predisposing factor in the development of DOD in the horse, particularly for osteochondrosis and physitis.

Primary copper deficiency was experimentally induced in foals fed milk replacer containing 1.7 mg Cu kg^{-1} DM and 154 mg Zn kg^{-1} DM (Bridges and Harris, 1988), resulting in severe, generalised osteochondrosis with microfractures of articular and physeal cartilage. It was hypothesised that copper deficiency resulted in defective function of lysyl oxidase, a metalloenzyme enzyme necessary for crosslinking collagen for which copper is an essential cofactor, based on an increase in aortic and articular cartilage collagen solubility of copper deficient foals. Chickens require little dietary copper to maintain normal connective tissue synthesis and cross link formation, even when tissue lysyl oxidase activity is reduced (Tinker and Rucker, 1985). Despite the extremely low level of dietary copper in the study of Bridges and Harris (1988), which is impossible to achieve in nature, clinical signs took many months to become apparent, and the high glucose diet may have further reduced copper availability (Cymbaluk and Smart, 1993). There are no definitive reports on the copper concentration at which lysyl oxidase activity is impaired in mammals, although in chickens decreased collagen cross-linking does not occur until dietary copper is substantially less than 1 mg kg^{-1} diet DM (Rucker et al. 1998). Chickens normally require 5 to 10 mg Cu kg^{-1} diet DM for optimal

growth. Copper deficiency results in reduced lysyl oxidase activity but not lysyl oxidase protein concentration in the rat (Rucker et al. 1996).

Skeletal abnormalities have been reported in horses in association with excessive dietary zinc, presumably inducing secondary copper deficiency. Severe skeletal abnormalities were observed clinically in foals fed high dietary zinc (3600 mg kg^{-1} diet DM) (Willoughby et al. 1972). Eamens et al. (1984) and Gunson et al. (1982) reported skeletal abnormalities including generalised osteochondrosis in horses grazing pastures contaminated by zinc from local smelters and industrial plants. Chronic zinc intoxication was associated with tibiotarsal effusion in three horses, and radiographic evidence of osteochondritis dissecans (Messer, 1981). Chronic cadmium toxicosis may also result in secondary copper deficiency in horses and cattle (Swerczek, 1997; Smith, 1998), and generalised osteochondrosis (Kowalczyk et al. 1986), possibly concurrent with secondary zinc deficiency (Swerczek, 1997). Zinc induced copper deficiency was implicated in the development of osteochondrosis in unweaned foals (Bridges et al. 1984). Bridges and Moffitt (1990) fed high zinc diets (1000 to 2000 mg kg^{-1} DM) to foals for 14 weeks, resulting in lameness within weeks, and at post-mortem osteochondrosis and articular and physeal cartilage fractures were noted. It was concluded that high dietary zinc induced secondary copper deficiency. However, in experiments involving young and mature ponies, high dietary zinc concentrations did not alter copper absorption (Coger et al. 1987; Young et al. 1987).

Mercury, cadmium, or lead excesses can also result in skeletal lesions in horses (Casteel, 2001).

The association between naturally occurring osteochondrosis and osteochondrosis induced experimentally by severe copper deficiency has been questioned (Hurtig and Pool, 1996). Experimentally induced copper deficiency results in tibial dyschondroplasia in rapidly growing chickens and turkeys (Leach and Gay, 1987), but the naturally occurring disease does not respond to copper supplementation (Kronfeld et al. 1990). Dyschondroplastic cartilage from copper deficient birds has a very different cross-linking pattern from

dyschondroplastic cartilage of birds with genetically induced tibial dyschondroplasia (Orth et al. 1994). This supports the hypothesis that development and elaboration of tibia dyschondroplasia can be induced by a number of very different mechanisms, perhaps relating to the age of the animal (Lilburn et al. 1989). It is highly probable that this holds true for osteochondrosis in mammals.

Epidemiological studies in America and Japan have reported negative associations between dietary copper and perceived incidence and severity of DOD, in particular physitis (Knight et al. 1985; Fujikawa et al. 1993; Asai et al. 1993). However, Knight et al. (1985) adjusted dietary copper for molybdenum, although there is little evidence that molybdenum affects the absorption or utilisation of dietary copper in horses (Cymbaluk et al. 1981b; Strickland et al. 1987; Pearce et al. 1999; Rieker et al. 2000). Kronfeld et al. (1990) claimed that removal of two outlier values of copper in the data of Knight et al. (1985) resulted in the correlation between dietary copper and observed DOD becoming non-significant. After dietary evaluation and correction of 17 farms in Kentucky and Ohio the dietary copper concentration increased by 53 % to 28 mg kg^{-1} DM, and clinically assessed severity of DOD decreased (Gabel et al. 1987). However, the dietary correction had also resulted in large changes in the dietary concentration of calcium, phosphorus, zinc and iron, and dietary energy intake also decreased.

Clinical reports have shown indicated that increasing dietary copper may alleviate or promote resolution of DOD in single animals, in particular physitis. Carbery (1978) in New Zealand and Egan and Murrin (1973b) in Ireland reported painful physeal enlargements improved and resolved after injection of calcium copper edetate. Hildebran et al. (1986) reported similar results in a foal with physeal enlargements and tendon contracture that was supplemented with copper orally. All of these clinical reports used treatments that altered more than one factor, so the role of copper supplementation in resolution of the clinical signs is unclear.

Several studies have examined the influence of dietary copper levels on development of bone and cartilage abnormalities. Cupps and Howell (1949) investigated the effects of feeding supplemental copper to foals. A control animal fed 8 mg Cu kg^{-1} DM had eroded articular cartilage at multiple sites when euthanased while a foal fed 100 mg Cu kg^{-1} DM had only one articular cartilage lesion observed. As only 2 animals were examined at post-mortem, and no histological examination took place, no conclusions could be made about the relationship between dietary copper and cartilage lesions. However, as the foals were assumed to show normal growth, the control diet copper concentration was considered satisfactory to meet requirements of growing foals. Cymbaluk and Smart (1993) noted that the foals in the trial of Cupps and Howell (1949) were also supplemented with vitamin A during the trial, which, if excessive, can result in osteochondrosis-like lesions (Donoghue et al. 1981).

Foals fed diets containing 25 mg Cu kg^{-1} DM for six months had less evidence of DOD than foals fed diets containing 8 mg Cu kg^{-1} DM (Hurtig et al. 1993). However, some of the foals in the lower copper group had evidence of DOD at the start of the trial, at 3 months of age. Severely affected foals from the lower copper group had reduced collagen cross-linking in cartilage, and the authors proposed an association between low copper intake in rapidly growing horses, inferior collagen quality, biomechanically weakened cartilage and osteochondrosis.

Knight et al. (1990) examined the effects of dam copper supplementation during last 3 to 6 months of gestation, and in the first three months of lactation, and foal copper supplementation from birth on the prevalence of cartilage lesions at 90 or 180 days of age. Mares were fed rations containing 13 or 32 mg Cu kg^{-1} DM, and foals were fed rations containing 15 or 55 mg Cu kg^{-1} DM. Statistical comparisons were not made, but foals fed higher levels of copper had more osteochondrosis lesions at 90 and 180 days of age. The prevalence of lesions was considered to be low. The effects of mare and foal dietary copper level could not be separated, and the authors concluded that daily consumption of an additional 235 mg of copper by mares, and 100 mg copper by their foals appeared to reduce the severity and number of cartilage lesions in foals.

The experimental design of Pearce et al. (1998a) allowed the effects of mare and foal copper supplementation to be examined separately. Mares were kept at pasture containing 4.4 to 8.6 mg kg^{-1} DM and were either treated thrice-weekly with oral copper sulphate, equivalent to 0.5 mg Cu kg^{-1} liveweight each day or 30 mg kg^{-1} DM for the last 13 to 25 weeks of gestation, or were not supplemented with copper. Foals were randomly allocated to copper supplemented and control groups, with supplemented foals receiving 0.2 mg Cu kg^{-1} liveweight each day from 3 weeks of age, increasing to 0.5 mg Cu kg^{-1} liveweight each day from 7 weeks of age. Supplementation of mares in late gestation with oral copper was found to have a protective effect on the severity of third metatarsal bone physitis assessed by whole bone radiographs and frequency of gross articular cartilage lesions at post-mortem in foals at 5 months of age. However, there was no effect of copper supplementation of the foal on the severity of metatarsal physitis determined radiographically and frequency of articular cartilage lesions at 5 months of age. It was concluded that mare copper supplementation during late gestation may be useful to prevent DOD in foals at 5 months of age, but copper supplementation would not prevent all DOD, highlighting the multifactorial nature of the condition. None of the foals developed clinical signs of DOD, and at post-mortem the incidence and severity of lesions was considered low, with 1.6 and 3.3 lesions per foal from copper supplemented and unsupplemented dams respectively. There were no cervical vertebral lesions found in the foals of Pearce et al. (1998a), in contrast to the studies of Knight et al. (1990); Hurtig et al. (1993); and van Weeren and Barneveld (1999), who reported a high number of cervical vertebral osteochondrosis lesions and suggested this was a predisposition site for copper responsive lesions.

The NRC (1989) recommends 10 mg Cu kg^{-1} DM for all classes of horses. Cymbaluk et al. (1981a) and Pagan (1994) calculated the daily maintenance copper requirement for mature ponies and horses as 3.5 and 7 mg Cu kg^{-1} DM day^{-1} respectively. Based on the results of copper supplementation studies, and clinical and anecdotal reports linking apparent copper deficiency and DOD, several authors have questioned the adequacy of NRC (1989) recommendations. Knight et al. (1990) and Cymbaluk and Smart (1993) recommended diets for

growing animals and 'susceptible breeds' include 20 to 25mg Cu kg^{-1} DM, while Lewis (1995) recommended diets for unweaned and weaned horses include 50 and 25 mg Cu kg^{-1} DM respectively.

Despite widespread adoption of dietary copper supplementation at rates in excess of NRC (1989) recommendations in the last 15 years, the incidence of DOD is perceived to be increasing (Jeffcott, 1997), and copper deficiency in foals is still perceived to be a common occurrence (Jeffcott and Davies, 1998). It is curious that there are aspects of copper deficiency in horses that are very different to copper deficiency of other species. In most species, bone lesions associated with copper deficiency occur after growth delay and vascular abnormalities (Tinker and Rucker, 1985), but growth delay and vascular abnormalities have not been reported in the horse. The histopathology of bone lesions vary with species, age and the aetiology of the copper deficiency (Cymbaluk and Smart, 1993; Underwood and Suttle, 1999). Typically bones from copper deficient animals may have thinner cortices and wide epiphyses than bones from normal animals, and primary copper deficiency frequently results in osteoporotic lesions in humans (Allen et al. 1982), ruminants (Suttle et al. 1972; Suttle and Angus, 1976) and dogs (Baxter and Van Wyk, 1953). However, there are no reports in the literature of such lesions in horses with suspected copper deficiency.

There is no consensus on what molecular mechanisms are involved in the development of cartilage lesions in copper deficient animals (Jeffcott and Davies, 1998). It is unlikely to be a single biochemical lesion affecting the bone and cartilage of copper derived animals (Underwood and Suttle, 1999). Much remains to be elucidated about the role of copper in DOD of horses.

1.6 Copper

In the recent years, much attention has been focussed on the association between copper and DOD in horses, particularly osteochondrosis. Copper nutrition will be reviewed in this section.

Copper is essential for the growth and development of all organisms (Kim et al. 2002), and deficiency can result in skeletal abnormalities in many species, including the horse (Bridges and Harris, 1988). Copper is a transitional metal, widely distributed in nature, with an atomic mass of 63.54. In biological systems it is present in the + 1 and + 2 valence states, with the latter more common. The major functions of copper metalloproteinases involve oxidation-reduction reactions. Copper is also an integral part of many metalloenzymes, and a number of pathological states result from loss of cuproenzyme activity (Section 1.6.5, page 94). Although copper is involved in various biologic functions, it must be properly sequestered to avoid toxicosis (Brewer, 1987).

1.6.1 Copper metabolism

In many monogastric species, including humans and horses, there is relatively little information available on copper requirements and metabolism, with much information extrapolated from studies in laboratory animals, ruminants and humans.

1.6.1.1 Absorption

Most copper absorption occurs in the small intestine of monogastric animals, while in ruminants the large intestine is of greater significance (Gooneratne et al. 1989). The precise mechanism of absorption in adults is not clearly understood, but there is a passive and unsaturable component, and an active and saturable component (Bronner and Yost, 1985). Firstly, copper crosses the brush border into the cells of the intestinal mucosa, which is probably by non-mediated diffusion, the copper is transferred across the serosa for entry into the blood which is mediated by an energy dependant saturable carrier (Linder and

Hazegh-Azam, 1996). Variable amounts of copper diffuse into mucosal cells, and some copper is retained in the cells by proteins such as metallothionein (Linder, 1991). Copper retained in metallothionein in enterocytes is passed in the faeces after enterocytes slough.

The rate of copper absorption varies inversely with copper intake (Turnlund et al. 1985; Turnlund et al. 1989). In humans, a theoretical maximum absorptive capacity of 67% has been calculated, although with typical diets the average true absorption is 30 to 40% (Wapnir, 1998).

Age has a profound effect on copper absorption. All newborns can absorb large proteinaceous complexes by pinocytosis, so the copper contained in colostrum is readily absorbed (Underwood and Suttle, 1999). Milk from most species is low in copper (Lonnerdal et al. 1981), but it has high bioavailability in all young animals (Wapnir, 1998). For example, milk-fed lambs can absorb up to 85% of the copper ingested, while weaned lambs absorb less than 10% (Suttle, 1974). Similarly, oral copper supplementation at a rate equivalent of 30 mg Cu kg^{-1} DM resulted in increased absorption and storage of copper in the livers of unweaned foals, but not in dams (Pearce et al. 1998b; Pearce et al. 1998c). In Thoroughbred mares the mean (± standard error) copper concentration in colostrum 0.76 ± 0.82 mg L^{-1} compared to 0.19 mg L^{-1} in milk from 55 to 150 days (Grace et al. 1999a).

Antagonists that compete for common transport mechanisms reduce copper absorption, and may include divalent cations such as zinc, iron or calcium, dietary components that form insoluble ligands with copper such as phytate, or by dietary anatagonists that induce copper binding ligands such as metallothionein induction by zinc or cadmium (Harris, 1991; Cymbaluk and Smart, 1993). Metallothionein thus retards the passage of copper from the mucosa into the blood, and it is eventually lost in the faeces. The relative importance of antagonists is species dependant, so care must be taken in extrapolation of research on copper antagonists from one species to another. For example, although molybdenum is a major copper antagonist in cattle and sheep

it is not a copper antagonist in horses (Strickland et al. 1987; Pearce et al. 1999; Underwood and Suttle, 1999; Rieker et al. 2000).

1.6.1.2 Transport

After intestinal absorption copper rapidly enters the blood and is transported to the liver and kidney primarily by plasma protein carriers such as albumin, and transcuprein, the primary components of the exchangeable plasma copper pool (Linder and Hazegh-Azam, 1996). A very small amount of absorbed copper is bound to small peptides and amino acids (Linder, 1991), and other proteins for transportation may exist (Harris, 1991). Under normal dietary conditions most copper entering the liver and kidney from the diet re-emerges in the plasma incorporated in caeruloplasmin to be transported to other tissues (Linder et al. 1998). It may also be transported in association with albumin and transcuprein in the post-hepatic circulation (Luza and Speisky, 1996). Caeruloplasmin copper is not part of the exchangeable plasma copper pool, and does not directly bind or incorporate the metal when exposed to copper ions (Linder and Hazegh-Azam, 1996), but allows delivery of copper to tissues for uptake.

1.6.1.3 Storage

The liver is the main organ of copper storage and homeostasis. In the liver newly absorbed copper is incorporated into several different compartments, including endogenous copper enzymes, copper requiring proteins, and bile in the liver (Linder et al. 1998). The mechanism by which copper enters into hepatocytes from transcuprein and albumin has not been studied adequately (Linder and Hazegh-Azam, 1996). Metallothionein is the major copper binding protein in the liver, but there are marked species differences in the ability to incorporate copper into metallothionein, with the dog and pig particularly effective (Bremner, 1991).

Diets high in copper can cause marked increases in liver copper concentration, but most non-ruminant species contain between 10 and 50 mg Cu kg^{-1} liver DM (Beck, 1956; Davis and Mertz, 1987). Sheep and cattle appear to have a

superior ability to store copper in the liver, with a normal range of 100 to 400 mg Cu kg^{-1} DM (Davis and Mertz, 1987), but may also have a lower capacity for excretion than other species. Sex differences in liver copper values have been reported only in salmon (Beck, 1956).

There are significant age effects on liver copper storage. The copper concentration of the foetus increases during gestation, but decreases in the maternal liver in sheep and cattle (Gooneratne and Christensen, 1989). In most species liver copper concentrations are higher in newborn animals adults, although foetal liver copper accumulation may not particularly marked in sheep (Gooneratne et al. 1989). Human babies have liver copper concentrations six to eight times greater than adults (Klein et al. 1991), while foals may have more than ten times the concentration of copper in the liver of adult horses (Pearce et al. 1998c). In newborn ruminants, a store of 300 mg Cu kg^{-1} liver DM at birth is considered the critical minimum (Gooneratne and Christensen, 1989). As most copper accumulates in the last trimester of pregnancy, gestational age has an important effect on liver copper stores of neonates. Premature human infants at risk of copper deficiency due to reduced copper stores at birth, and higher requirements due to growth rate (Widdowson, 1974; Olivares and Uauy, 1996).

Various suggestions have been made to account for the high liver copper concentration in neonates, and the rapid decline during the suckling period. Accumulation of copper in metallothionein in the liver of the foetus may act as a store for cysteine during the neonatal period (Andrews et al. 1987). The rapid decline in hepatic copper concentration during the suckling period may be due to the combined influence of the redistribution of hepatic copper to other organs, the postnatal growth spurt, and the relatively low copper concentration of milk (Luza and Speisky, 1996). The high levels of liver copper at birth may be used for cupro-protein synthesis, but the newborn must also obtain copper from the milk (McArdle, 1995). Copper may accumulate in the foetal liver because the excretion mechanism in bile is not patent during intrauterine life (Cymbaluk and Smart, 1993; McArdle, 1995; Beshgetoor and Hambidge, 1998).

1.6.1.4 Excretion

A large proportion of dietary copper is not absorbed, and is excreted in the faeces. Active excretion of absorbed copper occurs in the bile, and is important in the homeostatic control of copper retention. Endogenous copper excretion increases when dietary copper is high, and decreases when dietary copper is reduced (Turnlund et al. 1998). The mechanism of copper transport from tissues to the liver for biliary excretion is unclear (Linder and Hazegh-Azam, 1996).

Copper is also released into the digestive tract with saliva, as well as gastric, intestinal and pancreatic secretions, of which most is reabsorbed (Linder and Hazegh-Azam, 1996).

Urinary excretion of copper is small and constant, and generally unaffected by copper intake in all species (Underwood and Suttle, 1999). In surgically cannulated ponies 1 to 2 % of absorbed copper was excreted in urine, compared to 50% of absorbed copper excreted in the bile (Cymbaluk et al. 1981a).

1.6.1.5 Maternal-foetal copper metabolism

The essentiality of copper in foetal development is well established (Hidiroglou and Knipfel, 1981). The foetus is fully dependant on the maternal copper supply, and pregnancy may be associated with increased maternal copper retention, due in part to decreased biliary excretion induced by hormonal changes (Mason, 1979), and increased absorption (Linder, 1991). Serum copper and caeruloplasmin concentration rise significantly in women during pregnancy (McArdle, 1995), but the rise in caeruloplasmin may be related to its role as an acute phase protein. Caeruloplasmin is likely to be the major source of copper for transfer to the foetus (Linder, 1991), although in the placenta the mechanism for transfer of copper remains a mystery (McArdle, 1995).

Most of the copper in the foetal liver is associated with metallothionein (Andrews et al. 1987). The subcellular distribution of copper in livers of

foetuses and newborns differ from adults. In newborns, 80% of liver copper is associated with the lysosome-rich fractions, which decrease as liver copper concentration decreases postnatally (Luza and Speisky, 1996).

Dietary supplementation with copper during pregnancy can result in increased accumulation of copper in the liver of the foetus, as evidenced in sheep (Langlands et al. 1982; White et al. 1989), cattle (Allcroft and Uvarov, 1959) and horses (Pearce et al. 1998b). High concentrations of dietary copper antagonists during pregnancy, such as zinc, reduce foetal copper accumulation (Barone et al. 1998).

1.6.2 Altered copper metabolism

1.6.2.1 Genetic variation in copper metabolism

Genetic variation in copper metabolism is well recognised in sheep, probably due to differences in the efficiency of absorption by certain breeds (Gooneratne et al. 1989; Gooneratne et al. 1994), but may also involve partitioning of absorbed copper (Woolliams et al. 1982). For example, the Texel breed accumulates high concentrations of copper in the liver, and is more vulnerable to toxicity (Underwood and Suttle, 1999). In contrast, the Scottish Blackface breed does not accumulate to produce high liver copper concentrations, and when dietary copper is low the breed are more vulnerable to copper deficiency (Woolliams et al. 1986). No genetic differences in copper absorption by cattle have been reported, but there are breed differences in biliary copper excretion (Gooneratne et al. 1987; Gooneratne et al. 1994). Breed variations in plasma copper concentration have been reported in pigs (Reetz et al. 1975) and cattle (Wiener et al. 1980; Rowlands et al. 1983).

1.6.2.2 Menkes disease and Wilson's disease in humans

Two autosomal recessive conditions in humans result in abnormal copper metabolism. Both diseases involve faulty copper transporting membrane proteins. In Menkes disease, an X-linked autosomal recessive disease, the

copper transporting protein ATPase7A is defective. This results in copper accumulation in enterocytes and other tissues, with the notable exceptions of the brain and liver, which have little or no expression of the APTase7A gene (Vulpe et al. 1993). There is reduced copper transport from intestinal enterocytes into the bloodstream, and reduced copper transport across the blood brain barrier to the central nervous system (Kim et al. 2002). The clinical features observed in Menkes patients result from a deficiency of copper and its effects on the function of copper dependant enzymes (Bankier, 1995). Clinical symptoms include connective tissue abnormalities with deformities of the skull, long bones and ribs, including osteoporosis and flared metaphyses of long bones, as well as neurological degeneration, mental retardation and hypopigmentation (Kim et al. 2002). There is growth retardation and steely kinky hair (pili torti), due to defective disulphide bonding in keratin, as seen in copper deficient sheep. Depigmentation is presumably due to impaired tyrosinase activity, and parallels albinism. The disease is usually lethal in early childhood, but parenteral copper supplementation may reduce the neurological defects and prolong life expectancy (Kaler, 1998). There is a milder form of the disease known as occipital horn syndrome, which is primarily a connective tissue disorder (Mercer, 1998). Mottled mutant mice are used in animal models of Menkes disease (DiDonato and Sarkar, 1997), in particular brindled mice (Mercer, 1998).

Wilson's disease or hepatolenticular degeneration is an inherited autosomal recessive disease, in which ATPase7B is defective, which is essential for copper transport into the cytomembrane network of the hepatocyte. This results in decreased biliary excretion of copper and reduced caeruloplasmin production. Patients have low plasma caeruloplasmin and variable plasma copper, but increased concentrations of copper in the liver, kidney, brain and urine (Brewer, 1998). There is resultant liver inflammation and cirrhosis, and spongy degeneration of the central nervous system. Affected patients typically present with liver disease, neurologic symptoms or psychiatric disturbance (Brewer, 1998). Treatment may include zinc and tetrathiomolybdate to reduce intestinal copper absorption, and trientine or D-penicillamine for systemic chelation therapy (Brewer, 1998). Thiomolybdate complexes have been used in the initial

treatment of Wilson's disease, because thiomolybdates ingested with feedstuffs effectively scavenge dietary copper, which cannot be absorbed (Wapnir, 1998). High dietary sulphur and molybdenum in the diets of ruminants form thiomolybdates that react with copper, resulting in secondary copper deficiency (Grace, 1994).

The Long Evans Cinnamon rat, and toxic milk mutant mouse are used as animal models for investigation into Wilson's disease (DiDonato and Sarkar, 1997). Liver lesions similar to those seen in Wilson's disease have been reported in a calf (Wada et al. 1995).

1.6.2.3 Copper toxicity in dogs

A homozygous recessive disorder in Bedlington terriers leads to the accumulation high concentrations of copper in the liver, due to abnormal biliary excretion of copper. Up to 60% of the breed may be affected (Rolfe and Twedt, 1995). The defective gene has not been identified, but is not the same gene affected in humans with Wilson's disease (Brewer, 1998). When liver copper in a dog reaches 2000 mg kg^{-1} DM, multifocal hepatitis develops (Thornburg et al. 1990). Several other breeds of dogs are reported to accumulate very high concentrations of copper in the liver, which does not always lead to disease (Thornburg, 2000). The mode of inheritance for other breeds with copper toxicosis is not known (Thornburg et al. 1990).

1.6.3 Assessment of copper status

Assessment of copper status is essential to determine if an animal is in a state or copper deficiency, depletion, adequacy or toxicity. The definition of copper deficiency is somewhat controversial, but can be defined as a state in which any one or more of a criteria, such as enzyme function, are consistently and significantly impaired, and if the impairment is preventable by supplementation with physiological amounts of copper (Underwood and Mertz, 1987). An adequate copper status exists when tissue concentrations are sufficient to ensure

that biochemical and physiological function are not impaired, and supplementation does not improve performance or health of the animal.

Copper deficiency and toxicity occur in grazing animals in many parts of the world, and development of either syndrome depends on not only total dietary copper, but also other factors that influence the absorption and availability of copper (Gooneratne et al. 1989). Most trace element deficiencies are marginal rather than severe, without specific clinical signs, so there is a need to accurately diagnose the deficiency using biochemical criteria which have been established by relating the various tissue values to the performances of supplemented and unsupplemented animals (Grace, 1994). Much research has been conducted in ruminants, but indices of performance are more difficult to assess in other species such as companion animals and humans. Precise copper requirements have not been established in humans, partly because the mechanisms of normal copper metabolism and regulation are not understood, and partly because molecular and biochemical responses to copper restriction (particularly mild to moderate restriction) have not been described fully (Levenson, 1998).

1.6.3.1 Copper and caeruloplasmin in the blood

Caeruloplasmin, also known as ferroxidase I, is an α-2 glycoprotein which comprises about 90% of copper in the plasma or serum from rats or humans (Linder, 1991). In the horse caeruloplasmin is an α-1 glycoprotein in contrast to other species (Okumura et al. 1991) and comprises about 70% of the copper in the plasma (Auer et al. 1988). The remaining copper in the blood is bound less firmly to albumin, and amino acids to a lesser extent.

Caeruloplasmin is the main protein involved in copper transport, and is also a free radical scavenger, and catalyses oxidation of ferrous ion (2 + valency) to ferric ion (3 + valency) which can bind the iron transport protein transferrin. Dietary or genetic caeruloplasmin deficiency is accompanied by accumulation of iron in the liver (Linder and Hazegh-Azam, 1996). Caeruloplasmin is synthesised in and secreted almost entirely from the liver, but lung and

lymphocytes may also contribute (Linder, 1991). Caeruloplasmin contains six to eight copper atoms per molecule, but they are not directly available to binding substances, or exchangeable with copper ions (Linder, 1991; Linder et al. 1998).

Genetic polymorphisms of caeruloplasmin have been identified in many species, including the horse. Horse breeds identified with caeruloplasmin polymorphisms include the Icelandic Toelter, (Juneja et al. 1984), and several breeds from the Soviet Union and Europe (Kambegov and Lukash, 1982, cited by Schleger, 1974; Juneja et al. 1984), but caeruloplasmin polymorphisms were not detected in Thoroughbreds in New Zealand (Bolter, 1995). In Arab horses, serum copper concentration was apparently influenced by caeruloplasmin phenotype, with three phenotypes reported (Skripnichenko et al. 1980, cited by Auer et al. 1988). There are no other reports in the literature concerning the effect of caeruloplasmin polymorphism on copper metabolism in horses.

Plasma copper and caeruloplasmin levels (measured as the protein or as oxidase enzyme activity) are the most frequently used biochemical markers of copper status (Hambidge, 2003). In deficiency states they are depressed, but levels plateau when copper intake is adequate, and do not reflect the magnitude of copper intake beyond this point (Hambidge, 2003). Copper may be measured in whole blood, serum or plasma (Underwood and Suttle, 1999), as they are regarded as roughly equivalent (Solomons, 1979).

Caeruloplasmin is an acute phase protein, so is elevated during infections, inflammatory processes and other stress circumstances. For example, injection of the irritants such as oil of turpentine or Freud's adjuvant resulted in elevated serum copper and caeruloplasmin, and caeruloplasmin activity in ponies and horses (Smith and Cipriano, 1987; Auer et al. 1989). Oestrogens also influence caeruloplasmin synthesis, so caeruloplasmin is elevated during pregnancy and with use of oral contraceptives in humans (Fisher et al. 1990; DiSilvestro and Marten, 1990). Protein deficiency may reduce synthesis of caeruloplasmin and other hepatic proteins, resulting in reduced circulating levels of plasma copper and caeruloplasmin that are not the result of copper deficiency. Administration

of corticosteroids and adrenocorticotropic hormones reduce serum copper concentrations (Solomons, 1979).

Sex and age differences have also been noted in plasma copper and caeruloplasmin in adult humans (Johnson et al. 1992), as well as diurnal variation (Lifschitz and Henkin, 1971). Seasonal variations in blood copper values have been reported in horses (Stubley et al. 1983; Gromadzka-Ostrowska et al. 1985; Auer et al. 1988b) and were presumed to reflect alterations in dietary copper concentration. Age, breed and pregnancy related changes in plasma or serum copper have also been noted in horses (Cymbaluk et al. 1986; Auer et al. 1988a; Auer et al. 1988b). However, great individual variation exists in blood copper (Auer et al. 1988b).

Newborns of many species have low concentrations of serum copper (Amer et al. 1973; Keen et al. 1981), including the horse (Bell et al. 1987; Okumura et al. 1998; Pearce et al. 1998b). In many species adult values are reached in one month or more. Low serum copper at birth is due to delayed synthesis of caeruloplasmin by the body (Chang et al. 1976), as it is a maturation dependant process (Bell et al. 1987).

The lack of specificity of copper and caeruloplasmin in the blood, confounding factors including physiological factors that complicate the interpretation of data, and limited sensitivity, may diminish the value of these biomarkers to assess copper status (Hambidge, 2003). In normal Thoroughbred horses there was no relationship between caeruloplasmin and serum copper (Smith et al. 1983).

1.6.3.2 Erythrocyte Cu/Zn superoxide dismutase

Erythrocyte Cu/Zn superoxide dismutase (SOD) is a major antioxidant enzyme, catalysing the destruction of superoxide radicals. Around 60 % of copper in red blood cells is present as Cu/Zn SOD (Danks, 1988). Superoxide dismutase is produced at the same time of erythrocyte formation, so erythrocyte Cu/Zn SOD declines at a slower rate than plasma or serum copper during deficiency (Suttle and McMurray, 1983), and low values may confirm a prolonged deficiency

(Underwood and Suttle, 1999). Cu/Zn SOD may be a useful indicator of copper status in early life (Lonnerdal, 1998) and SOD levels or activity can serve as a measure of long-term copper nutrition (Sauberlich, 1999). Unlike serum or plasma copper, or caeruloplasmin, SOD does not seem to be affected by age, gender or hormone use in adult humans (Milne and Johnson, 1993). However, SOD may be affected by conditions other than copper status (DiSilvestro, 1988). Also, units of measurement for activity are not quantitative, and vary between methods (Underwood and Suttle, 1999). It is suggested that although SOD activity is reduced with severe dietary copper restriction, it offers no advantage over plasma copper because of a lack of adequate sensitivity (Hambidge, 2003). Others propose that as SOD activity is highly correlated with red cell copper, measurement of red cell copper is less problematical (Arthington et al. 1996). White blood cells also contain SOD, and may provide more information on recent copper intake and status as they have a shorter turnover rate than erythrocytes (Underwood and Suttle, 1999; Sauberlich, 1999).

1.6.3.3 Cytochrome c oxidase

Cytochrome *c* oxidase is the terminal electron acceptor in the mitochondrial respiratory chain, the cascade involved in oxidation of pyruvic acid to carbon dioxide and water (Brewer, 1987). Cytochrome *c* oxidase contains three copper atoms per molecule (Uauy et al. 1998), and its activity in platelets and leukocytes appears to offer a relatively sensitive biochemical marker (Milne, 1994). Cytochrome *c* oxidase is reported to be highly correlated with hepatic copper concentration (Milne, 1994). Cytochrome *c* oxidase and erythrocyte SOD activity appear to be more sensitive indicators of copper status than plasma copper concentrations or plasma caeruloplasmin concentrations (Sauberlich, 1999). However, there is wide subject-to-subject variability (Milne and Johnson, 1993) and more research is needed to determine guidelines for interpretation of results (Sauberlich, 1999). Erythocyte SOD activity may not be sensitive to small changes in copper status (Turnlund et al. 1990).

1.6.3.4 Lysyl oxidase

Lysyl oxidase is a cupro-protein enzyme involved in the post-translational modification of collagen and elastin. It is essential for stabilisation of extracellular matrixes, specifically the enzymatic crosslinking of collagen and elastin (Gacheru et al. 1990). The functional activity may be influenced by hormones, nutritional factors and factors that influence collagen and elastin formation (Rucker et al. 1998).

Lysyl oxidase is concentrated in connective tissues, such as skin and tendon, with relatively lower concentrations in the lung and aorta, and may account for 10% or more of tissue copper (Romero-Chapman et al. 1991). Lysyl oxidase may be important in the normal export of copper from connective tissue cells.

Bone and connective tissue fragility in copper deficient animals has been attributed to defective lysyl oxidase function. Bridges and Harris (1988) measured the fractional percentage of salt-soluble collagen (an index of collagen maturation) in the aorta and articular cartilage of copper deficient and control foals. The copper deficient foals had greatly increased fractional percentages of collagen, and it was suggested that failure of collagen to form insoluble intermolecular cross-links was due to defective function of lysyl oxidase, associated with copper deficiency. In chickens fed copper deficient diets decreased lysyl oxidase activity was accompanied by increased collagen solubility (Rucker et al. 1969). However, Bridges and Harris (1988) did not measure lysyl oxidase concentration or activity.

Maturation of collagen in skin and tendon is not as responsive to copper deficiency as aorta and lung (Reiser et al. 1992). Chou et al. (1969) found similar collagen skin solubility of copper deficient and copper supplemented chicks, but found significant differences in aorta collagen solubility. Researchers have found as much as a ten-fold response in functional activity of lysyl oxidase in response to varying dietary copper (Rayton and Harris, 1979; Opsahl et al. 1982). Solomons (1979) suggested cutaneous assays for lysyl oxidase may prove useful in assessment of copper status in humans. Weanling

rats fed low copper diets had lower values for skin lysyl oxidase functional activity but there was little apparent effect on the production of lysyl oxidase (Romero-Chapman et al. 1991; Rucker et al. 1996). Werman et al. (1997) assessed human male subjects fed varying levels of dietary copper, and determined that lysyl oxidase activity decreased when the copper intake was inadequate and the activity increased when the subjects were repleted. Lysyl oxidase levels or activity have not been assessed in horses fed different dietary copper levels, or in horses with suspected copper deficiency.

1.6.3.5 Other biochemical markers

Given the limitations of current tests available, some authors consider there are no suitable biochemical indices of copper status in humans (Olivares and Uauy, 1996). However, a number of biomarkers may be useful indicators of copper status in the future (Prohaska et al. 1997).

In vitro studies suggest the change in activity of peptidylglycine α-amidating monoxygenase (PAM) with the addition of copper has the potential as a relatively sensitive biomarker (Prohaska et al. 1997; Failla, 1999). Plasma diamine oxidase is a copper pyridoxal enzyme found in many tissues and fluids (Smith et al. 1983). Plasma diamine oxidase activity increases in copper supplemented normal humans (Kehoe et al. 2000b) and decreases in copper deficient rats (Kehoe et al. 2000a). However, no relationships between the activities of plasma diamine oxidase and serum copper concentrations were detected in Thoroughbred horses (Smith et al. 1983).

The essentiality of copper for the maturation and signal-mediated activity of immune cells may provide potential for the development of novel biomarkers of copper status (Failla and Hopkins, 1998).

1.6.3.6 Hair copper

Hair analysis has been advocated as a means of evaluating copper status, but doubt has been expressed as to its usefulness in humans and animals (Olivares

and Uauy, 1996; Hintz, 2001). Cunningham and Hogan (1958) found no relationship between copper levels in hair and copper levels in liver or diet. The concentration of hair copper can vary as the result of the action of external agents, including environmental contamination with copper (Danks, 1988), and it may vary in horses as the result of age, season and dexamethasone administration (Cape and Hintz, 1982). Effects of breed, sex, coat colour and location of body hair have been noted in other species (Cunningham and Hogan, 1958; Combs, 1987; Wells et al. 1990).

1.6.3.7 Hepatic copper

The main advantage of liver copper concentrations over other indicators for assessing copper status is the ability of the concentrations, with few exceptions, to indicate the level of copper reserves (Paynter, 1987). Liver copper concentrations are proportional to dietary availability over a relatively wide range in ruminants, but this is not apparent in monogastric species until dietary copper concentration is very high (Underwood, 1977). This effect is moderated by age, with younger animals being capable of absorbing and storing more copper. For example, supplementation with copper at 30 mg kg^{-1} DM for 15 weeks or longer increased liver copper concentration in foals, but not their dams (Pearce et al. 1998b; Pearce et al. 1998c). Diets containing 262 mg Cu kg^{-1} DM or more fed for 12 weeks elevated liver copper concentration in yearling ponies (Smith et al. 1975b). In ruminants there is a marked seasonal variation in liver copper reserves, with lowest levels in winter (Grace, 1994), often concurrent with reduced DM intake.

Liver copper concentration may be determined in the whole liver after death, or in a harvested liver biopsy sample, which is assumed to represent whole liver copper. Liver biopsies are routinely harvested from cattle and sheep to determine copper status (Chapman et al. 1963). However, the distribution of copper within the liver may vary. The coefficient of variation of copper concentration in horse liver may vary from 10 to 40% for horses of unknown age (O Cuill et al. 1970), 15 to 24% in adult mares, and 5 to 9% in feral horses of unknown age (Pearce et al. 1997).

Reference values for liver copper concentration have been established for cattle (Smart et al. 1992). A curvilinear relationship exists between liver and plasma copper concentration in cattle and deer (Vermunt and West, 1994), while a weak linear relationship has been suggested in adult horses (R^2 = 0.168 P = 0.006) (Suttle et al. 1996). However, others have found no relationship between serum and liver copper concentrations in horses (Cymbaluk and Christensen, 1986; Pearce et al. 1998b). The storage form and site within hepatocytes may affect the availability of copper in the liver. Determining the subcellular compartmentalisation of copper may assist in interpretation of liver copper concentration to assess copper status (Cymbaluk and Christensen, 1986; Suttle et al. 1996).

1.6.3.8 Dose-response studies

The response to copper supplementation in well-controlled studies may provide clues to inadequate copper intake in the absence of detectable abnormalities in traditional biomarkers (Hambidge, 2003). Dose response trials are the most accurate way of diagnosing a trace element deficiency, but are very costly.

1.6.4 Copper deficiency

Copper deficiency is the consistent and reproducible reduction of a biological function from normal to subnormal as a result of inadequate copper. In animals, copper deficiency may be the result of inadequate dietary intake or an excess of competing minerals such as molybdenum or zinc. Copper deficiency is uncommon or rare in monogastric species (Underwood and Suttle, 1999). Humans at risk for copper deficiency include infants and/or young children on prolonged intravenous nutrition, and premature infants due to reduced copper stores at birth (Levy et al. 1984).

1.6.5 Diseases associated with copper deficiency

1.6.5.1 Anaemia

Anaemia may develop when copper deficiency is severe or prolonged in all species, including humans (Suttle and Angus, 1978; Danks, 1988; Underwood and Suttle, 1999), but has not been reported in the horse. When blood copper reaches very low levels haemopoiesis cannot be sustained, and anaemia, indistinguishable from iron deficiency anaemia, develops.

1.6.5.2 Bone and cartilage disorders

Skeletal abnormalities are common in copper deficient animals, but unrelated to the concurrent anaemia (Underwood, 1977). Osteoporosis is a common feature of copper deficiency in many species, and is probably due to decreases lysyl oxidase activity, resulting in abnormal collagen cross-linking. In chronic clinical cases reduced bone mass may be associated with cachexia, reduced lean mass and inactivity. A marked reduction in lysyl (amine) oxidase activity occurs in the bones of copper deficient chicks, and collagen extracted from bones is more easily solublised than normal bone (Rucker et al. 1975), similar to the reduction of lysyl oxidase in elastin of the lungs (Harris, 1986). Bone strength was reduced in rats fed copper deficient diets for 6 weeks, despite increased bone collagen, suggesting an unsuccessful compensatory response by bone to copper as the limiting nutrient (Roughead and Lukaski, 2003). In this study low dietary copper also resulted in non-collagenous matrices of bone, and a reduction in systemic IGF-1. Several authors have reported the association of copper deficiency and skeletal lesions in horses as discussed in Section 1.5.2.5, page 72.

1.6.5.3 Cardiovascular disorders

Cardiac enlargement has been recorded in copper deficient cattle, and when copper deficiency is prolonged or severe the cardiac involvement may result in death (Fell, 1987). This has eloquently been referred to as 'falling disease' in cattle (Bennetts and Hall, 1935). Anatomical studies of several species of

copper deficient animals, including pigs, rats and mice, have revealed aortic fissures and ruptures, cardiac enlargement and rupture, coronary artery thrombosis and myocardial infarction (Klevay, 2000). It has been suggested that copper deficiency may play a role in ischaemic heart disease of humans (Klevay, 1973). In copper deficient states decreased activities of lysyl oxidase and superoxide dismutase may result in failure of collagen and elastin cross linking, and impaired defence against free radicals (Klevay, 2000). Cardiac abnormalities have not been reported in copper deficient horses, but an association between copper and rupture of the uterine artery in foaling mares has been made (Stowe, 1968) presumably as a consequence of weakened elastin around the major blood vessels.

1.6.5.4 Depigmentation of hair and wool

Copper-containing polyphenoloxidases are involved in the production of melanin from tyrosine. Depigmentation of wool, hair and feathers is considered a sensitive index of copper deficiency (Fell, 1987), especially in sheep and cattle (Underwood, 1977; Gooneratne et al. 1989). However, depigmentation is not a sign specific to copper deficiency, and can also occur in association with many vitamin deficiencies and cobolt deficiency, and when animals shed their winter coat (Underwood and Suttle, 1999). Visible coat changes are not observed in many cases of copper depletion or deficiency that respond to copper supplementation, so coat changes are not a sensitive index of copper status. In the horse, depigmentation has not been reported in the literature in association with copper deficiency. An Arab horse showing signs of the depigmentation syndrome "fading Arab syndrome" was reported to respond to oral copper supplementation (McLean and Jones, 1983).

In addition to depigmentation alterations in crimping of wool fibres can occur in copper deficiency, resulting in 'steely wool', presumably due to impaired keratinisation. Copper is probably required for the formation or incorporation of disulphide groups in keratin synthesis. However, nutritional stresses can lead to temporary loss of wool strength and crimp. Humans with Menkes disease

may have 'pili torti', or kinky, steely hair, with defective disulphide bonding of keratin (Danks, 1988).

1.6.5.5 Diarrhoea

Diarrhoea is not a common manifestation of copper deficiency in most species (Davis and Mertz, 1987). Diarrhoea has been reported in sheep and cattle, in association with primary copper deficiency (Mills et al. 1976; Fell, 1987) and 'peat scours' in cattle grazing pasture high in molybdenum (Underwood and Suttle, 1999). Bridges and Harris (1988) reported diarrhoea in foals with severe induced copper deficiency. The mechanism by which copper deficiency induces diarrhoea has not been elucidated.

1.6.5.6 Growth responses

Reduced growth rates are a common feature of copper deprivation in ruminant, but are a late and mild feature in non-ruminants (Underwood and Suttle, 1999). No significant reduction in growth has been reported in supposedly copper deficient horses. The biochemical lesions that result in decreased growth have not been identified, and it has been questioned whether decreased growth rate is more related to excessive dietary molybdenum than reduced copper in ruminants (Underwood and Suttle, 1999).

Supplementation of copper to diets marginal or adequate in copper may result in altered body composition. In steers, copper supplementation resulted in decreased backfat depth, and a tendency to increased longissimus muscle area (Ward and Spears, 1997; Engle et al. 2000). Copper is frequently supplemented to pigs as a growth stimulant (Coffey et al. 1994), and may also be added to alter the hindgut flora and improve the odour characteristics of swine waste (Armstrong et al. 2000).

1.6.5.7 Immune function

Copper appears to be essential for the normal functioning of the immune system in laboratory animals and ruminants (Underwood and Suttle, 1999) and in humans (Kelley et al. 1995). Copper deficiency reduces humoral and cell mediated immunity, as well as decreasing phagocytic activity of macrophages and neutrophils. The mechanisms for changes in immunity are not understood, but may be due to a failure of cellular differentiation, or decreased cellular viability (Percival, 1995). Neutrophils are predicted to be an effective and valuable tool for assessing copper status in humans in the future, because they are short-lived and homogeneous cell populations (Percival, 1998).

1.6.5.8 Infertility

Foetal death and resorption can result from copper deficiency in rats (Howell and Hall, 1969). Low fertility has been observed in cattle grazing copper deficient pastures, associated with delayed or depressed oestrus (Bennetts and Hall, 1935). Copper supplementation may improve conception rates and litter size in cats (Fascetti et al. 1998; Fascetti et al. 2000). However, small amounts of copper in intrauterine contraceptive devices can prevent embryogenesis by blocking implantation and blastocyst development.

1.6.5.9 Neonatal ataxia

Copper is essential for normal foetal and neonatal development (Keen et al. 1998). Neurological effects are seen in sheep, mice and humans when copper deficiency commences prenatally (Danks, 1988). Enzootic ataxia or swayback of lambs is a naturally occurring form of the disease, resulting in extensive demyelination of the spinal tracts and neuronal loss in the cerebellum (Solomons, 1979). The biochemical defects responsible for the lesions are incompletely understood (Underwood and Suttle, 1999), but may result from deficiencies of cyctochrome-*c* oxidase and dopamine-β-hydroxylase (Prohaska and Wells, 1975; Prohaska and Bailey, 1993). The disease also occurs in pigs, goats and guinea pigs (Ashworth and Antipatis, 2001) and is characterised by

spastic paralysis, severe lack of coordination, and anaemia. A similar condition affects adult deer on copper deficient diets (Peet and Hepworth, 1993). Decreased peptidylglycine α-amidating monoxygenase has been reported in the brains of pups born to copper deficient rats (Prohaska et al. 1995). Long-term neurochemical and behavioural abnormalities persisted in rats after perinatal copper deficiency (Prohaska and Hoffman, 1996).

1.6.6 Prevention and treatment of copper deficiency

Pasture levels of copper vary widely, and in New Zealand may range from 3.5 to 18 mg Cu kg^{-1} DM with little seasonal variation (Grace, 1994). Generally pasture containing 5 to 6 mg Cu kg^{-1} DM and 8 to 10 mg Cu kg^{-1} DM for sheep and cattle respectively, is adequate, providing copper absorption is not affected by dietary antagonists and DM intake is adequate (Grace, 1994).

A 'normal' diet consumed by humans in Westernised countries is considered to contain adequate to marginal levels of copper (Linder and Hazegh-Azam, 1996), although clinical copper deficiency is relatively infrequent (Uauy et al. 1998). In an average diet of humans the principal contributors of copper are potatoes, fruits, bread, meats, fish and legumes, with animal livers and shellfish having very high copper concentrations (Danks, 1988). Drinking water may constitute an important source of copper for the adult population (Sharrett et al. 1982). Where copper intake is considered to be low, minor changes in types of food eaten will improve the dietary copper concentration. Infant and preterm milk formulas are supplemented with copper, the latter having a higher copper content (Olivares and Uauy, 1996).

1.6.6.1 Indirect oral supplementation

The application of copper-containing fertilisers may raise pasture copper sufficiently to raise liver copper concentration in ruminants and prevent primary copper deficiency. However, the response is not consistent and varies with soil type, pasture composition, climatic conditions, and from year to year. In soils high in organic matter, copper becomes fixed in unavailable humic acid

complexes (Underwood and Suttle, 1999). In some situations annual application is required, which can be costly. Copper containing fertilisers are not recommended when secondary copper deficiency occurs on pastures high in copper (McFarlane et al. 1990).

1.6.6.2 Direct continuous oral supplementation

The chemical forms of copper that can be given orally include copper complexed to sulphate, a chelate, proteinate or diamine peptides, which have differing bioavailabilities. Mineral and vitamin supplements sold over the counter for humans for daily use contain copper oxide, a form of copper that is poorly absorbed from the diet in all species (Baker, 1999). Inorganic copper chelates, or organic copper chelates of protein and amino acid diamines are suitable for use where dietary interactions occur (Smart et al. 1992).

Copper may be supplied in a grain/concentrate mix to animals. Prepared feeds for horses in New Zealand contain up to 200 mg copper per day, when fed at recommended rates. Therefore horse feeds have the potential for toxicity if fed to ruminants, especially sheep.

Copper may also be added to the water supply via metering devices, and in cattle is sufficient to prevent primary or molybdenum induced copper deficiency (Allen, 1987). However, copper supplementation via water relies on animals obtaining water only from the supplemented source, and not from pools of water, as commonly occurs. This may be especially true for sheep (Allen, 1987). Concentrations of copper above 5 g L^{-1} may be unpalatable (Ellison, 1994).

In New Zealand, daily oral drenching of dairy cattle with copper (usually 1 to 2 mg of copper sulphate) may be given sufficient to increase and maintain liver copper reserves (Ellison, 1994).

1.6.6.3 Discontinuous oral supplementation

Discontinuous methods of copper supplementation are suitable for ruminants, which have the ability to store excess dietary copper and later use liver reserves when dietary intake is inadequate. Monthly drenching may be satisfactory under some circumstances (Underwood and Suttle, 1999).

Discontinuous oral supplementation may also be of use in monogastric species to prevent copper deficiency, especially in the young. Pearce et al. (1998c) reported that foals from pregnant mares orally drenched thrice weekly with copper sulphate have elevated liver copper concentration at birth.

1.6.6.4 Slow release oral supplementation

Copper oxide needles are effective at preventing and treating copper deficiency in ruminants. The needles are rod shaped particles, between 1 and 10 mm long, composed of either copper oxide or oxidised copper wire, and are administered in a gelatin or similar capsule. Once administered the capsule dissolves and the particles escape, and move from the rumen into the abomasum. Particles trapped in the abomasum release copper in the acid environment, which can overcome the problem of secondary copper deficiency as a result of thiomolybdate complexes formed in the rumen. Copper oxide needles are reported to release copper for 12 to 14 weeks (Ellison, 1994), and elevate liver copper stores for 14 to 39 weeks (Langlands et al. 1986).

1.6.6.5 Parenteral supplementation

Parenteral administration bypasses the complex factors that limit the intestinal absorption of copper, and reportedly up to 75% of a single dose may reach the liver, in contrast to less than 10% of an oral dose (Allen, 1987). Nearly 80 different compounds have been assessed for their ability to protect ruminant from copper deficiency (Allcroft and Uvarov, 1959), including intravenous, subcutaneous and intramuscular routes of administration. Intravenous copper injections, usually as copper sulphate, were effective in raising liver copper

stores or alleviating hypocuprosis, but were not considered a practical method of supplementing livestock (Harvey and Sutherland, 1953). Harvey and Sutherland (1953) assessed the suitability of a number of compounds according to a number of criteria including injection site damage, amount stored in the liver, and margin between therapeutic and toxic dose. They concluded that copper glycinate was the most satisfactory, but a proportion of treated animals developed injection site abscesses. Calcium copper ethylenediaminetetra-acetic acid (calcium copper edetate) resulted in little tissue damage, but most sheep injected with 80 mg died. Methionate complexes are the least toxic, but cause the largest injection site reactions (Suttle, 1981). Calcium copper edetate was the most effective preparation to relieve copper deficiency in cattle (Suttle, 1981).

Injectable preparations containing copper glycine and calcium copper edetate are marketed for sheep, cattle and deer in New Zealand for the prevention and treatment of copper deficiency. The products differ in the rate of absorption of copper from the injection site to the liver, with 70% of copper edetate transferred to the liver in seven days compared to up to 21 days for copper glycine (Carmago et al. 1962; Harrison et al. 1989; Ellison, 1994). Typical doses of either product are 30 to 40 mg for sheep, and 120 to 240 mg for cattle (Cunningham, 1959; Carmago et al. 1962). Single doses of copper edetate given in mid-pregnancy are sufficient to prevent neonatal ataxia (swayback) in lambs (Hemingway et al. 1970).

The duration of effect of parenteral copper supplements include the amount of copper in body stores, the dietary copper intake, and the continuing daily requirements of the animal (Allen, 1987). Gleed et al. (1983) reported that rapidly growing calves on severely deficient pasture required 200 mg copper as copper edetate every 10 weeks. However, toxicity can occur when parenteral copper is administered at recommended therapeutic doses, especially in sheep (Wiener et al. 1970; Ishmael et al. 1971).

Use of copper edetate has been reported in horses (Toms, 1963; Egan and Murrin, 1973b; Carbery, 1978), but descriptions of injection site reactions, and

effects on parameters such as blood or liver copper concentration were not included. Cymbaluk and Smart (1993) advised that injectable copper preparations should not be used in the horses. There is anecdotal evidence that copper edetate injections are widely used in the United Kingdom, in response to apparently 'low' blood copper levels.

1.6.7 Copper toxicity

Ruminants are the most susceptible species to copper poisoning, with sheep being more susceptible than cattle. There are also breed variations in susceptibility, with Texel sheep for example more susceptible than other breeds, because they are more efficient at absorbing dietary copper (Grace, 1994). Pigs seem very tolerant of dietary copper, and rations often contain 250 mg kg^{-1} to promote growth (Underwood and Suttle, 1999). Horses, especially ponies, appear to be extremely resistant to copper toxicity, and tolerate dietary concentrations of at least 791 mg kg^{-1} DM (Smith et al. 1975b). Neonates and milk-fed animals are more susceptible to copper toxicity than adults, probably because of the high efficiency of copper absorption and immaturity of biliary excretory mechanisms (Bremner, 1998). Most toxic effects of copper probably result from the production of free radicals by copper chelates (Shah et al. 1992; Bremner, 1998).

Acute copper poisoning is relatively uncommon (Howell and Gooneratne, 1987) and occurs when animals are given excessively large therapeutic doses of copper, or gain access to contaminated feed or other sources of copper. Parenteral copper administration can result in acute toxicity, presumably due to the rapid release of copper from the site of administration, probably in its ionic form, and a subsequent sudden and massive increase of copper concentration in the blood (Allen, 1987). The route of parenteral administration influences the acute toxicity of copper, with intraperitoneal injections resulting in more chick deaths than intravenous injections (McCormick and Fleet, 1988). Subcutaneous and intramuscular injection of copper in sheep and cattle can result in toxicity (Gardiner, 1978; Sommerville and Mason, 1985; Sommerville and Burrough, 1985; Van Niekerk et al. 1994).

The clinical signs associated with acute copper toxicity are not constant, and vary between species but may include nausea, diarrhoea and haemolysis. In a horse with suspected acute copper toxicity following injection of copper D-penicillamine there was no evidence of haemolysis, but the horse showed signs of colic (apparent abdominal pain), and elevated liver enzymes, indicating hepatocyte injury (Auer et al. 1989). Acute copper toxicity in horses was also reported by Bauer (1975) resulting in acute gastroenteritis, intravascular haemolysis, jaundice, haemaglobinuria and death. The acute single oral dose of copper sulphate was estimated to be 125 mg kg^{-1} liveweight, equivalent to 2800 mg Cu kg^{-1} DM (Hintz, 1987). The maximum tolerable levels of dietary copper during growth are estimated to be 25, 250 and 1000 mg Cu kg^{-1} DM for sheep, pigs and rats respectively, compared to 800 mg Cu kg^{-1} DM in the horse.

Chronic copper toxicity follows the repeated ingestion of copper, often at levels not greatly in excess of requirements. Biochemical and morphological changes may be observed in the liver, kidney, blood muscle and the central nervous system, with copper accumulation predominantly in the liver and kidney (Howell and Gooneratne, 1987). The symptoms of chronic copper toxicosis are not constant and vary between species. In sheep, the most susceptible species, copper toxicity proceeds with a period of initial copper loading, followed by a haemolytic crisis as copper is released from the liver into the blood. Surviving animals enter a post-haemolytic phase, where biochemical parameters may return to normal, but the animal has varying degrees of liver and kidney damage. Copper toxicity in monogastric species follows a similar two-phase pattern to sheep, except that the haemolytic crisis is less pronounced, and growth retardation is more prominent (Underwood and Suttle, 1999). Bauer (1975) reported chronic copper toxicity in horses following high levels of copper sulphate in the diet for two or more months. Affected horses showed signs of loss weight and colic, multiple haemolytic crises and death after six months or more of copper supplementation. Details of the dietary copper concentration or liver copper concentration were not given, but liver copper levels were stated to be ten times normal.

Objectives of the research and thesis contents

There are very little data on the growth and development of young horses, especially those raised at pasture. Rapid growth rates, large body size and fatness have been implicated as risk factors for the development of osteochondrosis, physitis and other developmental orthopaedic diseases in young horses. However, these associations have not been adequately studied, partly because it is problematic to quantify what are rapid growth rates, large body sizes and fatness, due to the limited data available. No in vivo techniques to predict body fat in the young horse have been validated against body composition determined by chemical analysis or dissection.

In pasture-fed Thoroughbreds there is a 'protective' effect of dam oral copper supplementation during late gestation on evidence of physitis and articular cartilage lesions in foals at 150 days of age (Pearce et al. 1998a). However, the number of young horses affected with DOD is believed to be increasing worldwide, despite the widespread use of dietary copper supplementation above the NRC (1989) recommendations of 10 mg Cu kg^{-1} DM. Regardless of dietary copper concentration, the severity and prevalence of osteochondrosis lesions in TB foals raised at pasture in New Zealand was low compared to foals raised in other countries, particularly under European and North American management systems (Pearce et al. 1998a). The effect of nutrition level, growth rate and fatness may be more important factors influencing the expression of osteochondrosis in young foals.

The work in this thesis is centred on the following hypotheses:

In pasture-fed Thoroughbred foals:

1. There is a statistically significant relationship between the prevalence and severity of DOD lesions and the average weight gain in two-weekly intervals or relative weight gain, assessed by weighing at 2 weekly intervals from birth to 160 days of age

2. Body composition, in particular fatness, is highly predictable through application of in vivo techniques for ultrasonographic fat thickness measurement, clinical condition scoring, and bioelectrical impedance analysis

3. Copper administered by injections in mares in late pregnancy results in statistically significant improved copper status of their foals

4. Injectable copper supplementation of dams in late pregnancy is associated with a statistically significant reduction in incidence and severity of some forms of DOD in their foals

The aims of the work were to test the hypotheses by designing and implementing suitable experiments, interpret the results of all the studies and integrate the findings in the context of their possible association with DOD, and indicate the meaning and the importance of the findings to the equine industry

The following chapters describe the experimental work performed to test the hypotheses. Objectives for the experimental work include:

Monitor aspects of foal growth from birth to 160 days (weight, condition score and size changes) and at 160 days assess the relationship between these parameters and

i)	Fatness	*Chapter 2*
ii)	Developmental orthopaedic disease	*Chapters 5 & 7*

Assess the chemical body composition of foals at 160 days as an indicator of growth, and investigate the relationship with various in-vivo techniques used to predict body composition *Chapter 2*

Describe the decline in liver copper concentration from birth to 160 days of age in 2 crops of foals *Chapters 3 & 4*

Assess the effect of prenatal injectable copper supplementation of dams on

i) Foal and dam liver and plasma copper concentration *Chapters 3 & 4*

ii) Evidence of developmental orthopaedic disease *Chapters 5, 6 & 7*

General discussion and conclusions of this work are presented in a final chapter *Chapter 8*

The Massey University Animal Ethics Committee, Palmerston North, New Zealand approved all procedures involving the use of animals. The research was conducted using Thoroughbred foals born in 1997 and 1998, all of which were bred and raised at pasture at Flock House in the Manawatu, New Zealand. Each study in this thesis involved some or all of the foals born in one or both years.

Chapter 2

Chemical body composition of 20 Thoroughbred foals at 160 days of age, and preliminary investigation of techniques to predict body fatness[1]

[1] Results accepted for publication as: Gee, E.K., Morel, P.C.H., Grace, N.D., Firth, E.C. and Mogg, T.D. *New Zealand Veterinary Journal*

2.1 Abstract

Objectives To determine the chemical body composition of Thoroughbred foals born in two consecutive years, and investigate several techniques to predict body composition in foals born in the second year, with particular reference to fat.

Methods The chemical composition of 20 foals at around 160 days of age, born in two years, was determined. In vivo techniques to predict body composition were assessed in 23 foals born in Year 2 before and after post-mortem; 10 of these foals were used for chemical body composition analysis. Techniques used to assess body composition in vivo included liveweight, overall and regional condition scores, ultrasonographic fat thickness measurements of the ribs and rump, linear measurements and bioelectrical impedance analysis. Correlations were determined between ultrasonographic fat thickness, and bioelectrical impedance analysis, before and after post-mortem. Stepwise regression analysis was used to determine the relationships between in vivo techniques to assess body composition and the chemical body composition of 10 animals.

Results Foals used for analysis of chemical composition weighed between 220.5 and 260.0 kg before post-mortem. Fat content ranged from 5.5 to 13.0% of the partial empty bodyweight (liveweight less head, gastrointestinal contents, distal limbs and skin). Fillies had significantly more fat mass and percentage fat than colts (both $p = 0.031$, $n = 10$). The mean chemical composition of the fat-free partial empty body was 73.2% (SD 0.6) water, 22.7% (SD 0.9) protein and 4.1% (SD 0.4) ash. Most of the variation in the concentration of empty body water was associated with variation in the concentration of fat ($p < 0.001$). The live animal overall condition scores were correlated with fat mass and concentration ($p = 0.006$ and $p = 0.0125$ respectively, $n = 10$). Condition score of the rib region was highly correlated with fat mass and concentration ($p = 0.004$ and $p < 0.001$, $n = 10$). Ultrasound measurement of rump fat thickness (10 cm anterior to the tailhead, 4 cm off midline) was correlated with condition

score ($p = 0.001$), and explained 71% of the variation in body fat mass ($p = 0.002$, $n = 10$).

Almost 50% of the variation in fat-free mass and partial empty body water mass were associated with variation in the impedance indices calculated from length and bioelectrical impedance analysis measurements ($p = 0.023$ and $p = 0.026$ respectively; $n = 10$).

Conclusions: Fillies were significantly fatter than colts at around 42% of expected mature weight. Condition scores were correlated with partial empty body fat mass, and there was a trend for higher scores in fillies compared to colts. Much of the variation in water or protein mass of the partial empty body could be explained by variations in liveweight. Measurements of liveweight, rump fat and condition score are useful to predict the chemical composition of foals at 5 months of age.

Abbreviations

BIA	Bioelectrical impedance analysis
CS	Condition score/scoring
DOD	Developmental orthopaedic disease
LW	Liveweight
PEBW	Partial empty bodyweight
PNCC	Partial non-carcass component/s
US	Ultrasound/ultrasonographic

2.2 Introduction

Studies of the growth patterns of horses have been limited to recording bodyweight, and in some cases skeletal dimensions and condition scores. However, a more definitive description of growth must include the changes in composition, including the deposition of fat. Rapid growth rates and overfeeding are believed to be factors associated with developmental orthopaedic disease (DOD) in young horses (Stromberg, 1979). This may be related to the accumulation of fat, or to factors related to skeletal development. There is some evidence that fat young horses may not become good athletes and excessive fatness should be avoided (Jackson and Pagan, 1993).

There are limited data on the chemical body composition of horses. Weight alone is not an accurate indicator of fatness or fat-free mass in any species, as it represents the sum of the somatic chemical constituents. There are few objective methods to assess fatness (or leanness) in the live animal. These include condition scoring (CS), ultrasonographic (US) fat thickness and bioelectrical impedance analysis (BIA). None of these techniques have been validated in the young horse.

Condition scoring is a subjective indicator of fatness, and in horses incorporates both visual and palpable assessment of the whole animal (Henneke et al. 1983; Carroll and Huntington, 1988). Two methods of CS have been developed for use in adult horses using scales of 1 to 9 and 0 to 5. The 9-point system has been adapted by some researchers for use in young animals (Pagan et al. 1996; Hoffman et al. 1999). Both scoring systems include some objective criteria of physical attributes but also require some subjective assessments. Both evaluate fat deposition on the neck, withers, shoulders, ribs, back and pelvis.

Ultrasonographic fat thickness measurements are widely used in production animals as an indicator of carcass fatness (reviewed by Houghton and Turlington, 1992), and may be useful as an indicator of fatness in the adult horse (Westervelt et al. 1976; Kane et al. 1987). Ultrasonographic rump fat thickness

has been measured in newborn foals (Kubiak et al. 1988), but the relationship with total body fatness has not been determined.

BIA is a relatively cheap, non-invasive and quick technique to estimate body composition, and is often used in humans (Ellis, 2000), as well as assessing carcass fatness in live and dead sheep and pigs (Swantek et al. 1992; Berg and Marchello, 1994; Hegarty et al. 1998). BIA is based on the principle of conductance of an electrical current through body fluids (Lukaski, 1987). The fat-free body is composed mostly of water containing electrolytes, and is a highly conductive substance, whereas fat serves as an insulator that impedes the flow of an introduced current (Swantek et al. 1992). At 50 kHz, as used in single frequency units, the impedance value is for total body water (Chumlea and Guo, 1994), and a fatter animal should impede the transmission of the current more than a lean animal. Electrical impedance is dependent on the conductor length, cross-sectional area and signal frequency. Assuming a similar geometric shape in foals, and using a constant electrical signal, the components of impedance (resistance and reactance, R_s and X_c respectively) should be indicative of body composition. Recent work has shown that BIA may be useful in improving the predictive accuracy of non-invasive estimates of extracellular fluid volume and plasma volume in horses (Forro et al. 2000).

The aim of this study was to determine the chemical composition of 20 Thoroughbred foals, at 160 days of age, covering a range of weights, and to identify any differences due to gender or year of birth. The study also aimed to investigate several techniques for predicting fatness in both live and dead foals born in one season, and the relationships between these techniques and chemical composition.

2.3 Materials and Methods

2.3.1 Animals

Twenty Thoroughbred foals (10 males and 10 females) of similar age and month of birth were used for determination of chemical composition. Ten foals were

born in Year 1, and 10 in Year 2. Techniques to predict body composition were assessed in the 10 foals born in Year 2 used for chemical composition, and a further 13 foals also born that year (not used for chemical composition). All the foals were part of a study investigating the effect of copper supplementation of mares in late gestation on liver copper concentration in foals, and the effects on the prevalence of DOD. Foals were kept at pasture with their dams for the duration of the experiment. The foals were euthanased at around 160 days of age (mean 162, range 156 to 176 days). Those from Year 1 were euthanased by barbiturate overdose (sodium pentobarbitone 125 mg kg^{-1}) after sedation with 100 mg xylazine hydrochloride administered intravenously. In Year 2 foals were euthanased by free bullet after sedation with 100 mg xylazine hydrochloride administered intravenously.

2.3.2 Ante-mortem techniques

2.3.2.1 Weight and condition score

Each foal was weighed within 24 hours before euthanasia. In Year 2 foals were assigned an overall CS on a scale of 1 to 9 (ranging from poor to extremely fat), in a manner similar to that developed by Henneke et al. (1983) for use in broodmares, using 0.25 increments. The neck, shoulder, withers, ribs, back and pelvis were also scored individually by the same assessor, using a 9-point scale with 0.25 increments to provide regional condition scores.

2.3.2.2 Ultrasound fat thickness

Rump and rib fat thickness were assessed in all foals in Year 2 within 24 hours before euthanasia, using a Pie Medical Scanner 100LC™ and 6 MHz linear array probe. The rump was clipped with a number 10 blade in a 20 cm x 20 cm-square region cranial to the tailhead along the midline. The area was cleaned with aqueous hibitane and alcohol, before application of the electrode conductivity gel (ConMed™ electro-gel, Utica, NY). An area over ribs 16 and 17, on the left and right sides of the animal was clipped and similarly prepared,

from the midline to about 25 cm distal to the thoracic vertebral spinous processes.

Rump fat measurements were taken at 6 cm and 10 cm cranial to the tailhead and 4 cm from midline (Sites 1 and 2 respectively) on both the left and right. The US probe was positioned parallel to the midline, with the centre of the probe over the site to be measured. Measurements were made using the calliper function of the US machine. Rib fat thickness was measured caudal to rib 16, at 5 and 20 cm ventral to the midline thoracic vertebral spinous processes (Sites 1 and 2 respectively) on both the left and right side of the animal. The transducer was positioned parallel to rib 16.

2.3.2.3 Bioelectrical impedance analysis

Impedance measurements were determined from each foal in Year 2 using a four-terminal single frequency instrument (BIM4™, UniQuest Limited, Qld, Australia). A tetrapolar electrode configuration was used to minimise contact impedance and skin-electrode interactions (Hoffer et al. 1969; Foster and Lukaski, 1996). The electrode placement sites were related to a palpably identifiable anatomical point to ensure consistent terminal placement. On the forelimb the first electrode was placed distal to the lateral side of the head of the radius, and the second electrode 10 cm proximal to the first. On the hindlimb the first electrode was placed proximal to the lateral malleolus of the tibia, and the second 10 cm proximal to the first. The skin at the electrode placement sites was clipped closely and cleansed with aqueous hibitane and alcohol. A thin layer of electrode conductivity gel was applied to the skin before application of electrodes, which were held in place with rubber straps around the limb. An alternating current (50 kHz, 800 mA) was transmitted through the most distal electrode on each limb, and the voltage drop detected between the two proximal electrodes. The foals were encouraged to stand squarely and still on a rubber mat while at least six consecutive measurements were made, which took approximately 15 minutes for each animal (mostly due to animal movement).

Following BIA measurements, linear measurements were taken with a measuring tape, and recorded to the nearest 0.5 cm in all foals in Year 2. The measurements included distance from withers to tailhead (Length A), poll to tailhead (neck extended until the mane was horizontal to the thoracic spinal processes; Length B), greater tubercle of the humerus to the cranial greater trochanter of the femur (Length C), chest circumference and height at withers. The ambient air temperature and rectal temperature of the foals were also recorded. All measurements were taken in the morning, up to 24 hours before euthanasia.

The impedance data were first evaluated in a biological model. In this model the body is assumed to be a number of interconnected cylinders, and that the electrical impedance is dependent on conductor length, cross-sectional area and signal frequency.

The impedance (Z) of a cylinder is proportional to its length (L) squared, divided by the cross-sectional area (A), as shown in Equation 1. This can be rearranged to give the conductive volume of the cylinder (Equation 3) (Hegarty et al. 1998).

$Z \alpha L/A$ (1)

$Z = \rho L/A$ (2) where ρ is the specific resistivity

$V = \rho L^2/Z$ (3) where V is the conductive volume of body water

From Equation 3, total body water can be described as some function of L^2/Z. In humans, the height of the subject is used for the length. In the foals the three different linear measurements (described above) were used to create impedance indices, to determine which measurement of length was the most appropriate to use. Index A used Length A, Index B used Length B and Index C used Length C.

2.3.3 Post-mortem measurements

As no attempt was made to restrict feed, milk or water intake prior to euthanasia, foals were not stressed unnecessarily. Also, this prevented a

reduction in the volume of gastointestinal contents, which may have resulted in dehydration and hence affecting chemical body composition results.

Immediately after euthanasia the animal was hung from a rubber-coated gambrel, and exsanguinated by severing the cervical vessels; all blood was collected and weighed. Blood collected from 10 foals in Year 2 was frozen. The distal forelimbs were removed 10 cm proximal to the radiocarpal joint, and the hindlimbs at the level of the tarsometatarsal joint. Each limb was weighed and put on ice for tissue harvest for another study. The head was disarticulated at the occipitoatlantal joint, removed and weighed. All recordings were made within 30 minutes following euthanasia.

2.3.3.1 Bioelectrical impedance analysis

The four electrodes were attached to the limbs at the same sites as in the live animal. Impedance measurements were taken in triplicate on all carcasses, without removing the electrodes. Two length measurements were made on each carcass: the direct distance between the distal electrodes of each limb was recorded (L_d), and the sum of the distances from the distal forelimb electrode to withers, withers to tuber sacrale, and tuber sacrale to distal hindlimb electrode (L_i). Carcass temperature was recorded by digital thermometer, inserted to a depth of 4 cm in the biceps femoris muscle, immediately after impedance measurements were taken. Ambient temperature was also recorded.

2.3.3.2 Rump and rib fat thickness

The hide was carefully removed from the body, weighed and discarded. Actual fat thickness was measured by callipers at the rib and rump sites previously described for US. A section of thorax, including the sites of rib fat thickness measurements, was removed and chilled at 4 °C overnight. The rib sites were measured the next day to determine the difference in fat thickness between the hot and cold carcass.

2.3.4 Post-mortem examination

Weights were recorded for the unopened gastrointestinal tract, and individual abdominal and thoracic organs. Fat adhering to the abdominal and thoracic walls was removed manually and weighed. The remaining carcass was split in half along the vertebrae using an electric meat saw and each half weighed (hot carcass weight).

2.3.4.1 Sample preparation

Gut contents were manually stripped from the gastrointestinal tract, and the mesentery and omentum manually removed and weighed. The empty gastrointestinal tract was then washed and reweighed. After weighing, the liver was placed inside two plastic bags, the air removed manually, and frozen. The live bodyweight (LW), less the gastrointestinal contents, head, skin and excised limbs constituted the partial empty bodyweight (PEBW). The distal limbs were used in another study on DOD, and not included in the chemical analyses. The head and skin were unable to be ground in the grinder available, and were also not included in the chemical analyses.

The right side of the carcass and all abdominal and thoracic organs were used for analysis of chemical composition. The right carcass was divided into five sections after weighing, each section was placed inside two heavy-duty plastic bags (100 μm thickness), air was manually expelled, and they were weighed prior to freezing at –20 °C. All thoracic, reproductive and abdominal organs (except the liver), digestive tract, mesentery and omentum, thoracic and abdominal fat (hence referred to as the partial non-carcass component, PNCC) were collectively weighed inside two heavy-duty plastic bags, and frozen at -20 °C after excess air was manually removed.

The frozen carcass sections, liver and PNCC were reweighed to assess the weight loss due to freezing. The frozen carcass was cut into pieces less than 8cm x 8cm x 2cm using an electrically-driven bandsaw, and ground three times through a 10 mm aperture plate in a Wolfgang meat grinder. The tissue mass

was divided in two equal portions after manual mixing for 10 min. One half was discarded and the other half then passed three times through a smaller grinder (Hobart mincer, Hobart Manufacturing Co. Ltd., London) with successively smaller aperture plates each time (9, 5 and 2 mm aperture plates), to produce a well-mixed homogenous mass. Representative subsamples (approximately 1 kg) were kept and frozen. Three samples (approximately 50 g) were bagged and accurately weighed before being frozen at –20 °C until analysed.

The abdominal and thoracic contents were treated similarly, but with only two passes through the larger grinder, before grinding twice in the smaller grinder, and sampling as for the carcass. The liver was ground twice through the smaller grinder, using a 5 mm aperture plate, before three 50 g samples were accurately weighed as above and frozen.

2.3.5 Chemical composition

Samples from 20 foals comprising carcass, PNCC, liver and blood (the latter from foals from Year 2 only) were freeze-dried to determine moisture content, and analysed for crude fat (pet-ether extraction), ash (combustion in a muffle furnace at 600 °C overnight), and crude protein (by difference). Samples were analysed in duplicate.

2.3.6 Statistical analyses

Analyses were carried out using SAS version 6.12 (Statistical Analysis Systems, SAS Institute, Inc, Cary, NC). Linear regression equations were developed from partial empty body weight or liveweight for prediction of chemical composition components. When significant linear regressions had non-significant intercepts, regressions were run in Microsoft Excel 2000 data analysis tool, with no intercept. Values for R^2 were corrected for the mean.

Effects of sex, age and year of birth on partial empty body chemical composition, and various predictor variables were analysed using LW as the

covariate. All interactions were examined. Multiple regression equations were developed from the predictor variables from the live animals to predict the partial empty body composition. Stepwise regression was used to eliminate variables that did not significantly influence variation in the model (level for entry in the model was $p = 0.15$, level of significance for inclusion in the model was $p \leq 0.05$). The residuals were plotted against predicted values for each significant model, to determine if there was non-linearity or non-constant error variance. Spearman's rank correlation procedure was used to determine associations between CS and fat thickness measurements. Pearson's correlation procedure was used to determine associations between US and actual fat thickness measurements.

2.4 Results

2.4.1 Chemical composition of 20 foals

2.4.1.1 Mean age, weights and chemical composition

The LW of foals used for chemical composition ranged from 225.0 to 260.0 kg (mean 239.5, SD 13.0 kg). Simple means of age and weights of body components are presented in Table 2.1. There were no significant differences in age, LW or PEBW due to year or sex. There were significant differences in the amount of blood collected each year, due to the method of euthanasia ($p < 0.001$). The gut contents, expressed as a percentage of LW, ranged from 5.2 to 11.0% (mean 8.3, SD 1.8%).

Fat mass ranged from 5.5 to 13% of the PEBW. The mean chemical composition of the fat-free partial empty body (PEBW less fat) was 73.2% (SD 0.6) water, 22.7% (SD 0.9) protein and 4.1% (SD 0.4) ash. Most of the variation in the concentration of partial empty body water was associated with variation in the concentration of fat ($p < 0.001$).

Table 2.1 Means and standard error of the differences (SED) for parameters measured post-mortem of 20 Thoroughbred foals (10 in Year 1, 10 in Year 2) used for analysis of chemical composition. Significant differences between years are indicated by superscripts, p<0.001.

Parameter	Mean		SED
	Year 1	Year 2	
Age (days)	161.4	161.6	1.6
Weight (kg)			
Liveweight	238.9	240.1	6.0
Gastrointestinal tract contents	18.4	21.2	1.8
Partial empty bodyweight (hot)[1]	190.8	189.8	5.8
Carcass (hot)	148.9	147.1	5.1
Blood	9.9[a]	13.9[b]	1.0

[1] Partial empty bodyweight = liveweight – (skin + head + distal limbs + digesta)

2.4.1.2 Predictions of partial empty body, carcass and liver weights, and fat-free mass from LW

The data were fitted to the linear model of $Y = a + bX$; where Y is the weight of the component (kg), a a constant (intercept), b the slope (regression coefficient) and X the liveweight. The hot PEBW, carcass weight and fat-free body mass could be accurately predicted from LW, with small residual standard deviations (Table 2.2).

Table 2.2 Regression relationships, standard errors (SE), and residual standard deviation (RSD) between weights of chemical components and liveweight (LW) of 20 Thoroughbred foals.

Weight (kg)	Regression relationship	SE *b*	SE *a*	R^2	P-value	RSD
Partial empty body	Y = 0.795LW	0.004		0.857	<0.001	4.7
Carcass	Y = 0.619LW	0.005		0.808	<0.001	4.8
Fat-free body	Y = 0.721LW	0.004		0.867	<0.001	4.8
Fat	Y = 0.0539LW + 4.66	0.080	17.8	0.029	0.48	17.8
Water	Y = 0.528LW	0.003		0.833	<0.001	3.6
Protein	Y =0.164LW	0.002		0.663	<0.001	1.7
Ash	$Y_{Year2} = 0.0188LW + 3.12$	0.0051	1.23	0.873	<0.001	0.3
	$Y_{Year1} = Y_{Year2} - 1.29$		0.13			

a was a constant (intercept), *b* was the slope (regression coefficient)

2.4.1.3 Predictions of partial empty body chemical components from LW and PEBW

The data were fitted to the linear model, and the effects of sex, year and interactions examined. The prediction models are given in Tables 2.2 and 2.3. There was little or no benefit in using PEBW over LW to predict the major chemical components. Variation in LW accounted for most of the variation of the chemical components, with the exception of fat mass. Fillies had significantly more partial empty body fat mass and percentage fat than colts ($p = 0.031$ and $p = 0.031$ respectively). There were significant differences in partial empty body ash mass and percentage due to year (both $p < 0.001$), and there was a negative relationship between partial empty body fat percentage and ash percentage in each year. Eighty percent of the variation of partial empty body ash percentage was associated with year and partial empty body percentage fat ($p < 0.001$). The adjusted mean values of the chemical components are given in Table 2.4, at a mean LW of 239.5 kg. The water to fat-free mass ratio was 0.73 and 0.74 in Year 1 and Year 2 respectively ($p < 0.001$).

Table 2.3 Regression relationships, standard errors (SE), and residual standard deviation (RSD) between weights of chemical components and partial empty bodyweight (PEBW) of 20 Thoroughbred foals.

Weight (kg)	Regression relationship	SE *b*	SE *a*	R^2	P-value	RSD
Fat	Y = 0.122PEBW - 5.55	0.077	13.9	0.135	0.12	4.0
Water	Y = 0.664PEBW	0.004		0.856	<0.001	3.4
Protein	Y = 0.206PEBW	0.002		0.770	<0.001	1.4
Ash	Y_{Year2} = 0.0141PEBW + 4.93	0.006	1.18	0.825	<0.001	0.4
	$Y_{Year1} = Y_{Year2}$ - 1.33		0.15			

Table 2.4 Adjusted mean values, and standard error of the difference (SED), at 239.5 kg liveweight for partial empty body chemical components for 20 Thoroughbred foals in Years 1 and 2, and 10 fillies and 10 colts.

	Year				Sex			
Weight (kg)	Year 1	Year 2	SED	P-value	Fillies	Colts	SED	P-value
Fat:	19.3	15.9	1.8	0.08	19.6	15.6	1.9	0.03
Carcass fat	13.1	10.9	1.0	0.05	13.0	11.0	1.0	0.62
PNCC fat	6.0	4.8	0.9	0.20	6.4	4.5	0.6	0.02
Water	125.2	127.7	1.5	0.11	126.4	126.6	1.8	0.91
Protein	40.5	38.0	0.5	<0.001	39.4	39.0	0.8	0.58
Ash	6.3	7.6	0.1	<0.001	6.9	7.1	0.3	0.52

PNCC: Partial non-carcass component = thoracic, reproductive and abdominal organs (except the liver), digestive tract, mesentery and omentum, thoracic and abdominal fat

2.4.2 Prediction of fatness in live animals

2.4.2.1 Condition scores in live foals in Year 2

The overall CS of the Year 2 foals used for chemical composition ranged from 4.0 to 7.0, with mean overall CS of 6.2 and 5.2 for fillies and colts respectively. Spearman's rank correlation coefficients for overall CS and regional CS with partial empty body fat mass were all significantly different from zero. The highest correlations were between overall CS and rib CS with partial empty body fat mass ($r = 0.791$, $p = 0.006$ and $r = 0.788$, $p = 0.007$ respectively, $n = 10$).

2.4.2.2 Linear measurements

The weight-adjusted mean linear body measurements are presented in Table 2.5. Colts were longer and taller than fillies. Inclusion of linear measurements with LW in stepwise regression analysis did not significantly improve the prediction models for partial empty body fat mass or fat-free body mass.

2.4.2.3 Ultrasonographic and actual fat thickness

Measurement of rib fat thickness by US was poorly correlated between the left and right sides for Site 1 ($r = 0.300$, $p = 0.18$; $n = 23$), but were better correlated at Site 2 ($r = 0.791$, $p < 0.001$; $n = 23$). Correlations between ultrasonographic and actual rib fat thickness were poor at Site 1 on the left and right sides ($r = 0.291$, $p = 0.21$ and $r = 0.180$, $p = 0.42$ respectively; $n = 23$), and poor at Site 2 on the left side ($r = 0.298$, $p = 0.20$; $n = 23$) but better on the right side ($r = 0.437$, $p = 0.02$; $n = 23$). Correlations between hot and chilled rib fat thicknesses were also poor ($r = 0.227$, $p = 0.32$ and $r = 0.534$, $p = 0.02$ for Sites 1 and 2 on the right side respectively).

Ultrasonographic and actual rump fat thickness measurements differed greatly between the left and right rump in some individuals. For this reason the left and right measurements were averaged. Adjusted mean values of rump fat thickness measured by US were greater in fillies than in colts at Site 1; but fat thickness

measured immediately post-mortem was greater in fillies than colts at Site 2 (Table 2.5). Ultrasound rump fat thickness measurements at Site 2 explained 71% of the variation in partial empty body fat mass.

$$Y = (1.562 \text{ x rump Site 2}) + 8.20$$

$$SE\ b = 0.355,\ SE\ a = 1.98,\ p = 0.001,\ RSD = 2.00$$

Spearman's rank correlation coefficients for comparison of overall CS and US rump fat thickness measurements were highest at Site 2 ($r = 0.688$, $p < 0.001$, $n = 23$).

Table 2.5 Mean linear and rump fat thickness measurements, and standard error of the difference (SED), adjusted to an average weight of 233.8 kg for 23 colts and fillies (Year 2).

Measurement	Fillies	Colts	SED	P-value
Linear (cm)				
Length A	82.1	86.4	1.3	0.004
Length B	165.5	168.9	1.5	0.03
Length C	115.9	115.9	1.9	0.99
Height	131.7	134.4	1.2	0.05
Chest	130.4	133.2	2.5	0.28
Average ultrasound rump fat thickness (mm)				
Site 1	6.2	3.9	1.1	0.05
Site 2	5.4	4.0	1.0	0.22
Average rump fat thickness (mm) at post-mortem				
Site 1	8.3	6.6	1.4	0.26
Site 2	5.9	4.0	0.6	0.007

Length A = withers to tailhead; Length B = poll to tailhead (neck extended until crest parallel with thoracic spinous processes); Length C = greater tubercle of humerus to cranial greater trochanter of femur. Site 1 = mean of left and right measurements taken 6 cm anterior to tailhead, 4 cm off midline; Site 2 = mean of left and right measurements taken 10 cm anterior to tailhead, 4 cm off midline.

2.4.2.4 Bioelectrical impedance analysis

Values for impedance and resistance in the live animal and post-mortem were correlated ($r = 0.554$, $p = 0.006$ and $r = 0.556$, $p = 0.006$ for impedance and resistance respectively, $n = 23$), and there were no significant differences in mean impedance or resistance due to sex.

Index C explained 50% of the variation in partial empty body water mass ($R^2 = 0.50$, $p = 0.02$; $n = 10$), while Index B explained 48% of the variation in fat-free mass of the partial empty body ($R^2 = 0.484$, $p = 0.03$, $n = 10$). However, stepwise regression of the impedance indices from the live animal did not improve the model to predict empty body water mass (or fat-free mass) over using LW alone. Similar indices were calculated using the two different lengths (L_d and L_i) measured at post-mortem as estimates of conductor length; in stepwise regressions neither index improved the model to predict the water or fat-free mass of the empty body.

The impedance data were then examined without consideration of the biological model to explore relationships between chemical body composition, impedance measurements and other data collected from the live animal. Data were reanalysed using multiple regressions to determine the most appropriate empirical model for predicting water, fat, and fat-free body mass, in a similar manner to Berg and Marchello (1994) and Hegarty et al. (1998). The additional parameters did not improve the prediction models over those using LW alone.

2.4.2.5 Predictors of fat, water, protein, ash and fat-free mass in the live animal

All parameters measured in the live animal (LW, linear measurements, CS, US rump fat thickness and impedance measurements), adjusted and unadjusted for LW, were examined together using stepwise regression to predict chemical composition. There was no improvement from the earlier models to predict fat or water mass.

2.5 Discussion

The prediction equations generated in this study were linear, as the animals used were of a similar age with a narrow range of liveweight, and equations are therefore were limited to similar populations of animals. With wider ranges in age and size the relationship between weight and body components is usually curvilinear, and allometric equations are more appropriate (Harries and MacFie, 1977). In this study the use of PEBW offered little advantage over liveweight in the prediction of partial empty body chemical composition, despite the wide range in weight of gastrointestinal contents as a proportion of liveweight. The weight of the partial empty body was measured within 4 hours after euthanasia, as a hot weight, so the normal weight loss associated with subsequent cooling and shrinkage was not allowed for.

Fat is the most variable component of the body, while in comparison composition of the fat-free body is relatively constant (Lohman, 1971). When partial empty body fat mass was corrected for LW in this study the variability in fat was still large, and mainly due to sex effects, which has been noted in other species (Berg and Butterfield, 1976). The body fat mass of fillies was significantly higher than colts at the same age and weight. The higher amounts of fat in the fillies were associated with PNCC, mostly from the abdominal cavity. At approximately 45% of mature weight (225 kg liveweight if the mature weight is assumed to be 500 kg) heifers had more fat than bulls (Berg and Butterfield, 1976). In this study, where differences in fat due to gender were present, the 20 foals used for chemical composition were at around 42% of their mature weight (if the mature weight of the Thoroughbred is assumed to be 575 kg (Pagan et al. 1996)). The percentage of fat may vary from 1 to 62% in the empty body (Reid et al. 1968). The total empty body fat mass ranged from 5.5 to 13.0% in this study, similar to that reported in mixed-age horses and ponies by other authors (Robb et al. 1972; Schryver et al. 1974; Westervelt et al. 1976; Kane et al. 1987).

The chemical composition of the fat-free mass of the 20 foals in this study was similar to the chemical composition of the lean empty body that included the

skin, head and distal limbs of 11 ponies reported by Robb et al. (1972). The composition of the fat-free mass of the foal is similar to the composition of cattle, sheep and pigs (Reid et al. 1968).

Most in vivo body composition methods rely on hydration of the fat-free body being constant (around 73%) to provide a means of estimating total body fat. Fat-free mass hydration information from in vitro studies is limited, particularly concerning humans and large animals. In this study the mean hydration of the fat-free body was 73.2% (SD 0.59), in close agreement with chemical analyses of other mammals (reviewed by Wang et al. 1999). Sex and body fat mass had no significant effect on the hydration status of the fat-free mass.

During growth and development the concentration of water decreases, while fat-free dry matter increases. It has been assumed that the ratio of ash to protein is relatively constant in fat-free dry matter in adult animals, which was described by Moulton (1923) as 'chemical maturity'. However, the protein to ash ratio is subject to manipulation by nutritional factors, physiological state and sex (Clawson et al. 1991). In this study the percentage ash ranged from 3.0 to 4.5% and was significantly higher in foals in Year 2. Although the samples for each year were analysed on separate occasions, they were performed in the same laboratory, using the same analytical methods. The authors can find no suitable explanation for the difference in ash percentage between the two years. There was a negative association between fat percentage and ash percentage, which occurred in foals born in both years. Obesity in rats is associated with a decrease in ash percentage of the fat-free dry body (Clawson et al. 1991).

Condition scoring is subjective, and intended to summarise the degree of fat cover in relation to the size of the animal (Evans, 1978). In this study overall and regional CS were correlated with partial empty body fat mass. Fillies tended to have higher CS than colts in all individual regions scored. It was expected that fillies would have more subcutaneous fat than colts because of the higher CS but there was no significant difference in carcass fat by chemical analysis. The individual regional CS are all auto-correlated; an overall score is probably still the most appropriate to use in clinical settings.

Ultrasound rib fat thickness measurements were a poor predictor of actual fat thickness or partial empty body fat mass. This may have been due to the very small amount of fat stored at this site, and the inability of both the transducer to image the small amount of fat, and the operator to measure the fat accurately. Ultrasound rump fat thickness measurements were generally less than the actual fat thickness; the difference was less at Site 2. This may indicate operator error in measurement of fat by US, or imaging errors with the position of Site 1. Rump fat thickness measurements at Site 2 were reasonable predictors of body fat mass.

Prediction models derived from BIA measurements for determination of fat-free mass are highly correlated in humans with hydrodensitometry methods (Lukaski et al. 1985). In this study, BIA measurements did not improve models using liveweight for prediction of fat-free mass or water mass of the empty body. The impedance indices calculated from different lengths measured in each animal are analogous to the human impedance index (height squared divided by impedance), which is correlated with body water and fat-free mass. Increases in the live animal impedance indices tended to be associated with increases in body water. BIA may be affected by hydration status, consumption of food and water, skin and air temperatures, recent physical activity, race (and probably breed), and patient size, age and positioning (Deurenberg et al. 1988; Kushner et al. 1996). Standardisation of measurement of BIA is important for these reasons, but may be difficult to achieve under field conditions. BIA measurements were easily taken in the live animals.

A limitation of this study was the small numbers of animals used, which is not unusual in this species, partly due to the cost and technical difficulties of working with large animals. However, the chemical composition data of the 20 foals contributes to the limited data available on horses, and showed significant differences in fat mass due to sex. Techniques used in vivo to predict fatness were compared to chemical composition in 10 animals, and results support the use of CS and US rump fat thickness to assess fatness in young animals. Further studies on the use of BIA to predict body water in horses is warranted due to good results obtained in other species, and the relative ease of use. Further

work to investigate sex differences in body fat over a range of ages and weights, and the implications of the differences in fat distribution of colts and fillies, especially with respect to DOD, are justified.

Chapter 3

Changes in liver copper concentration of Thoroughbred foals from birth to 160 days of age and the effect of prenatal copper supplementation of their dams[2]

[2] Results published as Gee, E.K., Grace, N.D., Firth, E.C. and Fennessy, P.F. (2000) *Australian Veterinary Journal* **78**, 347-353.

3.1 Abstract

Objectives To monitor the change in liver copper concentration of ten Thoroughbred foals from birth to 160 days of age and to determine the effects of supplementation by two injections of calcium copper edetate given to dams in late gestation on the liver copper concentration of their foals at birth.

Methods Ten mares pregnant to the same stallion were allocated into two groups on the basis of age, liver copper concentration and expected foaling date. The treatment group mares were given 100 mg and 250 mg calcium copper edetate intramuscularly during the ninth and tenth months of gestation respectively. Foals had liver biopsies taken weekly in the first month of life, then monthly for four months. At 160 days of age liver samples were taken at post-mortem and the copper concentrations were determined.

Results Two distinct patterns of age dependant decline in liver copper concentration were evident. The mean (± SD) liver copper concentration of the foals was high at birth (374 ± 130 mg kg^{-1} DM), and for 7 foals it declined to adult values by 160 days of age (21 ± 6 mg kg^{-1} DM). The remianing three foals showed a slower rate of decline than in the other foals, and at 160 days of age the mean concentration was 162 ± 32 mg kg^{-1} DM. Repeated measures analysis showed significant differences between each biopsy ($p < 0.01$) and between foals with 'normal' and 'slow' pattern of liver copper excretion ($p < 0.002$). Copper injections given to mares in late pregnancy had no effect on the liver copper concentration of foals at birth.

Conclusions The significance of the two patterns of age-dependant decline in liver copper concentration is unknown. Parenteral copper supplementation of the dam in late gestation had no effect on the liver copper concentration of the foal at birth, or the rate of decline in liver copper concentration.

Abbreviations

ANOVA	Analysis of variance
Cu	Copper
DM	Dry matter
DOD	Developmental orthopaedic disease
ICP/AES	Inductively coupled plasma atomic emission spectrometry

3.2 Introduction

The liver is the main storage organ for copper (Cu) in adult domestic livestock (Underwood, 1977), including horses (Smith et al. 1975b; Meyer and Ahlswede, 1978). Liver Cu concentration varies with species, age and diet, and individual variation is high in all species (Underwood, 1977). In all species studied, the total foetal Cu content rises during gestation, with most Cu being stored in the foetal liver (McArdle, 1995). At birth most mammalian neonates have much higher liver Cu concentrations than adults. This has been noted in humans (Underwood, 1977), cattle (Allcroft and Uvarov, 1959; Gooneratne and Christensen, 1989), and horses (Egan and Murrin, 1973a), although lambs may be an exception (Underwood, 1977). The high liver Cu concentrations of neonates decrease to adult levels over time. In humans and rats adult Cu levels are not attained until after sexual maturity and cessation or stabilisation of growth (Linder, 1991), but in foals the adult values of 20 to 35 mg kg^{-1} dry matter (DM) may be reached by 3 to 4 months of age (Cymbaluk and Smart, 1993). Copper supplementation of foals from 3 weeks of age leads to higher liver Cu concentrations at 5 months of age (Pearce et al. 1998b). The pattern of decline in liver Cu concentration under defined dietary conditions has not been reported in horses.

Inadequate Cu has been implicated as a causal factor of developmental orthopaedic disease (DOD) in young horses (Bridges et al. 1984; Asai et al. 1993; Fujikawa et al. 1993; Hurtig et al. 1993). Knight et al. (1986) reported that DOD was less severe on farms with higher dietary Cu concentration than on

farms with lower dietary Cu concentration. Several authors have suggested feeding foals and mares Cu at higher than National Research Council (1989) recommendations (Knight et al. 1990; Jackson and Pagan, 1993; Lewis, 1995). However, no controlled trials have been published showing any significant effect of higher dietary Cu levels on the prevalence or severity of DOD.

In New Zealand it has recently been shown that increasing dietary Cu intake may be more effective in reducing the severity of DOD when increased dietary Cu is given to the mare in late gestation rather than to the foal after birth. Pearce et al. 1998a found that oral Cu supplementation of mares in late gestation (0.5 mg Cu kg^{-1} day^{-1}) reduced the radiographic and pathological evidence of physitis and articular cartilage lesions in foals. Oral Cu supplementation of foals (0.2 mg Cu kg^{-1} day^{-1} from 3 weeks of age, increasing to 0.5 Cu kg^{-1} day^{-1}) did not affect bone or cartilage compared with untreated cohorts.

A regimen of thrice weekly oral Cu supplementation as used by Pearce et al. 1998a is time consuming and impractical on most New Zealand stud farms, many of which are pasture based and do not feed mares daily supplements during the last 25 weeks of gestation.

Copper injections are widely used in ruminants requiring Cu supplementation. They improve dam and neonate Cu status if given in appropriate amounts at the appropriate stage of gestation. Such supplementation is effective in preventing swayback in lambs (Hemingway et al. 1970), and can improve the Cu status of calves. It has been recommended that Cu injections not be used in horses (Cymbaluk and Smart, 1993). Firth, Pearce and Grace (unpublished observations) found that calcium Cu edetate injections (Coprin®) given subcutaneously in the pectoral region markedly elevated liver Cu concentrations in young horses, but caused unacceptable local tissue reactions. Copper given by deep injection in the pectoral muscles in young horses resulted in low-grade inflammation at the injection site (Gee, Firth and Grace, unpublished observations). The inflammation usually resolved within 10 days, with no abscess formation. Suspected acute toxicity has been reported after injection of

Cu d-penicillinamine in a horse (Auer et al. 1989), although yearling ponies tolerated high levels of dietary Cu (Smith et al. 1975b).

A wide range of liver Cu concentrations has been reported in young horses at post-mortem (Egan and Murrin, 1973a; Meyer and Ahlswede, 1978; Hebeler et al. 1996; Grace et al. 1999b), although the pattern of change from birth has not been described. The aim of this experiment was to monitor the change in liver Cu concentration in pasture fed Thoroughbred foals, from birth to 160 days of age, and to determine the effect of two Cu injections given to dams in late pregnancy on the liver Cu concentration in their foals at birth.

3.3 Materials and Methods

3.3.1 Animals

3.3.1.1 Mares

Ten Thoroughbred mares, aged 9 to 16 years (mean age 12 years) at the time of parturition, all in foal to the same Thoroughbred stallion, were grazed at pasture. They were allocated into two groups on the basis of age, last service date, and liver Cu concentration at the start of the experiment. Mares were treated with an anthelmintic at recommended doses every eight weeks before foaling and every six weeks thereafter, and received 25 mg selenium selenate orally every 4 weeks as the pasture and soil were deficient in selenium (Grace, unpublished observations). The condition score of mares was maintained at a minimum of 5.5 (Henneke et al. 1983) for the duration of the experiment.

3.3.1.2 Foals

Ten foals (5 male and 5 female) born to the above mares, between mid September and late November 1997, were kept with their dams at pasture. The foals were weighed every two weeks. Conformation assessment and condition scoring was done at least monthly. Foals were treated with anthelmintic at recommended doses every six weeks during the experiment.

3.3.2 Diet

Mares and foals grazed ryegrass/white clover swards, with some chicory present. Lucerne hay at up to 1 kg day^{-1} for 21 days was fed during the winter when the supply of pasture was inadequate. No grains were offered, except during a period of 4 to 10 days post-foaling when foals were required to be stabled after minor surgery for a concurrent experiment. During this period pasture still formed the major part of the diet.

3.3.3 Treatment

The treatment group of mares received 100 mg calcium Cu edetate (Coprin®, 50 mg mL^{-1}, Schering-Plough Animal Health Ltd) in the ninth month of gestation and a further 250 mg during the tenth month of gestation, calculated from last service dates. The two doses were administered by deep injection in the left and right pectoral regions using 18 gauge 40 mm needles, after clipping and preparation of the skin site with aqueous hibitane and alcohol. The control mares received a similar volume of saline given in the same sites. These doses of Cu were calculated to provide at least twice the amount estimated to be accumulated by the foal in that month (1.1 and 4 mg Cu daily in the ninth and tenth months respectively (Meyer and Ahlswede, 1978)). No data were available on the efficacy or kinetics of Cu injections in horses.

3.3.4 Samples

3.3.4.1 Pasture

Pasture samples were collected at least monthly during the experiment and hay samples were taken during the periods it was fed. Samples were frozen at –20 °C until analysed.

3.3.4.2 Blood

A jugular venous blood sample was taken from mares into lithium heparin tubes at the start of the experiment and before Cu or saline injections and post-foaling

liver biopsy. Foals were sampled in the first week of life, then weekly for three weeks, then monthly for 4 months (Table 3.1) using the same method as in the mares. Blood was centrifuged at 3000 revolutions per minute for 10 minutes and the plasma was frozen at –20 °C until analysed, in two batches, up to 3 months later.

3.3.4.3 Liver

Liver samples were obtained from mares and foals by percutaneous biopsy technique with a 14 gauge 150 mm Tru-cut needle (Baxter Healthcare Corporation, Deerfield, IL, USA), after visualisation of the liver using a 5 MHz linear ultrasound probe. Before biopsy, mares and foals were sedated with 0.3 to 0.5 mg kg^{-1} xylazine hydrochloride intravenously. Three mares were also given 10 mg butorphanol tartrate intravenously, due to intractability. No other restraint was required. Biopsy samples were collected from foals at weekly intervals in the first month of life, then monthly until 4 months of age (biopsies 1 to 8, Table 3.1). Biopsy was attempted in mares at the start of the experiment, at the time of the second injection and post-foaling.

Repeated passes of the biopsy needle obturator yielded 30 to 60 mg of liver. The samples were washed with deionised water, blotted dry and stored in capped plastic vials at –20 °C until analysed.

Table 3.1 Age of foals when liver biopsy and plasma samples harvested (n = 10).

Biopsy or plasma sample number.	Mean age (days)	Range
1	4	2-6
2	11	9-13
3	19	16-20
4	26	23-29
5	54	51-56
6	82	79-88
7	110	106-114
8	139	135-143
Post-mortem	161	155-172

3.3.5 Post-mortem

The 10 foals were euthanased with barbiturate after sedation with 100 mg xylazine hydrochloride. The liver, diaphragm and thoracic wall were examined in situ for evidence of the biopsy needle entry sites. The liver was removed and a video record made of the diaphragmatic surface; alterations in colour or surface texture were recorded. If any lesions were present a deep incision was made perpendicular to the dorsal border of the lobe, through the alteration closest to the hilus. The cut surfaces were visually examined for lesions and a 2.5 cm cube of liver adjacent to the superficial lesion was taken and stored in 10% buffered formalin for histological examination. Tru-cut needle samples were harvested adjacent to the sites of previous biopsies.

3.3.6 Analytical

All glassware was acid washed and care was taken to prevent chemical contamination from outside sources at all times.

3.3.6.1 Liver copper concentration

Liver samples were freeze-dried, dry matter determined, and weighed samples of 6 to 10 mg were taken. Concentrated hydrochloric acid was added (0.5 mL), and then samples were wet ashed using a closed system microwave furnace. Analytical grade reagents were used at all times. The sample was made up to 5 mL with deionised water. Copper was assayed by inductively coupled plasma atomic emission spectrometry (ICP/AES) (Applied Research Laboratories ARL-34000 ICP/AES) (Lee, 1983). Samples were run with a liver standard of known Cu concentrations (National Bureau of Standards bovine liver 1577-B). The limit of detection was 5 μg Cu L^{-1}. Liver Cu concentration was expressed on a dry matter basis.

3.3.6.2 Plasma copper concentration

One mL of plasma was mixed with 1mL concentrated nitric acid in a 10 mL plastic tube and left overnight. Samples were heated in a waterbath at 70 °C for about 3 hours. One mL of 30% hydrogen peroxide was added and the samples kept in the waterbath for a further hour. The sample was made up to 7mL with 2 mol L^{-1} hydrochloric acid and capped. The Cu concentration was then determined by ICP/AES.

3.3.6.3 Pasture and hay

Pasture and hay samples were dried for 48 hours in an oven at 60 °C then ground through a mill with stainless steel blades and a 1 mm sieve. The mill was carefully cleaned between samples to reduce the risk of cross contamination. An accurately weighed sample (approximately 0.5 g) was mixed with 5 mL of concentrated nitric acid and was left overnight. Samples were refluxed at around 120 °C for 1 to 2 hours, then kept at 200 °C for 2 to 3 hours before they were evaporated to dryness, allowed to cool, made up to 10 mL with 2 mol L^{-1} hydrochloric acid, and kept in capped plastic tubes until the Cu concentration were determined by ICP/AES.

3.3.7 Statistical Analysis

All analyses were performed using the computer programme, SPSS 9.0 for Windows. The effect of Cu supplementation of mares on foal parameters was analysed by analysis of variance (ANOVA), as was the effect of mare parameters on the liver Cu concentration of foals at birth. The effect of season was also examined, as was the relationship between average daily weight gain and liver Cu concentration at the final biopsy. The effect of mare treatment on foal liver Cu concentration was examined eight times during the first 5 months of life, using general linear model repeated measures analysis. The relationship between mare and foal parameters and the average daily decline in liver Cu concentration from biopsy 1 to biopsy 8 was examined using ANOVA. Means are presented with standard error of the mean (SEM) or standard deviation (SD).

3.4 Results

3.4.1 Bodyweight

The average foal weight was 57 kg (range 50 to 63 kg) at birth and 239 kg (range 221 to 254 kg) at post-mortem (mean 161 days of age), which are within the expected ranges for Thoroughbreds (Jelan et al. 1996). The average daily weight gain of the foals from birth to post-mortem was 1.15 kg day^{-1} (range 1 to 1.2 kg day^{-1}). All mares and foals remained healthy throughout the experiment, except one foal that developed chronic diarrhoea at 4 weeks of age. The diarrhoea was unresponsive to treatment, and persisted until the animal was euthanased. The aetiology of the diarrhoea was undiagnosed. Despite the diarrhoea the foal had a final weight of 230 kg and an average daily gain of 1.1 kg day^{-1}.

3.4.2 Copper injections

All mares showed dose-related soft swelling near the injection site within 24 hours. Palpation of the pectoral muscles did not elicit pain, and there was no lameness. Within 48 to 72 hours the swelling had become dependant, and

within 7 to 10 days had usually resolved. At foaling no fibrous tissue was detected by physical (including ultrasonographic) examination. Two mares showed mild colic within 4 hours of the 250 mg injection and attempted liver biopsy. Heart rates were mildly elevated, and the mares were in sternal recumbency or pawed the ground. One was treated with 500 mg flunixin meglumine intravenously and quickly recovered. No treatment was given to the other, as the mare appeared normal after 1 hour and resumed grazing.

3.4.3 Liver biopsies

Biopsies were obtained from all mares at the start of the experiment, but from only six mares in the ninth month of gestation and four post-foaling. The reason for failure was the inability to visualise liver ultrasonographically. All foals had eight liver biopsies.

3.4.4 Post-mortem

All foal livers had a roughly circular area up to 6 cm diameter of fibrinous material on the capsule of the diaphragmatic surface of the right lobe. Cuts made through the capsule did not reveal any parenchymal colour or textural changes.

3.4.5 Liver copper concentration

The mean (± SD) liver Cu concentration in mares at the start of the experiment was 24 ± 5.3 mg kg^{-1} DM, and did not differ significantly between control and treatment groups. Later analyses were not possible because of the low number of successful biopsies.

Foals had high mean (± SD) liver Cu concentrations at birth (374 ± 130 mg kg^{-1} DM) (Figure 3.1). Copper supplementation of the mares during late pregnancy had no significant effect on their foals' liver Cu concentration at birth, birth weight, weight at post-mortem, or average daily growth rate. There was no significant effect of the dam's age or parity, or the foal's gestational age at the

first biopsy, post-partum age, sex, or birth date, or birth weight on the liver Cu concentration in the foal in the first week of life (Biopsy 1).

Repeated measures analysis showed significant differences between mean liver Cu concentrations at the eight different times of liver biopsy ($p < 0.010$), but no differences related to the treatment of the mares. The average daily weight gain between birth and euthanasia had no significant effect on liver Cu concentration at the last biopsy (Biopsy 8), or at post-mortem.

Three foals (the 'slow' decline group) had a slower decline in liver Cu concentration than the other foals (the 'normal' decline group), clearly evident from the sixth biopsy (Figure 3.1), and had higher liver Cu concentrations at post-mortem. The data were re-examined for any difference between these two naturally occurring groups. Repeated measures analysis revealed a significant difference in liver Cu concentration between the normal and slow Cu decline foals ($p < 0.002$) (Figure 3.2). ANOVA revealed that the gestation of the slow Cu decline foals was significantly longer than for the normal Cu decline foals (mean 361 ± SD 5 days vs. 344 ± 6, $p < 0.003$). None of the other factors examined (see above) had any significant effect on the difference in the liver Cu concentrations of the two groups. The dams of the slow Cu decline foals showed a significant trend to higher liver Cu concentrations than the dams of normal Cu decline foals at the start of the experiment (29 ± 7 mg kg^{-1} DM vs. 22 ± 2 mg kg^{-1} DM, $p = 0.026$).

3.4.6 Plasma copper concentration

The observed changes in the plasma Cu concentration of the foals are shown in Figure 3.3. Plasma Cu increased in a linear fashion ($R^2 = 0.71$) from birth to 3 weeks of age, after which it did not significantly rise (mean plasma Cu concentration after 3 weeks of age 1.3 mg L^{-1}). There was no significant relationship between the plasma and liver Cu concentrations (Figure 3.4) or between foal plasma Cu concentration and mare treatment.

Immediately prior to the first and second Cu injections and post-foaling the mean plasma Cu concentration for mares (± SD) was 1.2 ± 0.3, 1.2 ± 0.4, 1.2 ± 0.4 and 1.0 ± 0.3 mg Cu L^{-1} respectively. There was no significant difference due to the mares' treatment or stage of pregnancy. There was no significant relationship between Cu concentration in plasma and liver.

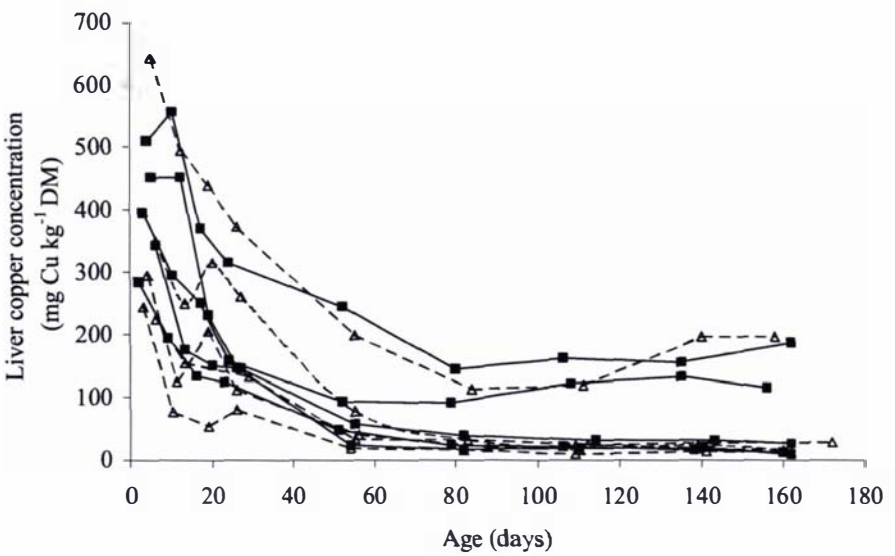


Figure 3.1 Liver copper concentration (mg Cu kg^{-1} DM) in each of 10 foals. Foals from copper treated dams indicated by solid lines, foals from saline treated dams (control) indicated by interrupted lines.

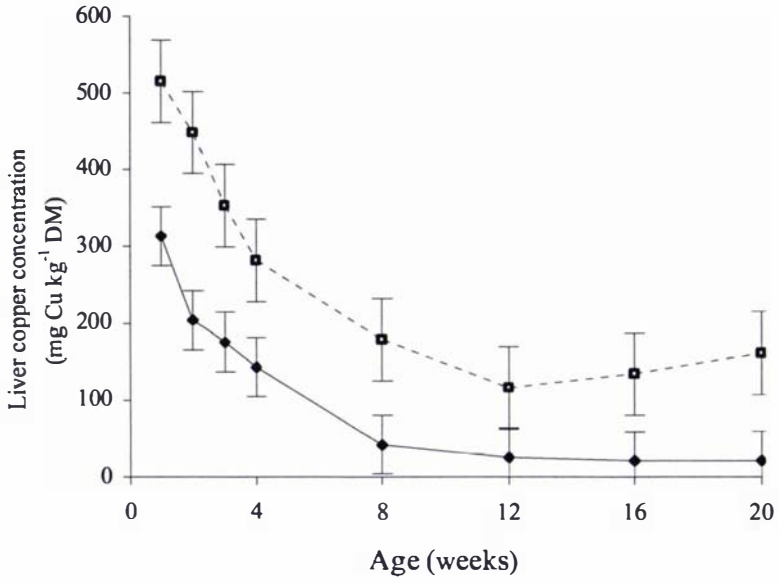


Figure 3.2 Mean liver copper concentration (mg Cu kg^{-1} DM ± SEM) in 7 'Normal' copper decline foals and 3 'Slow' copper decline foals (normal = solid line; slow = interrupted line).

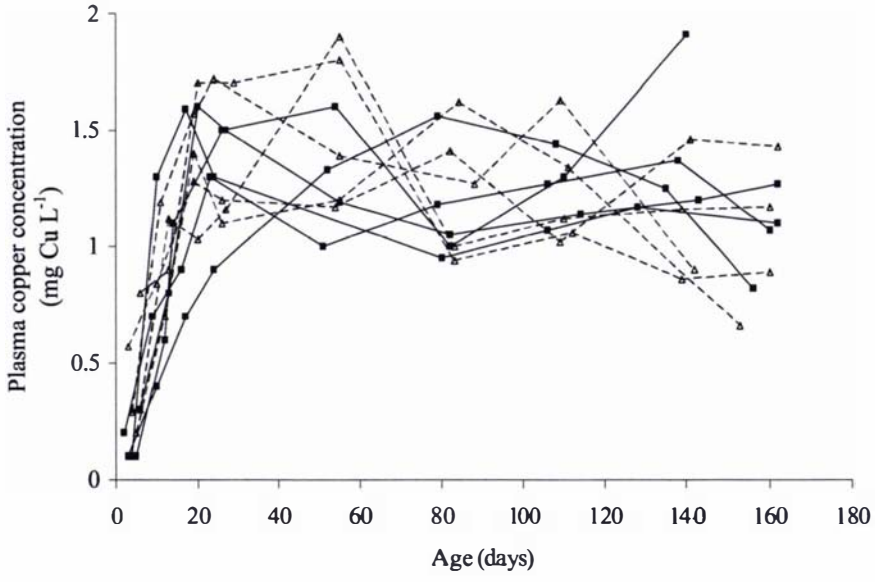


Figure 3.3 Plasma copper concentration (mg Cu L^{-1} plasma) for each of 10 foals. Foals from copper treated dams indicated by solid lines, foals from saline treated dams (control) indicated by interrupted lines.

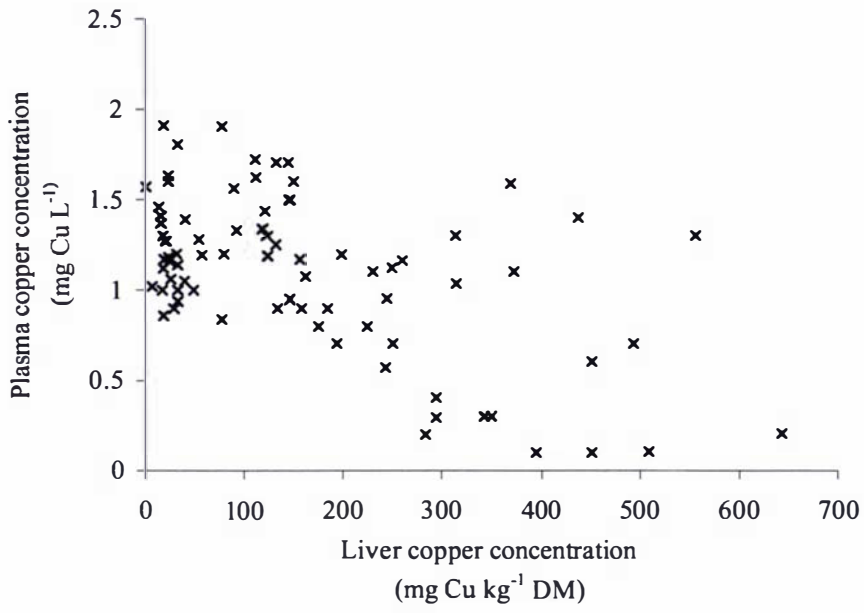


Figure 3.4 Plasma and liver copper concentration in 10 foals at each of eight samplings.

3.4.7 Pasture mineral composition

The mineral composition of pasture from June to February is given in Table 3.2. Mean pasture Cu concentration (± SD) was 7.8 ± 1.6 mg kg^{-1} DM.

Table 3.2 Mineral composition of ryegrass/clover mixed sward pasture on a dry matter basis. Pooled monthly results.

Month	Na	K	Ca	P	Mg	S	Cu	Fe	Zn	Mn	Mo	Co	Se
	g kg^{-1}						mg kg^{-1}						
Jul	1.9	30	4.9	3.3	1.7	2.8	6.7	92	22	76	0.5	n/a	n/a
Aug	2.0	43	3.5	4.7	2.0	3.8	9.0	324	31	72	1.8	0.2	0.03
Sep	2.2	39	4.1	4.3	1.9	3.3	9.1	145	28	77	1.4	0.1	0.01
Oct	1.9	40	3.2	4.7	2.0	3.6	10.7	399	29	94	3.0	0.2	0.01
Nov	2.9	37	3.5	3.5	1.8	2.7	7.4	175	25	75	1.1	0.2	0.01
Dec	2.1	20	3.3	2.5	1.7	2.4	6.6	111	23	93	1.3	n/a	n/a
Jan	3.1	29	4.9	2.5	2.4	2.3	7.0	129	24	70	0.8	0.2	0.00
Feb	1.3	21	4.2	2.4	2.3	2.5	6.2	209	28	137	0.6	0.2	0.10

n/a = not analysed

3.5 Discussion

Foals were born with high liver concentrations of Cu, with a wide variation between individuals (mean 374 mg kg^{-1} DM, range 225 to 643). This is consistent with other studies (Egan and Murrin, 1973a; Pearce et al. 1998c), even in greatly varying horse breeds and management systems. Liver Cu concentrations of less than 400 mg kg^{-1} DM in newborn foals may reflect inadequate absorption or intake of Cu by the dam (Meyer and Tiegs, 1995). Using this criterion our 'normal' foals had insufficient reserves to cover their requirements in the first month of life. As no clinical signs of Cu deficiency were detected in any of our foals, which grew at acceptable rates and were healthy, this minimum value for Cu reserves at birth may need to be reconsidered. Copper deficiency was experimentally induced in foals fed a low Cu milk replacer diet (1.7 μg Cu g^{-1}) from birth, although Cu deficiency did not develop for 4 to 7 months (Bridges and Harris, 1988). This may indicate that in these animals the liver Cu stores were sufficient for at least the first three months of life when fed an extremely low Cu diet.

Foetal Cu stores have several functions. They may be used for cupro-protein synthesis in the postnatal period although the newborn still requires Cu from the maternal milk (McArdle, 1995). The liver may synthesise metallothionein to act as a store for cysteine during the neonatal period (Andrews et al. 1987). Alternatively Cu accumulation in the foetus may simply result from the biliary excretory system not being patent (McArdle, 1995; Cymbaluk and Smart, 1993).

Injection of dams with 350 mg calcium Cu edetate in late gestation did not lead to increased liver Cu concentration in their foals at birth. This apparent lack of effect may be due to inappropriate dose, timing or frequency of injection. Alternatively the mare may excrete the Cu. Due to the small number of liver biopsies harvested from mares it was not possible to determine if Cu injections improved its storage in the mare liver. The limited results obtained tend to indicate that there was no effect.

No relationship was found between mare age and liver Cu concentration in the foal at birth, in contrast to the previous finding that foal liver Cu concentration tended to be higher in foals from older dams (Pearce et al. 1998c). The inconsistency may be due to the small number of animals in the current experiment.

Suttle et al. (1996) concluded that blood Cu concentration was reflective of the liver Cu stores in racehorses, but this does not appear to be accurate for broodmares or foals up to 160 days of age. Bridges and Harris (1988) reported very low plasma Cu concentrations when liver Cu stores were severely depleted in young horses with experimentally induced Cu deficiency. High levels of dietary Cu increased the liver Cu concentration of ponies, but had no effect on serum Cu (Smith et al. 1975b). Okumura et al. (1998) suggested that foals are in a critically marginal Cu status in the early stage of growth because of low serum Cu, caeruloplasmin and oxidase activity found in the first few weeks of life.

In the present study two patterns of decline in liver Cu concentration were identified. In cattle (Gooneratne et al. 1987), and sheep (Woolliams et al. 1983), there are breed differences in the ability to store Cu, possibly due to multiple genes causing differences in Cu absorption, retention or both. Grace et al. (1999b) have described differences in tissue Cu concentration in horses that could not be explained by variation in dietary content might be due to breed factors.

Egan and Murrin (1973a) reported liver Cu concentrations from horses at post-mortem, including 13 foals aged 4 to 6 months. When these data for are divided into two groups on the basis of high and low liver Cu concentrations ANOVA reveals the differences in mean liver Cu concentration are highly significant (high mean 554 mg kg^{-1} DM; low mean 55 mg kg^{-1}). Interpretation is difficult as dietary and medical history is not known for any of these animals.

Pearce et al. (1998b) reported that dietary Cu intake of up to 28 mg kg^{-1} DM from 3 weeks of age resulted in a mean liver Cu concentration of 69 mg kg^{-1} DM, which was significantly greater than the liver Cu concentration of 25 mg kg^{-1} DM for dietary intakes of 8 mg kg^{-1} Cu DM in 5-month-old foals. The differences in liver Cu concentration related to different dietary Cu concentrations are small compared to the differences observed in the present study between the slow and normal Cu decline subgroups.

The results from this experiment indicate the regimen used for Cu supplementation of dams in late gestation did not have an effect on the liver Cu concentration in their foals. Further work is needed to evaluate the effectiveness of Cu injections given to mares in late gestation in improving foal Cu stores.

At no stage was a significant relationship seen between liver and plasma Cu concentration, which brings into question the usefulness of plasma indices in evaluating Cu status unless animals are in a state of severe deficiency. Three animals were identified that differed in the pattern of decline in liver Cu concentration from the other seven foals. The significance of the higher Cu

concentrations in these individuals is unknown, but they may indicate that further studies are required to determine if these differences are present in other horse breeds, and what happens to the liver Cu concentration of slow decline animals after 5 months of age.

Chapter 4

Liver copper kinetics in Thoroughbred foals at pasture from birth to 160 days of age[3]

[3] Results submitted as: Gee, E.K., Morel, P.C.H., Mogg, T.D., Firth, E.C., Grace, N.D. and Fennessy, P.F. *New Zealand Journal of Agricultural Research*

4.1 Abstract

Objectives To model the decline in liver copper concentration of foals from birth to 160 days of age, and to determine the repeatability of transthoracic percutaneous liver biopsies of foals for the measurement of liver copper concentration.

Methods Pregnant mares were treated with injectable copper or saline, in two different regimens, in two consecutive years. Mares and foals had liver biopsies harvested to determine the effect of mare copper supplementation on liver copper concentration, and to identify normal patterns of decline in foal liver copper concentration from birth to at least 100 days of age. The repeatability of liver Cu concentration in biopsy samples was also investigated.

Results Injectable copper supplementation had no significant effect on dam or foal liver and plasma copper concentration. Two patterns of decline in liver copper concentration were evident in foals from each year. A mathematical model to describe the decline in liver copper concentration of foals was constructed. The concentration of copper in liver biopsies showed high repeatability (87%), and was strongly correlated with total liver copper concentration.

Conclusions Two patterns of decline in liver copper concentration have been identified in Thoroughbred foals born in two consecutive years. Liver biopsies harvested by a transthoracic, percutaneous method have a high repeatability for determination of copper concentration.

Abbreviations

Cu	Copper
CuS	Copper supplemented
DM	Dry matter
DOD	Developmental orthopaedic disease
ICP/AES	Inductively coupled plasma atomic emission spectrometry
NCu	No copper supplementation
NLCu	Normal pattern of decline of liver copper
NRC	National Research Council
SLCu	Slow pattern of decline of liver copper

4.2 Introduction

Copper (Cu) deficiency in monogastric species is rare (Underwood and Suttle, 1999). However, there is widespread Cu supplementation of young horses following studies that reported an association between low dietary Cu intakes and higher incidences of developmental orthopaedic disease (DOD) (Knight et al. 1986; Gabel et al. 1987; Asai et al. 1993; Fujikawa et al. 1993), a group of diseases affecting bone and cartilage of young horses. Experimental studies in New Zealand have supported this association. Pearce et al. (1998a) found a decrease in the number of articular cartilage lesions and radiographic physitis scores in 5 month old foals from dams that received oral Cu during late gestation. It has been suggested that suboptimal Cu nutrition of the mare during gestation results in less than adequate liver Cu stores in the foal (Meyer and Tiegs, 1995), which may predispose the foal to DOD. Hence, the adequacy of the National Research Council (NRC) recommendation (1989) of 10 mg kg^{-1} DM for dietary Cu in horses has been questioned.

The liver is the major storage organ of Cu. In 5 month old foals the liver contains 9 to 26% of the total body Cu, depending on dietary Cu intake (Grace et al. 1999b). However, there is limited information on the Cu concentration of the liver in healthy foals. The concentration of Cu in the liver and total body of

foetuses from 7 months gestation and newborn foals that were aborted, stillborn, or died (Meyer and Ahlswede, 1978), and foals of various ages with unknown nutritional history at post-mortem (Egan and Murrin, 1973a) have been reported.

Serial liver biopsies from 10 Thoroughbred foals harvested from birth to approximately 140 days of age revealed two patterns of age-dependant decline in liver Cu concentration (Chapter 3, page 129). At approximately 160 days of age 7 foals had mean (± SD) liver homogenate Cu concentrations of 21 ± 6 mg kg^{-1} DM, while 3 foals had liver Cu concentrations of 162 ± 32 mg kg^{-1} DM.

The objective in the present study was to model the decline in liver Cu concentration of foals from the previous study, and an additional 23 foals born in the following year. The relationship between the Cu concentration of the homogenised entire liver and the Cu concentration of small liver samples from defined sites, mimicking in vivo liver biopsies, was also examined.

4.3 Materials And Methods

4.3.1 Animals

A total of 33 Thoroughbred foals were used. Ten foals born in Year 1 (5 females and 5 males) and 23 were born in Year 2 (11 females and 12 males). All mares and foals were kept at pasture for the duration of the experiments and treated with an anthelmintic at recommended doses every six to eight weeks. All Year 1 foals and 18 Year 2 foals were by the same sire. The remaining Year 2 foals were by 3 different sires.

Mares received 25 mg selenium selenate orally every 4 weeks, as the pasture and soil on the property was known to be deficient in selenium (Grace, unpublished observations). Mare condition score was maintained at 5.5 or better (Henneke et al. 1983) during pregnancy, and at 4.5 or better post-partum.

Foals were weighed every two weeks using electronic platform scales, and hooves were trimmed regularly.

4.3.2 Treatment

Details of the Year 1 experiment have been described (Chapter 3). In Year 2, 23 mares were randomised into 2 groups on the basis of age, expected foaling date and sire of the foetus. The 11 mares in the treatment group (CuS) were given 250 mg calcium Cu edetate (Coprin®, Schering-Plough Animal Health Ltd.) at approximately 220, 248, 276 and 304 days gestation and then every two weeks until foaling. The 12 mares in the control group (NCu) were given saline injections of similar volume and in the same sites, at similar gestational times.

4.3.3 Liver biopsies and plasma samples

Liver samples were obtained by percutaneous, transthoracic biopsy (Pearce et al. 1997) using a 14 gauge 150 mm Tru-cut biopsy needle (Baxter Healthcare Corporation, Deerfield, IL, USA), harvesting at least 30 mg of tissue. Liver biopsies were harvested from all mares at the start of the experiment in Year 1, at an average gestational age of 205 days (range 163 to 236), and within the first 6 days post-partum. In Year 2 a liver biopsy sample was harvested from mares at approximately 7 and 8 months gestation, and within the first 4 days post-partum.

A total of 8 liver biopsies were harvested from Year 1 foals for determination of Cu concentration (weeks 1, 2, 3, 4, 8, 12, 16, and 20 of age), and 7 liver samples from Year 2 foals (weeks 1, 2, 3, 4, 5, 9 and 15 of age). Blood samples were taken at the time of each liver biopsy in lithium heparin tubes, and plasma collected after centrifuging at 3000 revolutions per minute for 10 minutes. Samples were stored at –20 °C until analysed.

4.3.4 Whole liver

Foals were euthanased at a mean age of 161 and 163 days in Year 1 and 2 respectively. The liver was examined for abnormalities, weighed, and samples obtained from the diaphragmatic surface using a Tru-cut biopsy needle. In Year 1 a liver sample was taken from a site identified 4 cm from lateral border of the right lobe and 4 cm ventral to the right triangular ligament. In most livers the sampling site was adjacent to a roughly circular region (often with fibrinous material and tags on the surface) associated with the region from where most ante-mortem biopsies were collected (Chapter 3). A second sample was taken from the most ventral part of this circular region. In Year 2 samples were obtained adjacent to the proximal, distal, and medial border of the circular area (associated with the region where most ante-mortem biopsies were collected). Samples were stored at –20 °C until analysed. The entire liver was frozen, and later homogenised for determination of the total liver Cu concentration.

4.3.5 Pasture

Pasture samples were collected at around 6 week intervals for determination of pasture Cu and zinc concentration. Samples were stored at –20 °C until analysed.

4.3.6 Analytical

Liver biopsies and homogenates, plasma and pasture samples were assayed for Cu by inductively coupled plasma atomic emission spectrometry (ICP/AES) (Applied Research Laboratories ARL-34000 ICP/AES) (Lee, 1983). All glassware was acid-washed and care was taken to prevent chemical contamination from outside sources at all times. Liver biopsy samples were prepared for ICP/AES analysis by freeze-drying and digestion in concentrated Analar nitric acid; the digest residue was dissolved in 2 mol L^{-1} hydrochloric acid. Liver biopsy samples were run with a liver standard of known Cu concentrations (National Bureau of Standards bovine liver 1577-B). Plasma samples were prepared by digestion in concentrated Analar nitric acid and

hydrogen peroxide. Liver homogenates, and dried and ground pasture samples, were digested with nitric acid; the residues were dissolved in 2 mol L^{-1} hydrochloric acid. Plasma Cu was expressed in mg L^{-1}. Liver and pasture Cu concentration was expressed on a dry matter (DM) basis.

4.3.7 Statistics

Statistical analyses used included analysis of variance (ANOVA), linear regressions and non-linear regressions using procedures of Statistical Analysis System (SAS) version 6.12 (SAS institute Inc. Cary, N.C., USA). Foals were classified as 'normal' liver Cu decline (NLCu) or 'slow' liver Cu decline (SLCu) on the basis of the average liver Cu concentration from around 15 weeks of age onwards (NLCu < 100 mg kg^{-1} DM, SLCu ≥ 100 mg kg^{-1} DM).

4.4 Results

4.4.1 Pasture copper and zinc

The mean pasture Cu and zinc concentrations are given in Table 4.1.

Table 4.1 Pasture copper and zinc concentrations

	Copper (mg kg^{-1} DM)		Zinc (mg kg^{-1} DM)	
	Mean	**Range**	**Mean**	**Range**
Year 1	7.8	5.2 – 10.2	26.2	22.0 – 31.1
Year 2	7.4	6.2 – 10.7	37.1	31.0 – 42.0

4.4.2 Plasma copper

Foal plasma Cu concentrations are given in Figure 4.1. There were no significant differences in foal plasma Cu concentration due to year, dam Cu treatment, foal liver Cu concentration or pattern of decline in liver Cu concentration.

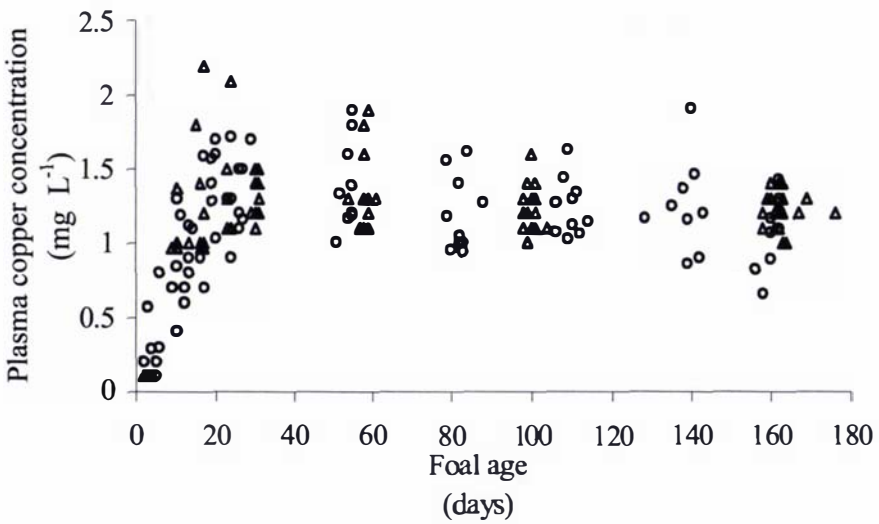


Figure 4.1 Plasma copper concentration from of 2 crops of Thoroughbred foals (○ Year 1, *n*=10; Δ Year 2, *n*=23).

4.4.3 Liver biopsies

The data from 2 animals were excluded from all analyses as suspected outliers. The first was an 18 year old Cu treated mare in Year 2 with a liver Cu concentration of 143 mg Cu kg^{-1} DM from a biopsy harvested 3 days after foaling. It was 11 days since the last Cu injection. The liver biopsy results for this mare at around 7 and 8 months gestation were 12 and 36 mg Cu kg^{-1} DM respectively. The second suspected outlier was from a foal born to a NCu mare. The foal's liver Cu concentration in the first week of life that was not consistent with subsequent biopsies: the liver biopsy results at 2 days of age was 1084 mg Cu kg^{-1} DM compared to 205 and 325 mg Cu kg^{-1} DM at 9 days and 16 days of

age respectively. This was a much more rapid decline in liver Cu concentration in the first week of life, compared to other foals.

Least squares means for liver Cu concentration of mares and foals by dam treatment were calculated with year as a fixed effect, and treatment nested within year (as the supplementation regimens were different in each year). Results are presented in Table 4.2. Least squares means for liver Cu concentration of dams and foals by pattern of liver Cu concentration decline were calculated using year and decline pattern (NLCu or SLCu) as fixed effects with interactions; results are given in Table 4.3. In each year there was no significant effect of mare Cu supplementation on mare or foal liver Cu concentration. There were significant differences in foal liver homogenate Cu concentration when grouped by NLCu and SLCu. (R^2 = 75%, p = 0.0001).

Table 4.2 Effect of 2 regimens of mare copper supplementation during late gestation on least squares means for liver copper concentration of mares and foals, in two years. CuS:dam given injectable copper in late gestation; NCu: dam not given with injectable copper in late gestation. Values with different superscripts in the same row are significantly different (p < 0.05). RV: residual variation

		Liver copper concentration (mg kg^{-1} DM)								
		Year 1				Year 2				
Dam treatment		**n**	**CuS**	**n**	**NCu**	**n**	**CuS**	**n**	**NCu**	**RV**
Animal										
Mare	Pregnant	5	26.0^{a}	5	20.0	9	11.3^{b}	9	18.5	10.3
	Post-foaling	3	12.3	1	14.0	7*	23.6	9	20.6	20.1
Foal	Newborn	5	396	5	351	10	349	11*	388	171
	160 days of age	5	121^{a}	5	85.4	11	42.2^{b}	12	43.1^{b}	68.5

*Results not included from one mare post-foaling (143 mg kg^{-1} DM) and one newborn foal (1084 mg kg^{-1} DM)

Table 4.3 Least squares means for liver copper concentration of mares and foals, in two years, grouped into 'Slow' copper decline (>100 mg kg^{-1} DM) and 'Normal' copper decline based on liver copper concentration of foals at 80 days or older. Values with different superscripts in the same row are significantly different ($p < 0.05$). RV: residual variation.

		Liver copper concentration (mg kg^{-1} DM)								
		Year 1				Year 2				
Foal group		**n**	**Slow**	**n**	**Normal**	**n**	**Slow**	**n**	**Normal**	**RV**
Animal										
Mare	Pregnant	3	26.5	7	21.5	2	8.8	16	15.8	10.6
	Post-foaling	1	14.0	3	12.3	3	19.0	13*	22.5	12.7
Foal	Newborn	3	516^{a}	7	313^{b}	3	589^{a}	18*	333^{b}	143
	160 days of age	3	254^{a}	7	38.5^{b}	3	127.8^{c}	20	29.9^{b}	24.2

* Results not included from one mare post-foaling (143 mg kg^{-1} DM) and one newborn foal (1084 mg kg^{-1} DM)

On 17 occasions liver biopsies were unable to be obtained from mares due to failure to visualise the liver ultrasonographically, 12 in the post-foaling period, and 5 during gestation.

The Cu concentrations of liver biopsies harvested from 33 foals are given in Figure 4.2.

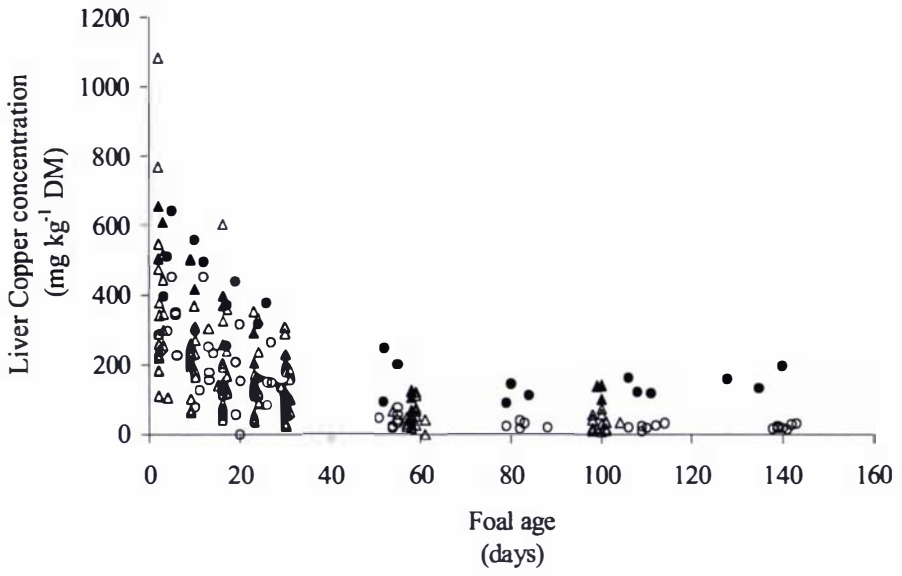


Figure 4.2 Liver copper concentration from biopsies of 2 crops of Thoroughbred foals grouped into 'Slow' decline of copper (● Year 1, *n*=3; ▲ Year 2, *n*=3) and 'Normal' decline of copper (○ Year 1, *n*=7; Δ Year 2, *n*=20) based on liver copper concentration at 100 days of age or older

Non-linear regression models (asymptotic, allometric, exponential decay and logistic regression) were fitted to the foal liver data. Data were examined by year and pattern of decline in liver Cu concentration (normal or high), and mean square errors were examined to determine the most appropriate model. Residuals were examined for bias due to foal age.

The best model to describe the decline in liver Cu concentration was an exponential decay function with 3 parameters.

$$\textit{Liver Cu concentration (mg kg}^{-1}\textit{ DM)} = \beta_0 + \beta_1(e^{-\beta 2\, age})$$

where β_0 corresponds to the liver Cu concentration at 15 to 20 weeks of age (or the asymptote)

β_1 corresponds to the liver Cu concentration at birth

β_2 describes the rate of decline

age is the foal age in days

The model was used to generate parameters for each animal; ANOVA was used to test for significant differences in the parameters due to year and grouping by NLCu or SLCu. Results are presented in Table 4.4 and Figure 4.3.

Table 4.4 Non-linear regression parameters (± standard error of the mean) to describe the decline in liver copper concentration of foals (y) from birth to approximately160 days, where age is in days in the model $y = \beta_0 + \beta_1(e^{-B_2 \text{ age}})$. Values with different superscripts in the same column are significantly different ($p < 0.05$). β_0 corresponds to the liver copper concentration at 15 to 20 weeks of age (or the asymptote), β_1 corresponds to the liver copper concentration at birth, β_2 describes the rate of decline.

Foal Group	Year	β_0	β_1	β_2
Normal	1	20.60 ± 7.81^a	374.0 ● 56.1	0.06138 ± 0.01314
	2	21.98 ± 4.62^a	343.5 ± 33.2^d	0.05720 ± 0.00778
Slow	1	166.4 ± 11.9^b	459.3 ± 85.7	0.05679 ± 0.02007
	2	109.7 ± 11.9^c	565.3 ± 85.7^e	0.06859 ± 0.02007

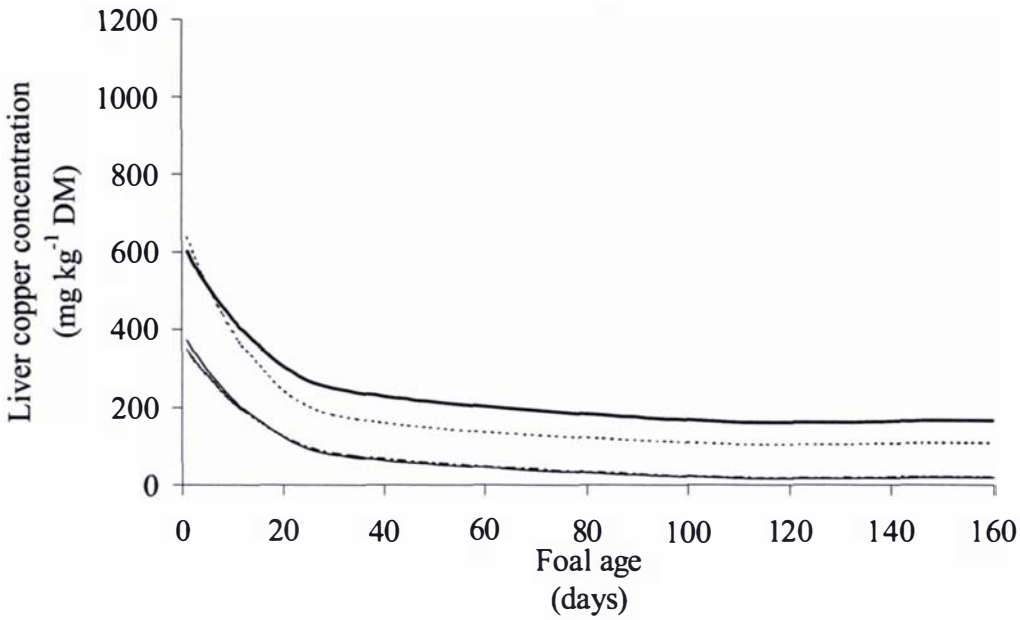


Figure 4.3 Predicted liver copper concentration of 2 crops of Thoroughbred foals grouped into 'Slow' decline of copper (— Year 1; ... Year 2) and 'Normal' decline of copper (—Year 1; •– · Year 2) based on liver copper concentration at 80 days of age or older, using the model $y = \beta_0 + \beta_1 * e^{-B_2 \text{ age}}$

4.4.4 Total liver, homogenates and samples

The repeatability of the liver Cu concentration from biopsy samples harvested at post-mortem examination was examined using the following formula:

$$\begin{aligned} \textit{Repeatability} &= \sigma^2 X_1 / (\sigma^2 X_1 + \sigma^2 X_2) \\ &= 2088/(2088 + 314) \\ &= 0.87 \end{aligned}$$

where $\sigma^2 X_1$ is the variance between foals

$\sigma^2 X_2$ is the variance within foals.

The repeatability was high, indicating the within foal variation was small, compared to the large between foal variation.

The relationship between the average Cu concentration of the post-mortem liver biopsies and the whole liver homogenates of foals grouped by NLCu or HLCu was examined by regression. The relationship for each group is described below:

'Normal' group homogenate Cu concentration (mg kg^{-1} DM)

$= 0.79 (\pm 0.12) * b + 15.0 (\pm 2.8)$

$R^2 = 0.68,\ p = 0.0001$

'High' group homogenate Cu concentration (mg kg^{-1} DM)

$= 1.79 (\pm 0.33) * b - 62.3 (\pm 48.7)$

$R^2 = 0.88,\ p = 0.006$

where b is the average Cu concentration of the biopsies obtained at post-mortem.

The intercept was higher than 1 for both groups, indicating a bias in the homogenate results. The liver homogenates had higher Cu concentration than the average of the biopsies taken at post-mortem examination for 31 of the 33 animals.

The mean foal liver weight (± SD) was 3.250 kg ± 0.320, with no significant differences in weight due to foal age, sex, year of birth, or pattern of decline in liver Cu concentration. The average liver DM was 27.8 ± 1.03%, and the average total liver Cu was 28.6 ± 11.4 mg and 174 ● 71.2 mg for NLCu or SLCu foals respectively.

4.5 Discussion

The validity of biopsy samples as an indicator of total liver Cu storage has been questioned for many species including horses, due to regional variability of Cu within the liver (O Cuill et al. 1970; Bingley and Dufty, 1972; Egan and Murrin, 1973a; Diaz et al. 1990). The distribution of Cu in the liver may reflect the site of Cu absorption from the gastrointestinal tract, and a consequence of portal

streaming (Haywood, 1981). Differences in Cu storage within adult horse livers have been described, with coefficients of variation of less than 9% for 50 g samples, and 21% for 30 to 60 mg samples (Pearce et al. 1997) and coefficients of variation up to 47% for 1000 mg samples (O Cuill et al. 1970). Results from the present study suggest 30 to 60 mg liver samples from foals at around 160 days of age are highly repeatable for Cu concentration, and were strongly correlated with total liver Cu, as determined in homogenates of the whole liver.

Distribution of Cu in the liver may reflect the site of Cu absorption (Haywood, 1981), and may also be influenced by dietary and physiological factors (Pearce et al. 1997). The average Cu concentration of biopsies harvested from liver at post-mortem was lower than liver homogenate for NLCu foals. Similar results have been reported in a single calf liver (Bingley and Dufty, 1972), and in two of three horse livers (Pearce et al. 1997). In the SLCu foals of the present study the average Cu concentration of biopsies was higher than the liver homogenate. Sheep liver biopsies overestimate hepatic content by almost 5% (Donald et al. 1984).

Three foals in each year were identified as showing a different pattern of decline in liver Cu concentration by 100 days of age, but the physiologic significance is unknown. One mare gave birth to a SLCu foal in each experimental year (the two foals were full siblings), which raises the possibility of a genetic component. Differences in Cu metabolism have been reported in different breeds of sheep, which may be due to different absorption efficiency, or partitioning of absorbed Cu (Woolliams et al. 1983). Welsh Mountain ewes have higher blood Cu concentrations than Scottish Blackface ewes in spite of Cu injections and seasonal variation in blood Cu concentration (Wiener et al. 1969; Wiener et al. 1970). The breed of the dam is an important determinant of lamb Cu status at birth and in early life (Wiener et al. 1984), with lambs born to Welsh Mountain ewes having higher blood and liver Cu concentrations than those from Scottish Blackface sheep. Genetic differences in Cu metabolism may also exist in cattle (Gooneratne et al. 1994). Several dog breeds, including the Bedlington terrier, accumulate Cu in the liver throughout life (Thornburg, 2000).

In the current study there were no differences in plasma Cu concentration between NLCu and SLCu foals. Plasma Cu increased rapidly from very low levels at birth to levels similar to adults by approximately 30 days of age. This is consistent with earlier reports (Bell et al. 1987; Pearce et al. 1998b). In foals fed milk based diets containing only 1.7 μg Cu kg^{-1}, serum Cu increased for the first week of life, but dropped between the 10^{th} and 20^{th} week, reaching less than 0.1 mg L^{-1} by the 18^{th} week (Bridges and Harris, 1988). In the latter study the mean liver Cu concentration of the low dietary Cu foals was 8.3 mg kg^{-1} DM at post-mortem (4 to 7 months of age), compared to 29.5 mg kg^{-1} DM of age-matched control foals.

In the present study foal liver Cu concentration at birth varied as much as seven-fold, even when the highest value was removed from the analysis. Others have reported similar values for foals of different breeds, and under different management systems (Egan and Murrin, 1973a; Meyer and Tiegs, 1995; van Weeren et al. 2003). In the current study there were no significant differences in foal liver Cu concentration between years, or with dam Cu supplementation, indicating that two different regimens of injectable Cu supplementation of dams had no effect on foal liver Cu storage in the first 5 months of life. Conversely oral Cu supplementation of mares in the last 15 to 25 weeks of gestation increased the liver Cu concentration of foals at birth, but this effect was not apparent at 5 months of age (Pearce et al. 1998b).

Failure to obtain liver biopsies in mares has been reported previously (Pearce et al. 1997), and may be due to atrophy of the right lobe which occurs commonly in older horses (Sisson, 1975), or displacement of abdominal organs with advancing pregnancy. In Year 2 treated mares received a total of 1250 to 1500 mg Cu prior to foaling, with no resulting increase in liver or plasma Cu. The exception was one mare that received 1250 mg Cu in total and had a considerably higher liver Cu concentration (143 mg kg^{-1} DM) compared to all other mares. The interval between the last Cu injection and foaling in this mare was 8 days, which was the average interval for all treated mares, but no other mares showed a high liver Cu concentration. Thus the high post foaling liver Cu concentration in this mare does not seem to be explained by the interval

between the last injection and foaling. The results show that for most mares parenteral supplementation during pregnancy is not able to improve liver Cu concentration of either the mare or foal.

Pasture Cu and zinc concentrations were lower than current recommendations (10 mg Cu kg^{-1} DM and 40 mg zinc kg^{-1} DM, NRC, 1989) in both years of the experiment. Diets containing high levels of zinc may induce secondary Cu deficiency in horses (Gunson et al. 1982; Eamens et al. 1984). Liver Cu concentrations of 16.6 and 18.5 mg kg^{-1} DM have been reported for mares with equivalent dietary Cu concentrations of 8 and 30 mg kg^{-1}DM respectively in New Zealand (Pearce et al. 1998c), where the latter group received oral Cu sulphate thrice weekly. Liver Cu concentrations of 18 to 22 mg kg^{-1} DM have been reported for yearling horses, consuming diets containing 7 to 15 mg Cu kg^{-1} DM (Cymbaluk and Christensen, 1986), and liver concentrations of 12 to 26 mg Cu kg^{-1} DM for yearlings grazing New Zealand pasture with or without supplementary Cu (8 and 30 mg Cu kg^{-1} DM respectively) (Grace et al. 2002). These results are similar to the findings of this study, where mares grazed pasture containing less than 10 mg Cu kg^{-1}DM. This suggests that diets containing less than the recommended dietary Cu levels (NRC, 1989) do not result in liver Cu depletion in adults, and therefore dietary Cu of less than 10 mg Cu kg^{-1}DM may be considered as adequate for pregnant Thoroughbred mares. In mature ponies the maintenance dietary Cu requirement of 3.5 mg kg^{-1}DM was estimated to be adequate (Cymbaluk et al. 1981).

The importance of Cu supplementation of pregnant mares at pasture, in order to raise foal liver stores at birth, may be greatly overestimated. The route of Cu supplementation is likely to affect the efficacy of supplementation, and genetic influences may greatly affect the concentration of Cu in foal liver during the first 160 days of life. The relationship between dam Cu supplementation in late gestation, foal liver Cu concentration and DOD is the focus of ongoing research.

Chapter 5

Enlargements of the distal third metacarpus and metatarsus in Thoroughbred foals at pasture from birth to 160 days of age

5.1 Abstract

Objectives The objectives of this study were to assess the relationship between the radiographic and microscopic appearance of the distal metacarpal and metatarsal (Mc3 and Mt3) physeal region of Thoroughbred foals at 160 days of age born in Year 1 and 2, and to clinically assess changes in the contour of the distal Mc3 and Mt3 physeal region in foals from birth to 160 days of age (Year 2). The relationships between maximum clinical physis scores and age, time of year, foal sex, condition score, growth rate, liver copper concentration of the foal, and dam copper supplementation in late gestation were also assessed.

Methods Ten foals were examined every 2 weeks for evidence of distal Mc3/Mt3 pain and lameness in Year 1. Twenty-three foals were examined every 2 weeks from birth to 160 days, and a clinical physis score for distal Mc3/Mt3 given in Year 2. Cabinet radiographs of sagittal slices of the distal Mc3/Mt3 physeal region at around 160 days of age were given a radiographic physis score. Physes were examined histologically for evidence of abnormal endochondral ossification.

Results Enlargements of the distal Mc3 and Mt3 were observed in all foals in this study, but were not associated with lameness, pain or inflammation. The most severe clinical physis scores occurred over two months in late summer/autumn, and were not influenced by the foal growth rate, sex, liver copper concentration or dam treatment in late gestation. The clinical physis score was highly correlated to radiographic evidence of shouldering in the forelimb and hindlimb (both $p < 0.0001$). Focal disturbances in endochondral ossification were evident radiographically and histologically in the some of the physes at 160 days of age.

Conclusions The distal Mc3 and Mt3 enlargements were not consistent with the previous descriptions of physitis. Results suggest that while many Thoroughbred foals at pasture will have visible bony distal Mc3/Mt3 enlargements in the first 5 months of life, few have physeal cartilage

abnormalities, or significant compromise of endochondral ossification. The importance of these clinical swellings may be overestimated, and they may more appropriately be called physiological enlargements associated with bone remodelling.

Abbreviations

Cu	Copper
CuS	Copper supplemented
DOD	Developmental orthopaedic disease
FP	Total forelimb clinical physis score
FP_{max}	Maximum forelimb clinical physis score
HP	Total hindlimb clinical physis score
HP_{max}	Maximum hindlimb clinical physis score
LDS	Last date of service
Mc3	Third metacarpal bone
Mt3	Third metatarsal bone
NCu	No copper supplementation

5.2 Introduction

Despite widespread occurrence, purported increasing prevalence of physitis in young horses (Sherrod, 1975; Thompson et al. 1988), and related severe clinical effects (Frankeny et al. 1994), there are few reports describing visible changes in limb contour over time, or the radiographic and the microscopic abnormalities. Various authors have suggested that the characteristic bony swellings may be due to abnormal growth and thickening of the metaphyseal cartilage and/or excessive bone formation (Rooney, 1963; Williams et al. 1982; Thompson et al. 1988; Hurtig and Pool, 1996). Although the exact aetiology is unknown (Turner, 1987), it has been suggested that the physeal changes may be due to excessive loading of the physis in association with excessive weight, exercise or conformational abnormalities, or structurally deficient bone that results in cartilage retention or poor trabecular bone formation (Bramlage,

1993). Genetic predisposition and rapid growth rate may also influence the likelihood of the disease (Turner, 1987), and fillies may be more severely affected (Hunt, 1997).

Physitis involving the distal third metacarpus and metatarsus (Mc3 and Mt3) peaks in foals at 4 to 8 months of age (Coffman, 1973), when physeal closure is initiated (Wagner and Watrous, 1990). Distal radial physitis is more likely to occur in yearlings and horses up to 2 years of age (Turner, 1987).

Negative correlations between the severity of fetlock physitis in foals and pasture copper (Cu) concentrations have been reported (Asai et al. 1993; Fujikawa et al. 1993). In New Zealand, oral Cu supplementation of dams during late pregnancy was associated with less severe radiographic physitis scores of the hind fetlocks in their foals at 5 months of age (Pearce et al. 1998a). Copper supplementation of horses suffering from presumed Cu deficiency appeared to hasten the resolution of physitis (Egan and Murrin, 1973b; Carbery, 1978).

Physeal dysplasia may be a more appropriate term than physitis or epiphysitis for the underlying changes resulting in bony enlargements at the distal ends of long bones in foals (Jeffcott, 1993), as the primary cause is unlikely to be inflammatory in origin (Brown and MacCallum, 1976; Firth, 1990). However, this terminology is not in widespread use (Hurtig and Pool, 1996), probably due to disagreement as to whether all distal long bone enlargements are manifestations of physeal dysplasia or if some enlargements are part of a normal physiological response to weight-bearing (Bramlage, 1993). Physeal dysplasia is considered one of the developmental orthopaedic diseases (DOD) (Hurtig and Pool, 1996). The DOD complex is characterised by disturbances in growth cartilage (Jeffcott, 1997). There is further speculation that physitis/physeal dysplasia may be a manifestation of osteochondrosis (White, 1980), or a result of it (Rejno and Stromberg, 1978). In pigs, lesions associated with the physes are considered to be different to lesions associated with the articular-epiphyseal cartilage complexes (Hill et al. 1990). The lesion found in foals may have no

relation to clinical disease (Firth and Poulos, 1984).

The objectives of this study were to assess the relationship between the radiographic and microscopic appearance of the distal Mc3/Mt3 physeal area of Thoroughbred foals at 160 days of age born in two successive years (Year 1 and 2), and to clinically assess the distal Mc3/Mt3 physeal area in foals from birth to 160 days of age (Year 2). The relationships between clinical physis scores and age, time of year, sex, condition score, growth rate, liver Cu concentration of the foal at birth and 160 days, dam Cu supplementation in late gestation, and radiographic physis scores, closure scores and histology of the physis were also assessed.

5.3 Materials and Methods

5.3.1 Animals

A total of 33 Thoroughbred foals were used in this study, 10 foals born in Year 1 (5 females and 5 males) and 23 born in Year 2 (11 females and 12 males). All mares and foals were kept at pasture and treated with an anthelmintic every 6 to 8 weeks. Pasture samples were collected every 4 to 8 weeks for determination of mineral concentrations. Mares received 25 mg selenium selenate orally every 4 weeks as the pasture and soil was known to deficient in selenium (Grace, unpublished observations). Mare condition score was maintained at 5.5 or better (Henneke et al. 1983) during pregnancy, and at 4.5 or better during lactation. Foals were weighed every two weeks using electronic platform scales, and hooves were conventionally trimmed every 2 to 6 weeks.

Details of the Year 1 foals and dams have been described in Chapter 3. Briefly 10 Thoroughbred mares, aged 9 to 16 years (mean age 12 years) were allocated into two groups on the basis of age, last date of service (LDS), and liver Cu concentration at the start of the experiment. The treatment group of mares (CuS) received 100 mg calcium Cu edetate (Coprin®, 50 mg/ml, Schering-Plough Animal Health Ltd) in the ninth month of gestation (calculated from the LDS) and a further 250 mg during the tenth month of gestation. Copper doses

were divided in two, and administered by deep injection in the left and right pectoral regions. The control mares (NCu) were given saline injection using similar volume, times and sites.

In Year 2, mares were allocated into 2 groups on the basis of age, LDS and sire of the foetus. Injections of 250 mg calcium Cu edetate (Coprin®, Schering-Plough Animal Health Ltd.) were given to CuS mares (n = 11) at around 220, 248, 276 and 304 days gestation and then every two weeks until foaling. Saline injections using similar volume, times and sites were given to NCu mares (n = 12).

5.3.2 Clinical examination

Foals born in Year 1 were examined for conformational abnormalities and evidence of distal Mc3/Mt3 pain and lameness every 2 weeks from birth to 160 days of age, abnormalities were described, but no clinical physis scores were assigned.

Foals in Year 2 were condition scored (Henneke et al. 1983) and clinically scored for evidence of distal Mc3/Mt3 physeal swelling every 2 weeks from birth to 160 days of age, by the same observer. Clinical scoring was usually on the same day as weighing. The subjective scoring system (Table 5.1) was similar to that of Cymbaluk and Christison (1989b) and Hoffman et al. (1999), based on visual examination of physeal region contour when viewed from in front of the animal so the most medial and lateral part of the distal Mc3 and Mt3 was visible, at a distance of 1 metre or less. Half scores were used. A medial and lateral score were given to every limb (i.e. every foal had 8 single physis scores at every examination). Combining the left and right, medial and lateral forelimb scores gave the total forelimb physis score (FP); similarly the single scores of the hindlimbs were combined to give a total hindlimb physis score (HP).

Liver samples were harvested by percutaneous biopsy from foals in Year 1 during weeks 1, 2, 3, 4, 8, 12, 16, and 20 of age for determination of Cu

concentration, and from foals in Year 2 during weeks 1, 2, 3, 4, 5, 9 and 15 of age (Chapters 3 and 4).

Table 5.1 Subjective clinical scores for evaluation of distal third metacarpal and metatarsal regions. Medial and lateral aspects are scored independently and half scores used when required.

Clinical abnormality	Score
No contour changes	0
Mild change in contour visible, but no extensive flaring	1
Moderate, clearly visible changes, with substantial metaphyseal flaring that alters the configuration of the joint	2
Severe contour change of physeal region, with extensive metaphyseal flaring, but not lame	3
As above but animal lame, and physeal region is painful to palpate	4

5.3.3 Post-mortem

Foals were euthanased at around 160 days of age, by intravenous barbiturate or free bullet, after sedation with intravenous xylazine hydrochloride. At post-mortem forelimbs were removed at distal radius, and hindlimbs removed at the tarsometatarsal joint. The liver was weighed, and homogenised for determination of Cu concentration. Samples were stored at -20 °C until analysed.

5.3.4 Preparation of physes

Both Mc3 and Mt3 of the Year 1 foals, and left Mc3 and Mt3 of the Year 2 foals were defleshed, the length measured; then each was sawn transversely at approximately 130 mm from the distal end of the condyle. The distal portions were fixed in 10% buffered formalin solution and were sawn frontally, the first cut through the most distal portion of each condyle. The next cut was 2 mm palmar/plantar to the first, and the thickness of each slice was measured laterally and medially at the level of the physis, with a sliding calliper. Differences of

more than 2 mm between the medial and lateral side were equalised by sanding with wet and dry paper. The slice was washed under running water.

5.3.5 Radiographic examination

Cabinet radiographs of the 2 mm slice were taken (55 kV, 3 mA and 30 seconds) using a Torrex 150 machine (Torr X Ray Corp., California, USA). The slabs and slices were stored in alcohol until further analyses were performed. The radiographs were scored for morphological abnormalities in a manner similar to that described by Pearce et al. (1998a) for whole bones, without the use of a hot light (Table 5.2). Scores for morphological abnormalities were added together to give a radiographic physis score for each limb. Physeal flaring was defined as a local widening of the physis medially or laterally; beaking was defined as a bony protrusion at the level of the physis, with a concavity of the adjacent metaphysis and epiphysis; metaphyseal sclerosis was defined as an increase in radiodensity proximal to the physis; metaphyseal shouldering was defined as an abnormal convex contour of the metaphyseal cortex proximal to the physis; epiphyseal and diaphyseal malalignment were defined as angulation between a line drawn through the midpoint of the most distal and proximal aspect of the epiphysis and an axial line through midpoints in the metaphysis and diaphysis.

The degree of radiographic physis closure was assessed using a quantitative grading system of the proportion of the measured distance of physeal width in the slice that was closed (i.e. no radiolucency between the metaphysis and epiphysis). Grade 1 was 'closed' (more than 80% of the physis), Grade 2 was 'closing' (more than 20% of the physis was closed) or Grade 3 was 'open' (less than 20% of physis was closed). These grades correspond to grades A, B and C used by others (Reed, 1965). The cartilage thickness, degree of undulation (Firth and Poulos, 1984), presence of cartilage cores and epiphyseodeses were also noted.

Table 5.2 Subjective scores for radiographic evaluation of distal Mc3 and Mt3 physeal region slices

Radiographic abnormality	Score for each physis
Focal cartilage widening	0 = not present, 1 = mild, 2 = moderate, 3 = severe
Physeal flaring	0 = not present, 1 = present
Metaphyseal sclerosis	0 = not present, 1 = present
Metaphyseal shouldering[a]	0 = not present, 1 = mild, 2 = moderate, 3 = severe
Epiphyseal and diaphyseal malalignment	0 = not present, 1 = mild, 2 = moderate, 3 = severe

[a] Medial and lateral scores were added together for each physis

5.3.6 Histopathology

The physeal regions of the 2 mm Mc3 and Mt3 slices were cut into 3 sections (lateral, central and medial), decalcified in formic acid/sodium formate, and vacuum-embedded in paraffin wax. Sections (6 μm) were stained with haematoxylin and eosin (H&E) and alcian blue. Each physis was assessed for the evidence of closure and cartilage or bone abnormalities.

5.3.7 Statistical analysis

Data were statistically analysed by ANOVA, using the GLM Procedures of SAS version 8.2 (SAS institute Inc. Cary, N.C., USA). A 5% level of statistical significance was chosen. The raw data are presented as arithmetic means and standard deviations (SD). The growth data were analysed as: absolute weight; average daily weight gain (ADG) from birth to the time of clinical scoring; ADG in the two weeks before each clinical score; change in ADG between ADG in the two weeks before clinical scoring, and ADG 2 weeks earlier; and the percentage weight change between each 2 week period. Other variables examined included foal sex, condition score at the time of clinical scoring, change in condition score between at the time of clinical scoring and condition

score 2 weeks earlier, Cu or saline treatment of the dam during late gestation, and liver Cu concentration of the foal at birth and at post-mortem. Regression analysis was used to determine the relationship between variables and maximum clinical physis scores. Mixed linear models with interactions were fitted to determine variables that had significant influences on clinical physis scores. Residual standard deviation (RSD) was calculated for significant models. Spearman rank correlations were determined between clinical physis scores prior to post-mortem and radiographic physis scores, and individual scores that made up the radiographic shouldering scores.

5.4 Results

The pasture mineral concentrations are given in Table 5.3. The mean foal birth weight, and weight and age at post-mortem examination are given in Table 5.4 for fillies and colts in each year. Foals were born over a period of 8 and 16 weeks in Year 1 and 2 respectively. There was no significant differences in foal weight at birth (or at euthanasia) due to the year of birth or gender.

In Year 1, one foal had bilateral carpal valgus angular limb deformity, and was treated conservatively with appropriate hoof trimming and exercise restriction. In Year 2, two foals had hemicircumferential periosteal transection and periosteal elevation of the distal medial radius before 3 months of age. Another foal had congenital flexural deformity of one hind fetlock, which improved within 1 week with the use of splints, intravenous oxytetracycline and toe extensions. One foal showed signs of bilateral acquired flexural deformity of the forelimbs in the two weeks prior to euthanasia; the animal responded satisfactorily to restricted exercise.

Table 5.3 Mineral composition of ryegrass/clover mixed sward pasture in each year.

		Na	K	Ca	P	Cu	Fe	Zn
		g kg^{-1} DM				mg kg^{-1} DM		
Year 1	Mean	2.2	32	3.9	3.5	7.8	198	26
	SD	0.58	8.6	0.68	1.0	1.6	109	3.2
Year 2	Mean	2.7	26	4.2	3.6	7.4	225	37
	SD	2.1	6.3	2.3	1.1	1.8	95.3	5.6

SD standard deviation

Table 5.4 Mean weights of foals in Year 1 and 2 at birth and euthanasia, and age at euthanasia

		n	Birth weight (kg)	SD	Weight at euthanasia (kg)	SD	Age at euthanasia (days)
Year 1	Colts	5	57.8	1.6	242.8	5.6	156 - 162
	Fillies	5	56.8	5.4	235.0	12.1	160 - 172
Year 2	Colts	12	53.0	5.5	229.4	14.9	159 - 164
	Fillies	11	55.0	4.8	238.5	18.6	158 - 176

SD standard deviation

5.4.1 Clinical physis scores in Year 2 foals

5.4.1.1 All clinical scores

The data were examined with foal age as a repeated measure using a linear mixed model, with age and scoring site as fixed effects (forelimb or hindlimb and medial or lateral physis, left or right limb), and interactions between age and scoring site (forelimb or hindlimb and medial or lateral physis), and with foal as a random effect. All main effects and interactions were significant

($p < 0.001$) with the exception of side (left or right), which was removed from the model. The model accounted for 61% of variation in clinical physis scores ($p < 0.0001$, $n = 2512$). Adjusted mean clinical scores for the medial and lateral physis of forelimbs and hindlimbs were calculated, and are presented in Figure 5.1. All clinical physis scores increased from birth to 15 weeks of age, then remained at a similar score, or slowly decreased. Hindlimb physes had higher mean clinical scores than forelimb physes at each age. Forelimb lateral clinical physis scores were significantly higher than forelimb medial clinical physis scores from 15 weeks of age.

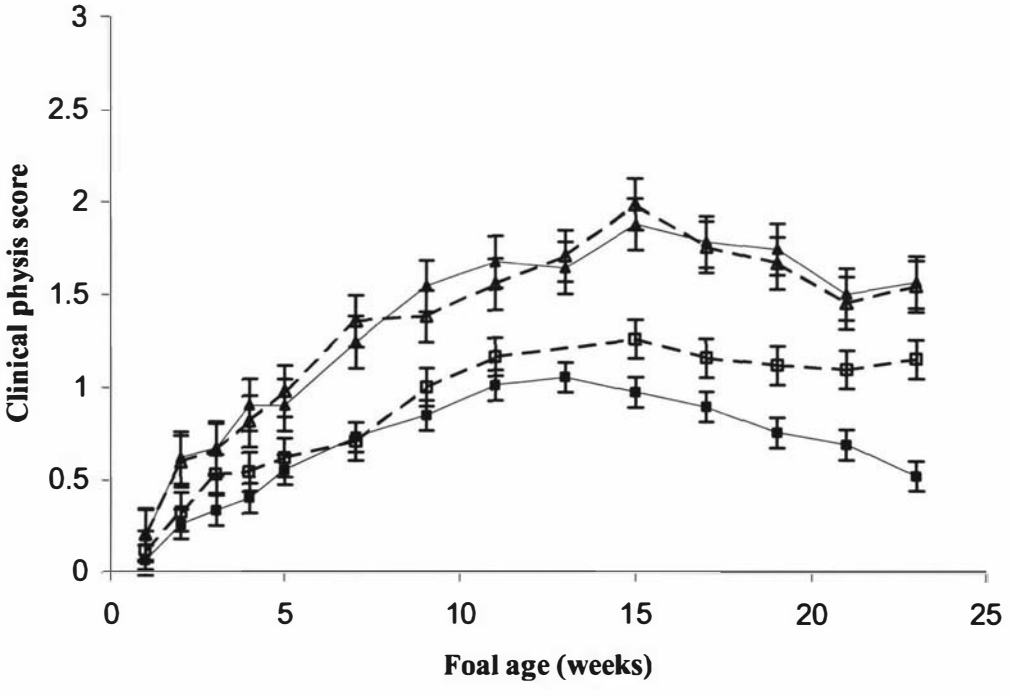


Figure 5.1 Adjusted mean clinical physis scores (± standard error of the mean) for foals in Year 2 (n = 23) of lateral forelimb (□– –□), medial forelimb (■—■), lateral hindlimb (Δ– –Δ) and medial hindlimb (▲—▲)

When foal age was replaced in the model with week of the year, the interaction between experimental week and clinical scoring site (forelimb or hindlimb and medial or lateral) became non-significant, and the model accounted for 64% of

the variation in clinical physis scores ($p < 0.0001$, $n = 2512$). The effect of age and week of year were difficult to separate, but a season effect did appear to influence the clinical physis scores. An example of enlargement of the distal Mc3 physeal region is shown in Figure 5.2.

5.4.1.2 Maximum clinical scores

The mean FP_{max} and HP_{max} were 6.2 and 8.2 respectively. With non-significant effects removed the FP_{max} could be described by the following regression equation

$$FP_{max} = 1.1 (\pm 0.5) * b + 0.17 (\pm 0.07) * c$$

$$R^2 = 41\%, p = 0.005, \text{ RSD } 1.15, n = 23$$

where b is the change in condition score between condition score at the time of FP_{max} and the previous scoring occasion (2 weeks earlier), and c is the week of the year (i.e. a seasonal effect). The severity of FP_{max} was associated with a positive increase in condition score of the foal in the previous 2 weeks, and there was also a strong seasonal effect. The sex of the foal, ADG from birth, ADG in the previous 2 weeks before FP_{max}, or change in ADG at the time of FP_{max} and the previous scoring occasion, dam treatment in late gestation and liver Cu concentration at birth or 160 days had no significant effects on the severity of the score.

The HP_{max} could be described by the following regression equation.

$$HP_{max} = 0.39 (\pm 0.15) * c$$

$$R^2 = 24\%, p = 0.020, \text{ RSD} = 1.7, n = 23$$

where c is the week of the year. This indicates a season effect on the severity of HP_{max}. The sex of the foal, ADG from birth, ADG in the previous 2 weeks before HP_{max}, or change in ADG at the time of HP_{max} and the previous scoring occasion, condition score at the time of HP_{max} or change in condition score between the time of HP_{max} and the previous scoring occasion, dam treatement in late gestation and liver Cu concentration at birth or 160 days had no significant effect on the severity of the score.

The time at which the maximum physis score occurred was defined for each animal as the first occasion the highest forelimb or hindlimb physis score was achieved. FP_{max} occurred between the 18^{th} and 25^{th} weeks of the experiment in 16 of the 23 foals that were aged between 10 and 16 weeks at that time. HP_{max} occurred between weeks 20 and 24 of the experiment, and in foals aged between 9 and 17 weeks, for 16 of the 23 foals.

5.4.2 Physis radiographs

Examples of 2 mm slice radiographs are given in Figure 5.3. All forelimb physes were either closed (31 of 43) or closing (12 of 43), while hindlimb physes were open (18 of 43), closing (17 of43) or closed (8 of 43). Normal physeal closure began in the central region of the physis. However, in 9 of 86 limbs different patterns of closure were seen, either as one side of the physis closed with the other open, or more commonly closure only of the medial or lateral trough, with the central region remaining open. The physes varied in the degree of undulation and cartilage thickness, with steeper inclination more common in closing physes. Focal increases in cartilage height were common in closing physes in the lateral or medial troughs or the margins, and usually extended into the metaphysis, but occasionally into the epiphysis.

Slight beaking was present in most limbs, both medially and laterally. An increase in concavity of the epiphyseal contour of the epiphysis (scalloping) was more pronounced in Mt3 compared to Mc3, but was unrelated to physeal abnormalities. Metaphyseal sclerosis was present in 21/40 Year 1, and in 12/46 Year 2 physis radiographs. Sclerosis was presents in the medial aspect in 22 physes and in the lateral aspect of 15 physes. Radiographs of 3 limbs, 2 from the same animal, showed marked epiphyseal malalignment.

The mean radiographic scores at post-mortem were 1.3 for forelimbs (n = 43) and 3.4 for hindlimbs (n = 43). There were no differences in forelimb or hindlimb radiographic physis scores due to experimental year, foal sex, weight at post-mortem, average daily weight gain from birth to post-mortem, liver Cu concentration at birth or post-mortem, dam treatment in late gestation or change

in liver Cu concentration of the foal from birth to post-mortem. There was a small negative effect of age at post-mortem on Mt3 radiographic score.

$$\text{Hindlimb radiographic physis score} = -0.17\ (\pm 0.05) * d + 31.1 \pm 8.6$$

$$R^2 = 20\%,\ p = 0.003,\ RSD = 1.37,\ n=43$$

Where d is the age of the foal in days at post-mortem. Older foals at post-mortem had less severe radiographic physis scores, indicating the distal Mt3 enlargements were starting to reduce or resolve. There were no significant differences in mean radiographic scores between foals born in Year 1 and 2.

Clinical physis scores of foals immediately prior to post-mortem were correlated with radiographic physis score ($r = 0.636$, $p = 0.001$, $n = 23$ and $r = 0.484$, $p = 0.019$, $n = 23$ for forelimbs and hindlimbs respectively). Shouldering was the only component of the radiographic score that was positively correlated with clinical physis score ($r = 0.851$, $p < 0.001$, $n = 23$ and $r = 0.514$, $p = 0.012$, $n = 23$ for forelimbs and hindlimbs respectively). The high correlation between clinical physis scores and radiographic shouldering scores indicated it was most likely that the distal Mc3/Mt3 enlargements scored clinically were apparent as shouldering radiographically.

There was a strong correlation between physis closure score and radiographic evidence of shouldering ($r = 0.764$, $p < 0.0001$, $n = 43$ and $r = 0.803$, $p < 0.001$, $n = 43$, forelimb and hindlimb respectively). Shouldering was more prominent in physes that were open or partially closed. However, shouldering was still present in some limbs with closed physes.

5.4.3 Histological examination of physes

There was variation in the histological appearance of the physis, with some physes very wide and with little undulation, while others were of variable thickness with marked changes in inclination and irregularity. The physeal width varied within the physis, between limbs and between animals. Eosinophilic streaks varied in frequency, usually coursing parallel to the chondrocyte columns. Chondrocytes adjacent to streaks were often irregularly shaped in contrast to the more normal oval shape, and abnormally oriented.

Occasionally single large oval-shaped chondrocytes were observed on the epiphyseal border of the physis, either singularly or in groups.

Closure of the physis began in the central third of the physis, where cartilage structure became very disorganised. In physes with the central region closed, focal areas of epiphyseal and metaphyseal bone fusion were relatively common on steep inclinations of the physis, separated by islands of disorganised cartilage. Epiphyseodesis extending from the epiphyseal side (complete or partial) was common in closing physes, and considered normal. Areas of fusion were sometimes adjacent to focal areas of cartilage retention.

Focal increases in cartilage height of the physis were relatively common, often in the distal troughs and often in association with eosinophilic streaks (Figure 5.4). The cartilage in these areas contained mainly hypertrophic cells, and in large lesions there was often eosinophilic staining of matrix. In most areas of increased cartilage height the hypertrophic cells were of normal appearance, although in some large lesions there were many vacuolated cells, chondrocytes with eosinophilic staining cytoplasm, and eosinophilic shrunken nuclei. Occasionally chondrocyte clusters (chondrones) were present. Generally the chondrocytes were aligned normally in areas of increased cartilage height, but were occasionally disorganised. Two areas of increased cartilage height contained focal areas of calcification. Primary spongiosa adjacent to increased cartilage height was either normal in appearance, or less commonly was associated with short, stubby and irregularly shaped trabeculae. One physis had a small area of cartilage necrosis with associated metaphyseal and epiphyseal fibroplasia.

The metaphyseal cortex and primary and secondary spongiosa proximal to the medial and lateral margins of the physes showed no abnormalities. Similarly the perichondrial/periosteal region had no evidence of abnormality or disruption, with oval cells in the ossification groove and collagen fibres in the perichondrial ring.

There was no significant effect of year, foal sex, average daily weight gain, condition score, dam treatment in late gestation or Cu concentration of the foal liver at birth or at 160 days of age on the number of histological abnormalities.

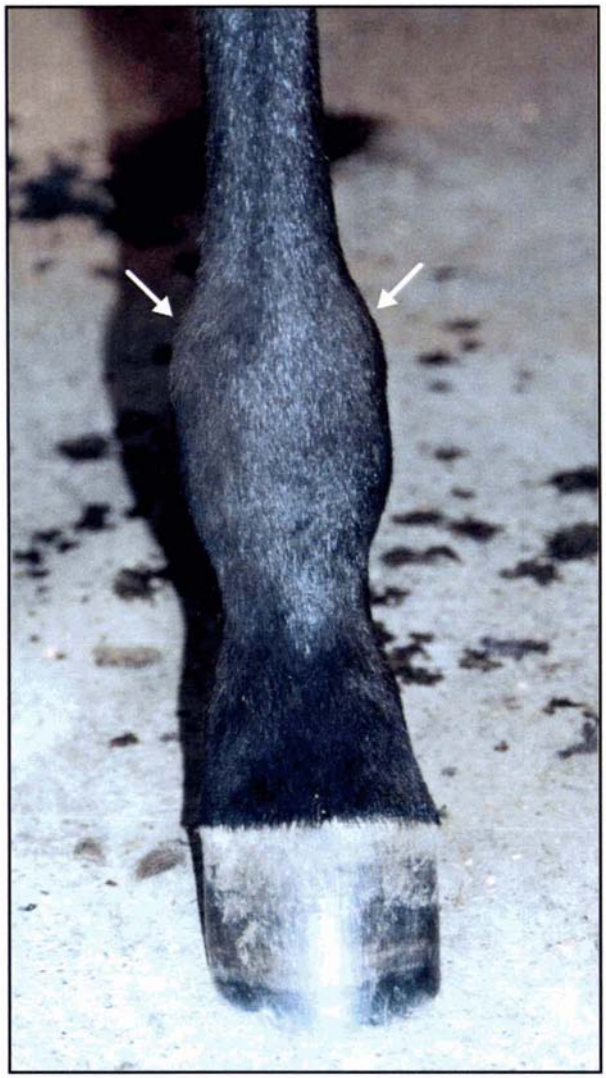


Figure 5.2 Enlargements of the left third metacarpal bone distal metaphysis in a 5 month old foal, clinically scored 2 both medially and laterally.

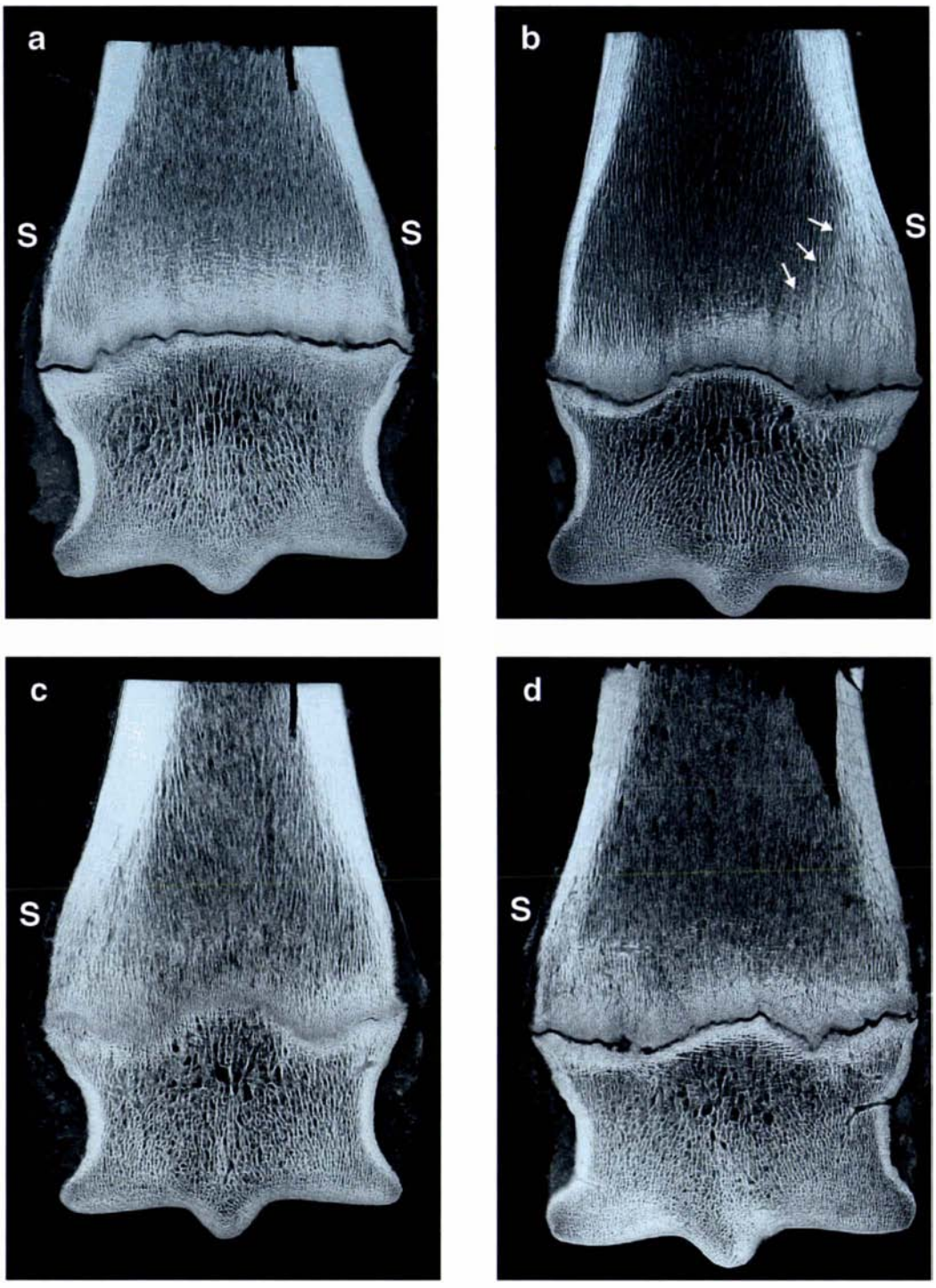


Figure 5.3 Cabinet radiographs of 2 mm frontal slices of the left distal third metatarsal bones at around 160 days of age, showing physes with varying degrees of inclination and closure; the physis in c) is completely closed. Evidence of shouldering (S) is present in all radiographs. In b) there is sclerosis (arrows). Focal areas of increased cartilage height are evident in b) and d). Medial is left, lateral is right.

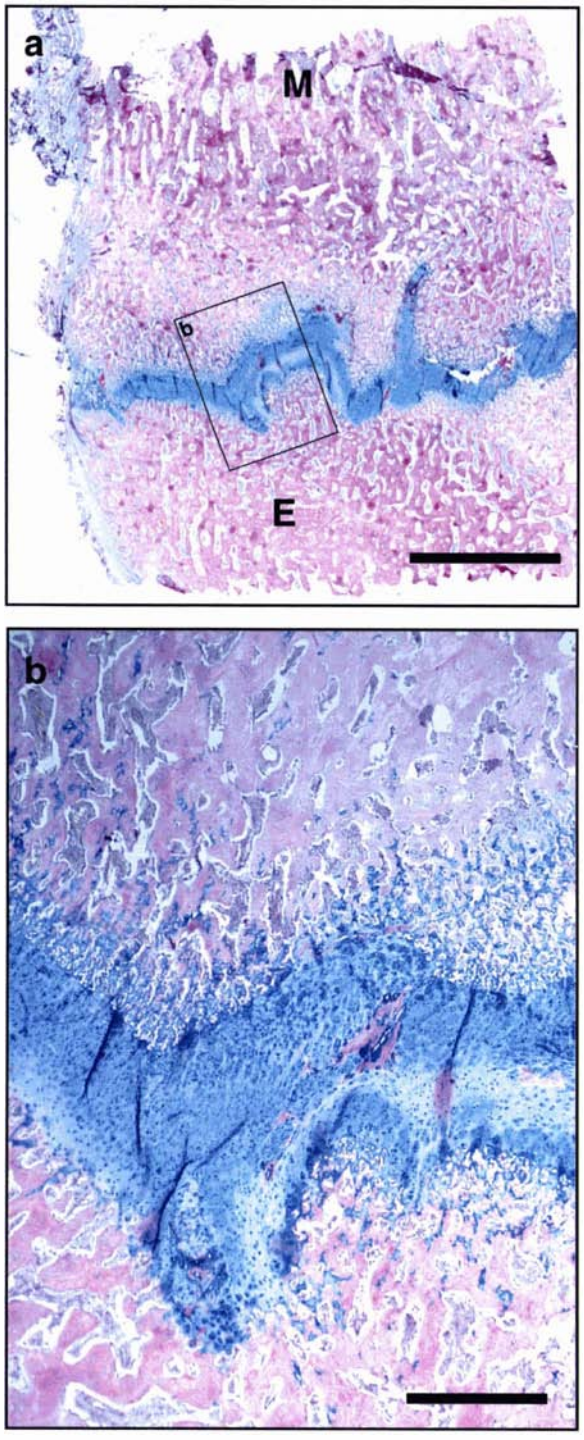


Figure 5.4 a) Low power micrograph showing increased cartilage thick ness in the medial physis of a distal third metatarsal bone of a foal aged 158 days, BAR = 5 mm. b) Area of increased cartilage thickness, BAR = 2 mm. M = metaphysis, E = epiphysis. Haemotoxylin, eosin and alcian blue.

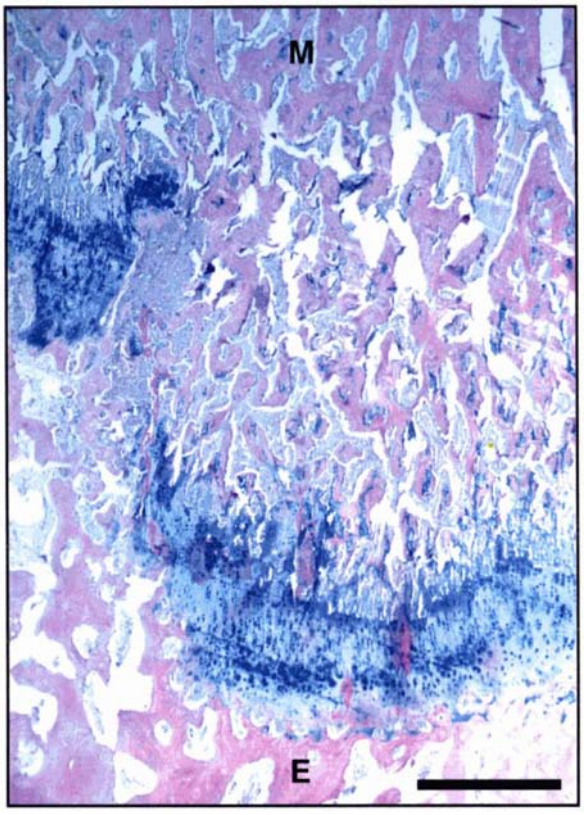


Figure 5.5 Photomicrograph of the distal third metatarsal physis of a foal aged 161 days, showing a small area in early stages of closure. BAR = 1 mm, M = metaphysis, E = epiphysis. Haemotoxylin, eosin and alcian blue.

5.5 Discussion

In this study all foals in Year 2 showed visible enlargements of the physeal region of Mc3 and Mt3, that tended to progress in severity from the first few weeks of life (n = 23). However, none of the 33 foals in this study became lame as a result of these enlargements. One foal had flexural deformity in association with high clinical physis scores, but there was no pain on palpation of the physeal region, or lameness. Cymbaluk and Christison (1989b) noted in weanlings fed concentrate and forage diets that flexural deformity was concurrent with apparent physitis of the distal Mc3/Mt3 or distal radius in most cases.

There was no statistical relationship between clinical physis scores and sex, growth rate, or liver Cu concentration of the foals in the present study, in contrast to suggestions by other authors (White, 1980; Turner, 1987; Lewis, 1995; Hunt, 1997). The lack of a universally accepted scoring system makes it difficult to explain the disparate outcomes. It is possible the degree of physitis were more severe in previous studies, or possibly the same diseases were not observed. The feeding of concentrates and mineral mixes (which were not given to foals in the present study) may alter the intake and absorption of macro- or micro- minerals, which may affect the degree of bony enlargements. Differences in dietary calcium were almost as large as differences in dietary Cu after dietary correction between the first and second year of epidemiological studies by Gabel et al. (1987). It has been suggested that physeal swelling may reflect temporal changes in pasture nutrient supply and spring pasture growth (Hoffman et al. 1999). In the present experiment pasture-fed foals achieved good growth rates, but growth rate did not influence the severity of the physis score. The highest clinical physis scores occurred in late summer and early autumn, but it was difficult to separate the time of year and age effects.

Visible enlargements of distal Mc3 and Mt3 persisted after physeal closure, as reported by others (Hoffman et al. 1999), but it is unclear how long they would have remained (Ueda et al. 1983). Clearly physitis is an inappropriate term to describe these enlargements, given that they persist after the physis has closed,

with no evidence of inflammation. There was a highly significant relationship between the clinical physis score with the radiographic evidence of metaphyseal shouldering, indicating the bony enlargements observed in live foals relate to bone above the physis, and not the physis itself. Metaphyseal shouldering need not be due to adjacent inflammation, and may be a consequence of a lack of bone remodelling (White, 1980) or a normal physiological response of modelling/remodelling bone that results in a temporarily altered morphology.

The radiographic appearance of physitis varies with the severity and duration of the disease (White et al. 1984), and the originating cause (Bramlage, 1993). Periosteal bone production of variable degrees contributes to beaking (or lipping) of the metaphysis and epiphysis (Rooney, 1963; Smith et al. 1975a; White, 1980) and was common in slice radiographs. Beaking was not associated with cartilage changes or epiphyseodesis, in agreement with Brown and MacCallum (1976). Beaking did not contribute to the clinical physis score, and is not associated with the clinical signs of enlargement of the distal Mc3 and Mt3 in the present study.

Metaphyseal sclerosis was observed in at least one limb of all foals from Year 1, but only in the left fore or hindlimb of 9 animals from Year 2. This may be related to less foot trimming in Year 1 compared to Year 2, allowing minor conformational aberrations for longer intervals, and increased weight bearing on one side of limb. However, there was no significant difference in mean radiographic score (which include a score for metaphyseal sclerosis) between foals born in Year 1 and 2.

Pearce et al. (1998a) found a statistically significant reduction in the hindlimb radiographic physitis score of foals at 150 days of age in association with oral Cu supplementation of the dams in late gestation compared to foals from unsupplemented dams, but not with foal Cu supplementation, or foal liver Cu concentration at birth. In the same study there was no effect of dam oral Cu supplementation on the number of Mc3/Mt3 physeal lesions observed at 150 days. The radiographic scoring systems used in that experiment and the present were very similar, but in the present experiment scored radiographs taken of

bone slices, rather than whole bones. Enlargements of distal Mc3 and Mt3 were most obvious at the medial and lateral margins, so the enlargements were always included in the bone slices. Dam Cu supplementation by injection did not alter the liver Cu concentration of the foal, or have a protective effect against distal Mc3/Mt3 enlargements in the present experiment.

Radiographic physeal defects could be classified into three groups. The first group showed abnormal physis closure: either one side of the physis was in a more advanced state of closure than the other, or closure was present in the distal troughs or steep inclinations, but not centrally in the physis. The latter pattern of closure has been noted by others and considered normal (Brown and MacCallum, 1976).

The second group of physeal abnormalities was apparent as focal increases in cartilage height, generally in the metaphysis, but occasionally in the epiphysis. Firth and Poulos (1984) showed metaphyseal cartilage retentions in the distal radial physis, but were unable to determine their relationship with clinical disease features. Histologically, focal areas of increased cartilage height in the present study varied from containing large numbers of hypertrophic chondrocytes of normal appearance and columnar orientation, to containing mostly abnormal chondrocytes, with at least one of the following abnormalities: eosinophilic cytoplasm, eosinophilic shrunken nuclei, eosinophilic matrix staining, or loss of normal columnar organisation of lacunae. Similar findings have been reported in physes of normally growing pigs aged 25 to 169 days (Hill et al. 1984).

The third group of abnormality was epiphyseodesis, which was considered abnormal only when not associated with normal physis closure (orderly narrowing of the physis and initiation of central closure).

The possible significance of the clinical, radiographic and histological physeal abnormalities in these foals is unclear. Given that there was no evidence of lameness, pain or inflammation the abnormalities appear to be of extremely little significance. Histological and radiological findings similar to the present

study have been reported by Brown and MacCallum (1976), suggesting these physeal abnormalities may indicate a 'subclinical' physitis. The histological and radiological findings in the present study were considered to be of low significance. Other authors have described bony enlargements of distal Mc3 and Mt3 for many months after physeal closure (Ueda et al. 1983; Hoffman et al. 1999). Physeal cartilage thickness was not related to the clinical appearance of distal Mc3 and Mt3. The maximal distal Mc3 and Mt3 enlargements (FP_{max} and HP_{max}) between birth and 160 days were more severe in hindlimbs than forelimbs. Although the reasons for this are not apparent, we noted that distal Mc3 physes closed (or began closing) earlier that distal Mt3 physes.

It is highly probable that all the cartilage lesions resolve with complete closure of the physis. Bramlage (1993) suggested that structurally weakened bone may be a consequence of physeal cartilage lesions, but this was not observed in the present study. It may be that different authors are referring to not only different types of physeal cartilage abnormality, but also different degrees of severity. For instance, in this study there were no primary spongiosa microfractures as described by Firth and Poulos (1984) in the distal radial physis of foals. Therefore, it is proposed that all metaphyseal enlargements be described and scored accurately, greatly reducing possible confusion in the future. The distal Mc3/Mt3 enlargements observed clinically may represent a normal physiological phenomenon that is strongly related to both season and foal age, but unrelated to growth rate (when assessed at 2 weekly intervals). Hoffman et al. (1999) concluded that seasonal changes had larger effects on physitis scores of than did dietary supplementation with sugar and starch or fat and fibre. Changes in clinical physitis scores of the distal radius of yearlings over 3 summer months have been reported (Grace et al. 2002), indicating season may be important in appearance of distal radial enlargements also.

Enlargements of distal Mc3 and Mt3, which resemble those included in descriptions of physitis, are very different from the previously reported severe physitis that is painful and results in lameness, and even fractures (Frankeny et al. 1994). Results from the present experiment suggest that while many Thoroughbred foals at pasture will have visible bony distal Mc3/Mt3

enlargements in the first 5 months of life, few have cartilage abnormalities, or true physeal dysplasia with failure of endochondral ossification. These enlargements are unrelated to growth rate, and in foals raised at pasture the condition appears to be limited or benign. The importance of these clinical swellings is probably overestimated. This coincides with the anecdotes of studmasters who believe the enlargements are not associated with permanent conformational or functional defects, but presence of the enlargements prevents the affected animal being sold at weanling sales. This condition of distal Mc3 and Mt3 enlargements needs renaming to reflect the affected site and lack of pathological changes. Physitis and physeal dysplasia do not appropriately describe these enlargements; physiological distal Mc3/Mt3 remodelling may be more accurate. Further research to determine the relationships between bony enlargements of the distal radial physis with radiographic and histologic pathology and growth rate are warranted, due to the economic losses when affected animals are withdrawn from sales.

Chapter 6

Histology of articular cartilage at osteochondrosis predisposition sites from foals out of copper supplemented dams[4]

[4] Results submitted as Gee, E.K., Davies, M.E., Firth, E.C., Jeffcott, L.B., Fennessy, P.F. and Mogg, T.D. *The Veterinary Journal*

6.1 Abstract

Objectives The effect of copper supplementation of dams during late gestation and the pattern of liver copper decline in foals, on histological appearance of articular cartilage at 4 sites predisposed to osteochondrosis was investigated in 22 Thoroughbred foals at 160 days of age. The expression of cathepsin B and alkaline phosphatase in normal and dyschondroplastic cartilage was also examined.

Method Copper supplemented mares received calcium copper edetate injections in late gestation (250 mg at around 220, 248, 276 and 304 days gestation, then every 2 weeks until foaling). Mares and foals grazed pasture containing 5.2 to 9.1 mg copper kg^{-1} dry matter. Articular cartilage was harvested from defined sites predisposed to the development of osteochondrosis from foals at around 160 days of age.

Results The prevalence and severity of cartilage irregularities and histologic abnormalities were considered minor. Copper supplementation of the dam, or pattern of liver copper decline of the foals, had no significant effect on the prevalence or severity of cartilage irregularities or abnormalities observed grossly or histologically. Cathepsin B was expressed strongly in chondrocyte clusters of dyschondroplastic cartilage. Alkaline phosphatase expression was strong in chondrocyte clusters within the hypertrophic zone of dyschondroplastic cartilage.

Conclusions Minor histological cartilage abnormalities were observed in grossly normal cartilage from osteochondrosis predisposition sites, that might be 'early' dyschondroplastic lesions. There was no statistical relationship between lesion numbers and copper supplementation of the dam, or pattern of copper decline of the foal.

Abbreviations

ALP	Alkaline phosphatase
BSA	Bovine serum albumin
Cu	Copper
CuS	Copper supplemented
DCP	Dyschondroplasia
DM	Dry matter
ECM	Extracellular matrix
H&E	Haematoxylin and eosin
NCu	No copper supplementation
NLCu	Normal decline of liver copper
OC	Osteochondrosis
PBS	Phosphate buffered saline
SLCu	Slow decline of liver copper

6.2 Introduction

The first case of osteochondrosis (OC) was reported in the horse over 50 years ago (Nilsson, 1947) and since then the economic and welfare importance of OC has been well documented for many species selected for rapid growth (Olsson, 1978; Jeffcott, 1991). In the horse OC develops because of a focal failure of endochondral ossification at predilection sites in articular/epiphyseal cartilage (Jeffcott and Henson, 1998). The primary lesion frequently occurs in the hypertrophic or proliferative zone, and therefore it has been suggested these early lesions are more appropriately termed 'dyschondroplasia' (DCP), and lesions that progress to more extensive pathology be described as osteochondrosis dissecans and osteochondritis dissecans (Jeffcott, 1997).

Many factors have been implicated in the causation of OC, including growth rate, genetic influences, dietary and mineral imbalances, endocrinological factors and biomechanical trauma (Jeffcott, 1991; Hurtig and Pool, 1996). There are clinical or experimental reports of OC induced by copper (Cu)

deficiency in swine, dogs, calves, chickens, deer, humans and horses (Teague and Carpenter, 1950; Baxter and Van Wyk, 1953; Gallagher, 1957; Irwin et al. 1974; Smith et al. 1975a; Bridges and Harris, 1988; Thompson et al. 1994).

Copper supplementation trials have yielded variable results. A decrease in the prevalence and severity of OC has been reported in association with Cu supplementation of mares during late gestation and foals from 90 to 180 days of age (Knight et al. 1990), and Cu supplementation of foals only (Hurtig et al. 1993). An experimental design that allowed separation of the effects of mare and foal Cu supplementation showed that Cu supplementation of pregnant mares reduced the prevalence of some forms of developmental orthopaedic disease in their foals at 5 months of age (Pearce et al. 1998a). The mechanisms of the Cu requirement for skeletal development are unknown.

Investigations of the requirement of Cu for cartilage extracellular matrix (ECM) metabolism, Davies et al. (1996) showed that in vitro Cu has a slight stimulatory effect on proteoglycan synthesis in normal cartilage and is able to reverse the proteoglycan depletion induced by synovial tissue, and reduce the chondrocyte expression of the ECM-degrading lysosomal proteinases, cathepsins B and D. These lysosomal proteinases, together with the metalloproteinases, act to degrade collagenous and non-collagenous components of the ECM (Murphy and Reynolds, 1993). There is recent evidence to suggest that cathepsins B and L have a role in endochondral ossification in the horse, and that cathepsin B may be important in mechanically induced turnover of ECM (Gläser et al. 2003). It has been proposed that DCP cartilage defects may arise from alterations in the matrix-degrading enzymes produced by the chondrocytes (Hernandez-Vidal et al. 1996). High levels of cathepsin B have been described associated with chondrocyte clonal clusters (chondrones) in DCP equine cartilage (Hernandez-Vidal et al. 1998). Normally the enzyme is located intracellularly as a lysosomal digestive enzyme, playing a role in normal proteoglycan turnover (Palmer and Bertone, 1994). Cathepsin B may be secreted by chondrocytes under certain pathological conditions, such as inflammatory arthritis (Van Noorden et al. 1988; Buttle et al. 1993; Buttle et al. 1995), where it contributes to destruction of ECM.

DCP is definitively diagnosed by the presence of a retained cartilage core (Savage et al. 1993). Histological confirmation of DCP/OC is also based on the presence or absence of chondrocyte clusters, chondronecrosis, increased spatial separation of chondrocyte columns, haemorrhage, dissection and reparative signs including fibrocartilagenous proliferative reactions and woven bone formation (Savage et al. 1993). Others have also noted the presence of degenerative foamy matrix, microfractures (Hurtig et al. 1993) and myelofibrosis (Salisbury et al. 1991). Despite the range of histological appearances it remains difficult to identify the DCP/OC lesions accurately (Jeffcott and Henson, 1998).

The objective of the study was to identify early cartilage abnormalities and established DCP lesions by examining histological, histochemical and immunocytochemical differences in articular cartilage harvested from OC predisposition sites of foals whose dams were either supplemented with Cu, as calcium Cu edetate, (CuS), or unsupplemented (NCu), during late gestation. The effect of foal liver Cu concentration at birth and 160 days of age on cartilage abnormalities and DCP lesions was also examined.

6.3 Materials and Methods

6.3.1 Source of samples

Twenty-three Thoroughbred mares were randomised into 2 groups on the basis of age, expected foaling date and sire of foetus. The 11 CuS mares were given 250 mg calcium Cu edetate (Coprin™, Schering-Plough Animal Health Ltd.) divided between two pectoral sites intramuscularly at around 220, 248, 276 and 304 days gestation and then every two weeks until foaling. The 12 NCu mares were given saline injections, at the same times. The 23 foals had liver biopsy samples taken in the first week of life for determination of Cu concentration. The liver of each foal was examined at post-mortem (mean 163 ± 4 days of age) and 3 samples taken for determination of Cu concentration. Mares and foals were grazed at pasture for the duration of the experiment. Pasture samples were

taken at least monthly during the experiment, frozen, and analysed together at a later date. Mineral levels were determined by inductively coupled plasma atomic emission spectrometry (Lee, 1983), and are presented in mg kg^{-1} dry matter (DM).

6.3.2 Samples

Foals were euthanased at around 160 days of age by free bullet after sedation with 100 mg xylazine hydrochloride given intravenously. The left and right shoulder, elbow, hock and stifle were removed from the carcass without opening the joint and stored on ice for transport. Within 6 hours joints were carefully opened using a scalpel blade, and the joint surfaces, capsule synovial linings, synovial fossae and synovial fluid examined for visual abnormalities under bright light. Thickening, folding, fissures or pitting of the left or right glenoid, humeral head, distal humerus, proximal radius, distal tibia, talus, distal femur, patella and proximal tibia was described, measured, and counted as an 'irregularity'.

Samples of articular cartilage and subchondral bone were harvested from the left side only of the animal, using a 9 mm diameter trochar. Samples were taken from the middle third of the femoral lateral trochlear ridge, mid-lateral talar trochlear ridge, tibial cranial intermediate ridge and the central humeral head.

6.3.2.1 Snap freezing

Samples in optimum cutting temperature compound (OCT, Tissue-Tek, UK) were snap frozen in isopentane (BDH Laboratory Supplies, UK) cooled in liquid nitrogen, and stored at –80 °C, before being transported to the UK on dry ice, and then stored at –20 °C until analysed.

6.3.2.2 Sections

Serial sections were cut (8 μm) using a cryostat (Shandon, Bright Instruments, Huntingdon, UK) with a working temperature of –30 °C. Slides were pretreated

with 0.01% (v/v) poly-L-lysine (0.1% v/v, Sigma, UK) for 5 minutes at room temperature, and dried for 1 hour at 65 °C or left overnight, then stored at 4 °C for up to 2 weeks to ensure adherence of sections to slides. Sections were fixed for 20 minutes in 4% paraformaldehyde, and washed in phosphate buffered saline twice, for a total of 20 minutes. Slides were stored in humidified containers at 4 °C for up to 2 weeks or frozen at –20 °C.

6.3.2.3 Histological staining

Fixed sections were stained with toluidine blue and with haematoxylin and eosin (H&E), using established methods. Sections were examined for the appearance of chondrocyte clusters, chondronecrosis, disruption in normal transitions of chondrocytes from resting through proliferation and into hypertrophy, cartilage canals, fissure formation and proteoglycan depletion (as evidenced by decreased toluidine blue staining). An 'abnormality' was defined as the presence of one or more of the following: retained cartilage core, chondronecrosis, fissure formation, or disrupted normal transition of chondrocytes.

6.3.2.4 Histochemistry

Alkaline phosphatase activity was detected using a substituted naphthol reaction as described by Bancroft (1996). Control samples were incubated with levamisol, an alkaline phosphatase inhibitor, before staining (Henson et al. 1995).

6.3.2.5 Immunocytochemistry

Immunofluorescence

Cartilage sections were treated with blocking buffer (1% bovine serum albumin (BSA), 10% foetal calf serum), in phosphate buffered saline (PBS) to minimise non-specific binding of primary antibody. Sections were rinsed in PBS, and 25 µl of sheep anti-human cathepsin B antibody (donated by Dr J. Buttle, Sheffield

Medical School), diluted 1:50 in wash buffer (1% BSA in PBS), was added. Sections were incubated overnight at 4 °C or for 1 hour at room temperature.

Unbound antibody was removed by washing sections 3 times in PBS for a total of 15 minutes. Sections were treated for 15 minutes with blocking buffer, and washed again in PBS. Sections were incubated with 25 μL fluorescein isothiocyanate-conjugated rabbit anti-sheep immunoglobulin (Dako, UK), diluted 1:200 in wash buffer, for one hour at room temperature. Controls were included in which primary antiserum was replaced by normal sheep serum diluted at the same concentration as the primary serum. Sections were washed 3 times in PBS for a total of 15 minutes, then nuclei were counter-stained with 0.01% (w/v) methyl green solution for 2 minutes, before rinsing in PBS and coverslip mounting in Citifluor (UKC, UK) to enhance fluorescence. Positive staining was viewed using a Nikon Diaphot microscope fitted with epifluorescent illumination (Hernandez-Vidal et al. 1996).

Biotin-streptavidin-horseradish peroxidase staining

Slides were placed in 70% ethanol for 5 minutes, then 1% hydrogen peroxide in methanol for 10 minutes to remove endogenous peroxidase, rehydrated in 70% ethanol for 5 minutes, followed by distilled water for 5 minutes. Primary antibody was added as above, after treatment with blocking buffer. The secondary antibody used was biotinylated donkey anti-sheep immunoglobulin (Sigma, UK), diluted 1:250 in wash buffer. For controls the primary antibody was replaced with normal sheep serum at the same dilution, or PBS. Unbound antibody was removed by washing for 5 minutes in PBS, then twice for a total of 10 minutes in 0.1 mol L^{-1} Tris-HCl buffer, pH 7.6. Sections were treated with 25 μL streptavidin-horseradish peroxidase complex (Sigma, UK), diluted 1:100 in 0.1 mol L^{-1} Tris-HCl buffer containing 1% BSA for 30 minutes, washed twice in distilled water for 2 minutes, then twice in 0.1 mol L^{-1} Tris-HCl buffer for 5 minutes. Sections were stained using diaminobenzidine (Vector Laboratories, UK: DAB substrate kit) for peroxidase for 5 minutes, then washed in 0.1 mol L^{-1} Tris buffer twice for 2 minutes and distilled water for 2 minutes, and coverslip mounted in Apathy's mounting medium (BDH Laboratory Supplies, UK) and examined using a light microscope.

6.3.3 Statistical analysis

Differences in the mean number of gross cartilage irregularities and histological abnormalities in foals from CuS and NCu dams, and foals with normal or slow pattern of liver Cu decline (NLCu and SLCu respectively) (Chapter 4), were examined by analysis of variance (ANOVA). A 5% level of significance was chosen. Arithmetic means are presented with standard deviations (SD).

6.4 Results

6.4.1 General observations

The mean pasture Cu level was 7.4 mg kg^{-1} DM (range 5.2 to 9.1). The mean growth rate of the 23 foals was 1.1 kg day^{-1} (± 0.08), with mean final weight of 234 ± 17 kg. No joint effusions were noted except in one foal with septic arthritis. Two foals had surgical correction of forelimb carpal valgus angular limb deformities by 3 months of age.

6.4.2 Liver copper concentration of foals

Mean liver Cu concentration at birth was not significantly different due to dam treatment (mean 349 ± 158 and 447 ± 280 mg Cu kg^{-1} DM for foals from CuS dams and NCu dams respectively, $p = 0.614$), or at 160 days of age (37.6 ± 37.4 and 31.6 ± 28.8 mg Cu kg^{-1} DM for foals from CuS and NCu dams respectively, $p = 0.668$). Three foals, 2 from CuS dams, had a highly significantly different mean liver Cu concentration at 160 days age compared to the other 20 foals (mean 107.9 ± 15.1 and 23.5 ± 15.0 mg kg^{-1} DM for SLCu and NLCu foals respectively, $p = 0.0001$. This was unrelated to mare treatment during late gestation. Removal of the SLCu animals from the analysis did not result in a statistical difference in foal liver Cu concentration at birth or 160 days due to mare Cu treatment.

6.4.3 Gross lesions

Results are presented in Tables 6.1 and 6.2. All foals had at least one irregularity present, mostly in the glenoid cavity, and in the talus, which were usually bilateral. Lesion number and site was not different in the foals from CuS or NCu dams, or between SLCu or NLCu foals, and was not correlated with liver Cu concentration of the foal at birth.

Wear lines were present in the left and right elbow joints of 2 animals (both from CuS dams), and in one hock joint of two animals (both from NCu dams), one of which had septic arthritis of both hocks soon after birth. This foal had 4 gross cartilage irregularities, including changes on the cranial aspect of the intermediate ridge of the distal tibia that was suggestive of OC or a fracture; the fragment was adhered to the parent bone by a strong fibrous union. This abnormal area was not included in the cartilage harvested for snap freezing.

Table 6.1 Total number of irregular cartilage surfaces per foal at 160 days of age.

Number of irregular articular cartilage surfaces per foal	All foals (n = 23)	Foals from control dams (n = 12)	Foals from copper treated dams (n = 11)	Foals with 'normal' decline of liver copper (n = 20)	Foals with 'slow' decline of liver copper (n = 3)
1-3	7 (30%)	3 (25%)	4 (36%)	5 (25%)	2 (67%)
4-6	13 (57%)	8 (67%)	5 (45%)	11 (60%)	1 (33%)
7+	4 (17%)	2 (17%)	2 (18%)	4 (20%)	0

Table 6.2 Number of foals with gross articular cartilage irregularities at 160 days of age, by site.

Site of articular cartilage irregularity	All foals (n = 23)	Foals from untreated dams (n = 12)	Foals from copper treated dams (n = 11)	Foals with 'normal' decline of liver copper (n = 20)	Foals with 'slow' decline of liver copper (n = 3)
Humerus	8 (35%)	4 (33%)	4 (36%)	8 (40%)	0
Glenoid cavity	18 (78%)	9 (75%)	9 (82%)	17 (85%)	1 (33%)
Proximal radius	5 (22%)	3 (25%)	2 (18%)	5 (25%)	0
Talus	17 (74%)	9 (75%)	8 (73%)	14 (70%)	3 (100%)
Tibia	4 (17%)	2 (17%)	2 (18%)	4 (20%)	0
Distal femur	6 (26%)	4 (33%)	2 (18%)	6 (30%)	0

NB All foals had more than 1 site with cartilage irregularities

6.4.4 Histological examination of H&E and toluidine blue stained cartilage

Cartilage samples from one foal (NCu dam) were not successfully snap frozen, and hence histological cartilage data are from 22 foals only. The details of histological abnormalities and the types of abnormalities in cartilage harvested from 4 sites in 22 foals are in Tables 6.3 to 6.5, and examples given in Figures 6.1 and 6.2. There was no evidence of a reduction in the number of sites with abnormalities in foals from CuS dams, or in the 3 SLCu foals at 160 days of age.

Most abnormalities were present in the humeral head and the talus. The site distribution of cartilage abnormalities was not different in the foals from CuS or NCu dams. Three cartilage samples harvested from the humeral head, and one from the mid-lateral trochlear ridge of the talus, had visible cartilage irregularities and microscopic abnormalities. All other 18 samples that had microscopic abnormalities were grossly normal at the time of harvesting. No

foal had histological abnormalities in all 4 cartilage samples, but one SLCu foal, from a NCu dam, had histological abnormalities in 3 of the 4 sites.

Table 6.3 Total number of sites with histological cartilage abnormalities (from 4 selected sites) per foal at 160 days of age.

No. of sampled sites with abnormalities	All foals (n = 22)	Foals from control dams (n = 11)	Foals from copper treated dams (n = 11)	Foals with 'normal' decline of liver copper (n=19)	Foals with 'slow' decline of liver copper (n = 3)
0	6 (27%)	3 (27%)	3 (27%)	4 (21%)	2 (67%)
1	12 (55%)	6 (55%)	6 (55%)	12 (63%)	0
2	3 (14%)	1 (9%)	2 (8%)	3 (16%)	0
3	1 (5%)	0	0	0	1 (33%)

Table 6.4 Number of foals at 160 days of age with histological cartilage abnormalities, by site.

Articular cartilage sampling site	All foals (n = 22)	Foals from control dams (n = 11)	Foals from copper treated dams (n = 11)	Foals with 'normal' decline of liver copper (n = 19)	Foals with 'slow' decline of liver copper (n = 3)
Left lateral trochlear ridge of femur	7 (32%)	3 (27%)	4 (36%)	5 (26%)	2 (67%)
Left central humeral head	11 (50%)	6 (55%)	5 (45%)	10 (53%)	0
Left intermediate ridge of distal tibia	1 (5%)	1 (9%)	0	1 (5%)	0
Left lateral trochlear ridge of talus	2 (9%)	1 (9%)	1 (9%)	1 (5%)	1 (33%)

NB Foals were considered to have a cartilage abnormality when 1 or more of the following were present: retained cartilage core, chondrocyte clusters, chondronecrosis, loss of normal cellular transitions or fissures.

Table 6.5 Details of histological cartilage abnormalities, cartilage canals and proteoglycan depletion at the 4 selected sites of all foals (n = 22) at 160 days of age. Results for foals from copper treated dams are given in brackets (n = 11), followed by foals with slow decline of liver copper (n = 3).

		Cartilage abnormalities			Cartilage canals	Protoeoglycan depletion
Cartilage sampling site	**Retained cartilage core**	**Chondrocyte clusters**	**Chondro-necrosis**	**Fissure**		
Left lateral trochlear ridge of femur	1 (0, 0)	4 (1, 0)	1 (1, 0)	1 (1, 0)	12 (7, 1)	9 (7, 1)
Left central humeral head	2 (2, 0)	1 (1, 0)	8 (4, 0)	0	0	20 (9, 3)
Left intermediate ridge of distal tibia	0	1 (0, 0)	0	0	0	0
Left lateral trochlear ridge of talus	0	1 (1, 0)	2 (1, 0)	1 (1, 0)	0	0

NB Some foals had more than one cartilage abnormality at each cartilage sampling site

6.4.5 Histochemistry

Alkaline phosphatase was visualised as a red/pink precipitate in the chondrocytes and occasionally as a pale pink precipitate in the surrounding matrix. Chondrocytes in the deep hypertrophic zone of the articular/epiphyseal complex stained strongly for ALP in all sites; sections of cartilage pre-incubated with levamisole showed no staining. Chondrocyte clusters in the hypertrophic or deep proliferative zones stained very strongly for ALP. One abnormal area with a concentration of apparently necrotic cells in the proliferative zone stained positively, but all other areas of chondronecrosis were negative, as were retained cartilage cores and the apparent fissures. Occasionally ALP staining occurred in small cells surrounding cartilage canals. The four samples that had grossly visible irregularities and histological abnormalities did not stain differently from other samples.

The depth of hypertrophic zone ALP staining varied between sites and also between individuals at the same site, and was unrelated to the presence of cartilage abnormalities. Staining intensity or depth in foals was not affected by SLCu at 160 days, or dam CuS (results not shown).

6.4.6 Immunocytochemistry

Chondrocytes positive for cathepsin B by immunofluorescence appeared bright green in colour when viewed under epifluorescence (Figure 6.3). Using the horseradish peroxidase method cathepsin B appeared as brown intracellular staining of chondrocytes. The distribution of cathepsin B immunoreactivity was similar with both staining methods. Control sections treated with normal sheep serum were negative, and no ECM staining was observed. Cathepsin B expression varied between sites (Table 6.6), but was always present at the articular surface. The resting zone of all 4 sites showed moderate immunoreactivity, while the hypertrophic zone stained strongly, as in previous studies (Hernandez-Vidal et al. 1998).

In 7 samples that contained chondrocyte clusters in association with other cartilage abnormalities high levels of cathepsin B expression was observed (Figure 6.3). In areas of cartilage abnormalities where no chondrocyte clusters were present cathepsin B was not expressed. Cathepsin B expression was not affected by dam CuS, or SLCu of the foal at 160 days of age.

Table 6.6 Cathepsin B activity in normal and dyschondroplastic cartilage from 4 sites of all foals (n = 22). Activity graded on the intensity of cell staining, from – (no staining) to +++ (strong staining) in normal chondrocytes and chondrocyte clusters.

	Resting Zone		Proliferative Zone		Hypertrophic Zone	
Cartilage sample site	**Normal chondrocytes**	**Clusters**	**Normal chondrocytes**	**Clusters**	**Normal chondrocytes**	**Clusters**
Left lateral trochlear ridge of femur	++	+	+	+++	++	+++
Left central humeral head	++	NP	+	NP	++	+++
Left intermediate ridge of distal tibia	++	+	++	NP	+	NP
Left lateral trochlear ridge of talus	++	NP	++	NP	+	+++

NP: chondrocyte clusters not present in this zone

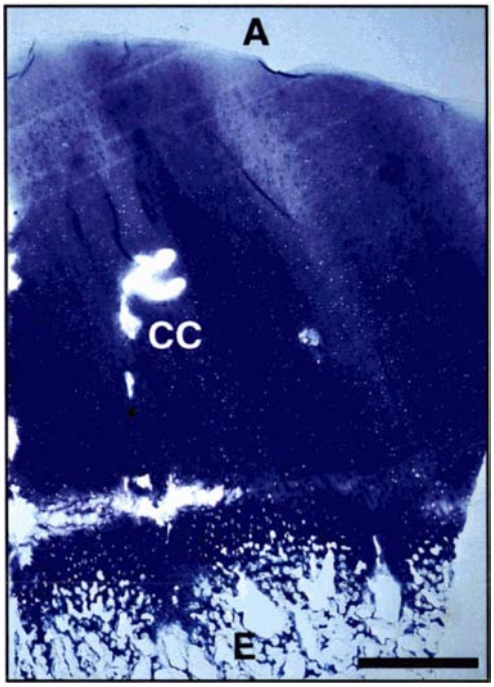


Figure 6. 1 Low power photomicrograph of undecalcified articular/epiphyseal cartilage from the femoral lateral trochlear ridge of a foal aged 161 days of age. Cartilage canals are present (CC). The discontinuity may be a microfracture, but it is more likely to be artifactual. BAR = 1.0 mm; A = articular surface and E = epiphyseal bone. Toluidine blue.

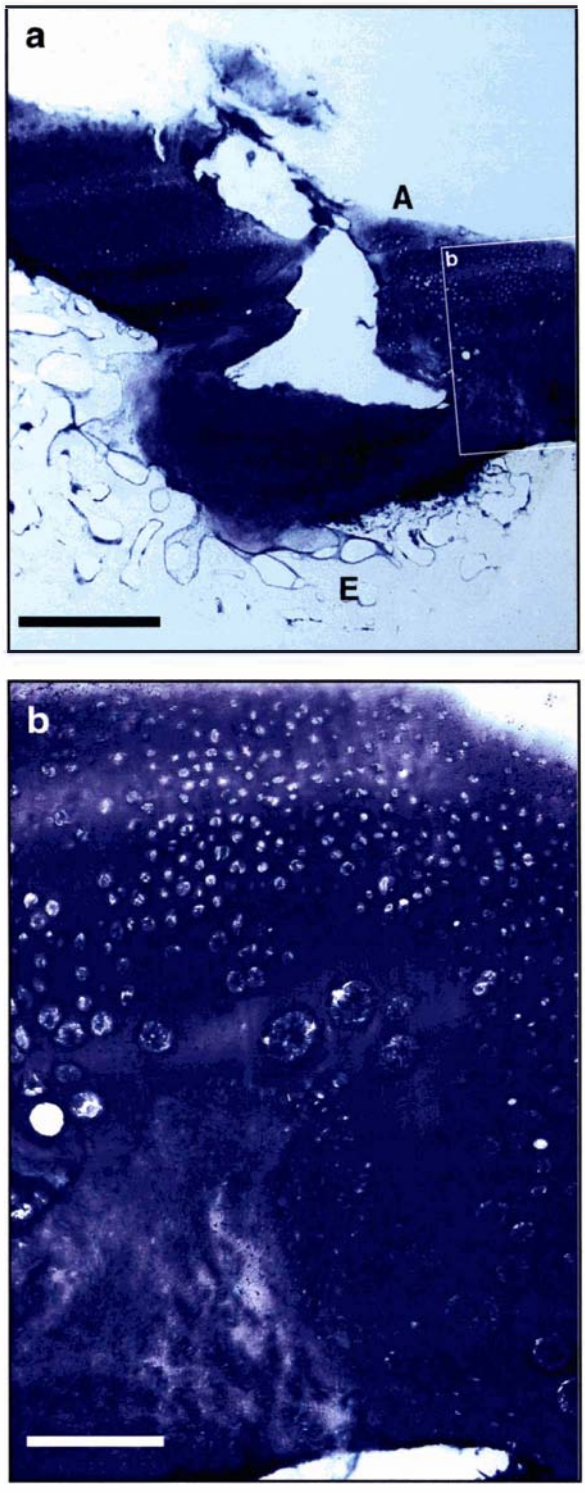


Figure 6.2 Low power photomicrograph of undecalcified articular/epiphyseal cartilage from the medial trochlear ridge of the talus of a foal age 160 days. The cartilage was irregular and discoloured on visual inspection. BAR = 1 mm. b) Severe disorganisation is present, with an area of necrosis bordered by chondrocyte clusters (chondrones). A = articular surface, E = epiphyseal bone, and BAR = 0.25 mm. Toluidine blue.

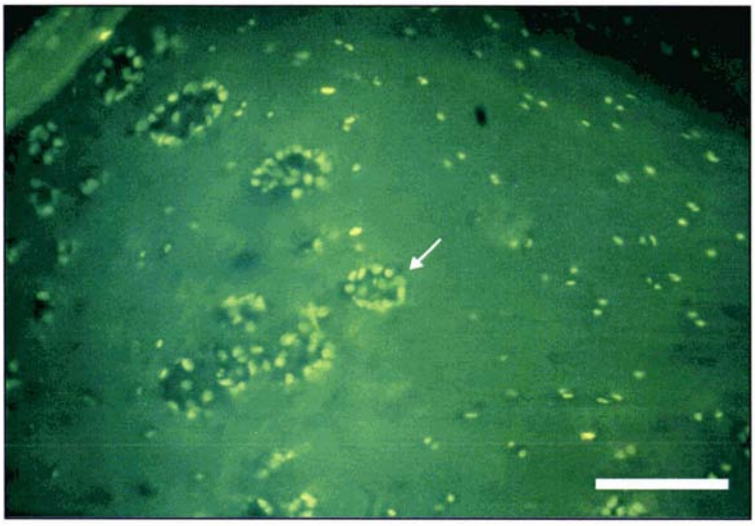


Figure 6.3 Fluorescent immunostaining for cathepsin B in chondrocyte clusters (chondrones) (arrows) from cartilage shown in Figure 6.2, BAR = 0.1 mm

6.5 Discussion

The experiment set out to examine the prevalence of early cartilage abnormalities and DCP lesions in foals from CuS and NCu dams. Injectable Cu supplementation of the dam during late pregnancy had no significant effect on the liver Cu concentration of the foal at birth or 160 days of age, the prevalence of gross cartilage irregularities, or histological abnormalities in cartilage taken from 4 OC predisposition sites. Pearce et al. (1998a) found a significant 'protective' effect of dam oral supplementation with Cu sulphate, reducing the prevalence of articular cartilage lesions in the foal at 5 months of age. In the present study the prevalence and severity of cartilage irregularities observed at post-mortem examination was considered minor, as found in previous studies of pasture-fed Thoroughbreds in New Zealand (Pearce et al. 1998a), but in contrast to North American and Dutch studies (Knight et al. 1990; Hurtig et al. 1993; van Weeren and Barneveld, 1999). In the present study all foals had minor irregularities at the cartilage surface, the majority being present, usually bilaterally, in the glenoid cavity and talus. Histological abnormalities were more common in the humeral head and lateral trochlear ridge of the femur. The Cu concentration of the foal at birth and at post-mortem had no significant effect on the prevalence or location of these gross irregularities or microscopic abnormalities.

As expected, the 4 samples with gross irregularities also showed abnormalities microscopically. Retained cartilage cores were identified in only 3 samples, but other abnormalities often associated with DCP (as used in the criteria of Henson et al. 1997b) were evident in a further 18 cartilage samples microscopically. The microscopic abnormalities were unaccompanied by gross irregularity or retained cores of cartilage, may be 'early' DCP lesions, which may resolve, or progress to OC.

Decreased toluidine blue staining, interpreted as proteoglycan depletion, was most common in cartilage from the humeral head, detected in both normal cartilage and in cartilage showing histological abnormalities. Not all microscopic abnormalities were associated with decreased toluidine blue

staining. The significance of proteoglycan depletion in cartilage of normal appearance is not known.

Cartilage canals were present only in the lateral femoral trochlear ridge. Cartilage canals contain the vascular elements necessary for the maintenance of epiphyseal growth cartilage (Shingleton et al. 1997). No cartilage canals were observed in normal articular--epiphyseal cartilage from this site in horses older than 6 to 7 months of age (Salisbury et al. 1991; Shingleton et al. 1997). In the present study canals were not associated with DCP/OC lesions, and necrotic cartilage canal blood vessels were not observed. In contrast, (Salisbury et al. 1991) found that all OC lesions in foals from 3 weeks to 5 months of age were only associated with necrotic cartilage canals and not with normal or chondrifying cartilage canals. The absence of cartilage canals in all samples from 3 sites may indicate that the time frame for lesion development had passed at these sites. There would appear to still be potential for lesion development at 160 days in lateral femoral trochlear ridge of the femur.

Chondrocyte clusters were observed around areas of chondronecrosis, and sometimes in areas of otherwise normal appearing cartilage. Clusters appeared as a roughly circular, tightly packed group of cells with variable amounts of matrix. Chondrocyte clusters are a common pathological indicator of osteoarthritic degeneration, but little is known of their formation or function (Poole et al. 1991). They may represent non-specific responses in attempt to repair damaged cartilage (Henson et al. 1997b). Chondrocyte clusters in the hypertrophic zone stained strongly for ALP, but did not stain when present in more superficial zones. All chondrocyte clusters had relatively strong expression of cathepsin B. Expression of cathepsin B by normal chondrocytes was generally low, and expression varied between the zones of cartilage, but was common at the articular surface, and in the hypertrophic zone, as noted by (Gläser et al. 2003). The importance of high levels of cathepsin B in chondrocyte clusters is not known, but could play an undetermined role in ECM turnover (Hernandez-Vidal et al. 1998; Kostoulas et al. 1999).

Dietary Cu levels below National Research Council (1989) recommendations of 10 mg Cu kg^{-1} DM were not associated with a high prevalence of DCP/OC in 5-month-old TB foals raised at pasture. The results of this study suggest that injectable Cu supplementation of the dam in late gestation had no effect on the development of DCP in the 4 sites examined. The histological cartilage abnormalities observed in grossly normal cartilage might be early DCP lesions, which could resolve or progress. Further work is needed to investigate the range of microscopic abnormalities in grossly normal cartilage, and the progression or resolution of these lesions, in order to determine their significance in the pathogenesis of DCP/OC. The possible role of Cu in the pathogenesis of equine DCP/OC remains poorly understood.

Chapter 7

Articular/epiphyseal osteochondrosis in Thoroughbred foals at 5 months of age: influences of foal growth and prenatal maternal copper supplementation

7.1 Abstract

Objectives The influence of copper supplementation by injection of mares in late gestation on the distribution and severity of osteochondrosis lesions in their foals at around 160 days of age was investigated. The study also aimed to determine if there was any influence of foal liver copper concentration, growth rate, sex, or year of birth on the distribution and severity of OC lesions.

Methods Thirty-three Thoroughbred foals, born in 2 consecutive years, were weighed every 2 weeks from birth. The dams had been supplemented with parenteral copper or saline during late gestation. Foals had liver biopsies harvested at birth for determination of liver copper concentration. At 160 days of age the foals were euthanased, and articular cartilage of long bones was examined. Gross lesions were counted and scored, then sawn and radiographed, and processed for histology. Lesions were given radiographic scores, and histological scores. Maximum scores for each lesion were combined to give a total osteochondrosis score for each joint and each foal.

Results Dam copper supplementation had no significant effect on liver copper concentration of the foal at birth, or the prevalence or severity of articular cartilage lesions at 160 days of age. Cartilage lesions were considered to be minor. The number of tibiotarsal lesions and the tibiotarsal OC score at 160 days were positively associated with average daily weight gain in the previous 4 weeks ($p = 0.005$ and $p = 0.001$ respectively). There was no significant effect of foal sex or year of birth on the prevalence and severity of osteochondrosis lesions.

Conclusions Dam copper supplementation, or liver copper concentration of the foal at birth did no have any statistical effect on the prevalence of osteochondrosis lesions at 160 days of age. The prevalence of cartilage lesions in Thoroughbred foals at 5 months of age was considered minor. Rapid growth rate may increase the number of tibiotarsal lesions at 160 days of age.

Abbreviations

ADG	Average daily weight gain (kilograms)
CF	Coxofemoral joint
Cu	Copper
CuS	Copper supplemented
FP/FT	Femoropatellar/femorotibial
H&E	Haematoxylin and eosin
HR	Humeroradial
MCP	Metacarpophalangeal
MTP	Metatarsophalangeal
NCu	Not copper supplemented
OC	Osteochondrosis
SH	Scapulohumeral
TT	Tibiotarsal

7.2 Introduction

Osteochondrosis (OC) is a disease characterised by disturbance of cellular differentiation in growing cartilage (Olsson and Reiland, 1978), which occurs in a large number of domestic species including horses. OC is of great importance to the equine industry (Rossdale, 1999) due to wastage, economic losses and welfare issues. The incidence of the disease may be increasing worldwide (Jeffcott, 1996).

There is considerable confusion regarding the definition and pathogenesis of OC (Ekman and Carlson, 1998). In all species the disease is considered to be multifactorial, while the aetiology is contentious (Hurtig and Pool, 1996; Jeffcott and Henson, 1998). Aetiopathogenetic factors include rapid growth, genetic predisposition, nutritional excesses or imbalances, and trauma (Jeffcott, 1991).

In domesticated species such as cattle, swine, poultry and large breeds of dogs, OC occurs in young, rapidly growing animals (Reiland et al. 1978), which may

also be true in the horse (Stromberg and Rejno, 1978; Fischer and Barclay, 1984). However, few studies conclusively support the role of large body size and rapid growth rates as a predisposing factor in the development of equine OC (Savage et al. 1993a; Pagan and Jackson, 1996; Jelan et al. 1996). Standardbred foals with radiographic evidence of OC had higher birth weights and growth rates than unaffected foals (Sandgren et al. 1993b). In a group of Dutch Warmblood foals genetically predisposed to OC, high growth rate in the 3rd and 5th months of life was associated with evidence of OC in the femoropatellar joint (Firth et al. 1999; van Weeren et al. 1999).

A sex predilection to OC may also exist, possibly due to faster growth rates and larger body size (Fischer and Barclay, 1984). Male horses may be more likely to be affected than females (Sandgren et al. 1993a). There are limited data on normal growth patterns of Thoroughbred foals (Hintz et al. 1979; Jelan et al. 1996; Pagan et al. 1996), so identification of fast growth rates for individuals may be problematic.

Various nutritional factors have been implicated in the pathogenesis of OC. In poultry, pigs and horses there are strong correlations between dietary energy intake and the occurrence of OC lesions (Olsson and Reiland, 1978; Hurtig and Pool, 1996); although high dietary energy intake is not always associated with rapid weight gain in horses (Savage et al. 1993a). The source of the excess dietary energy may be important. Associations between hyperglycaemia following large starch meals and the development of OC have been proposed (Glade and Belling, 1986; Ralston, 1996).

Several mineral imbalances have been implicated in the development of OC in the horse, including excessive calcium or phosphorus (Savage et al. 1993b) and copper (Cu) deficiency (Knight et al. 1986; Gabel et al. 1987; Bridges and Harris, 1988; Knight et al. 1990; Hurtig et al. 1993). Very severe experimentally induced Cu deficiency resulted in severe, debilitating OC lesions in foals (Bridges and Harris, 1988), although naturally occurring Cu-responsive disorders are rare in monogastric species (Underwood and Suttle, 1999). Epidemiological studies indicated a relationship between low dietary Cu

concentrations and higher yearlings scores for developmental orthopaedic disease, including osteochondrosis (Knight et al. 1986; Gabel et al. 1987). Cu supplementation trials in North America indicated decreased numbers of OC lesions in foals with higher dietary Cu levels (Knight et al. 1990; Hurtig et al. 1993). However, studies in New Zealand demonstrated no decrease in the number of articular cartilage lesions in Cu supplemented Thoroughbred foals, but oral Cu supplementation of dams during late gestation reduced the prevalence of articular cartilage abnormalities in their foals at 5 months of age (Pearce et al. 1998a). Recently it has been suggested that high liver Cu concentration at birth may have a positive effect on the number of radiographic OC lesions that repair between 5 and 11 months of age in Dutch Warmblood foals (van Weeren et al. 2003).

The objective of this study was to investigate the influence of Cu supplementation by injection of mares in late gestation on the distribution and severity of OC lesions in their foals at around 160 days of age. The study also aimed to determine if there was any influence of foal liver Cu concentration, growth rate, sex, or year of birth on the distribution and severity of OC lesions.

7.3 Materials and Methods

7.3.1 Animals

In Year 1 a total of 10 foals were born (5 males and 5 females) while in Year 2 a further 23 foals were born (11 males and 12 females). All dams and sires were Thoroughbred, with all 10 foals in Year 1 and 18 in Year 2 by the same sire. The remaining 5 foals were by 4 different sires.

All mares and foals were kept at pasture for the duration of the experiment, in groups of 10 or less mare and foal pairs. The paddocks were around 2 hectares, with ryegrass or mixed ryegrass/clover swards. Pasture samples were taken at least every 8 weeks for analysis of mineral concentrations. Appropriate proprietary anthelmintics were given to mares and foals every 6 to 8 weeks, and

hooves were trimmed regularly. Mares received 25 mg sodium selenate orally every 4 weeks, as the pasture and soil were low in selenium (Grace, unpublished observations).

7.3.2 Mare treatment

In Year 1 mares were assigned to treatment or control groups on the basis of age, last date of service, and liver Cu concentration. The five treatment (CuS) mares were given 100 mg calcium Cu edetate intramuscularly in the 9th month of gestation and a further 250 mg in the 10th month. Control (NCu) mares were given a similar volume of saline in the same sites. In Year 2 mares were assigned to CuS and NCu on the basis of age, last date of service, and sire of the foal. Injections of 250 mg calcium Cu edetate were given intramuscularly to CuS mares in the 8th, 9th and 10th month of gestation, then every 2 weeks until foaling. Control mares were given a similar volume of saline at the same stages of gestation. Mares were maintained at a condition score (Henneke et al. 1983) of 5.5 or better during pregnancy, and 4.5 or better during lactation.

7.3.3 Clinical data

Foals were weighed within 12 hours of birth, weekly in the first month of life, and then every 2 weeks thereafter, using electronic platform scales. Clinical examinations for evidence of joint effusion and lameness were conducted at least monthly in Year 1, and two-weekly in Year 2. Liver biopsies were harvested from foals in the first week of life (Chapters 3 and 4).

7.3.4 Post-mortem and cartilage lesions

Foals were euthanased at around 160 days of age. Livers were harvested and later homogenised for determination of Cu concentration (Chapters 3 and 4). The distal forelimbs were removed 10 cm proximal to the radiocarpal joint, and the distal hindlimb removed at the tarsometatarsal joint. The distal limbs were stored on ice for transport to the laboratory. The scapulohumeral (SH), humeroradial (HR), metacarpophalangeal (MCP), coxofemoral (CF), femoropatellar/femorotibial (FP/FT), tibiotarsal (TT) and metatarsophalangeal

(MTP) joints were opened immediately or stored on ice and opened within 6 hours at the laboratory. Articular cartilage samples were harvested from selected sites in the right side joints of foals born in Year 2, and snap-frozen for use in another study (Chapter 6). In Year 2 the right MCP and MTP joints were not opened immediately and instead the right distal limbs were wrapped in plastic film and frozen for tomography scanning for another study. The limbs were later thawed, and joints opened and examined by the same two examiners, and thereafter treated as for fresh samples.

The same two examiners assessed all macroscopic cartilage irregularities, in both years. Each irregularity was described, measured, and recorded on video. Selected irregularities were photographed. Scoring classifications for macroscopic irregularities were based on those of van Weeren and Barneveld (1999) (Table 7.1). Small cartilage irregularities (often less than 2 mm diameter) that were in close proximity to each other (<5 mm) were counted as one lesion; cartilage irregularities more than 5 mm apart were considered as single lesions, regardless of their size.

Joint surfaces containing macroscopic cartilage lesions were either immediately sawn in a sagittal plane into bone-cartilage slices 3 to 5 mm thick, or were sawn after fixation in formalin. A cabinet radiograph (Torrex 150 machine, Torr X Ray Corp., California, USA) of each slice was given a radiographic score, based on the classification system of changes in bone and cartilage described by Dik et al. (1999) (Table 7.2). The bone and cartilage slices were stored in formalin or 70% alcohol, before decalcification in formic acid/sodium formate. The decalcified samples were washed, vacuum embedded in paraffin wax, and stained with haematoxylin and eosin (H&E) and alcian blue. Selected sections with suspected microfractures were stained with Lendrum's acid Picric Mallory. Each lesion was scored on histological criteria, using the classification system of van Weeren and Barneveld (1999) with minor changes (Table 7.3). Macroscopic irregularities or radiographic abnormalities that did not have histological changes associated with OC as described by Henson et al. (1997b) (locally thickened cartilage, loss of normal columnar arrangement of chondrocytes, chondronecrosis, chondrocyte cluster (chondrone) formation and

the variable presence of fissures) were considered to be due to another process. For irregularities that were not due to OC the macroscopic, radiographic and histological scores were adjusted to zero.

Each lesion was given an OC score, being the highest macroscopic, radiographic or histologic score. In most lesions the highest score was the histological score. Each lesion with an OC score of greater than one was considered to be OC positive, while lesions with scores of zero or one were classified as OC negative. A joint OC score was calculated for the SH, HR, MCP, FP/FT, TT and MTP joints of each foal, by summation of the OC scores of OC positive lesions (i.e. if a lesion had a score of 0 or 1 it was not included in the joint OC score). A total OC score was the sum of the joint OC scores for each foal.

The chemical body composition of all foals in Year 1, and 10 foals from Year 2 matched for sex and date of birth with foals born in Year 1, was determined (Chapter 2).

Table 7.1 Macroscopic classification of articular cartilage irregularities

Classification	Characteristics
1	Flattening or discolouration of cartilage only
2	Irregular cartilage (mild), usually <3 mm, shallow indentation when viewed under bright light
3	Irregular cartilage (severe), usually >5 mm, obvious cartilage invagination or erosion
4	'Classic' lesion with osteochondral fragment, or cyst

Table 7.2 Radiographic classification of articular cartilage and bone slices

Classification	Characteristics
0	Normal
1	Smooth flattening of contour of subchondral-cartilage junction
2	Irregular flattening of contour and/or an obvious ill-defined subchondral lucency
3	Small rounded irregular concavity and/or well defined local subchondral lucency
4	Large concavity with extensive subchondral lucency with or without a fragment

Table 7.3 Histologic classification of cartilage irregularities

Classification	Characteristics
0	No abnormalities
1	Local thickening of cartilage, with normal chondrocytes
2	Local thickening of cartilage with loss of normal columnar arrangement of chondrocytes and chondronecrosis
3	Fissures, necrosis and intact articular surface
4	Fissures, necrosis and articular surface not intact. Includes all osteochondral fragments and cysts.

7.3.5 Statistical analyses

The data were normally distributed, and so parametric tests were used for analysis. All data were examined using linear models, with year of birth, dam treatment within year, and foal sex as fixed effects, and their interactions were fitted. To standardise weighing intervals, linear extrapolation between consecutive weighings was required for some data to construct average daily weight gain in 28 day intervals for each foal, as the actual intervals between weighing were not exactly the same for all foals in all cases. The relationships between ADG in each 28 day interval, birth weight, liver Cu concentration, post-mortem weight and fatness (as determined by chemical body composition)

with the number of OC lesions or OC score were examined using analysis of covariance or linear regression analysis. Means are presented with standard deviations (SD), and slopes (*b*) are presented with standard errors (SE). The level of significance was set at $p < 0.05$. All tests were run using The SAS System 8.2 (SAS Institute Inc. Cary, N.C., USA).

7.4 Results

7.4.1 Pasture copper concentration

The average Cu concentration of pasture consumed by the mares and foals was 7.8 mg Cu kg^{-1} DM (range 5.2 to 10.2) and 7.4 mg Cu kg^{-1} DM (range 6.2 to 10.7) in Years 1 and 2 respectively.

7.4.2 Clinical data

The average foal birth weight was 55 kg (SD 5), with no significant difference in birth weight due to year of birth, dam treatment within year, or foal sex. There was a small but significant effect of dam age on foal birth weight, with foals from older dams tending to be heavier at birth ($p = 0.029$, $R^2 = 0.029$, $b = 0.5 \pm 0.2$)). Prior to euthanasia (mean age 162 days, SD 4) the average foal weight was 235 kg (SD 16), with no significant difference in weight due to sex or year. There was a positive statistical relationship between birth weight and weight prior to euthanasia ($p < 0.0001$, $R^2 = 0.47$, $b = 2.1 \pm 0.4$).

The average daily weight gains of foals in each 28-day period ($ADG_{1\text{-}6}$) during the study are given in Table 7.4. Higher birth weight was associated with more rapid weight gain in periods ADG_1, ADG_2 ADG_4 ($p = 0.018$, $R^2 = 0.17$, $b = 0.02 \pm 0.01$; $p = 0.009$, $R^2 = 0.20$, $b = 0.01 \pm 0.00$ and $p = 0.040$, $R^2 = 0.13$, $b = 0.01 \pm 0.00$ respectively). Dam age, foal sex or year of birth was not statistically related to ADG in any of the 6 periods. Joint effusions were observed in only one Year 2 foal, which had bilateral septic arthritis of the TT joint in the first week of life; the effusions resolved after antibiotic therapy and joint lavage.

The mean liver Cu concentration at birth was 394 mg kg^{-1} DM (SD 205) with no significant differences due to year of birth, sex of foal or dam treatment within year.

Table 7.4 Birth weight, average daily growth rate in 28 day intervals, and weight prior to post-mortem in foals born in Year 1 and Year 2 (± standard deviation) (n = 10 and n = 23 respectively).

Period	Age (days)	Year 1	Year 2
Birth		57.3 ± 4.1	54.0 ± 5.3
ADG_1	1 - 28	1.7 ± 0.2	1.6 ± 0.2
ADG_2	29 - 56	1.4 ± 0.1	1.2 ± 0.2
ADG_3	57 - 84	1.1 ± 0.2	1.1 ± 0.1
ADG_4	85 - 112	1.0 ± 0.1	1.0 ± 0.1
ADG_5	113 - 140	0.8 ± 0.1	0.8 ± 0.2
ADG_6	141 - 169	0.9 ± 0.3	0.8 ± 0.3
Post-mortem	156 - 176	238.9 ± 10.5	233.8 ± 17.4

7.4.3 Post-mortem examination of articular cartilage lesions

Fillies had significantly more fat that colts as determined by chemical body composition (mean percentage fat 10.2, SE 1.9 and 8.2, SE 1.9 for fillies and colts respectively, p = 0.030, n = 20) (Chapter 2). The mean Cu concentration of liver homogenate was 32, SD 19 and 191, SD 86 mg Cu kg^{-1} for foals with normal (n = 27) and slow (n = 6) decline of liver Cu respectively (Chapters 3 and 4). There was no significant difference in the Cu concentration of the liver homogenate due to year of birth, sex of foal, or dam treatment within year.

All 23 foals in Year 2, and 9 of 10 foals in Year 1, had evidence of irregular articular cartilage at post-mortem examination, that was confirmed histologically to be indicative of OC (Table 7.5). The distribution of lesions

within joints is given in Table 7.6. There was no significant difference in total number of OC lesions or OC score per animal due to sex of foal, dam treatment within year, percentage body fat, liver Cu concentration at birth or post-mortem, or pattern of decline in liver Cu concentration.

Macroscopically the cartilage lesions varied from 1 mm diameter 'indentations' and slight discolourations, to more classic lesions of more than 1 cm diameter, although the latter were very uncommon. Most lesions occurred in the SH, TT and MTP joints in foals in both years. No lesions were observed in FP/FT joint of foals born in Year 1 and the prevalence of lesions in this joint was low in Year 2. When cut, most lesions showed invagination of articular cartilage. Histologically lesions ranged from focal increases in cartilage depth with increased numbers of normal-appearing hypertrophic chondrocytes, to lesions characterised by necrosis, chondrocyte cluster (chondrone) formation and fissures.

Scapulohumeral joint lesions were present in 24 foals, mostly bilaterally, and were commonly seen as linear or stellar invaginations of cartilage in the glenoid cavity of the scapula, with evidence of retained cartilage on sectioning. The radiographic and histologic appearances of typical SH joint lesions are given in Figures 7.1 and 7.2. Only one foal had a severe SH joint lesion, present in the articulating surface of the proximal humerus, with a large eroded area (2.5cm diameter) centrally (Figure 7.1b).

In Year 1 the highest prevalence of lesions were in the TT joint, and most were classified as mild. Most of the TT joint lesions were present on the medial trochlear ridge of the talus, and infrequently at the classical lesion sites of the intermediate ridge of the distal tibia, and lateral trochlear ridge of the talus. The lesions were mostly multiple small punctate lesions in close proximity to each other on the summit or the axial surface of the medial trochlear ridge, and 38 lesions were bilaterally symmetrical. The radiographic and histologic appearance of a typical TT joint lesion is given in Figure 7.3b and 7.5 respectively. The number of TT joint lesions and the TT joint OC score were

positively associated with ADG_6 ($p = 0.005$, $R^2 = 0.23$, $b = 1.6 \pm 0.5$ and $p = 0.001$, $R^2 = 0.29$, $b = 5.0 \pm 1.4$ respectively).

Twenty-four foals had lesions in the MTP joint. These lesions varied from small circular invaginations (< 2 mm diameter) in the medial and lateral articulating surface, or in the intermediate groove of the first phalanx, to irregular lesions (>3 mm diameter) on the medial or lateral dorsal eminence of the proximal phalanx (Figure 7.3a).

Table 7.5 Number of osteochondrosis cartilage lesions per joint, including the number of bilateral lesions, the mean number of lesions per foal (± standard deviation), the number and percentage of animals affected, and osteochondrosis score per foal in Year 1 (n = 10) and Year 2 (n = 23). Joints: scapulohumeral (SH), humeroradial (HR), metacarpophalangeal (MCP), coxofemoral (CF), femoropatellar/femorotibial (FP/FT), tibiotarsal (TT) and metatarsophalangeal (MTP) joint.

	Number of lesions		Number of bilateral lesions		Mean number of lesions per foal		Number of foals affected with osteochondrosis (%)		Osteochondrosis score per animal	
Year	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**	**1**	**2**
Joints										
SH	11	33	10	28	1.1 ± 1.1	1.4 ± 1.0	6 (60)	18 (78)	2.6 ± 2.5	3.3 ± 2.4
HR	6	14	2	8	0.6 ± 1.1	0.6 ± 0.9	3 (30)	7 (30)	1.5 ± 3.0	1.4 ± 2.6
MCP	0	5	0	0	0	0.2 ± 0.4	0 (0)	5 (22)	0	0.5 ± 1.1
FP/FT	0	13	0	10	0*	0.6 ± 0.8*	0 (0)	8 (35)	0	1.3 ±2.1
TT	21	32	16	16	2.1 ± 0.9	1.4 ± 1.1	9 (90)	18 (78)	5.2 ± 2.7	3.3 ± 2.9
MTP	9	35	4	6	0.9 ± 1.1	1.5 ± 1.1	5 (50)	19 (83)	2.7 ± 3.4	3.7 ± 2.6
Total	47	132	32	68	4.7 ± 1.1	5.7 ± 1.1	9 (90)	23 (100)	12.0 ± 8.3	13.6 ± 6.3

Significant differences between years are indicated. *$p < 0.05$.

Table 7.6 Distribution of osteochondrosis lesions in examined joints of foals born in Year 1 and 2.

Joint	Site	Number of lesions Year 1	Year 2
Scaupulohumeral	Glenoid cavity of the scapula	11	27
	Middle articulating surface of the proximal humerus	0	6
Humeroradial	Medial/lateral condyle of the distal humerus	1	0
	Medial/lateral articulating surface of the proximal radius	5	14
Metacarpophalangeal	Medial/lateral condyle or sagittal groove of the distal metacarpus	0	1
	Mediodorsal/laterodorsal margin or Medial/lateral articulating surface or intermediate groove of the proximal phalanx	0	4
Femoropatella/ femorotibial	Medial/lateral trochlear ridge	0	5
	Medial/lateral condyle	0	6
	Patella	0	2
Tibiotarsal	Intermediate ridge or articular grooves of the distal tibia	1	6
	Lateral trochlear ridge of the talus	1	2
	Medial trochlear ridge of the talus	19	24
Metatarsophalangeal	Medial/lateral condyle or sagittal groove of the distal metatarsus	1	7
	Mediodorsal/laterodorsal margin or medial/lateral articulating surface or intermediate groove of the proximal phalanx	7	25
	Sesamoid	1	3

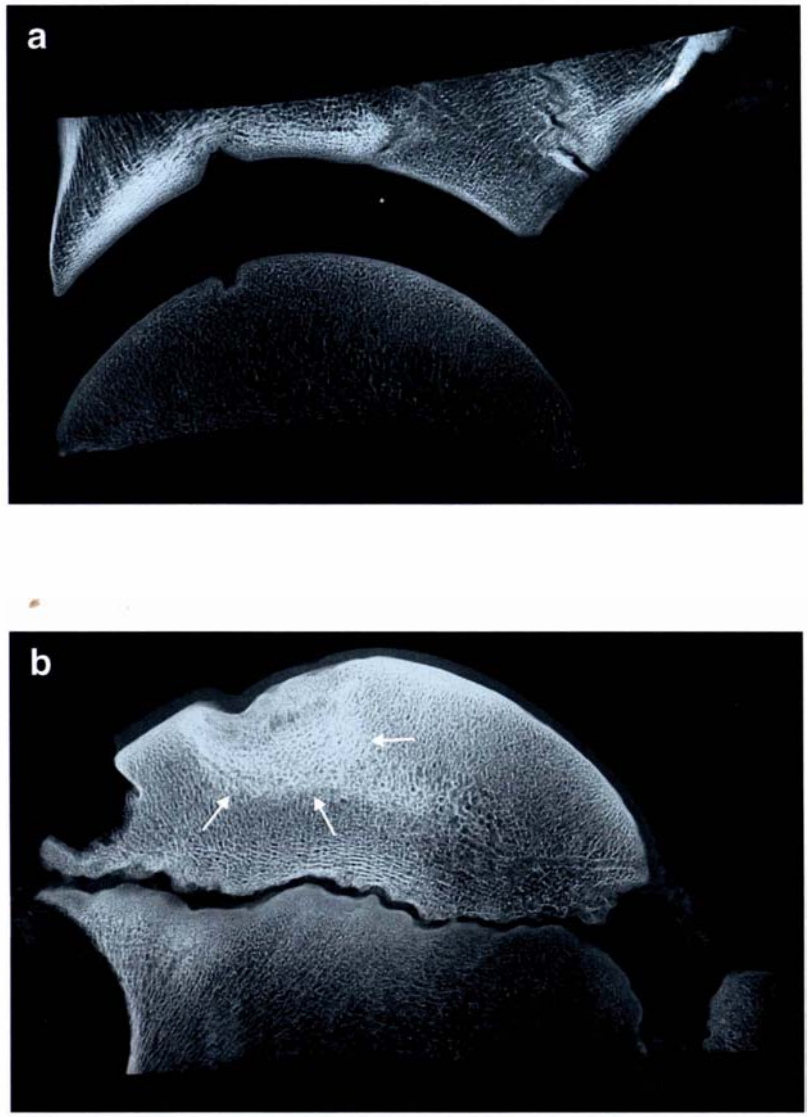


Figure 7.1 Cabinet radiographs of 2 mm osteochondral slices of the scapulohumeral joint, through a) adjacent lesions in the glenoid cavity of the scapula and proximal articulating head of the humerus, and b) a larger lesion in the proximal articulating head of the humerus which is not accompanied by glenoid cavity changes, from a foal aged 164 days. There is a depression of the joint surface with lucency and sclerosis in the subchondral area (arrows).

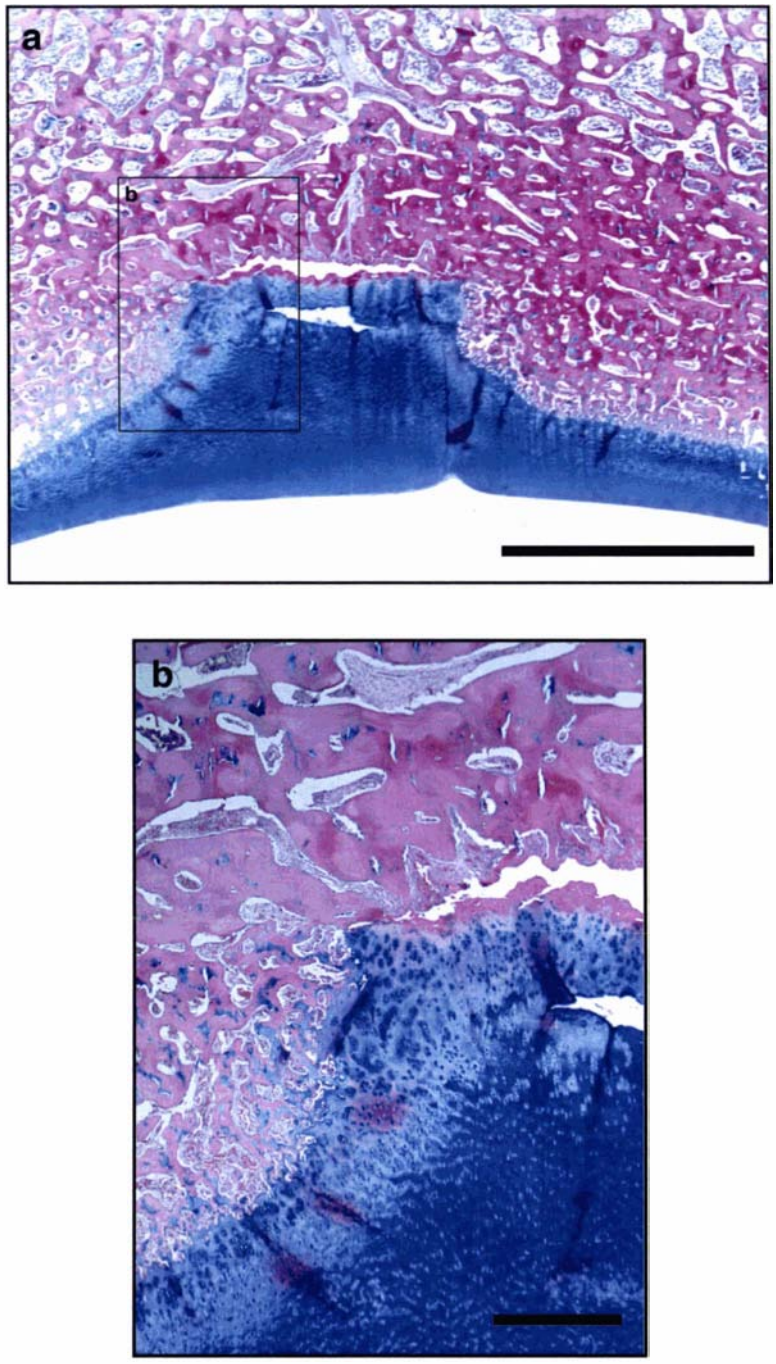


Figure 7.2 a) Low power photomicrograph of retained cartilage in the glenoid of a 160 day old foal, BAR = 5 mm. b) detail of retained cartilage, with apparent subchondral microfracture accompanied by chondrocyte clusters (chondrones) and subchondral sclerosis, BAR = 1 mm. Haemotoxylin, eosin and alcian blue.

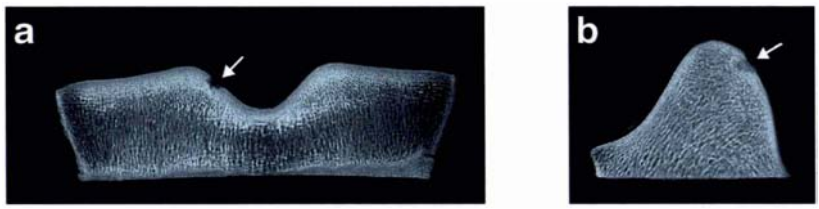


Figure 7.3 Cabinet radiographs of 2 mm slices of bone, through subtle punctate lesions (arrows), identified grossly in a) the median sagittal groove of the proximal phalanx, and b) the lateral aspect of the lateral talar ridge.

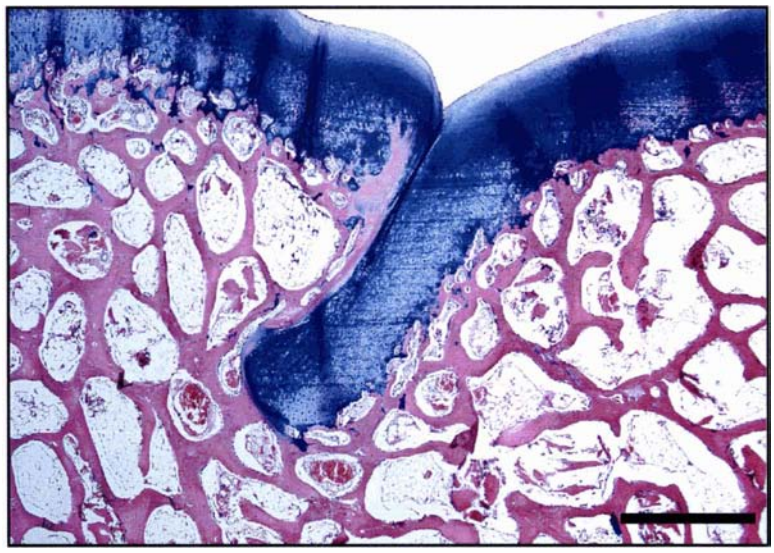


Figure 7.4 Photomicrograph of Figure 7.3 a) showing cartilage invagination with no evidence of subchondral sclerosis, BAR = 1 mm. Haemotoxylin, eosin and alcian blue.

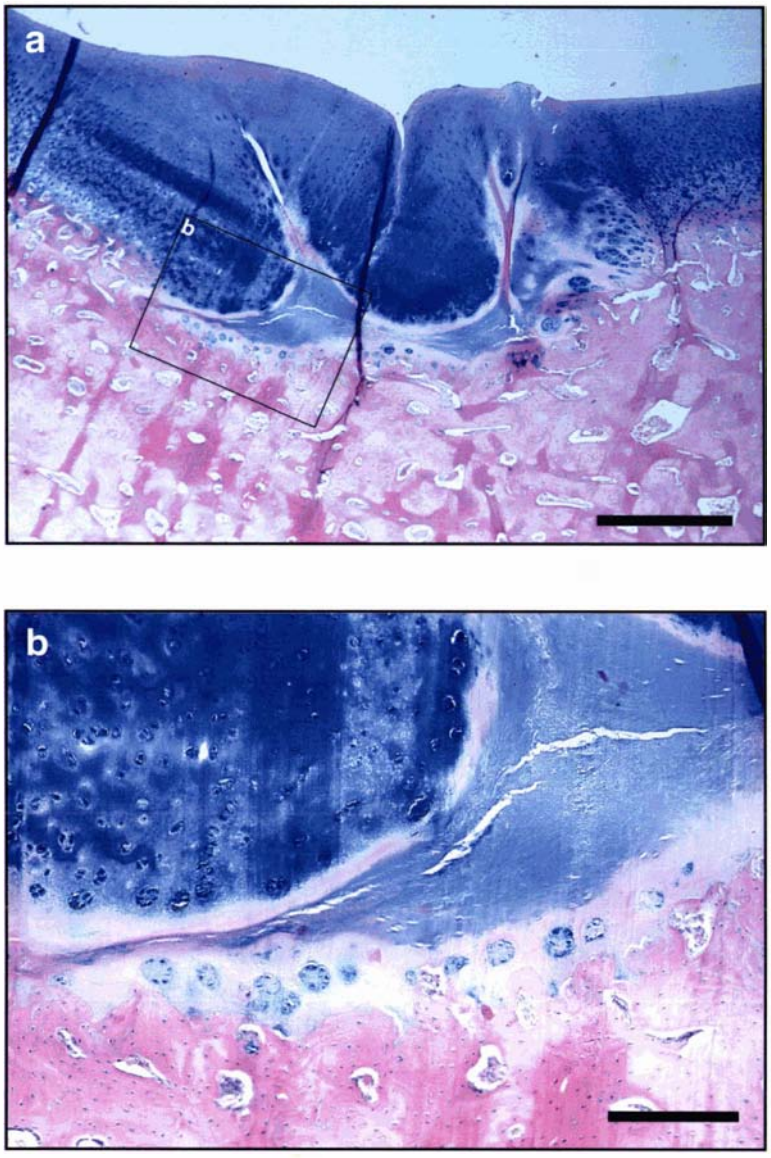


Figure 7.5 Photomicrograph of the cartilage lesion shown in Figure 7.3 b). a) Thickened cartilage and cartilage invagination, with chondrocyte cluster (chondrone) formation, myelofibrosis and subchondral sclerosis, BAR = 1 mm, and b) detail, BAR = 0.25 mm. Haemotoxylin, eosin and alcian blue.

7.5 Discussion

Lesions of OC are common in horses, pigs, poultry and large-breed dogs, but do not always result in clinical signs (Ekman and Carlson, 1998). In this study no foals showed clinical signs of lameness or joint effusion, but 32/33 foals had histological evidence of OC, using the criteria of Henson et al. (1997b). It is likely that many of these lesions would have resolved (Carlson et al. 1988; van Weeren and Barneveld, 1999) and never have caused clinical signs, so their significance is questionable. Using the definition of Jeffcott (1993), most of the cartilage irregularities were dyschondroplastic i.e. there were no clinical changes in the joint, and irregularities were 'early' OC lesions. Some authors have questioned whether dyschondroplastic lesions are the precursors of osteochondral lesions (Whitton, 1998), or whether dyschondroplasia may be a common feature of endochondral ossification in all growth cartilage, and not just at OC predisposition sites (Pool, 1993).

In this study the word 'lesion' was used to denote any abnormality, however slight, of the macroscopic, radiographic or histological morphology of articular cartilage and adjacent subchondral bone. The word did not imply that the abnormality was pathological, or would necessarily lead to clinical signs. In fact, many of the lesions were extremely subtle.

The high prevalence of retained cartilage in the glenoid cavity of the scapula observed in 24/33 foals in this study, without associated clinical signs, has not been reported before. Pearce et al. (1998a) observed SH joint lesions in 1/24 Thoroughbred foals at 5 months of age; Savage (1993a) observed SH joint lesions 0/10 and 1/11 foals at 3 and 6 months of age respectively; while van Weeren and Barneveld (1999) observed lesions in 1/24 and 3/19 Warmblood foals at 5 and 11 months of age. Frequently OC of the SH joint presents as debilitating lameness in 18 to 24 month old animals, when degenerative changes are already present (Hurtig and Pool, 1996). The significance of SH joint lesions in the current study is unknown, but their site and size suggest they are not linked with clinically important larger lesions on the mid and caudal humerus and scapula. Similar linear and stellar cartilage invaginations in the

central glenoid site are incidentally found in older horses, and appear not to be associated with clinical, radiographic or pathologic evidence of degenerative joint disease (Firth, unpublished observations). These 'lesions' may be another example of a morphology abnormality of developing cartilage, which has little significance in later life.

The relatively low numbers of FP/FT joint lesions in this study, and that of Pearce et al. (1998a), is in contrast to the high prevalence in genetically predisposed Warmblood foals at 5 months of age (van Weeren and Barneveld, 1999), and the apparent Thoroughbred breed predisposition for this site reported by McIlwraith (1993a) from clinical cases. Most FP/FT lesions in genetically predisposed Warmblood foals resolved by 11 months of age (van Weeren and Barneveld, 1999), indicating excellent reparative potential for lesions in this site in young foals.

The TT joint lesions reported in the present study are similar to those described by others (Hurtig and Pool, 1996; Pearce et al. 1998a; van Weeren and Barneveld, 1999). The high prevalence of lesions on the medial trochlear ridge of the talus is intriguing, as it is not considered a classic predisposition site for OC. These gross lesions were mostly very small (<1 mm) punctate irregularities, but histologically were consistent with OC as there was slight thickening of the cartilage. Foals with increased average weight gain in period ADG_6 had higher numbers of TT joint lesions and OC scores, indicating that perhaps the rapid gain resulted in the development of more lesions, or alternatively that rapid weight gain slowed the normal repair of these lesions at this age. The latter is supported by the findings of Dik et al. (1999), who determined that in genetically predisposed Warmblood foals OC lesions of the hock that were apparent on clinical radiographs do not change between 5 and 11 months of age. The very small size, and lack of information in the common literature that such lesions are common, progressive, or associated with diagnostic or treatment indication, implies that the clinical importance of medial talar trochlear ridge lesions in this study can only be minor or negligible.

The variation in liver Cu concentration of foals at birth was high, as described previously (Chapters 3 and 4) (Pearce et al. 1998c; van Weeren et al. 2003), with no significant effect of dam treatment. There was no evidence of a 'protective' or 'reparative' effect of injectable Cu treatment of the dam during late gestation on the prevalence and severity of OC lesions. In contrast, Pearce et al. (1998a) found that oral Cu supplementation of mares during late gestation resulted in fewer articular cartilage lesions of their foals at 5 months of age. The mechanism of this apparent 'protective' effect of oral Cu supplementation has not been elucidated, and could be breed specific (van Weeren et al. 2003). It was suggested that an association between higher liver Cu concentration of foals at birth (≥328 mg Cu kg^{-1}DM) with more lesions 'repairing' between 5 and 11 months of age may be important in Warmblood foals (van Weeren et al. 2003). However, it could be argued that liver Cu concentration at birth greater than 100mg Cu kg^{-1} DM in the Thoroughbred has either a protective or reparative role against the development of clinical OC in the first 5 months of life in the TB foal, as evidenced by no foals in the present study, or that of Pearce et al. (1998a) having clinical evidence of OC.

The growth indices measured in this study were comparable to data published for Thoroughbreds in Canada, USA and Ireland (Hintz et al. 1979; Jelan et al. 1996; Pagan et al. 1996), indicating that unweaned Thoroughbred foals achieved good growth rates grazing on pasture with Cu concentrations below the recommended 10mg Cu kg^{-1} DM (NRC, 1989). There was no evidence of Cu deficiency, or clinical OC, supporting the hypothesis that Cu is an overemphasised factor in the aetiopathogenesis of OC (Jeffcott and Davies, 2000; van Weeren et al. 2003). However, it is possible that Cu may have a role in repair of OC lesions, although the mechanisms of this effect have not been elucidated. Further research in this area may be justified for populations of horses predisposed to the development of clinically important OC.

Chapter 8

General Discussion

8.1 Introduction

This general discussion outlines the major findings, and relates the various studies to each other, and to general aspects of growth and development of the pasture-raised foal in a temperate environment. This is done under headings of experimental design, growth and development, copper storage and developmental orthopaedic disease. The discussion concludes with consideration of possible sources of error in the studies, recommendations for the New Zealand Thoroughbred industry, and areas for further research.

The original aims for the studies described in this thesis were to determine if:

a) parameters describing growth in pasture-fed Thoroughbreds in New Zealand were related to the incidence and severity of DOD lesions
b) parenteral copper supplementation of dams during late gestation reduced the incidence and severity of DOD in their foals
c) statistically significant relationships existed between in vivo techniques to assess body composition and chemical body composition

Testing of the aims was achieved by studies of parenteral copper supplementation of pregnant mares in two consecutive years, measurement of foal growth parameters, assessment of body composition and liver copper concentration, and assessment of evidence of DOD in foals, from birth to 160 days of age.

8.2 Experimental design

One reason for this investigation was based on concerns by the New Zealand Thoroughbred industry regarding wastage of animals due to DOD lesions. It had already been shown by Pearce et al. (1998a) that oral copper supplementation of mares during late gestation was associated with reduced evidence of DOD lesions in foals at 5 months of age. In New Zealand, broodmares are kept on pasture all year, and it was considered that thrice-

weekly oral copper supplementation for the last 15 to 25 weeks of gestation as described by Pearce et al. (1998a) would be too time-consuming and expensive. Using copper-supplemented feed instead of oral supplementation may not suit all breeders, being impractical, unnecessary or expensive. Injectable copper given during gestation is effective in raising foetal liver copper concentration in ruminants (Hemingway et al. 1970). Hence, the use of parenteral copper supplementation in pregnant mares was investigated.

Copper deficiency is only one of the many aetiologies that have been proposed for DOD, but its role has received the most attention in the past 25 years, mainly in North America and Europe. This has been due, in part, to the ease with which copper can be incorporated into grain feeds, and the relative safety of copper supplementation in the horse. The relationship between rapid growth and osteochondrosis is well established in other species such as chickens, pigs and large breeds of dogs (Olsson and Reiland, 1978; Dammrich, 1991), and is often suggested for horses (Stromberg, 1979; Fischer and Barclay, 1984). However, evidence for such an association is not strong.

Calcium copper edetate was chosen as the form of parenteral supplementation, as in cattle and sheep it causes less tissue reaction that other injectable copper preparations marketed in New Zealand, including copper glycinate. The intramuscular route was chosen for the studies reported in this thesis, after pilot work in which calcium copper edetate given intramuscularly to 3 weanlings resulted in minimal tissue reaction, which was undetectable within 7 to 10 days. Calcium copper edetate injected subcutaneously in a small number of horses had resulted in large tissue reactions in some animals (Pearce, S.G., Firth, E.C., and Grace, N.D. unpublished observations).

There were no data on efficacy or kinetics of calcium copper edetate, so doses were calculated using a combination of published ruminant data (Carmago et al. 1962), and copper accretion rates of the foal in late gestation (Meyer and Ahlswede, 1978). The frequency of copper injections increased in Year 2, based on the failure of two injections to improve mare or foal liver copper concentration. No more than 350 mg calcium copper edetate was given on any

occasion due to increased potential for tissue reaction with increased dose, based on results from previous studies in young animals (Pearce, S.G., Firth, E.C., and Grace, N.D., unpublished observations).

Mares and foals were kept at pasture for the duration of the experiments, supplemented with hay during winter when required. Monitoring of dietary energy intake per animal was not possible due to this extensive feeding system, and was a limitation of the study. Others have shown a strong relationship between dietary energy intake and the frequency and severity of osteochondrosis in foals (Savage et al. 1993). The quantity and quality of pasture available for consumption by mares and foals can vary in response to rainfall, ambient temperature and management (for example, the amount of residual post-grazing dry matter). Therefore, it is possible that dietary energy intake could be different in each year of the experiment.

This experimental design allowed the hypotheses to be tested in two consecutive years, two regimens of copper supplementation of mares to be investigated, and data on growth, body composition and evidence of DOD from two crops of foals to be compared. The design also allowed the influence of year effects to be statistically examined. Foals were euthanased at 160 days of age, allowing comparison between results of these studies and those of Pearce et al. (1998a), whose foals were also Thoroughbred, and raised on the same property. Comparison with data from 5 month old Warmblood foals predisposed to osteochondrosis was also possible (van Weeren and Barneveld, 1999; van Weeren et al. 2003).

8.3 Growth and Development

8.3.1 Techniques to assess fatness

Ultrasonographic rump fat measurements are reported to be highly repeatable, precise, accurate and easy to perform in horses (Westervelt et al. 1976; Kane et al. 1987; Kearns et al. 2001). In the studies in this thesis, ultrasonographic

measurement of rump fat thickness (10 cm anterior to the tailhead and 4 cm off midline) explained 71% of the variation in empty body fat mass, determined by chemical analysis in 20 foals at 160 days of age. Thus, ultrasonographic measurement of rump fat thickness is a good technique for in vivo prediction of body composition in foals at 160 days of age. However, the thickness of fat over the rump varied greatly in different locations; and in some animals there were significant differences between left and right sides. Position of the live animal or carcass can influence ultrasonographic measurements in pigs, and this is likely to also be true in cattle and sheep (Houghton and Turlington, 1992), and the horse. However, in the present study in some individuals differences in fat thickness between the left and right sides were detected both ultrasonographically and at post-mortem, indicating the differences were real, and not solely related to stance and positioning of the live animal or carcass. For the group of foals the mean differences in rump fat thickness were not significantly different between left and right. Fat thickness varied greatly with rump site, being thickest where two muscle groups meet, for example, in the division between the vertebral head of the biceps femoris and the semitendinosus muscles.

The rump fat thickness measurement site needs to be carefully described, so different operators can use the same technique, and results can be compared. In the foals in the present study, a site 10 cm anterior to the tailhead and 4 cm off midline, with the average of left and right measurements, had the highest correlation with body fat mass. This site may not be the most suitable for horses of different ages or sizes, and so future work is required to identify the best site for measurement of rump fat thickness in mature horses. The influence of site preparation also needs to be investigated. In cattle, unclipped hair at the site, or the presence of dirt affected ultrasonographic measurements (Houghton and Turlington, 1992). In the present study foals the area was closely clipped and cleaned before ultrasonographic measurements were taken.

Condition scores, using the system described by Henneke et al. (1983), of foals at 160 days of age were significantly correlated with fat mass, and the percentage of fat in the partial empty body. These findings are in contrast to

suggestions by Kearns et al. (2002) that condition scores are not useful for estimating fat mass or percentage fat in all horses. In the foals of the present study, there was no advantage in using regional condition scores over overall condition scores, with the exception of condition scores given to the rib region, which had the highest correlation with fat mass and percentage fat. Thus, when assessing the overall condition score of foals, special attention should be given to the rib region. This is in contrast to condition scoring pregnant mares, where less importance is given to the ribs, as advancing pregnancy influences the apparent fat stores in this region (Henneke et al. 1983).

Bioelectrical impedance measurements could be easily obtained in foals, but 3 repeat measurements took about 15 minutes. The technique required co-operation from the animals to minimise movement during measurements, which were affected by both animal movement and position. In contrast, Forro et al. (2000) found that stance of the horse did not affect BIA measurements. This difference between the two studies cannot be explained, except that the movement of 3 adult, well-handled horses may have been of much less magnitude than movements in Thoroughbred foals of the present study.

In foals of the present study, bioelectrical impedance indices accounted for only 50% of the variation in the fat-free mass in foals at 160 days of age, and thus were less useful for prediction of body composition than ultrasonographic rump fat thickness measurements. Forro et al. (2000) found that BIA data in adult horses was no better than using length, or length plus height measurements in predicting total body water, and this is supported by findings in the present study.

In humans, BIA is widely used for prediction of total body water and fat-free mass (and consequently fat mass), most often using the impedance index calculated by height squared divided by resistance, accounting for 59 to 98% of total variability in these compartments (Kushner, 1992). However, it must be remembered that human data were validated against reference values from hydrodensitometric and hydrometric methods, and so include the inherent errors

associated with the assumptions for those methods (Barr et al. 1994). In contrast, studies in animals with technologies such as BIA are often validated against data from dissection or chemical composition, with less associated errors.

It has been suggested that the large volume of gastrointestinal fluid may influence BIA measurements in the horse (Kearns et al. 2002), and Forro et al. (2000) starved horses overnight before BIA measurements, but access to water was not restricted. In humans, consumed fluid does not significantly alter BIA measurements until it is absorbed into the bloodstream, thereby increasing total body water (Kushner et al. 1996). For this reason in humans it is recommended that BIA measurements are taken 8 or more hours postprandially. As horses at pasture spend most of the day eating, it is possible there are no large changes in total body water due to fluid shift from gastrointestinal tract to the bloodstream, as fluid absorption occurs throughout most of the day. Therefore, its seems unlikely that BIA measurements were greatly influenced through not restricting feed or fluid. If access to feed and fluid had been restricted in the foals for 8 hours, significant water conservation mechanisms may have been activated, altering the distribution of total body water, and therefore affecting BIA measurements.

In humans, BIA measurements are also affected by recent exercise, with the intensity of exercise, and degree of thermal changes and fluid loss determining resistance increases or decreases. In foals of the present study, all BIA measurements were taken after foals had been in yards for 1 hour or more, so it unlikely that exercise influenced the BIA measurements.

Forro et al. (2000) suggested BIA may be a useful technique to detect dehydration in horses in a clinical setting. It seems likely that more research will be directed in the field in the future, due to the relative ease of obtaining measurements, and minimal expense of equipment.

8.3.2 Fatness of pasture-fed Thoroughbred foals at 160 days of age

At 160 days of age fillies were significantly fatter (fat mass and percentage fat in the partial empty body) than colts. However, there were no significant differences in mean birthweight, growth rate or weight at 160 days of age in fillies and colts. Although there is increased deposition of subcutaneous fat at the time of puberty onset in human females, independent of age (Vizmanos and Marti-Henneberg, 2000), the greater fatness of fillies was not associated with puberty. In Thoroughbred horses raised on the same property, puberty occurred in spring, at around mid-October, in both fillies and colts, independent of their month of birth in the previous year (Brown Douglas, C., 2003, personal communication).

In foals of the present study, there was a trend for fillies to have higher condition scores at 160 days of age, using the system described by Henneke et al. (1983). There were no significant differences between sexes in the fat thickness over the ribs and rump regions at 160 days of age. However, fillies had significantly more intra-abdominal fat than colts at 160 days of age. In humans, accumulation of visceral fat is hypothesised to play a central and mediating role relating obesity and lifestyle to increased disease risk in children, particular diabetes mellitus and cardiovascular disease (Goran and Malina, 1999). The implications of greater fatness in fillies at 160 days of age are not known.

The mean fat percentage ranged from 5.5 to 13% in 20 foals of the same age, with a liveweight range of 40 kg at around 160 days of age (Chapter 2). On visual assessment the foals were not excessively fat, with fillies having an average condition score of 6.2 and colts 5.2 (classified as moderately fleshy and moderate respectively, Henneke et al. 1983). Fatness was not statistically associated with increased frequency or severity of DOD lesions in these pasture-fed foals at 160 days of age. However, this may not preclude a positive relationship between excessive fatness in young animals and the frequency of DOD lesions, since pasture-fed foals might not receive sufficient excess dietary energy to become excessively fat, in contrast to grain-fed animals. The

condition scores of Kentucky Thoroughbred foals that were fed grain in addition to pasture were similar to New Zealand Thoroughbreds in the present study at the same age. Kentucky fillies and colts had average condition scores of 5.7 and 5.5 respectively (Pagan et al. 1996) at 5 months of age, although grain intake had probably been restricted in animals considered to be too fat (Pagan and Jackson, 1996). Nonetheless, in the latter study the incidence of clinical osteochondrosis was as high as 10% in foals on one Kentucky stud over a 4 year period, and was reported to be associated with rapid growth rate. However, condition scores were not reported, so the association between osteochondrosis and fatness could not be tested.

Condition scoring is a useful technique to assess fatness of foals, but it must be remembered it assesses subcutaneous fat only, and is not indicative of other fat stores. In the present studies, fillies had significantly more abdominal fat than colts, but no significant differences in condition score or depth of rib or rump fat at 160 days. Condition scoring or ultrasonographic measurement of rump fat thickness will not detect these sex-related differences in total fatness. This highlights the importance of identification of other in vivo techniques for estimation of fat mass or percentage.

Foals in the present study were moderately fat, but there are no published data on the chemical fat percentage of other horses at the same age. Schryver et al. (1974) reported the mean fat percentage of 4 mixed-breed 4 month old geldings was 5.3%. Other authors have reported higher chemical fat percentages in older horses compared to foals in the present study (Robb et al 1972; Westervelt et al. 1976; Kane et al. 1987), and it is expected that the percentage of fat increases with increasing maturity. The only reported condition scores of horses at similar ages to the present study are those of Pagan et al. (1996), and these mean scores for over 200 animals were comparable with those in the present study. It is possible that young animals fed high-energy diets accumulate significant amounts of abdominal fat, long before subcutaneous fat increases. Increases in abdominal fat are not detectable by condition scores. If it is correct that abdominal fat stores increase greatly before subcutaneous fat thickness increases, and the hypothesis that increased fatness is strongly associated with

osteochondrosis, this may account for the much greater frequency and severity of osteochondrosis in young Thoroughbreds raised in North America compared to those raised in New Zealand, even though the mean subcutaneous fat stores (assessed by condition scores) are very similar in these two populations.

8.3.3 Growth of pasture-fed foals, and the relationships between growth and evidence of DOD

There are no published data for mature weights of Thoroughbreds in New Zealand. The liveweights of the small number of mares used in the present study indicated it is appropriate to use mature weights of 560 and 570 kg for mares and stallions respectively, based on Kentucky data (Pagan et al. 1996). In the 33 foals used in the present study, fillies and colts were 240 and 232 kg at 160 days of age, achieving 43 and 41 % of their predicted mature weights respectively. Similarly, Thoroughbred fillies and colts in Kentucky had average weights 230 and 234 kg respectively, at 5 months of age (Pagan et al. 1996), and in Canada 212 and 221 kg respectively (Hintz et al. 1979). In Ireland, Thoroughbred foals had average weights of 241 kg at 6 months of age (Jelan et al. 1996). This implies that growth rates of Thoroughbred foals in the first 5 months of life under different management regimens are similar, but there are insufficient published data for statistical comparison.

In foals of the present study there was a significant relationship between tibiotarsal (TT) joint lesions (and TT joint osteochondrosis scores) at 160 days of age, and rapid average daily weight gain in the previous month. Although the relationships were highly significant, rapid average daily weight gain in the month prior to euthanasia accounted for less that 30% of the variation in frequency of TT joint lesions, or in TT joint osteochondrosis scores. The significant association between rapid growth rate in a defined time-frame and frequency of TT joint lesions (or TT joint score) possibly support a specific 'window of vulnerability' (Pool, 1993) for this bone site to develop osteochondrosis in pasture-fed Thoroughbred foals.

In Warmblood foals, predisposed to development of osteochondrosis, higher average daily weight gain in the 5th month of life was not associated with increased frequency of TT joint lesions diagnosed at post-mortem and radiographically (van Weeren et al. 1999). However, in the same group of foals, evidence of femoropatellar joint osteochondrosis at 5 or 11 months of age was associated with significantly higher average daily gain in the 3rd and 5th months of life, or between birth and 11 months of age, compared to foals with no evidence of femoropatellar joint osteochondrosis. Affected foals were also taller than unaffected foals at 11 months of age, but condition scores were not assessed, so it is not clear if animals were larger and fatter, or only larger.

It is difficult to compare the results of the Dutch studies and the present study, as the populations studied were different breeds, with greatly different predisposition to osteochondrosis. Warmblood foals had higher birthweights and higher average daily weight gains compared to Thoroughbred foals. For example, the Dutch foals were gaining 1.1 kg per day between 4 and 5 months of age, compared to 0.8 to 0.9 kg per day for Thoroughbred foals over the same period in the present study. However, the percentage increase in bodyweight from birth to 5 months of age in Warmblood and Thoroughbred foals were very similar, with mean weight increases of 338% in Warmbloods (calculated from data of van Weeren et al. 1999), compared to mean increases of 317% and 333% in Thoroughbred foals born in Year 1 and 2 respectively of the present study. Perhaps the emphasis on growth rate is overemphasised: the multifactorial pathogenesis of osteochondrosis may be more closely related to hereditary aspects in Dutch Warmbloods, compared to Thoroughbreds. The heritability of osteochondrosis in Scandinavian Trotters and Warmblood horses is high (Philipsson, 1996), but the heritability in Thoroughbred horses has not been reported.

With the exception of tibiotarsal lesions, there was no relationship between growth rate (absolute or relative) and evidence of DOD lesions at 160 days of age in foals of the present study. In other species, such as poultry and pigs, increased growth rates are associated with greater number and severity of lesions (Olsson and Reiland, 1978). The growth rates of foals in the present

study were very similar to growth rates of Thoroughbred foals in North America (Hintz et al. 1979; Pagan et al. 1996), but the prevalence and severity of osteochondrosis at post-mortem is much lower in New Zealand (Chapter 7; Pearce et al. 1998a) than in North America or Europe. This indicates that growth rate is unlikely to be a major risk factor for the development of osteochondrosis in Thoroughbred foals in the first 5 months of life, with the exception of TT joint lesions. In any case, the TT lesions observed in foals of the present study may not be particularly significant in terms of later clinical relevance, since they were very small, and were not observed to result in fragmentation, only retained cartilage.

8.4 Copper storage

8.4.1 Parenteral copper supplementation of mares, and the effects on mare liver and plasma copper concentration

In the studies reported in this thesis, parenteral copper supplementation of mares during late gestation did not raise liver copper concentrations of mares. Two regimens of injections were used, at doses considered to meet or exceed the requirements of cows and calves during pregnancy. During late gestation, mares were given total doses of 350 mg calcium copper edetate in Year 1, and between 1250 and 1500 mg in Year 2. Typically, a total dose of 100 to 400 mg of calcium copper edetate (in 100 to 200 mg divided doses) given to cattle grazing copper deficient pastures during late pregnancy is considered sufficient to meet the requirements of the dam and the foetus (Ellison, 1994).

The failure of calcium copper edetate to raise liver copper concentrations of mares was unexpected. A liver biopsy 4 to 8 weeks after injection of mares was considered a suitable time frame to detect any changes in liver copper concentration. A small number of mares in Year 2 were biopsied 10 days after the first copper injection, but no increase in copper concentration was detected. In ruminants, elevation of liver copper concentration occurred within 1 to 2 weeks of injection with calcium copper edetate (Bohman et al. 1984; Harrison et

al. 1989), with 75 to 90 percent of the copper dose present in the livers of sheep and deer within a week (Harrison et al. 1989).

The liver copper concentration of mares is similar to that for adults of most monogastric species, between 15 and 30 mg Cu kg^{-1} liveweight, but the amount of copper stored in the liver is much lower than in ruminants. The differences in storage are probably related to inherited patterns of excretion (Davis and Mertz, 1987). If the liver of an adult Thoroughbred horse weighs between 5 and 6 kg, and contains 30% DM, 20 mg Cu kg^{-1} DM, it contains only 30 to 46 mg copper. Any increase in liver copper storage as a result of copper injection should be detectable by serial liver biopsies, as shown in ruminants (Harrison, 1989). Even if only 10% of a 250 mg injection was stored in the liver of mares the copper concentration would increase to between 37 and 38 mg Cu kg^{-1} DM, and likely to be detectable by biopsy (CV 10 to 20%, Pearce et al. 1997).

In the present study calcium copper edetate was chosen as the form of injectable copper, as in cattle it resulted in fewer abscesses than other injectable copper preparations, such as copper methionate (Suttle, 1981). In ruminants, injection site abscesses are reported to result in encapsulation of the administered copper, so it is unavailable for transfer to the liver (or other tissues) (Carmago et al. 1962; Suttle, 1981). Injection of calcium copper edetate in the mares was at a depth of 40 mm in the pectoral region, but there was no evidence of abscess formation, induration or fibrosis by palpation or ultrasound imaging at the injection site after the mare foaled. There was no increase in plasma fibrinogen monitored at weekly intervals for up to 4 weeks in 6 mares after the first copper injection, indicating there was no significant inflammatory response associated with copper injection. It thus seems highly unlikely the injected copper was sequestered at the injection site.

Calcium copper edetate is injected subcutaneously in cattle, due to a higher incidence of injection site reactions when given intramuscularly. However, the increase in liver copper concentration in cattle was similar when calcium copper edetate was given subcutaneously or intramuscularly (Bohman et al. 1984), so

the injection site of calcium copper edetate in mares in the present study seems unlikely to have affected transfer to the liver.

Nor was there detectable rise in serum copper concentration in mares 2 to 4 weeks after injection of calcium copper edetate (up to 0.5 mg Cu kg^{-1} liveweight). In ruminants, increased plasma or serum copper concentration occur only after high doses of injectable copper (Bohman et al. 1984; Van Niekerk et al. 1994).

Copper injections did not result in an increase in mare liver or plasma copper concentration, and this indicates copper was most likely excreted in bile and faeces. As there was no increase in plasma copper concentration it is unlikely a significant amount of injected copper was incorporated into caeruloplasmin (or bound to albumin or amino acids) for transport to tissues other than the liver. No reports in the literature have shown increased liver copper concentration in adult horses (> 2 years old) in response to copper supplementation, with the exception of Bauer (1975) who did not report the actual dietary copper levels or liver copper concentration in horses that died of chronic copper toxicity.

8.4.2 Parenteral copper supplementation of mares, and the effects on foal liver and plasma copper concentration at birth

Calcium copper edetate injections given to mares in late gestation had no significant effect on foal liver or plasma copper concentration in the first week of life.

The range in liver copper concentration of foals in the first week of life was large (106 to 1084 mg kg^{-1} DM) and unrelated to dam copper treatment, dam age or experimental year. The variability in liver copper concentration was consistent with other studies in horses (Meyer and Tiegs, 1995; Egan and Murrin, 1973a; van Weeren et al. 2003), including studies where oral copper supplementation resulted in significantly higher foal liver copper concentration at birth (Pearce et al. 1998c).

Pearce et al. (1998c) showed that oral copper supplementation of pregnant mares did not influence liver or blood copper indices of mares, but did increase foetal liver copper concentration, as evidenced by very significant higher liver copper concentrations at 4 to 10 days of age. However, the effect was not predictable, with 4 of the 12 foals from dams supplemented with oral copper having liver copper concentrations of greater than 600 mg kg^{-1} DM, while the other 8 foals had liver copper concentrations less than 400 mg kg^{-1} DM.

The critical minimum liver copper concentration of a foal at birth has been questioned. Meyer and Tiegs (1995) proposed that stores of at least 400 mg Cu kg^{-1} DM were required for sufficient copper for the first month of life. Similarly, Knight et al. (1985) speculated that low neonatal hepatic stores may result from the dam receiving low levels of dietary copper during gestation, but this has not been proven. In contrast to these suggestions, results from the present study indicate that neonatal liver copper concentrations $\geq$ 140 mg Cu kg^{-1} DM (the lowest of any foal in the present study) are sufficient for normal growth and development, and are associated with low frequency and severity of DOD in the first 160 days of life. Also, there appears to be no effect of pregnant mares grazing pasture containing copper level below NRC recommendations (1987) on liver copper stores of the foal at birth.

Regardless of mare copper supplementation, neonatal foals had very low plasma copper concentrations, which reached adult values in the first month of life (Chapters 3 and 4), as previously described by Bell et al. (1987); Okamura et al. (1998) and Pearce et al. (1998b). Okumura et al. (1998) suggested that the limited synthesis of caeruloplasmin in the neonate might indicate foals are in a critical situation with regard to shortage of copper in the early stage of growth. However, this phenomenon of extremely low serum or plasma copper and caeruloplasmin concentration and caeruloplasmin activity is not restricted to the foal, and has been reported in many other species (Underwood and Suttle, 1999).

8.4.3 Liver biopsy in mares

Liver biopsy was not successful in all mares during pregnancy and post-foaling due to failure to identify the right lobe of the liver ultrasonographically, which has previously been reported (Pearce et al. 1997). Failure to obtain biopsies was more common in late gestation or post-foaling than in early pregnancy, indicating that with advancing pregnancy the position of the liver may be altered, moving it further (axially) from the right thoracic wall. The presence of intestine in the biopsy area increased the risk of intestinal perforation with the biopsy needle. The liver of horses also atrophies with age, reportedly due to pressure of the right dorsal colon and the caecal base (Dyce, Sack and Wensing, 1987). Two mares showed signs of mild colic following liver biopsy, but they also had copper injections given at the same time, so it was not possible to determine the cause of the pain. Symptoms of colic resolved in a few hours. Several mares showed signs of sharp pain as the biopsy instrument passed from the thoracic cavity through the diaphragm. It the needle was repositioned through the diaphragm, the mare showed no further discomfort. It is possible the biopsy needle was in contact with a phrenic nerve, resulting in a pain response.

Liver biopsy is a suitable, safe technique to obtain liver to determine copper concentration, however, the technique will not be successful in all mares, due to age-related and pregnancy-related changes in liver size or position.

8.4.4 Repeated liver biopsies of foals between birth and 160 days of age

The use of repeated liver biopsy in foals to describe the decline in liver copper concentration has not been reported before. The liver biopsy technique was suitable for use in sedated foals, with minimal additional restraint required. There were no apparent problems, with biopsies repeated at intervals as often as weekly in the first month of life in foals, and up to 8 liver biopsies harvested in the first 20 weeks of life. Use of ultrasound allowed accurate identification of the liver and the optimal biopsy site. Multiple passes with the very thin biopsy

needle were required to obtain sufficient tissue (30 to 60 mg) for determination of copper concentration.

At post-mortem, there was a roughly circular area, up to 6 cm diameter, of mild fibrous capsular thickening, irregularity and tags of the diaphragmatic surface of the right lobe of all foals' livers. Deep to the circular area there were no associated gross parenchymal changes on cut sections in any of the foal livers, and no histological change in the parenchyma of Year 1 foals.

Liver biopsy in foals showed high repeatability for determination of copper concentration (87%), and was strongly correlated with total liver copper at 160 days of age (determined in homogenised whole liver). This indicates liver biopsy is a good indicator of total liver copper concentration. Liver biopsy is considered the best indicator of copper status (Jeffcott and Davies, 1998). The liver copper concentration at which inadequacy occurs in horses, and associated reduction in plasma copper concentration and copper-dependant processes, has not been defined. Data from foals in Years 1 and 2 can be considered as 'normal' liver copper values for foals raised at pasture in New Zealand, as there was no evidence of copper inadequacy.

Two age-dependant patterns in decline of liver copper concentration were identified in foals born in Year 1 and Year 2, which have not been described before (Chapters 3 and 4). In most foals, liver copper concentration declined rapidly, and reached adult levels by 100 days of age. In 6 foals, a slower pattern of decline in liver copper concentration was present, with significantly higher liver copper concentration than normal at post-mortem (a 4-fold difference). No differences in the pattern of decline of liver copper concentration of 26 Warmblood foals between birth and 5 months of age (van Weeren et al. 2003). The two patterns of decline in liver copper concentration observed in foals in the present study may be breed specific.

It is not possible to determine if foals in the present study with a slower decline in liver copper concentration were less efficient at liver copper excretion than other foals, and/or if they were more efficient at absorbing and storing dietary

copper. It is not known whether the high liver copper concentration in foals at 5 months of age would have persisted into adult life, or when the typical, low adult values would have been reached. There was no effect of pattern of liver copper decline on plasma copper concentration, which ranged from 1.0 to 1.5 mg Cu L^{-1} at 160 days of age. The range in plasma copper concentration was consistent with other studies in foals (Pearce et al. 1998b). There are few other reports of genetic variation in copper metabolism in horses, but caeruloplasmin polymorphisms in Arab horses are thought to account for the large variability in plasma copper (Skripnichenko et al. 1980, cited by Auer et al. 1988b). Genetic variation in copper metabolism has been described between breeds in other species, such as in dogs, sheep and cattle (Wiener and Field, 1971; Gooneratne et al. 1994; Thornburg, 2000).

8.4.5 Neonatal liver copper stores and prevalence of DOD at 160 days of age

There was no statistically significant association between neonatal liver copper concentration and the frequency or severity of DOD lesions in foals at 160 days of age. However, van Weeren et al. (2003) suggested that high liver copper concentrations of Warmblood foals at birth might have a palliative effect on lesions present at 5 months of age, but this effect could not be determined in the present study.

8.5 Developmental orthopaedic disease

8.5.1 Low severity and prevalence of osteochondrosis lesions

The frequency and severity of osteochondrosis observed at post-mortem in foals aged 160 days old was considered to be low, as previously reported by Pearce et al. (1998a). The pasture contained between 5.2 and 10.7 mg Cu kg^{-1} DM, so mares and foals often grazed pasture containing less copper than recommended by NRC (1989). However, the so-called 'suboptimal' dietary copper levels were

not associated with severe lesions, as reported by others (Knight et al. 1990; Hurtig et al. 1993), or any clinical evidence of osteochondrosis.

If is difficult to compare New Zealand and overseas osteochondrosis studies, as the relationship between minor abnormalities and lesions that have clinical consequences has not been established. Also, others studying the very obvious lesions may have overlooked or ignored the very subtle post-mortem lesions investigated and recorded in the present study. There is good evidence that a large number of osteochondrosis lesions identified at 5 months of age in Warmblood foals (radiographically and at post-mortem), regress by 11 months of age, and do not become clinically evident (Dik et al. 1999; van Weeren and Barneveld, 1999; van Weeren et al. 2003). The decrease in number of lesions between 5 and 11 months of age was most evident in the femoropatellar/femorotibial joints, but negligible in the tibiotarsal joint. Radiographic evidence of osteochondrosis was considered permanent if present in the tibiotarsal joint at 5 months of age, or 8 months of age for the femoropatellar/femorotibial joint (Dik et al. 1999). It seems unlikely that the subtle lesions characterised in the present study would progress to clinical lesions, but this can only be surmised.

The most common abnormality detected in foals was subtle tibiotarsal joint lesions, mainly on the medial trochlear ridge of the talus, in Years 1 and 2 (Tables 7.5 and 7.6, pages 227 and 228 respectively). This site is not classically described as a predilection site for osteochondrosis. However, these subtle lesions were not associated with clinical signs. In radiographic and post-mortem studies, tibiotarsal lesions present at 5 months of age were considered permanent in Warmblood foals, as there was little evidence of regression (or progression) at 11 months of age (van Weeren and Barneveld, 1999; Dik et al. 1999; van Weeren et al. 2003). If the tibiotarsal joint lesions in 5 month old New Zealand Thoroughbred foals act like those of Dutch Warmblood foals, it would be expected they would still be present at 11 months of age, but they would not have progressed, and therefore would not be associated with clinical signs. It is doubtful the lesions identified in the studies described in this thesis would have been apparent in radiographs in the live animals.

The high prevalence of scapulohumeral joint lesions in foals at 160 days of age (Chapter 7) has not been described in other post-mortem studies. The lesions were mostly linear or stellate cartilage invaginations in the central glenoid, with retained cartilage, thus were characterised as osteochondrosis. The significance of these lesions is not known. Almost all published post-mortem and arthroscopic studies of osteochondrosis in the scapulohumeral joint describe severe (not subtle) lesions, associated with degenerative joint disease, most often in the caudal articular surface of the humeral head and the glenoid cavity (Nyack et al. 1981; Bertone and McIlwraith, 1987; Hurtig and Pool, 1996; Doyle and White, 2000). However, these reports do not provide a specific description of the site affected within the glenoid cavity, which is not surprising, when horses present for surgery the lesions usually chronic and large. There is not enough information to determine if the lesions reported by these authors are definitely in the same site as those identified in foals in the present study. In foals in the present study, cyst-like lesions in the glenoid cavity or lesions in the caudal humeral head were not present in contrast to other authors (cited above), and none were lame. It cannot ruled out that the lesions identified in the present study could progress and become clinically evident, although given the differences between most descriptions of lesions in clinical cases (of what is a relatively uncommon clinical disease), and the frequent but minor lesions at a specific site in the present study, it is considered unlikely. Alternatively, the lesions could resolve, as has been described at a number of sites (Dik et al. 1999).

It is possible that retained cartilage cores that are not associated with gross, or even other histological evidence of osteochondrosis (for example, cartilage necrosis) represent a 'normal' (i.e. occurring commonly) temporary physiological aberration of cartilage development (Pool 1993). If this is true, the presence of retained cartilage alone as evidence of osteochondrosis may need to be reconsidered. Identification of retained cartilage at sites that are not predisposition sites of clinically evident osteochondrosis would support the hypothesis that, at least in the absence of other abnormalities, retained cartilage cores are a normal physiological aberration, or a successful response to minor osteochondral insult (for example, a microfracture or infection).

8.5.2 Characterisation of distal metacarpal and metatarsal physeal enlargements

Enlargements in the metaphyseal region of the distal third metacarpal and metatarsal bones were clinically evident in all foals in these studies, but were not associated with signs of pain or lameness. There was no statistical relationship between growth rate (absolute or relative) and the clinical score given to the enlargements, both assessed at 2 weekly intervals. At 160 days of age, the metaphyseal enlargements were not associated with metaphyseal growth plate abnormalities, and were sometimes present in association with completely closed physes, if it can be assumed that closure in a mid-frontal slice through the physis is indicative of closure of the entire physis. There was no statistically significant association between metaphyseal bony enlargements and evidence of abnormal endochondral ossification in the metaphyseal growth plate.

These data suggest the enlargements are a normal physiological response in growing foals, and are not signs of the painful disease referred to as physitis repeatedly described in the literature (Wagner and Watrous, 1990; Frankeny et al. 1994). The bony enlargements of foals described in this thesis should thus not be called 'physitis', but rather considered as benign physiological enlargements associated with bone remodelling. The enlargements are self-limiting, and should not be considered to be of relevance to the sale potential or future athletic performance of an affected animal.

All metaphyseal enlargements of foals should be characterised accurately, so comparisons can be made between individual and different groups of horses. Further research to characterise the bony enlargements of the distal radius in older animals and their relationships to growth and endochondral ossification is required. 'Physitis' of the distal radius often presents clinically with signs of pain and lameness (Williams et al. 1982), and may be of great clinical significance during preparation of animals for yearling sales. The clinical signs are different to those associated with distal third metacarpal and metatarsal

enlargements of foals in the present study, and thus may be of considerable clinical significance, as opposed to a normal physiological change.

8.5.3 Cathepsin B expression and osteochondrosis/dyschondroplasia

Cathepsin B may be important in the post-natal, mechanically induced turnover of the cartilage extracellular matrix (Gläser et al. 2003). In Chapter 6 the immunolocalisation of cathepsin B in normal and osteochondrotic age-matched cartilage samples was compared. The level of cathepsin B expression varied between sites, and between the zones of cartilage, but in cartilage from all sites there was moderate expression in the resting zone, and strong expression in the hypertrophic zone, also noted by Hernandez-Vidal et al. (1998). All chondrocyte clusters (chondrones) had relatively strong expression of cathepsin B. The importance of high levels of cathepsin B in chondrocyte clusters is not known, but could play an undetermined role in extracellular matrix turnover (Hernandez-Vidal et al. 1998; Kostoulas et al. 1999). Increased expression of this degradative enzyme in dyschondroplastic/osteochondrotic cartilage may represent a mechanism to increase matrix turnover, and enable repair of some lesions.

In vitro, copper has been shown to reduce cathepsin B expression (Davies et al. 1996). However, in the present study there was no effect of dam copper supplementation, or foal liver copper concentration at birth, on the level of cathepsin B expression.

8.5.4 Potential sources of experimental and interpretative error

The experimental design allowed comparison of growth data, condition scores and liver copper concentration from birth to 160 days of age, in age-matched animals, born in two consecutive years. The animals in this study were grown on the same property in both years, and were managed similarly, thereby reducing differences in the measured parameters due to location or style of management. There were no significant differences in mean growth between the years.

Errors in measurement of live animals were minimised through use of the same well-trained technicians and standardised protocols for each foal measurement. Biases in assessment of distal third metacarpal/metatarsal enlargements, condition score and ultrasonographic fat thickness was minimised by using the same assessor (the author). Significant differences in assessment of ultrasonographic fat thickness between operators have been reported (Houghton and Turlington, 1992), and between veterinarians using the same system to assess clinical DOD scores (Knight et al. 1987).

Foals were euthanased at 160 days of age, allowing comparison between results of these studies and those of Pearce et al. (1998a), whose foals were also Thoroughbred, and raised on the same property. Comparison with growth and post-mortem data from 5 month old Warmblood foals predisposed to osteochondrosis was also possible (van Weeren and Barneveld, 1999; van Weeren et al. 2003). Comparisons between age-matched animals are important, especially for osteochondrosis data, as it has been shown to develop in different sites at different periods of time (van Weeren and Barneveld, 1999). Comparisons of disease prevalence data in foals of different ages could lead to erroneous conclusions. Caution has been employed in this thesis in comparisons made across breeds and across production systems.

In all areas of the present studies, except one, the numbers of animals were suitable to determine statistical relationships and differences, as the predictive power was greater than 0.8. An exception was the use of 10 animals only to determine the relationships between in vivo methods to assess body composition and chemical body composition. In Year 1, BIA and ultrasonographic rump fat thickness measurements were not carried out in all animals, due to technical problems with the ultrasound machine, and a delay in clearing the BIA machine through customs. However, the data from the small number of animals allowed identification of suitable sites for measurements in the following year, development of techniques, and identification of the most repeatable condition scoring method. All Year 1 animals were used for chemical body composition analysis. In Year 2, BIA and ultrasonographic measurements were made in 23 animals, allowing comparison of measurements

in live animals and at post-mortem. All 23 animals were condition scored, and 10 were selected for chemical composition. Although the numbers of animals used were small for determining the relationship between in vivo techniques to assess body composition and chemical body composition (n = 10), the data provide a valuable contribution to the limited equine data that exists. Although small, the sample sizes for investigation of the relationships between in vivo methods to predict body composition and chemical body composition resulted in significant correlations and regressions. This restriction meant that for improved predictive power of greater than 0.8 for the regression relationship between the fat-free mass (or total body water) and BIA measurements, more than 30 animals were required. This was not possible due to time, cost and ethical constraints of terminal studies. In spite of the small numbers the predictive power of the regression between ultrasonographic rump fat measurements and percentage fat was 0.7, and therefore acceptable.

8.6 Recommendations to the New Zealand Thoroughbred industry

The prevalence and severity of osteochondrosis in foals at 5 months of age, grazing pasture containing 5.2 and 10.7 mg Cu kg^{-1} in New Zealand, is low compared to overseas. However, owners should consider copper supplementation of all mares during late gestation, as this could lead to fewer gross articular cartilage lesions in foals at 5 months of age (Pearce et al. 1998a), or increase the number of gross articular cartilage lesions that resolve between 5 and 11 months of age (Dik et al. 1999; van Weeren and Barneveld, 1999; van Weeren et al. 2003).

Injectable calcium copper edetate or other injectable copper preparations should not be used. Instead mares should be orally given the equivalent of 0.5 mg Cu kg^{-1} bodyweight day^{-1} during late gestation.

Enlargements proximal to the fetlock regions, previously called 'physitis', are not pathological. In young, pasture-fed horses the enlargements are more likely to represent a normal physiological response of bone during growth. Animals with enlargements of the distal third metacarpus and metatarsus should not be considered abnormal, and management practices should not be altered, as the enlargements will resolve. Careful and regular farm system surveillance programmes should exclude faults in management and nutrition so that 'real' cases of physitis, and their causes, are identified promptly and corrected.

In New Zealand, there is no published evidence the diseases included under the DOD banner (such as osteochondrosis, physitis, flexural or angular limb deformities) have a common aetiopathogenesis. Most of these problems appear to be sporadic, and occur at a low prevalence. Therefore, each disease needs to be carefully defined, allowing accurate recording of the true incidence of clinical disease in New Zealand. Accurate recording systems for both growth and development and disease incidence would allow the relationships to be further investigated.

Condition scores were significantly correlated with partial empty body fat mass and concentration, and should used as an indicator of total body fatness. The condition scoring system for horses devised by Henneke et al. (1983) is well described, and can easily be learned and applied by horse-owners and veterinarians, without the need for special equipment. In young horses, special consideration should be given to fatness over the ribs when assessing the overall body condition.

Owners should weigh and condition foals at 4-weekly intervals, recording all results, so regular assessment is made of each foal's growth and development, with respect to fatness, weight gain and conformation. Such accurate monitoring will allow rapid identification of foals that are not growing as expected, so the reason for altered growth can be determined, and rectified where possible. Data collected will also be able to be used in a growth model for New Zealand foals, to be developed in the future.

Foals to 5 months of age can be successfully grown on a pasture-only diet with their dams, providing the pasture available is of suitable quality and quantity, without the requirement for grain.

8.7 Further research

These studies have identified areas of limited information, which beg further study in aspects of growth and development in the pasture-fed Thoroughbred foal. Further research should be directed to:

1. Identify appropriate means to implement systems that require owners and veterinarians to regularly assess and record fatness and growth of young horses

2. Determine the mature weight and height of Thoroughbred mares and stallions in New Zealand

3. Develop a growth model of horses in New Zealand, from birth to maturity

4. Develop suitable methodology for highly repeatable and reliable measurement of rump fat thickness in horses of all ages, as an indicator of total body fatness

5. Determine the prevalence of clinical DOD lesions in New Zealand, and investigate the relationship with recorded measurements of growth and fatness

6. Investigate the frequency of scapulohumeral lesions in Thoroughbred horses of all ages that present for post-mortem examination

7. Determine the critical liver and plasma copper concentration at which lysyl oxidase function (or other connective tissue indices) is impaired, resulting in collagen or other connective tissue abnormalities

8. Evaluate the effectiveness of copper-containing supplements fed daily to mares in late gestation in increasing foal liver copper concentration at birth, and the optimal daily Cu dose required

9. Investigate the growth plate changes associated with 'physitis' of the distal radius, and determine the relationships with feeding, management, weight gain and fatness

10. Determine if two patterns of decline in liver copper concentration occur in other Thoroughbred populations, and if there is evidence for a palliative effect on osteochondrosis lesions in foals with slow decline in liver copper concentration

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SUPERVISOR'S DECLARATION

This is to certify that the research carried out for the Doctoral thesis entitled 'Aspects of growth and development of the pasture-fed Thoroughbred foal in New Zealand' was done by Erica Kathleen Gee in the Institute of Veterinary, Animal and Biomedical Sciences, Massey University, Turitea Campus, New Zealand. The thesis material has not been used in part or in whole for any other qualification, and I confirm that the candidate has pursued the course of study in accordance with the requirements of the Massey University regulations.

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CANDIDATE'S DECLARATION

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CERTIFICATE OF REGULATORY COMPLIANCE

This is to certify that the research carried out in the Doctoral Thesis entitled 'Aspects of growth and development of the pasture-fed Thoroughbred foal in New Zealand' in the Institute of Veterinary, Animal and Biomedical Sciences at Massey University, New Zealand:

(a) is the original work of the candidate, except as indicated by appropriate attribution in the text and/or in the acknowledgements

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Massey University Animal Ethics Committee protocol number 97/108

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