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THE LIPID AND FATTY ACID COMPOSITION OF SEMEN IN RELATION TO FERTILITY IN THE MALE ANIMAL.

by

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SUMMARY

Spermatozoa are highly specialised cells which display a range of unique features associated with their crucial function of egg fertilisation. One of the most striking characteristics of spermatozoa, in biochemical terms, is the extremely high proportion of long chain highly polyunsaturated fatty acids present as components of the plasma membrane phospholipids. This high degree of unsaturation is almost unique amongst animal cells ; the only other cell types which display similar levels of these polyunsaturates are the neurons of the brain and retina. The reason why spermatozoa exhibit such an unusual fatty acid composition is not clear but it is feasible that the highly unsaturated phospholipids may confer a high degree of flexibility on the sperm plasma membrane as well as provide a potential energy source in order to facilitate the characteristic flagellar motion of these cells. There is also evidence that spermatozoa lipids play a crucial role in the membrane fusion and signal transduction events associated with the acrosome reaction and fertilisation.

Initial observations were made between the semen of domestic cockerel and bull with respect to animal ageing and semen quality. Within both species similar patterns were observed in that with age there was a loss of the long chain C20 and C22 polyunsaturated fatty acids accompanied by a loss of the major antioxidant enzyme systems. A decrease in phosphatidyl ethanolamine and an increase of phosphatidyl choline with age were also observed to be associated with a reduction in semen quality parameters and fertility in both species. In contrast cockerels displayed significant increases in spermatozoa and seminal plasma lipid levels where the bull exhibited losses, reflecting possible differences in spermatozoa metabolism and function.

From the initial results it was obvious that there was a dramatic difference between the spermatozoa of domestic mammals and birds in that the former display high proportions of n-3 polyunsaturates (especially docosahexaenoic acid, 22:6 (n-3)) whereas the latter are characterised by fatty acids of the n-6 family (particularly arachidonic, 20:4 (n-6) and docosatetraenoic, 22:4 (n-6)). However, since the only avian examples so far examined are domestic poultry species, it is conceivable that the reported n-6 predominance in birds could be an aberration due to the feeding of commercial diets which are relatively deficient in n-3 fatty acids. In order to test this possibility, two major experiments were performed. In the first of these,

cockerels were provided with a diet supplemented with linseed oil for 49 weeks during the reproductive period. Linseed oil is a rich source of alpha-linolenic acid (18:3(n-3)) which is the metabolic precursor for the biosynthesis of 22:6 (n-3). In spite of this sustained period of n-3 supplementation there was little or no increase in the proportion of 22:6 (n-3) in the spermatozoa phospholipid in comparison with the unsupplemented cockerels. The only effect of any note was the appearance of low levels of 22:5 (n-3) in the samples from the supplemented cockerels. This result indicates that the developing spermatozoa of the cockerels are effectively unable to convert 18:3 (n-3) to 22:6 (n-3) and/or to incorporate 22:6 (n-3) into phospholipid. In order to distinguish between these possibilities, a second experiment was performed in which cockerels were supplemented with tuna orbital oil, a rich source of 22:6 (n-3) for 48 weeks during the reproductive period. Although in some instances differences were small between unsupplemented and supplemented cockerels the changes in the lipid composition of the spermatozoa and seminal plasma of the cockerels in both experiments did result in improvements in semen quality and fertility. Supplementation with 22:6 (n-3) induced a significant but limited increase in the level of 22:6 (n-3) in the spermatozoa phospholipid. However, the maximum level of 22:6 (n-3) that was achieved by the supplementation was less than 10 % (w/w of fatty acids) and thus fell far short of the level routinely observed in mammalian spermatozoa (e.g. 60 - 70 %, w/w of fatty acid in the bull) even in the absence of n-3 supplementation. Moreover, 20:4 (n-6) and 22:4 (n-6) remained as the major polyunsaturates of the cockerel spermatozoa in spite of the n-3 supplementation. Thus it is clear that the n-6 predominance in domestic cockerel spermatozoa is extremely resistant to dietary manipulation and certainly does not switch to a "mammalian" pattern following dietary supplementation with n-3 fatty acids. The conclusion of this work is that the high levels of n-6 fatty acids in avian spermatozoa most likely represent the natural situation and are not an artefact due to the feeding of commercial diets. Thus the differential utilisation of n-3 versus n-6 fatty acids for spermatozoa phospholipid biosynthesis appears to be a true phylogenic difference between mammalian and avian species.

In contrast to the cockerel studies, as discussed the major fatty acid component of bull spermatozoa was 22:6 (n-3). Dietary supplementation of Holstein/Fresian bulls with tuna orbital oil for two months had no effect on the level of 22:6 (n-3) in the

total phospholipid of the spermatozoa but did produce a dramatic rearrangement of the distribution of this fatty acid between the different phospholipid classes. Most notably, the n-3 supplementation produced significant improvements in semen quality parameters of both fresh and frozen semen samples. Additional studies were performed to assess the effectiveness of the antioxidant vitamin E in the maintenance of semen quality during storage. Addition of vitamin E in the appropriate form was found to reduce lipid peroxidation and to improve fertility as assessed by artificial insemination.

In summary, these results highlight the possibility of improving semen quality and male fertility to varying degrees in commercial animal species by the manipulation of the lipid and antioxidant parameters of the semen.

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The encouragement from all members of my family in particular my husband and parents during the study period is highly appreciated for their good humour, support and criticisms and to my son, Christopher for his never ending patience and understanding which enabled me to undertake and carry on with this work.

DEDICATION

I dedicate this work to my

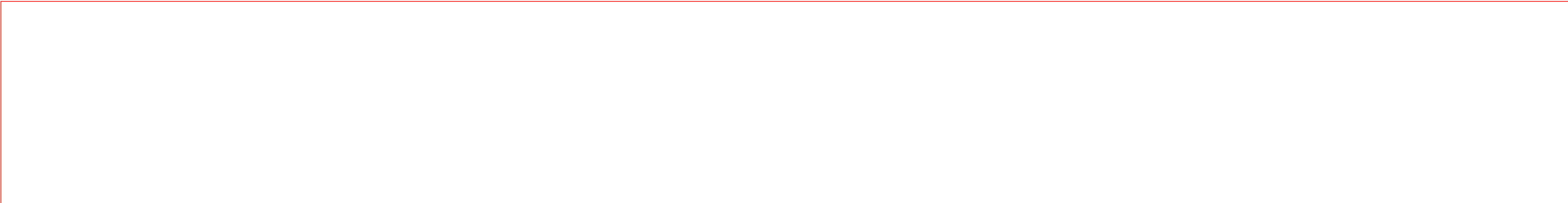
husband **GORDON KELSO** and son **CHRISTOPHER KELSO.**

and

MY PARENTS, ANNE, JOHN, MARGARET AND JOSEPH

DECLARATION

The contents of this thesis are the work of the author. The thesis has not been submitted previously for the award of a degree to any University.



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CHAPTER 1

INTRODUCTION

The term infertility is defined as the inability of an animal of any kind to produce offspring. For decades this phenomenon was looked upon as originating from the presence of disease, bacterial or viral, which was believed to suppress the female or males' natural mechanisms of fertilisation and conception. It is estimated contemporarily that such problems of infertility as a result of "the male factor", are responsible for some 25% of couples being unable to conceive (McLaughlin *et al.*, 1992 ; Aitken, 1994). In the agricultural world for the commercial farmer male infertility means something quite different. It signifies many hundreds of thousands of pounds lost in annual revenue as well as the unnecessary slaughter of male animals unable to perform to target figures (Lansbergen and Koorn, 1995 ; Redpath, 1995).

For centuries it has been known and reported that the testes of the male animal were responsible for the transmission of a biological secretion necessary for the production of viable offspring and that in the absence of this necessary anatomy conception was not possible. Following from these simplistic observations, knowledge continued to expand to include the detailed anatomy and physiology of the male reproductive organs, followed by intricate examinations of the semen itself to reveal the presence of individual, microscopic, self-propelled organisms "swimming" in a fluid medium (van Leeuwenhoek 1969, cited by Mann and Lutwak-Mann, 1981a). The early pioneers who realised the significance of this body fluid exclusive to the male animal soon began exploitation of *in vitro* procedures such as semen collection techniques, transportation and ultimately artificial insemination of female animals leading to successful conception rates in humans and a range of farm animals (Perry 1937, cited by Smith, 1988). Such developments eliminated the need for "natural" service and laid the foundations for the multimillion pound industries that exist in advanced societies and used by the vast majority of their commercial farming organisations.

There are many modern theories on male reproductive failure and infertility that embrace a range of factors that include nutritional disorders, environmental influences, the effects of disease as well as genetic features as summarised in Table 1.1. The modern theories of environmental oestrogens somehow suppressing the production of male androgen hormones thus affecting semen quality and quantity are in fierce debate (Vines, 1995)

Table 1.1. Various causes of reproductive failure in the male animal (after Jainudeen and Hafez, 1993 ; Jequier, 1995).

Major causes	Possible origin of disorders
Neuroendocrine	disorders of erection, ejaculation and impotence
Endocrine	deficiency of leutenising hormone or testosterone
Testicular	cryptorchidism, hypoplasia, infection or trauma. hypercurvature syndrome
Seminal	disorders of spermatogenesis, sperm defects, poor motility, antisperm antibodies and leukocytic reactive oxygen species
Accessory glands	trauma, infection and obstruction

In the 17th century it became apparent that spermatozoa had their own intricate biochemistry and that between species the spermatozoa morphology and semen metabolism were quite different. Differences were also noticed with regard to the viability of spermatozoa during freezing and in the ability of the spermatozoa to survive any great period of time *in vitro* during preparative techniques and analysis (Sarmah *et al.*, 1984 ; McLaughlin *et al.*, 1992). Early investigators were aware of the lipids as a possible source of variation and recognised, even from the mid 19th century, that various species possessed lipid fractions and associated parameters (Koelliker 1856, cited by Steinberger and Steinberger, 1975). The lipid interest was one which was to continue for decades, ever expanding and in ever more detail. In the present century the lipid composition of spermatozoa and seminal plasma from almost all farm animal species have been reported including the more detailed fatty acid profiles of the individual phospholipid classes (Neill and Masters, 1972, 1973 ; Darin-Bennett *et al.*, 1974 ; Jain and Anand, 1976a, b ; Ravie and Lake, 1985 ; Sebastian *et al.*, 1987).

Subsequent to these initial conclusions it soon became realised that the phospholipids of spermatozoa membranes contained very high levels of long chain fatty acids and that such types of membrane were particularly susceptible to lipid peroxidative damage (Aiken, 1994 ; Griveau *et al.*, 1995). Peroxidation became a key word in relation to male infertility with the recognition of a species difference in the range and extent of endogenous antioxidant protection which was offered by the spermatozoa and the seminal plasma in which they were bathed (Mennella and Jones, 1980 ; Beconi *et al.*,

1993 ; Cecil and Bakst, 1993 ; Meisel *et al.*, 1993). The maintenance of a specific role of polyunsaturated fatty acids requires its protection from peroxidation either through adequate levels of antioxidants or promotion of protective enzyme systems (Bunker, 1992 ; Surai and Ionov, 1992a, b ; Beconi *et al.*, 1993). From the evidence to date from a variety of farm animal species, inadequate protection of polyunsaturates in spermatozoa leads to accumulated peroxidation products exacerbating impairment of spermatozoa function (Aitken and Fisher, 1994). The functional role of the major C20 and C22 polyunsaturated fatty acids, in particular docosahexaenoic acid (22:6 n-3) and the required antioxidant protection to maintain male fertility has been the subject of several reports (Aitken, 1994 ; Aitken and Fisher, 1994 ; Griveau *et al.*, 1995). As a result there is now a focus of attention to try and alleviate the effects of peroxidative damage by improvements in semen collection and handling procedures, dietary supplementation of antioxidants, coupled by the use of cryopreservation technology, freeze curves and freezing diluents. However, in spite of such efforts in many areas of commercial semen handling and preservation, the problems associated with lipid peroxidation are still prevalent.

A major feature in relation to reduced fertility of fresh ejaculates and intolerance to cold storage has now also been shown to be related to the composition of the plasma membrane, in particular its phospholipid composition and associated polyunsaturated fatty acid components. Thus it was able to be shown that spermatozoa with differing lipid and polyunsaturated fatty acid compositions display a range of resistance to cold shock (Darin-Bennett *et al.*, 1974 ; Ravie and Lake, 1985). Data was also obtained to indicate that differences in phase transition temperature associated with the lipids of the sperm membrane were a major factor in acrosomal and membrane damage (Holt and North, 1986 ; Palleschi and Silvestroni, 1996). High concentrations of C20 and C22 polyunsaturated fatty acids of the n-3 series comprise a major constituent of mammalian spermatozoa and seminal plasma (Nissen *et al.*, 1981). Changes in concentrations of these polyunsaturated fatty acids, in particular their association with specific phospholipid moieties, in discrete molecular combinations, have now been shown to be associated with a range of morphological and biochemical features correlatable with a significant reduction in spermatozoa viability and fertility (Nissen and Kreysel, 1983)

The use of fresh or frozen semen for artificial insemination practices is heavily relied upon in the majority of intensive farm animal production systems to meet the needs and demands of todays agricultural environment. However, artificial insemination using frozen semen is much less widely practised with certain domestic animals, such as poultry, because cryopreservation protocols remain unavailable and thus an acceptable level of fertile spermatozoa is unable to be provided (Parks and Graham, 1992). The early reviews on general artificial insemination were concerned with the dog and horse and date back to the late 19th century (Heape 1897, cited by Smith, 1988) ; with early references from as far back as 1786. Smith (1988) reviewed that in the mid 20th century Perry visited Denmark, observing work by Larsen, thereby returning to the USA to form the first Artificial Insemination Centre. In the UK artificial insemination dates from 1942 with the establishment of Centres at Cambridge and Reading in order to promote food production during war times. This basic start within the agricultural industry saw rapid development resulting in more specialised centres, facilities and procedures that are the accepted features of modern day agricultural practice allowing the commercial farmer easy access to top quality semen at any time in the farming calendar (Woelders, 1995). Artificial insemination has thus become an established method of breeding livestock eliminating almost completely the need for natural service in cattle. In 1962 Melrose recorded the percentage of cows artificially inseminated in various parts of the world : Scotland, 19 %, England and Wales, 66 %, Ireland, 40 %, Canada, 10 % and Denmark, 98 %. Great Britain lagged behind for many years, however it is now a leader of top quality animals and in semen production and supply. The advantages of artificial insemination have been reviewed by Evans and Gareth (1987). They include : increased rates of genetic gain, easy transport of genetic material, increased efficiency of breeding, reduction or elimination of the need to maintain males on farm, prevention and control of disease, use of good yet sexually incapacitated males, accurate record keeping, use of synchronisation and out of season breeding and the use of a range of modern technologies such as superovulation and embryo transfer.

Today the problem of male infertility seems to primarily involve the refinement of *in vitro* fertilisation techniques to meet the special demands imposed by the spermatozoa of sub fertile males. This approach has been adopted in preference to the improvement of semen and increased quality of spermatogenesis where in fact the long term solution may lie. As a contribution to the latter approach it has been the objective of the present

study to delineate unique basic lipid compositional criteria of spermatozoa and the seminal plasma, in particular criteria involving C20 and C22 polyunsaturates and associated metabolites in relation to parameters of fertility of fresh and stored ejaculates.

The aims embraced the following investigations ;

i) the characterisation of simple and complex lipid moieties containing long chain polyunsaturated fatty acids in the spermatozoa and seminal plasma across species and their correlation with spermatozoa viability and storage

ii) the correlation of specific phospholipid molecular species and structure with cellular features associated with spermatozoa viability and function.

iii) the existence of oxygenated lipid by-products in stored semen with respect to membrane structure and function.

iv) the quantification of the spermatozoa and seminal plasma of major protective antioxidant systems such as vitamins A and E, trace elements and major enzyme systems.

v) the metabolic interrelationships between the above features and their combined role in spermatozoa viability and function.

vi) promotion of fertility by rectification of above parameters by appropriate means such as diet, diluent addition to ejaculates etc.

vii) assessment of improvements by dietary manipulation and diluent addition in viability of spermatozoa.

viii) to provide an increased understanding in the broader area of male infertility, including that of the human of lipid metabolic function.

CHAPTER 2

LITERATURE REVIEW

2.1 HISTORICAL BACKGROUND

Semen is a liquid or semi-gelatinous cellular suspension which contains the male gametes, or spermatozoa, in addition to secretions from the accessory organs of the male reproductive tract. Semen is a body fluid which has been a source of fascination ever since the earliest investigations of Aristotle who proposed that the embryo was formed from a coagulation of menstrual fluid induced by interaction with the semen (Peck, 1963).

Spermatozoa are intricate, complex, elegantly tailored and highly specialised microscopic organisms whose primary function is to deliver the male pronucleus to the female egg to produce a diploid embryo. Serious morphological observations and examinations of spermatozoa were not possible until the invention of the microscope by van Leeuwenhoek. Using a microscope consisting of a single, highly convex lens this early scientist was able to report some of the first findings on the structure of spermatozoa or "animalcules", observing simply a head and motile tail.

"Now, when a man is unable to beget children by his wife, although his virility is unimpaired, he is said in common parlance to have a cold nature. To my mind, however, it would be more apt to say that no animalcules will be found in the seed of such a man, or that, should any living animalcules be found in it, they are too weakly to survive long enough in the womb."

Quote by Antoni van Leeuwenhoek (1685), cited by Mann and Lutwak-Mann (1981a).

Following Leeuwenhoeks' pioneering observations, a more detailed description of spermatozoa morphology, including the identification of the head, plasma membranes and tail pieces, was able to be constructed by the endeavours of a series of scientists including Ballowitz, Jensen, Meves and Retzius (reviewed by Mann and Lutwak-Mann, 1981a). In spite of this increasing knowledge, there was still considerable debate and disagreement on the significance of spermatozoa in the reproductive process. However, in the early 19th century it was finally recognised that spermatozoa were the males'

contribution responsible for the fertilisation of the female egg. A historical review to the understanding of the reproductive process is provided by Asdell (1977). In the present century, the development of the electron microscope has enabled major advances to be achieved in the structural architecture of spermatozoa, whereas advances in biochemistry have similarly provided extensive information on spermatozoa metabolism (Fawcett, 1962 ; Phillips, 1975 ; Curry and Watson, 1995). Most notably, biochemical analysis of spermatozoa has revealed the importance of lipids, not only as structural components and energy substrates but also as a source of lipid peroxides and free radicals which play their own role in spermatozoa dysfunction. A list of the major contributors to the understanding of spermatozoa and male reproductive function is given in Table 2.1

Table 2.1. Major contributions made to the understanding of male reproductive function. Adapted from Steinberger and Steinberger (1975) and Mann and Lutwak-Mann (1981a).

Researcher	Date	Contribution to the understanding of male reproduction
Leeuwenhoek	1679	Spermatozoon morphology in the semen and testes
Hunter	1786	Testicular contribution to seminal plasma formation
Killer	1841	Identification of seminiferous tubules, site of spermatozoa development
Koelliker	1856	Presence of lipids in spermatozoa
Leydig	1857	Identification of spermatogenic support cells. Microscopic examination of interstitial cells
Sertoli	1865	Identification of spermatogenic support cells. Sertoli cells
Ebner	1871	Spermatogenic process: orderly aspects of
La Vallette	1876	Classification of spermatozoa morphology. Morphological classification of various germinal epithelium cells
Miescher	1878	Spermatozoa biochemistry. Lipids in spermatozoa from salmon
Benda	1887	Orderly aspects of spermatogenesis
Matthews	1897	Supported findings of Miescher and Koelliker on spermatozoa lipid components.
Regaud	1901	Spermatogenic cycle
Ebner	1902	Spermatogenic cycle
Ivanov	1907	Semen collection techniques
Sano	1922	Confirmed lecithin presence and small amounts of cephalin, sphingomyelin and cerebroside in spermatozoa
Gray	1931	Theories on spermatozoa senescence
Prawochenski and Walton	1935	Transportation of spermatozoa and artificial insemination procedures
Lardy and Phillips	1941	Phospholipids as a substrate for spermatozoa energy in bull
MacLeod	1943	First to suggest and describe reactive oxygen species in human spermatozoa
Others	to date	Major advances in sperm structure, biochemistry and composition

2.2 THE MALE REPRODUCTIVE SYSTEM

The reproductive anatomy of mammalian and avian species differ considerably in terms of anatomy and physiology of the testes and penis. A detailed representation of the reproductive systems of both the domestic avian and mammalian species are outlined in Figures 2.1 and 2.2a, b ; they illustrate the non-intromittent phallus of the avian (domestic bird) (King and McLelland, 1984a, b) and the intromittent penis of the mammal (bull) (Setchell, 1982 ; Ashdown and Hafez, 1993a).

2.2.1 Testicular anatomy and physiology

Both birds and mammals possess a pair of testes, of approximately equal size, which in the case of the mammal descend into a scrotum during foetal or early post-natal life (Hafez, 1993 ; King and McLelland, 1984a). Beneath these fibrous encapsulated testicles are tightly packed seminiferous tubules containing two types of somatic cells, namely myoid and sertoli. The testes are responsible for the production of spermatozoa and the male sex hormones, of which the most important is testosterone. Review of the spermatogenic function of the testes are given by Steinberger and Steinberger (1975) and Mann and Lutwak-Mann (1981b).

The seminiferous tubules open into the rete testis (see Figures 2.3 and 2.4) situated centrally in the testis of the ram, bull, boar and at one side of man and rodents. The efferent ducts lead from the rete testis to the epididymis where they unite to form the epididymal duct, a single more convoluted tube that eventually gives rise to the ductus deferens. The testicular differences displayed by domestic avian and mammalian species are detailed in Table 2.2. Myoid or smooth muscle-like cells form an important part of the seminiferous tubule walls. These smooth muscles are thought to be responsible for producing peristaltic-like tubular motions as well as exerting an important stimulatory influence on the sertoli cells, the main function of which is to create a favourable environment for germ cell differentiation. Sertoli cells possess a highly complex morphology, their ultrastructure and function being reviewed in detail by Fawcett (1975)

and Barratt (1995). Sertoli himself described these specialised cells as "nursing cells", which also exhibit phagocytic activity (Steinberger and Steinberger, 1975).

The seminiferous tubules are not penetrated by blood vessels or nerves, these are present in the spaces between the tubules forming part of the interstitial tissue. This interstitial tissue also contains the leydig cells, which are cholesterol-rich, and possess extensive smooth endoplasmic reticulum necessary for androgenic steroid synthesis within the testis (Wallace *et al.*, 1984 ; King and McLelland, 1984a ; Garner and Hafez, 1993).

Figure 2.1. Reproductive organs of the cockerel (lateral view) (see Van Krey, 1990)

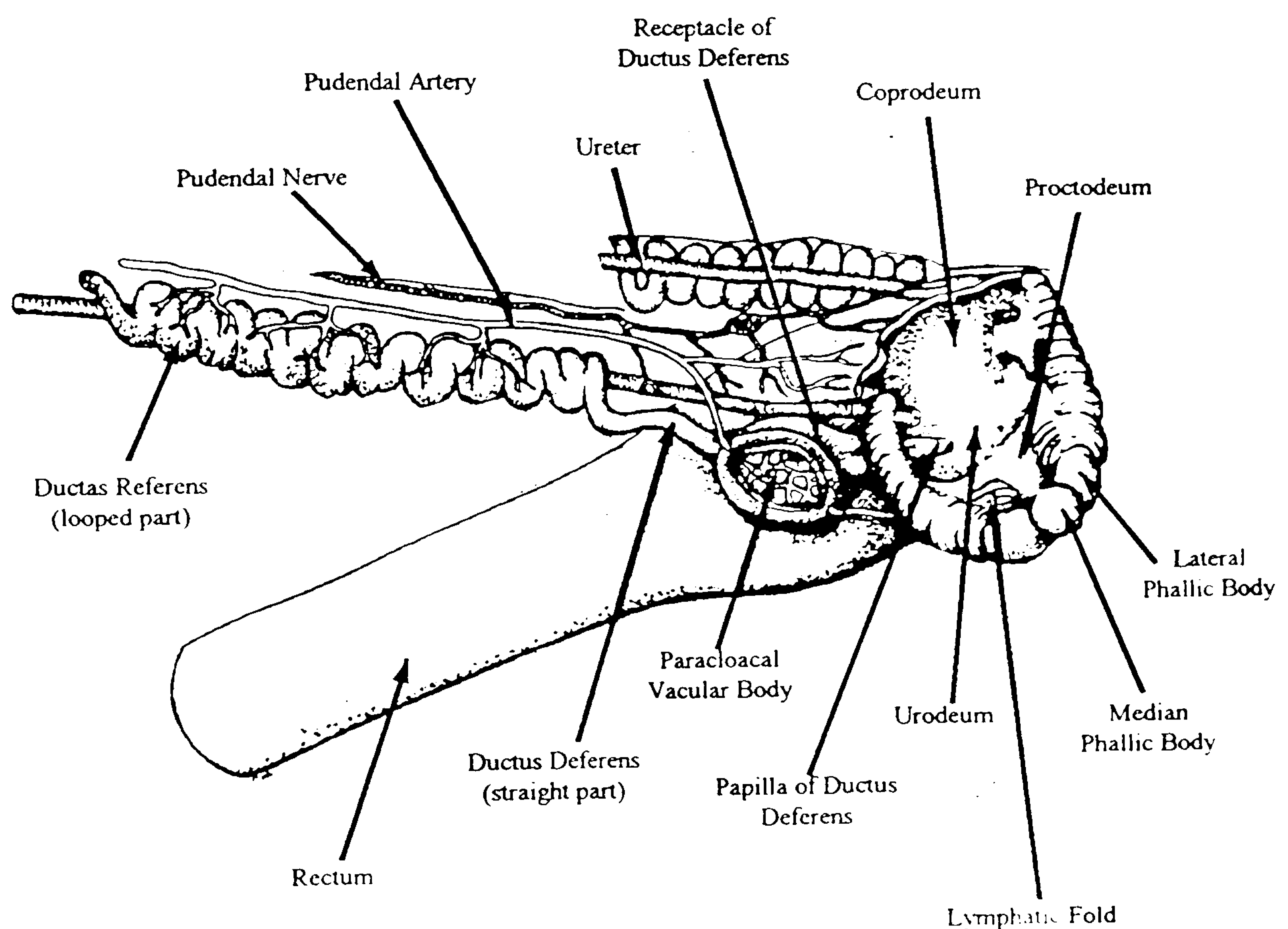


Figure 2.2a. Pelvic genitalia of the bull (dorsal view) (see Ashdown and Hafez, 1993a).

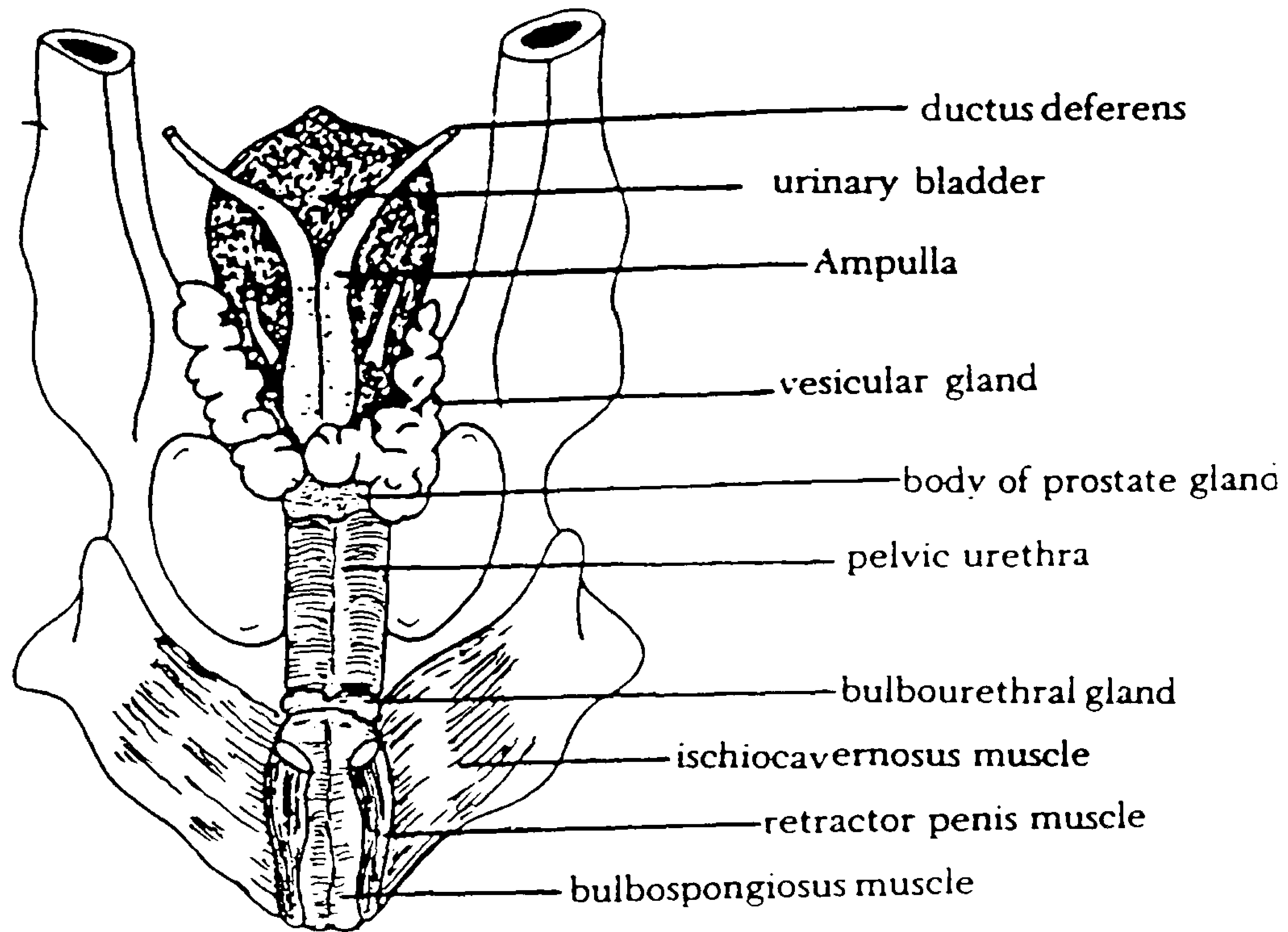
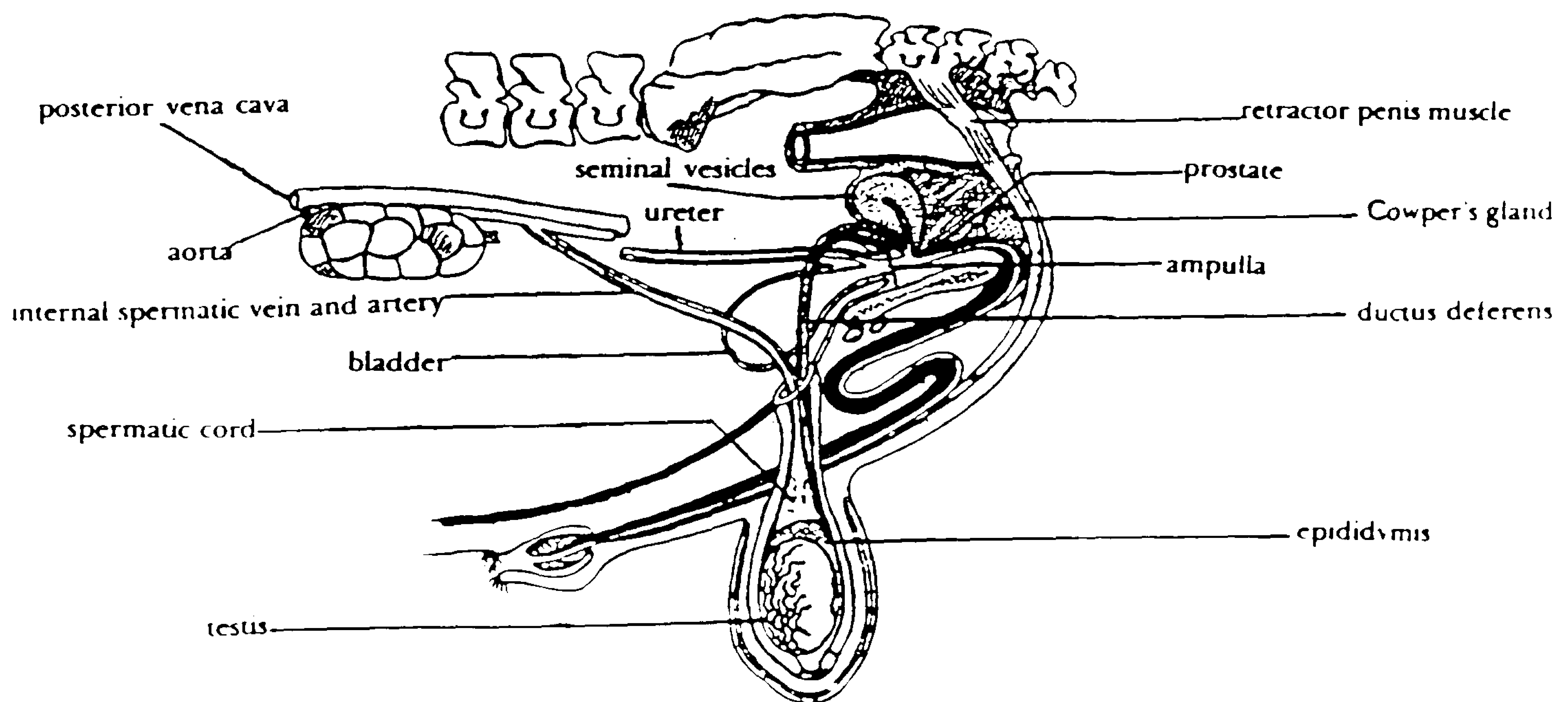


Figure 2.2b. General representation of the reproductive organs of the bull (lateral view) (see Setchell, 1982)



'able 2.2. A comparative review of testicular and spermatogenic features of the mammalian and avian species.

Feature	Mammal	Avian	Citation
'esticular	ovoid	bean-shaped	Lake (1971) ; Hafez (1993)
'estes composition	testes are encapsulated by a triple coating of dense fibrous connective tissue	testes are very soft due to lack of connective tissue.	Lake (1971)
ınatomical location of estes	testes are situated in external scrotal sac	testes are positioned in anterior abdomen suspended from the wall adjacent to anterior ends of the kidneys, just posterior to the lungs	Lake (1971)
Testes blood supply	testes possess a pampiniform plexus of blood vessels to maintain testicular temperature 6 - 8°C below that of the body in bull and 2 °C below in man	plexus is absent. It is believed that post-abdominal air sacs may keep the temperature 3 to 4 °C lower than the body	Lake (1971) ; Setchell (1982) ; Ashdown and Hafez (1993) ; Barratt (1995)
Seminiferous tubules	resemblance is similar, however anastomoses are much more numerous in the avian. The tubules are not grouped into evident lobules bounded by fibrous connective tissues like mammals		Lake (1971) ; King and McLelland (1984a)
Ductus epididymis	this region is very short in the avian compared to the mammal. The fowl also lacks extensive subdivided coiled ductus epididymis		Lake (1971)
Ductus deferens	avian lacks the thick muscular coat of the mammal where it is the main storage organ for spermatozoa		Lake (1971)
Testosterone	most important testicular androgen in both species with both possessing the two pathways of synthesis		Lake and Furr (1971) ; Bahr and Johnson (1991)
Spermatogenesis	spermatozoa appear 29 to 30 days in the ram and 60 days in the bull from the start of multiplication of primary spermatocytes	spermatozoa appear 13 to 15 days after the start of multiplication of the primary spermatocytes	Lake (1984)

able 2.2. (continued). **A comparative review of testicular and spermatogenic features of the mammalian and avian species.**

Feature	Mammal	Avian	Authors
permatozoa maturation	spermatozoa are not mature when released from the seminiferous tubules. Each contains a cytoplasmic droplet on the mid-piece and must undergo maturational changes to ensure changes for egg fertilisation	spermatozoa produced more rapidly and in larger number with quicker maturation. No capacitation, also lack a cytoplasmic droplet with only a spermatid droplet present at the posterior end of head. Spermatozoa are motile and fertile soon after leaving the testes	Lake (1971) ; Setchell (1982) ; Lake (1984)
permatozoa speed ım/sec)	bull - 120 ; stallion - 90 ; man - 35 to 50	rooster - 17	Nelson (1975)
Mitochondrial number	80 to 85 in the bull	approximately 30	Lake (1971)
Semen ejaculation	spermatozoa appear in the semen at 42 weeks in the bull, 18 in the ram, 22 in the boar and 64 to 96 in the stallion. Sexual maturity is considered to be 150 weeks in the bull, 24 in the ram, 30 in the boar and 90 to 150 in the stallion	spermatozoa are observed at 20 weeks of age with sexual maturity considered to be between 30 to 35 weeks of age.	Lake (1984) ; Hocking (1990) ; Ashdown and Hafez (1993a, b)
Storage in female tract	spermatozoa are stored in cervical crypts of mucosal folds with vaginal insemination in the bovine and ovine and uterine insemination in equine and porcine species	spermatozoa stored in spermatozoa storage tubules near utero-vaginal junction	Lake (1971) ; Pringel (1990) ; Hafez (1993)
Barriers in female tract	spermatozoa face pH, cervical and ovum barriers requiring female oestrus for AI to give watery mucous aided by female oestrogens	spermatozoa can fertilise 10 to 15 minutes after insemination but also face oviduct and secretion obstacles of female tract	Harper (1982) ; Hafez (1993) ; Bakst *et al.* (1994)
Lifespan of spermatozoa in female tract	Bovine - 90 hours Ovine - 72 hours Equine - 98 hours Porcine - 50 hours Human - 48 to 72 hours	Fowl 2 weeks Turkey 8 weeks	Harper (1982)

2.2.2 Accessory glands and secretions

Accessory glands in the mammal produce chemically different secretions which together comprise the seminal plasma. A bolus of epididymal fluid is ejected along the urethra resulting in a "sperm rich" fraction mixed with accessory gland secretions. The mammal possesses the following accessory glands (see Setchell, 1991);

i) vas deferens
ii) ampulla
iii) prostate
iv) seminal vesicle
v) bulbo-urethral or cowper's gland
vi) urethral or Littres gland

The avian species have no accessory glands resembling the prostate, seminal vesicle, bulbo-urethral or urethral glands of the mammalian species (Lake, 1971 ; Fujihara, 1992). The length of the tract between the epididymis and the claoca is taken up by the deferent duct, which is often convoluted with its terminal position distended into the so called "seminal vesicle" and which acts as a storage space, from here the semen is discharged into the phallus through the ejaculatory duct. Lymph-like fluid, used for phallic erection and arising from tumescent lymph folds, which lie on the floor of the proctodeal compartment of the cloaca, give the avian spermatozoa a fluid, plasma-like medium (Lake, 1971 ; King and McLelland, 1984a ; Blesbois, 1990). An anatomical representation for each species is given in Figures 2.3 (avian) and 2.4 (bull)

Figure 2.3. Diagram of the testis and epididymis of the domestic fowl (see King and McLelland, 1984a).

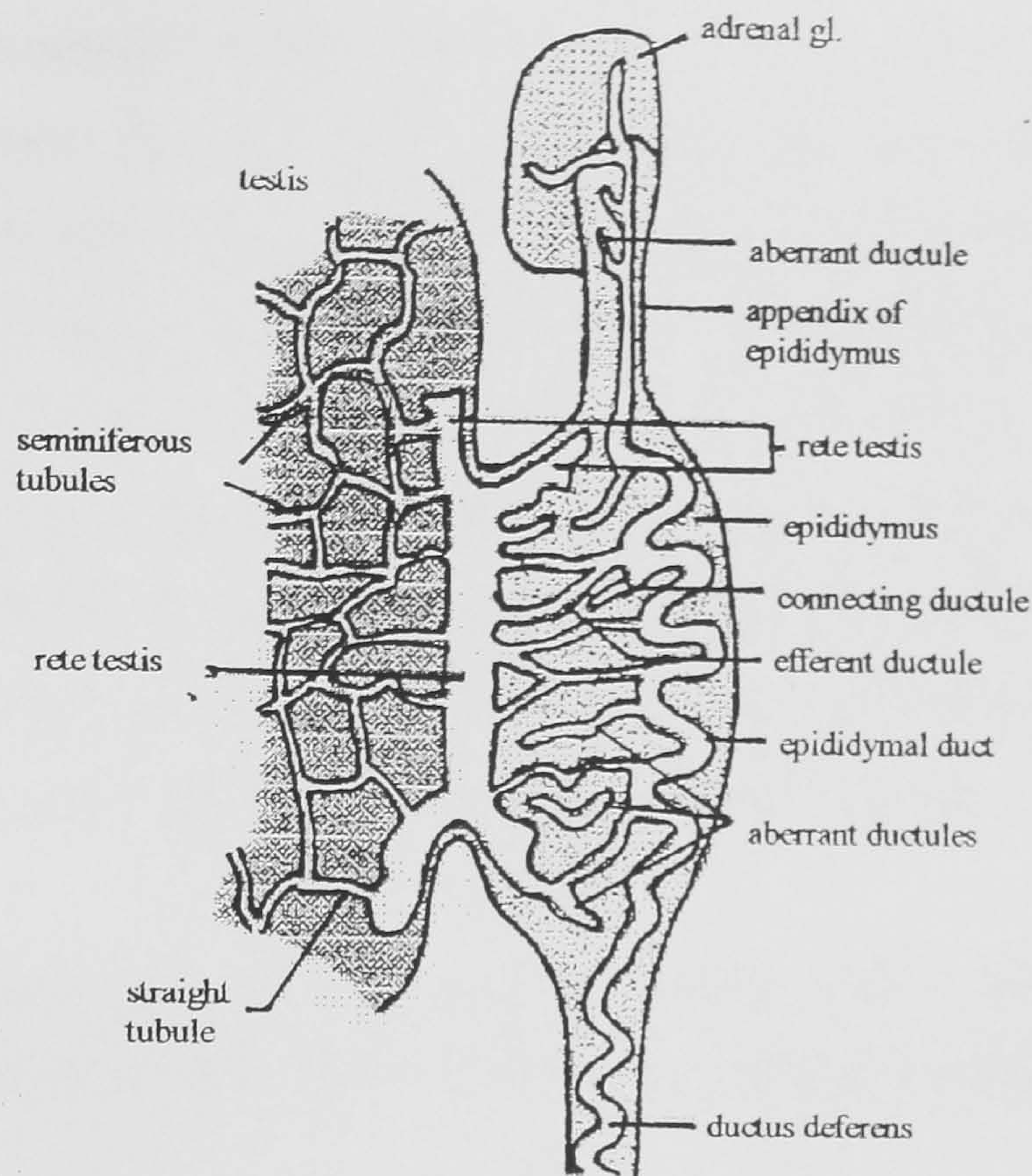
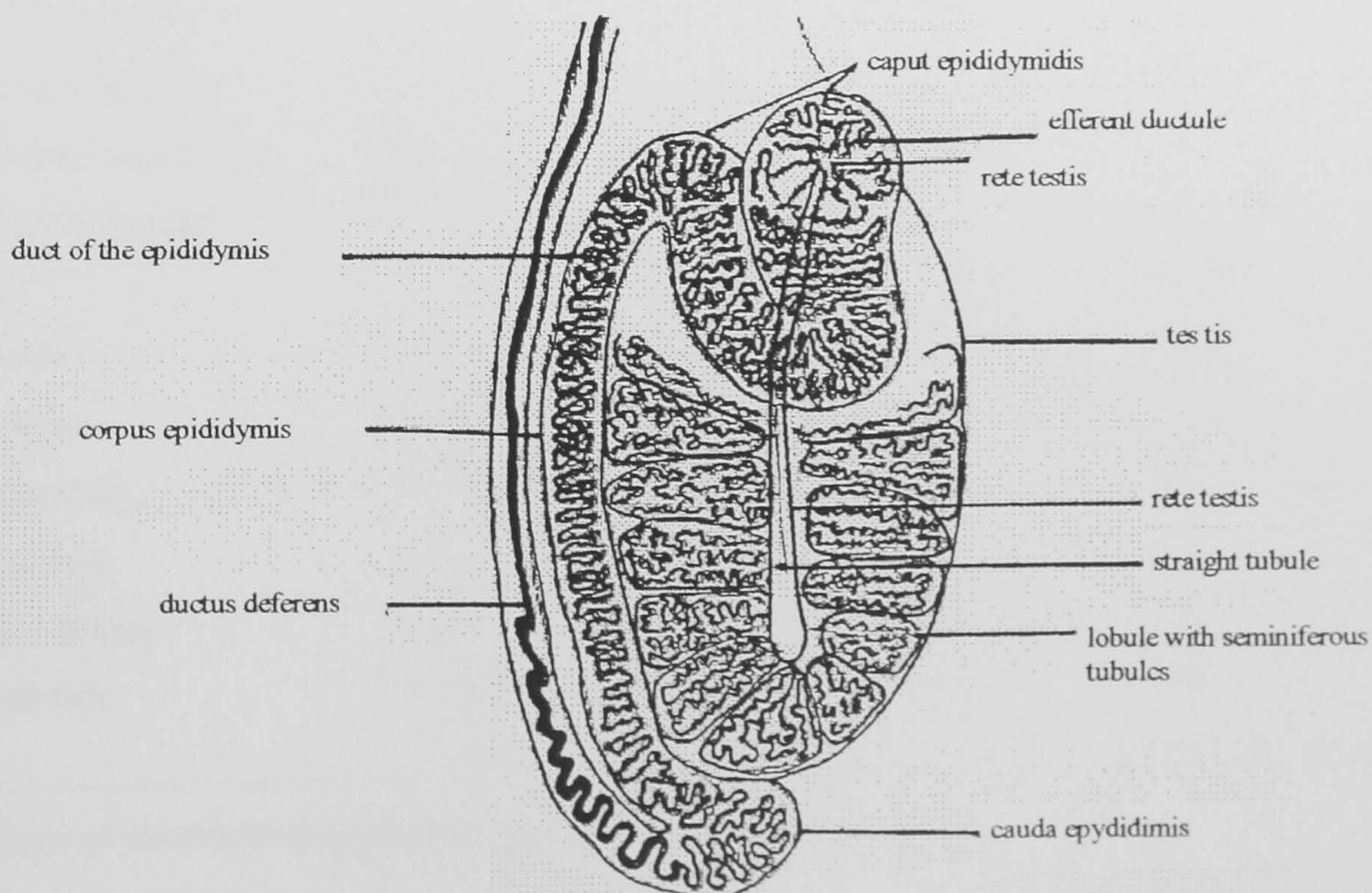


Figure 2.4. Schematic drawing of the tubular systems of the testis and epididymis of the bull (see Ashdown and Hafez, 1993a).



2.2.3 Semen characteristics

Semen varies considerably between species as discussed by Melrose and Laing, (1970). Bull semen has been described as an opaque homogenous creamy white fluid, the spermatozoa of the ram has been reported in a similar manner although it is normally denser and more opaque than the bull. Semen of the stallion has been described as greyish-white and variably opaque with boar semen exhibiting a similar greyish-white appearance but being more viscid possessing a variable quantity of "tapioca-like" material secreted mainly by the bulbo-urethral glands. The spermatozoa concentration and other important semen parameters also vary considerably between species. Differences between the major animal species are detailed in Table 2.3

Table 2.3. Important semen characteristics for major farm animal species. Adapted from Garner (1991), Hafez (1993) and Ashdown and Hafez (1993b).

Measurement	Species				
	Bovine	Ovine	Porcine	Equine	Avian
Volume/ml	5	1	225	60	0.5
Conc.10 ^{9}/ml	1.1	3.0	0.2	0.15	3.5
Total sperm 10 ^{9}	5.5	3.0	45	9	1.8
Motile sperm %	65 - 70	75	60	70	85
Normal sperm %	80 - 89	90	60	70	90
Ejaculates/week	4	20	3	3	3
pH	6.4 - 7.8	5.9 - 7.3	7.3 - 7.8	7.2 - 7.8	7.2 - 7.6
Fructose	460 - 600	250	9	2	4
Sodium	225 ± 13	78 ± 11	587	257	352
Potassium	155 ± 6	89 ± 4	197	103	61
Calcium	40 ± 2	6 ± 2	6	26	10
Magnesium	8 ± 0.3	6 ± 0.8	5 - 14	9	14
Chloride	174 - 320	86	260 - 430	448	147

Value of chemical components : mg/100 ml ± SE.

2.3 SPERMATOZOA

2.3.1 Spermatozoa morphology

The intricate morphology of the mammalian spermatozoa is illustrated in Figure 2.5a. The immediate structure of the spermatozoa appears to be rather simplistic being composed largely of a head, neck and tail. However, the internal organisation within this simple structure is far more complex. The spermatozoa head in mammals is typically about 5µm long, 2.5 µm wide and 1.5 µm thick followed by a tail or flagellum, both enclosed by a plasma membrane. The spermatozoon length is approximately 50 µm in humans, rabbits and domestic mammals, increasing to 150-250 µm in small rodent species. The longest spermatozoa belong to the tiny marsupial *Tarsipes rostratus* (Setchell, 1982 ; Garner and Hafez,1993). A general species comparison of spermatozoon morphology is shown in Figure 2.6 ; spermatozoa possessing the ability to internally fertilise the female being far more complex. Fossil relics of early spermatozoa are lacking and therefore the evolution of spermatozoa is based on current morphological data and are related to the taxonomic order of the species. A review of evolution of spermatozoa by Austin (1995) describes a large variation in spermatozoon morphology within the animal kingdom.

Figure 2.5. The internal structure of a typical spermatozoon with the plasma membrane removed (see Setchell, 1991)

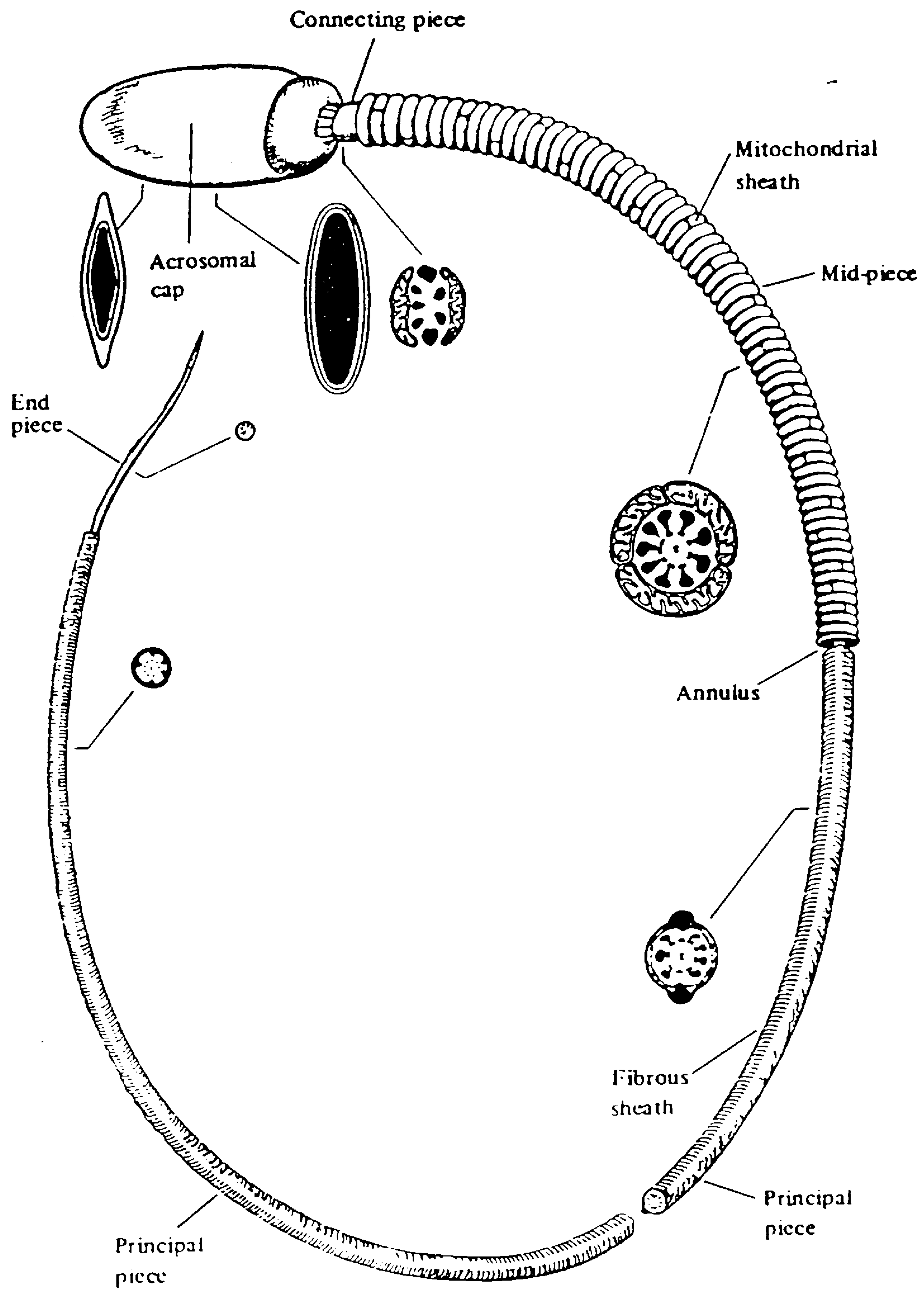
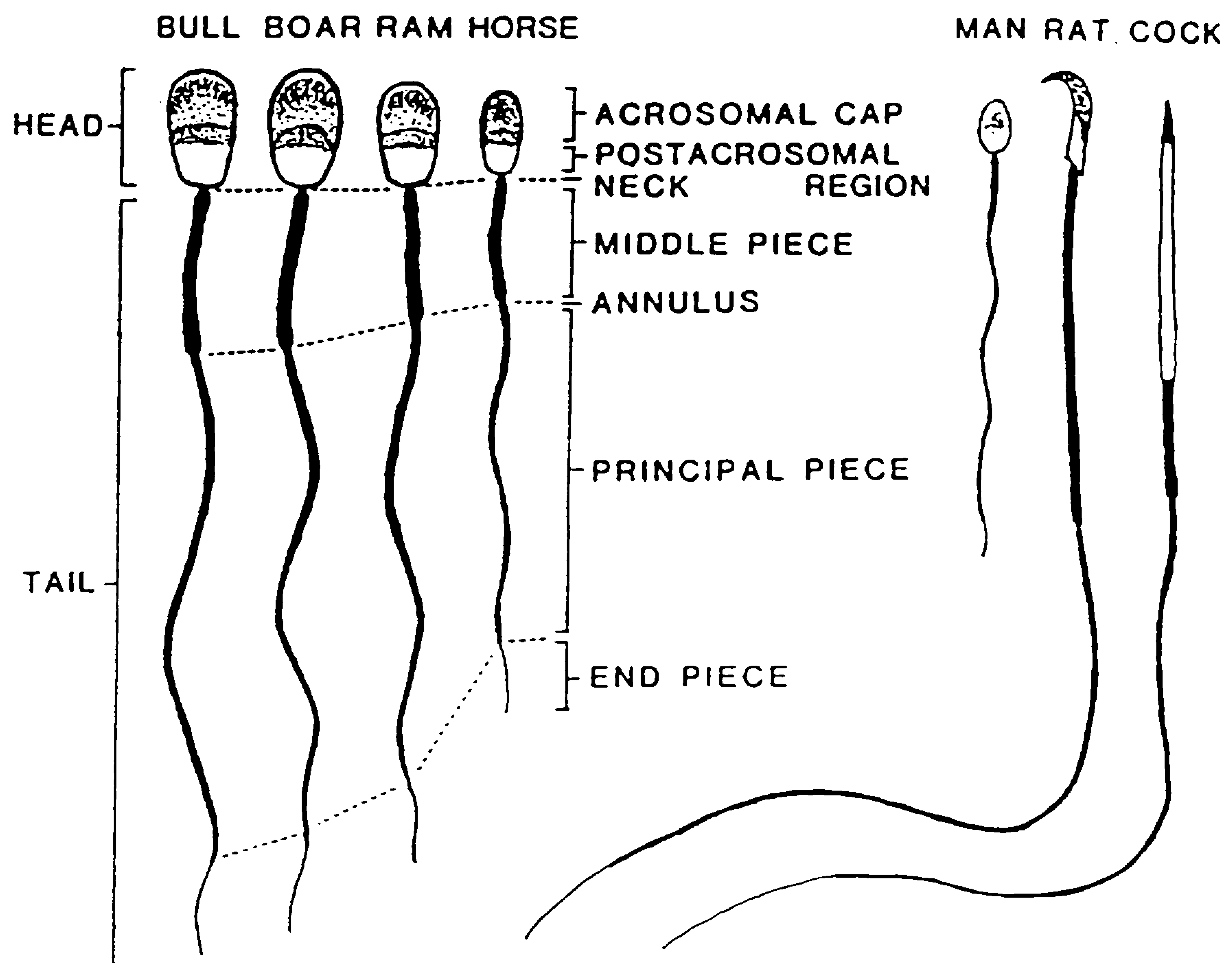


Figure 2.6. Comparison of spermatozoa of domestic animals and other vertebrates (see Hafez, 1993)



2.3.2 *The spermatozoon head*

The functions of the spermatozoa head are to contain and conserve cellular DNA and to deliver it at the time of fertilisation during fusion with the female gamete, namely the egg or ova. The haploid set of chromosomes present within the spermatozoa DNA are either X or Y in the mammalian male and ZZ in the avian male ; therefore in contrast to mammalian species avian sex determination is governed by the chromosomes of the female (Bloom, 1974 ; Shields, 1987). The sperm head region is covered by the acrosome, or "terminal body", an inverted sac of membrane of specific lipid and protein composition (Austin, 1995). The acrosome, believed to be a modified lysosome, contains hydrolytic enzymes necessary for penetration of the egg at fertilisation. Such enzyme systems include the following ; hyaluronidase, which degrades the matrix of the cumulus oophorus, phospholipases ; acrosin, a protease which disperses acrosomal contents and a selection of proteases, esterases and acid hydrolases (Mann, 1975 ; Bazer *et al.*, 1993). Fusion of male and female gametes occurs over the membrane covering the principal and apical segments of the spermatozoon head (Cummins, 1995). The spermatozoa acrosomal structure is detailed in a selection of reviews (Phillips, 1975, '77; Curry and Watson, 1995). The caudal area of the spermatozoon head is indented to allow for the attachment of the mid-piece and tail.

2.3.3 The spermatozoon tail

The tail or flagellar region of the spermatozoon is concerned with motility, containing both the site of energy production and propulsive apparatus for movement, with characteristics common to both mammalian and avian species (reviewed by Burkman, 1995 and Curry and Watson, 1995). The spermatozoa tail includes the neck region where it is attached to the implantation fossa of the head. The mid-piece of the tail contains a helix of mitochondria which generate energy for spermatozoa motility (Curry and Watson, 1995) ; their position on the plasma membrane of the mid-piece is such as to admit the passage of substrates to the mitochondrial sheath (Curry and Watson, 1995). The number of mitochondria present are related to specific animal species (see Table 2.2 and Figure 2.7a). Several findings suggest that spermatozoa mitochondria

have typical cell metabolic functions (Bellve and O' Brien, 1983 ; Eddy and O'Brien, 1994). The mid-piece of the tail terminates at the annulus and this is followed by the principal piece, the longest part of the spermatozoon which contains the axonema and outer dense fibres. At the principal piece a fibrous sheath replaces the mitochondrial helix of the mid-piece. The final terminal piece of the tail consists only of the axonema covered by the plasma membrane. Each region of the tail possesses a distinct anatomy related directly to its specific function (Fawcett, 1962 ; Gray, 1962 ; Curry and Watson, 1995).

2.3.4 Spermatozoon tail function

The axoneme which stretches the full length of the flagellum constitutes the major motor apparatus of the tail (Figure 2.5b). The movements of the microtubules of one half of the axoneme are in synchrony, but are out of phase with those of the microtubules in the other half, with the result that the sliding motion is converted to bending to provide movement (Curry and Watson, 1995). The flagellar protein which contributes most to the conversion of chemical energy to mechanical movement is called dynein which is located in the microtubules (Figure 2.7b)

The importance of tail motility has been the subject of many reviews (Fawcett, 1962 ; Gray, 1962). The acquisition of motility involving both maturational changes and a triggering event at ejaculation, (Jones, 1978 ; Gray, 1962). A range of both chemical and environmental conditions exert a considerable effect on spermatozoa motion. Thus Wishart and Ashizawa (1987), working on fowl spermatozoa, observed that spermatozoa became immotile when the temperature was raised from 30 to 40 °C and that motility was restored at 40 °C on the addition of caffeine or calcium in a dose dependent manner. The effect of caffeine was mediated by an increase in cellular cAMP levels. Simpson and White (1987) have also described the interrelationships between sperm tail motility, cAMP, respiration and calcium uptake in the ram and boar.

Figure 2.7a. The mid-piece of the spermatozoon with cell plasma membranes removed (see Setchell, 1982)

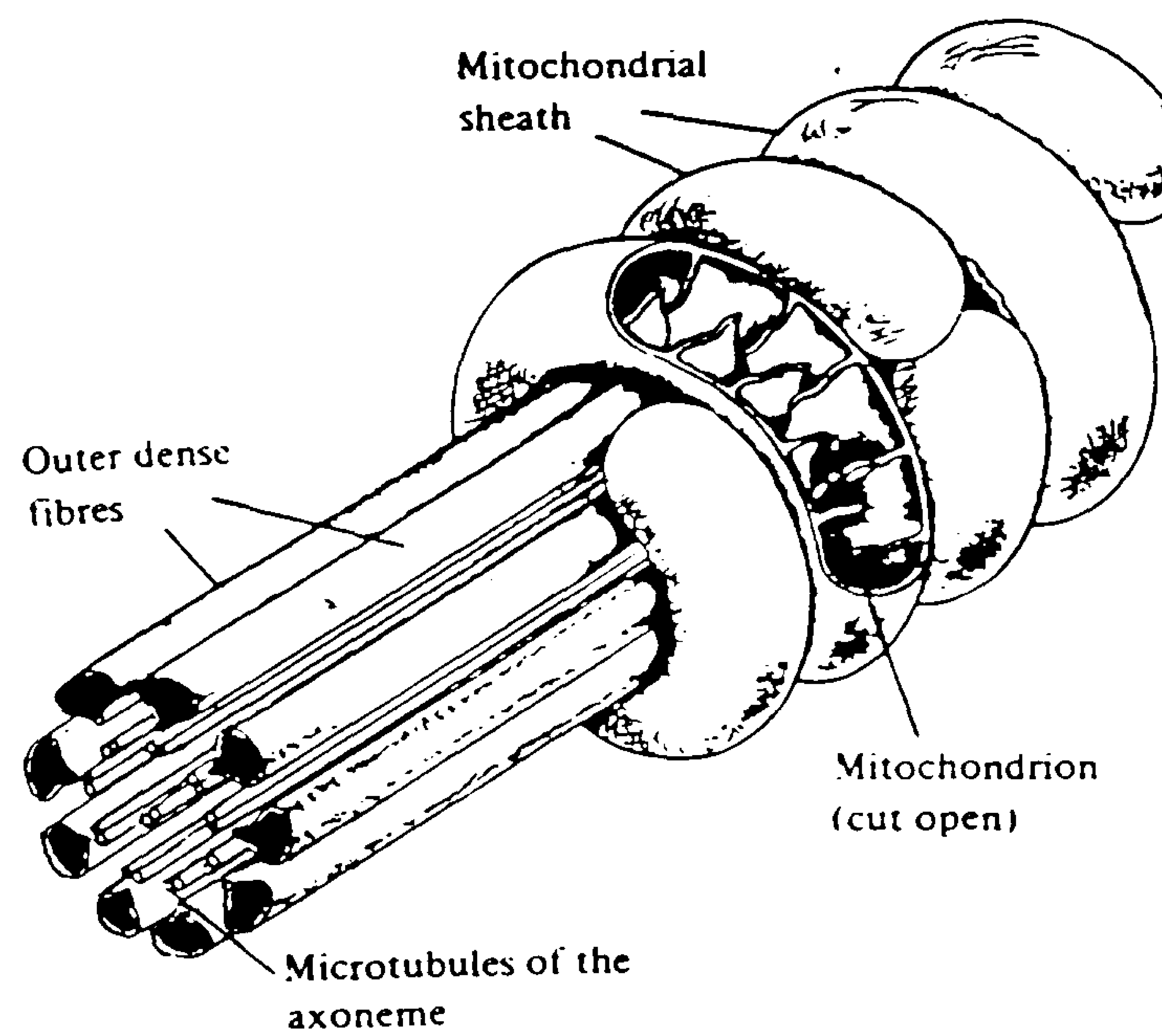
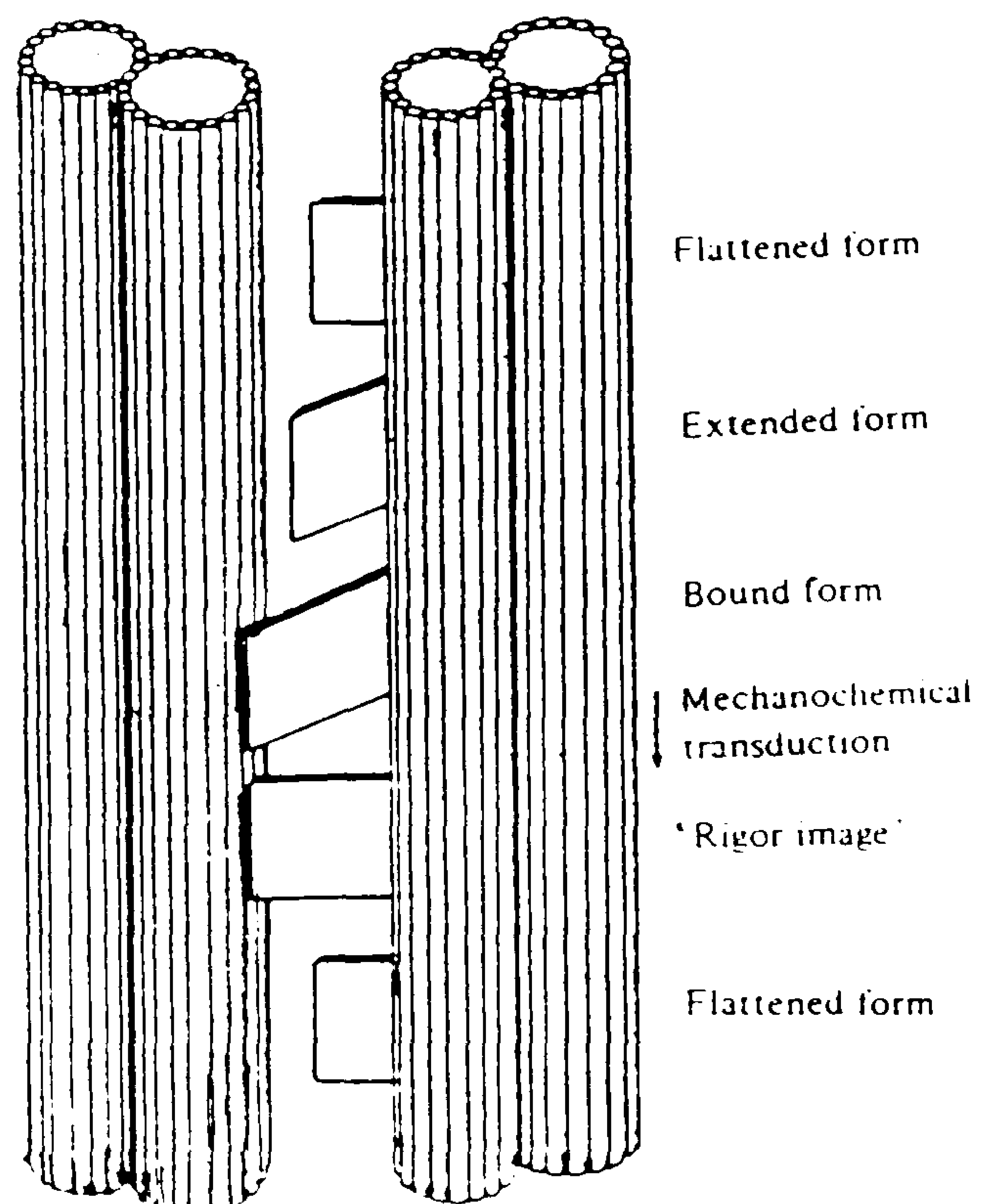


Figure 2.7b. Changes executed by dynein arms in association with movement of microfilaments (see Setchell, 1982)



2.4 SPERMATOGENESIS

The process of spermatogenesis has most recently been reviewed by Johnson (1991) and Barratt (1995). Spermatogenesis begins before birth when primordial germ cells of the early developing embryo migrate into undifferentiated foetal gonads from the yolk sac. Primordial germ cells undergo several divisions in the gonads to produce gonocytes within the foetal male sex cords. Gonocytes persist until puberty when differentiation to spermatogonia occurs and the first spermatozoa are released. The result of spermatogenesis is self-propelled, male genomic delivery devices which are then released in vast numbers to the tubular lumen. Spermatogenesis can be conveniently divided into 3 phases:

2.4.1 Phase 1

Spermacytogenesis (mitosis). This phase maintains the stem cell number in order to continue lineage throughout the adult life of the male animal. The number of spermatogonial divisions in various mammals, cockerels and fish have been quantified (Garner and Hafez, 1993).

2.4.2 Phase 2

Meiosis. This is the phase by which the process of genetic material is exchanged or crossed between homologous chromosomes to produce haploid spermatids, that is spermatocytes (Mann and Lutwak-Mann, 1981c). The first meiotic division produces secondary spermatozoa and the second division the spermatids.

2.4.3 Phase 3

Spermiogenesis. This phase is the morphological differentiation of spermatids to spermatozoa. The four phases discernible in spermatid differentiation are designated as golgi, cap, acrosomal and maturation phases (Mann and Lutwak-Mann, 1981c).

Upon completion of spermatogenesis the cytoplasmic extensions within the sertoli cells are broken and the spermatozoa are released ready for their journey (Johnson, 1991 ; Barratt, 1995)..

2.4.4 Spermatozoa movement and transportation within the female

Within the female reproductive tract it has been designated that spermatozoa must meet the following general criterion (Wishart and Steele, 1990 ; Mortimer, 1995) in order to be viable for fertilisation purposes.

- **i** display motility and successful transportation to and from female reservoirs.
- **ii** undergo capacitation with slow, prolonged release from sperm storage sites.
- **iii** recognise and bind to the zona pellucida of the ovum.
- **iv** undergo acrosome reaction.
- **v** bind to the oolemma and initiate gamete fusion.

A sequence of major physiological phenomena associated with spermatozoa transport in the male and female tract of the mammal has been described by Hafez (1993). A series of sperm selection processes are believed to operate but the suggestion is open to much debate. Selection can begin as early as ejaculation with a backflow of spermatozoa giving rise to immediate loss, remaining spermatozoa becoming trapped in cervical mucosal folds of the mammal or spermatozoa storage tubules of the avian. Uterine phagocytosis and the presence of leukocytes are also believed to play a role in spermatozoa loss (Aitken and West, 1990 ; Aitken *et al.*, 1992). The full extent of spermatozoa movement within the female tract for both mammalian and avian species has been extensively reviewed (Bedford and Cooper, 1978 ; Bazer *et al.*, 1993 ; Roldan, 1994 ; Bakst *et al.*, 1994).

2.4.5 Fertilisation in the female reproductive tract

As early as 1902 Theodore Boveri proposed that to achieve fertilisation gametes must somehow "activate" each other (see Roldan, 1994). Successful fertilisation is now described as the result of a series of complex molecular events that ultimately enable spermatozoa to recognise, bind and fuse with the egg (Bedford, 1982 ; Hafez, 1993). In order to achieve this goal the mammalian spermatozoa must undergo a plethora of cell surface modifications during their development from initial spermatogenesis to final fertilisation. Following spermatozoa capacitation and oocyte maturation, fusion of the male spermatozoon head and female cumulus oophorus occurs to initiate the process of gamete fusion and fertilisation (Roldan, 1994).

Activation of spermatozoa for the fertilisation process requires an early initialisation of Ca^{2+} from the extracellular space leading to a complex chain reaction resulting in the generation of diacylglycerol which in turn initiates phospholipase A_2 with the activation of fusogenic metabolites (Roldan, 1994). To avoid premature exocytosis intracellular Ca^{2+} levels of spermatozoa are known to be maintained by Ca^{2+} ATPase (Aitken, 1995). Fisher *et al.* (1995) reported that Ca^{2+} influx and sperm head exocytosis were related to the release of reactive oxygen species from the egg cumulus oophorus in response to acrosomal hyaluronidase. Oehringer *et al.* (1995) also concluded that hydrogen peroxide directly affected spermatozoa function in humans in a dose and time-dependent fashion ; low concentrations of hydrogen peroxide maintaining capacitation and high concentrations having a deleterious effects on spermatozoa function.

In addition to reactive oxygen species other important factors have been reported. Bazer *et al.* (1993) reported that specific spermatozoa receptors on the extracellular matrix zona pellucida of the egg were identified as ZP3. This zona pellucida specific receptor only reacted in response to sperm with intact acrosomes, through interaction with O-linked oligosaccharides. It was suggested that the presence of glycosyl transferase, proteinases and glycosidases on the plasma membrane of the sperm could be acting as a "lock and key mechanism", resulting in the development of pores on the sperm plasma membrane permitting acrosomal contents to escape. These sperm plasma membrane

receptors have been described as p95, sp56 or sperm surface galactosyltransferase or GalTase (Cummins, 1995). The other zona pellucida glycoprotein, namely ZP2 has been implicated in assisting the attachment of the spermatozoon and egg allowing spermatozoon penetration to the next phase of the perivitelline space (Aitken, 1995).

Complex events associated with spermatozoa exocytosis and spermatozoon-egg interaction are still poorly understood. Indeed it is generally agreed that there is a need for close synchrony between the cumulus penetration, the approach to the zona pellucida and ultimate completion of the acrosome reaction (Cummins, 1995).

2.5 LIPIDS

2.5.1 General overview

The general definition of a lipid is described as a biological material soluble in organic solvents such as chloroform, diethyl ether, hexane, benzene etc., however Gunstone and Herslof (1992) point out that there is as yet no exact succinct and agreed definition. A definition by Christie (1989) encompasses fatty acids, their derivatives and substances related biosynthetically or functionally to these compounds. Lipids can be divided into two major groups namely simple and complex lipids depending on the types of products produced upon hydrolysis.

2.5.2 Simple lipids

Simple lipids are those which yield one or two hydrolysis products per molecule namely, tri-, di- and monoglycerides, free and esterified cholesterol and free fatty acids (Noble, 1987 ; Christie, 1989).

2.5.3 Complex lipids

Complex lipids are dominated by phospholipids. The major classes are reviewed by Hanahan (1960) and Yeagle (1987). Phospholipids possess a phosphoric acid derivative which condenses with one of the -OH groups of the molecule instead of a fatty acid. The phosphate group may join to a variety of alcohols to produce the following very important constituents of animal membranes which include phosphatidyl inositol, (PI) , phosphatidyl serine, (PS), phosphatidyl choline, (PC), phosphatidyl ethanolamine, (PE) and sphingomyelin (Sph). Phosphatidyl choline is also commonly known as lecithin and phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol as cephalins.

Phospholipids contain high levels of polyunsaturated fatty acids with phosphatidyl ethanolamine, phosphatidyl choline and phosphatidyl serine functioning as important constituents of cell membranes (McDonald *et al.*, 1988). Phospholipids have also been implicated in creating the charge on the plasma membrane with sphingomyelin, phosphatidyl choline and phosphatidyl ethanolamine being neutral and phosphatidyl inositol, phosphatidyl serine and cardiolipin possessing a net negative charge. This poses significant differences in charge between the monolayers of the plasma membrane (Alberts *et al.*, 1983 ; Yeagle, 1987). The phospholipid molecular structures of spermatozoa are illustrated by Mann and Lutwak-Mann, (1981c).

2.5.4 Steroids

Steroids include cholesterol, derived sterols, bile acids, adrenaline and sex hormones. These are essential components of all eukaryotic cells but also act as precursors for hormones and bile acids. Cholesterol is the most important sterol of animal cell membranes (Christie, 1982).

2.5.5 *Fatty acids*

Free fatty acids are the hydrolytic products of both the simple and complex lipids with each lipid species exhibiting a characteristic fatty acid composition. The fatty acids present can be saturated or unsaturated and fatty acids can be subdivided into several families in accordance with the position of their double bonds. Non-essential fatty acids, that is those which can be biosynthesised by eukaryotes, include saturated fatty acids and acids of the n-9 and n-7 series. This designation is used to indicate the location of the carbon to carbon double bond from the methyl end of the molecule. Thus for an n-9 acid the double bond is situated between the 9th and 10th carbon atom. A short hand presentation based on this scheme is used to provide a description of all fatty acids. In this abbreviated formula the chain length is given followed by a colon which in turn is followed by the number of double bonds in the molecule. The position of the terminal double bond is often denoted by 'n'. A list of common and abbreviations for a selection of fatty acids are shown in Table 2.4.

Table 2.4. Nomenclature of major long-chain fatty acids.

Common name	Abbreviation
Palmitic acid	16:0
Palmitoleic acid	16:1 (n-7)
Stearic acid	18:0
Oleic acid	18:1 (n-9)
Vaccenic acid	18:1 (n-7)
Elaidic acid	*t*-18:1 (n-9)
Linoleic acid	18:2 (n-6)
Linoelaidic acid	*t*, *t*-18:2 (n-6)
Alpha-linolenic acid	18:3 (n-3)
Beta-linolenic acid	18:3 (n-6)
Stearidonic acid	18:4 (n-3)
Arachidic acid	20:0
Gondoic acid	20:1 (n-9)
Dihomo-linolenic acid	20:3 (n-6)
Mead acid	20:3 (n-9)
Arachidonic acid	20:4 (n-6)
Timnodonic acid	20:5 (n-3)
Behenic acid	22:0
Erucic acid	22:1 (n-9)
Adrenic acid	22:4 (n-6)
Docosapentaenoic acid	22:5 (n-6)
Docosapentaenoic acid	22:5 (n-3)
Clupanodonic acid	22:6 (n-3)

2.5.6 Fatty acid desaturation and chain elongation in animal tissues

All fatty acid series, n-9, n-7, n-6 and n-3 are non-interchangeable, that is only fatty acids of the n-3 series can be used for biosynthesis of longer chain fatty acids such as 18:3 (n-

3) to 22:6 (n-3) (Figure 2.8). The separate biosynthetic pathways for the 3 families of fatty acids share and compete for the same enzymes. Thus delta 6-desaturase can use either 18:1 (n-9), 18:2 (n-6) or 18:3 (n-3). The conversion of 18:1 (n-9) through to 20:3 (n-9) is usually only favoured when 18:2 (n-6) and/or 18:3 (n-3) are deficient. Likewise the synthesis of 22:4 (n-6) and 22:5 (n-6) often only occurs when n-3 fatty acids are deficient. Reviews of fatty acid biosynthesis have been produced (Brenner, 1989 ; Cook, 1991 ; Aveldano, 1992).

Figure 2.8. Major pathways of fatty acid biosynthesis by desaturation and chain elongation in animal tissues.

Enzymatic step	Oleate (n-9)	Linoleate (n-6)	Linolenate (n-3)
	18:1n-9	18:2n-6	18:3n-3
Δ6 Desaturation	↓	↓	↓
	18:2n-9	18:3n-6	18:4n-3
Elongation	↓	↓	↓
	20:2n-9	20:3n-6	20:4n-3
Δ5 Desaturation	↓	↓	↓
	20:3n-9	20:4n-6	20:5n-3
Elongation	↓	↓	↓
	22:3n-9	22:4n-6	22:5n-3
Δ4 Desaturation	↓	↓	↓
	22:4n-9	22:5n-6	22:6n-3
Elongation	↓	↓	↓
	24:4n-9	24:5n-6	24:6n-3
	(22:3n-9 →) 24:3n-9	(22:4n-6 →) 24:4n-6	(22:5n-3 →) 24:5n-3

2.5.7 General characteristics of cell membranes

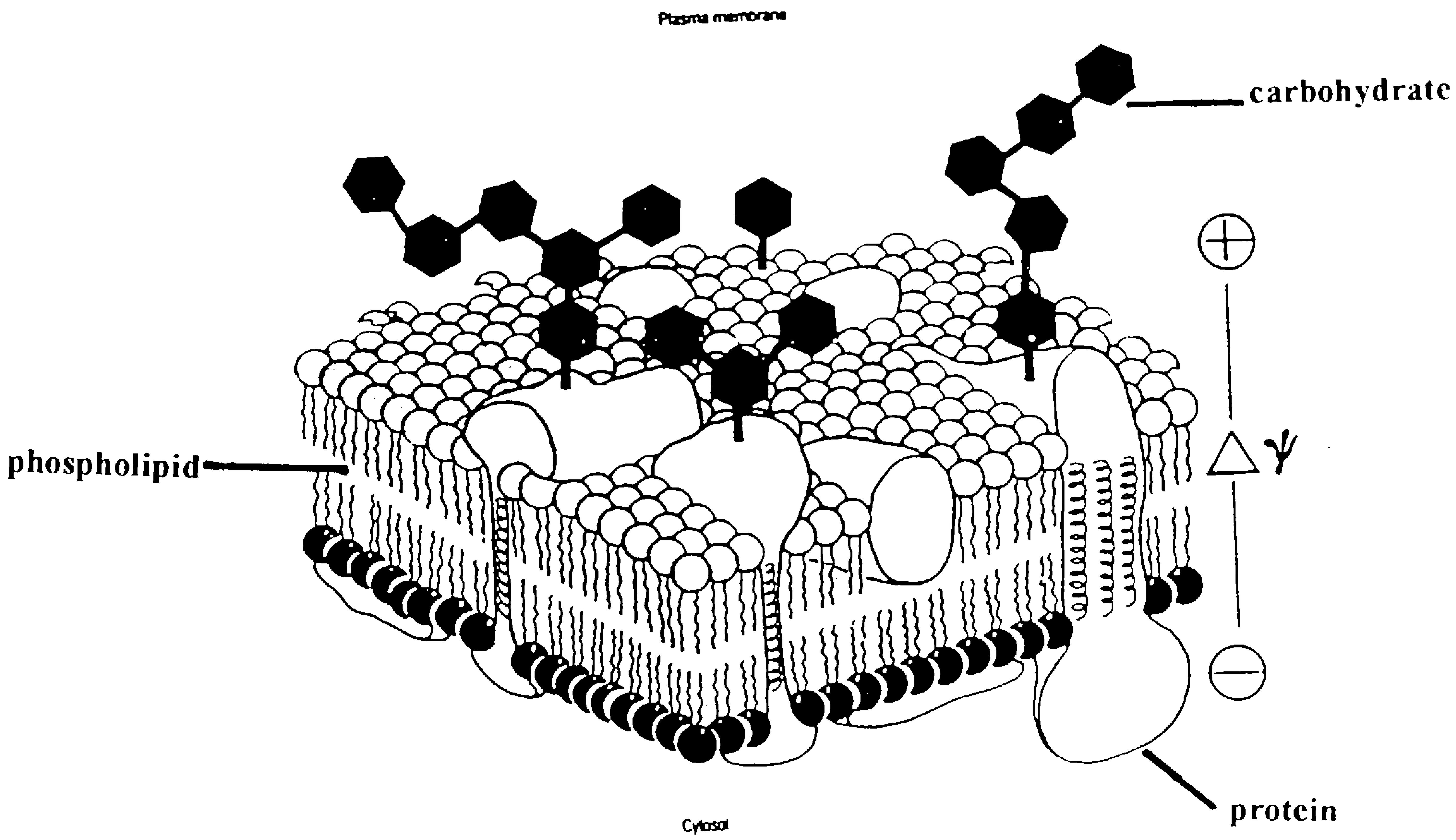
Cell membranes have become adapted during evolution with the result that there is no one standard membrane structure (Lockwood and Lee, 1978 ; Parks and Graham, 1992). Biological membranes vary widely in their general and intimate lipid composition to give them a high degree of specialisation and individuality.

Investigations into the major role of membrane lipids go back many years. As a result of work on the erythrocyte membrane Gorter and Grendell (1925) recorded sufficient

membrane lipid to provide a bilayer matrix for the cell. Subsequent observations led to the classic Singer and Nicholson (1972), fluid mosaic model as illustrated in Figure 2.10. It was concluded that the high electrical resistance of biological membranes and their impermeability to certain compounds was best explained by a central hydrophobic core of lipid arranged as a bilayer structure. The basic organisation is governed by the amphipathic characteristic of membrane lipids, illustrated by their hydrophilic ("water loving") head region and hydrophobic ("water hating") tail region (Alberts *et al.*, 1983 ; Gennis, 1989).

Within the bilayer naturally occurring unsaturated fatty acids containing double bonds can exist in two geometric forms, namely *cis* or *trans* configurations. The *cis* configuration is very important as it introduces a "kink" into the fatty acid sidechain encouraging a more open, fluid arrangement on the lipid aggregate. Bilayer lipids may also take one of two conformations ; firstly the beta or gel conformation (ordered or crystalline) where the hydrocarbons are stiff, their parallelism providing little mobility and secondly the alpha-conformation (random or liquid crystalline) which is highly mobile and easily altered by environmental factors. Phospholipids and cholesterol containing specific fatty acids as well as their relative proportions within a membrane are very important for membrane structure and function as well as contributing to characteristics of fluidity which is particularly important in terms of spermatozoa motility and function (Elias *et al.*, 1979 ; Bearer and Friend, 1982 ; Cossins, 1983 ; Holt and North, 1986 ; McDonald *et al.*, 1988 ; Parks and Graham, 1992). In addition, protein molecules are "dissolved" in the lipid bilayer and mediate the various functions of the membrane, for example ; transportation of specific molecules into or out of the cells, acting as enzymes to catalyse membrane associated reactions, acting as structural links between the cell cytoskeleton and the extracellular matrix and as receptors for receiving and transducing chemical signals from the cells internal environment (Alberts *et al.*, 1983 ; Cullis and Hope, 1991).

Figure 2.9 . Topography of membrane protein, lipid and carbohydrate in the fluid mosaic model of a typical eukaryotic plasma membrane (see Cullis and Hope, 1991).



2.6 LIPIDS AND SPERMATOZOA FUNCTION

There are three possible roles that lipids can play in the correct function of spermatozoa. These include ;

i membrane properties of the spermatozoa

ii energy source for spermatozoa metabolism

iii acrosome reaction/fertilising ability

Each of these lipid functions will be looked at in detail in the following paragraphs.

i) MEMBRANE PROPERTIES OF SPERMATOZOA

2.6.1 Sperm plasma membrane

As with all cells the sperm plasma membrane serves as a continuous limiting cell boundary, maintaining cell integrity and forming a dynamic interface between the cell and its immediate environment. Each spermatozoon is enveloped by a highly heterogeneous plasma membrane or plasmalemma which exhibits distinct functional regions, for example the plasma membrane covering the anterior head region participates in the acrosome reaction whilst the post acrosomal surface is considered to be responsible for the initial fusion of male and female gametes. Spermatozoa membrane lipids may also be localised in discrete domains (Elias *et al.*, 1979). Using a cholesterol probe, these workers identified complexes forming in the area of the plasma membrane overlying the acrosome of guinea pig sperm. Localised interaction of polymysin with the fusogenic membrane covering the acrosomal region and cytoplasmic droplet also suggested high local concentrations of anionic lipids (Bearer & Friend, 1982). Such lipid domains may be important in facilitating the membrane events attending capacitation and the acrosome reaction

2.6.2 Lipid composition of the sperm plasma membrane

A selection of techniques have been developed to investigate the composition of the isolated sperm plasma membrane (Ivanov and Profirov, 1981; Holt and North, 1985). Holt and North (1985), using a relatively simple technique showed the existence of specific differences between the phospholipid composition of whole ram spermatozoa and the isolated spermatozoa plasma membrane, the latter displaying a higher proportion of sphingomyelin than was present in the total spermatozoa lipid extract ; conversely, a significantly lower proportion of phosphatidyl ethanolamine was observed. Wolf *et al.* (1990) reported a very low percentage of sphingomyelin in the head region of the ram spermatozoa plasma membrane, whereas the proportion of phosphatidyl choline and cardiolipin were similar to those of whole spermatozoa.

Phospholipid and sterol in the plasma membrane of boar spermatozoa accounted for 76 and 13% respectively, a composition similar to whole spermatozoa (Nikolopoulou *et al.*, 1985). In this study phosphatidyl choline was found to be the major phospholipid followed by phosphatidyl ethanolamine. The major fatty acids detected were 16:0 and 22:5 with four fatty acids ; 16:0, 18:1, 22:5 and 22:6 comprising 90% of the total fatty acids present within the phosphatidyl choline fraction. However, the series of each fatty acid, n-3 and/or n-6, was not designated in this study. Holt and North (1985) detected a higher proportion of cholesterol to phospholipid in the spermatozoa plasma membrane of the ram compared to whole spermatozoa, contrasting with bovine spermatozoa in which the plasma membrane displayed much higher proportions of phospholipid relative to cholesterol when compared to whole spermatozoa (Parks *et al.*, 1987). In this study the phospholipid composition of the bull spermatozoa plasma membrane was only slightly different from that of whole spermatozoa ; phosphatidyl choline was the major fraction in both the membrane and whole spermatozoa with sphingomyelin and phosphatidyl ethanolamine present at much lower amounts. The percentage of sphingomyelin increased from 8.9 in whole spermatozoa to 12.6 in the plasma membrane and to 16.5 in the outer acrosomal membrane. Phosphatidyl ethanolamine levels decreased (11.8 - 9.9 - 6.8) in the corresponding membranes.

For the avian no current literature is available on the sperm plasma membrane composition, however, Ansah and Buckland (1982) concluded that high cholesterol levels in fowl spermatozoa may lead to lowered fertility in spite of cholesterol improving membrane integrity. Froman and Thurston (1984) reported the percentage of fertile eggs was greatly reduced in the fowl, when spermatozoa was treated by neuramidase and phospholipase C. The conclusions were that alteration of the carbohydrate and phospholipid moieties of the sperm plasma membrane decreased fertility without reducing spermatozoa motility.

2.6.3 Plasma membrane fluidity

At physiological temperatures most and usually all membrane lipids are fluid although most lipid species in isolation can undergo a transition from the frozen to the fluid state as temperature is increased. The temperature of this transition depends on both the head group of the phospholipid and on the fatty acids present. The longer the fatty acid chains the higher the transition temperature will be. The higher the proportion of unsaturated bonds the lower the transition temperature will be as unsaturated fatty acids, particularly in *cis* formation, make packaging of fatty acid chains more difficult. Polyunsaturated fatty acids also possess greater cross-sectional areas in the plane of the bilayer and a greater degree of molecular flexibility (taken from a review by Cossins, 1983).

Poikilotherms ("cold" blooded animals) respond to temperature changes by altering the unsaturated:saturated fatty acid ratio in favour of unsaturated fatty acids, helping to restore the physical properties of cooled membranes. This is achieved as the kinked unsaturated lipids pack less well and hence tend to increase the fluidity of the membrane (Lockwood and Lee,1978). The lipid composition of membranes can vary greatly among different cells or organelles and between different sides or monolayers of the same membrane. Membrane lipids satisfy demands related to membrane structure, fluidity, permeability as well as protein association and function. The subject is covered by a selection of reviews (Furth, 1980 ; Alberts *et al.*, 1983 ; Cullis and Hope, 1991)

Alterations in the organisation of sperm membrane lipids can be induced as a result of temperature-related phase transitions from the gel to the liquid-crystalline state and vice-versa. Such changes in the lipid phase have been recognised as having profound effects upon membrane properties including their permeability and ability to undergo membrane fusion reactions (Holt and North, 1986 ; Thaler and Cardullo, 1995 ; Palleschi and Silvestroni, 1996). It has been shown that sperm membrane ATPase activity is clearly modulated by the physical state of the lipid environment in the ram (Holt and North, 1986). It was shown in this study that there were at least three significant temperatures associated with changes in membrane conformation. Lipid-phase transitions occurred in the regions of 15-17 °C, 24-26 °C and 35-38 °C, and it was suggested that each transition point represented events in different membrane regions. The apparent phase transition at 35-38 °C was potentially very important because it was slightly above the normal testicular and epididymal temperature of 32-33 °C. It therefore appeared that ejaculated ram spermatozoa underwent a transition in at least part of the plasma membrane and that such changes in membrane lipid might promote the events which precede the acrosome reaction. When ram spermatozoa were incubated with cholesterol and liposomes containing phosphatidyl choline the phase transition temperature increased; from these results it was hypothesised that cholesterol or phosphatidyl choline were transferred from the liposomes to the membrane vesicles with the lipid environment surrounding the ATPase molecules being modified and becoming more viscous (Holt and North, 1986). Wolf *et al.* (1991), cited by Thaler and Cardullo (1995) identified phase transition temperatures in ram spermatozoa at 12 - 39 °C and 48 - 74 °C. The first transition temperature (12 - 39 °C) related to the passage of spermatozoa from the relatively low temperature environment of the cauda epididymis (30 - 33 °C) to the much warmer temperature of the female reproductive tract (37 - 39°C). It was suggested that this abrupt temperature increase upon ejaculation could possibly act as a "thermodynamic trigger" driving immotile, inactive spermatozoa into a dynamic and activated state - a requisite for fertilisation. Palleschi and Silvestroni (1996) also reported that the human sperm plasma membrane was a high rigidity structure in the temperature range 10 - 42 °C.

ii) LIPIDS AS AN ENERGY SOURCE FOR SPERMATOZOA METABOLISM

2.6.4 Use of lipids as an energy source

The difference in certain lipid metabolic features of bull and ram spermatozoa may be a reflection of the far greater amount of fructose available in bull seminal plasma per spermatozoon for energy metabolism. Indeed, on addition of fructose to whole ram semen the breakdown of phospholipids was suppressed (Scott and Dawson, 1968) suggesting that phospholipids were probably used only for energy production in spermatozoa in the absence of oxidisable soluble carbohydrate. Incubation of ram semen in the absence of any exogenous substrate, resulted in spermatozoa oxidising the long chain fatty acid resides from phosphatidyl choline which is a principal phospholipid of spermatozoa (Hartree and Mann, 1959, 1961). In the avian, Howarth (1981) reported that after incubating spermatozoa for 24 hours no substantial changes occurred in the content of total lipid, total phospholipid, neutral lipid or in the component phospholipids and it was therefore considered unlikely that any major endogenous phospholipid was utilised as an energy source for cockerel spermatozoa, such results however are conflicting as previous work showed a loss of spermatozoa lipid with *in vitro* storage (Howarth, 1978 ; Howarth, 1980). The long survival of avian spermatozoa in the hens oviduct is obscure but an involvement of exogenous lipids supplied by the oviduct is possible (Bakst *et al.*, 1994). Lardy and Phillips (1941) suggested that in the absence of carbohydrate, bull spermatozoa appeared to derive their energy from the oxidation of intracellular phospholipid, as the phospholipid content of washed bull and human spermatozoa decreases markedly on aerobic incubation (Poulos and White, 1973).

Bull, ram, dog, and cockerel spermatozoa also utilise short chain fatty acids as an energy source in the presence of oxygen from an exogenous substrate but with some differences between species (Minassian and Terner, 1966 ; Scott and Dawson, 1968 ; Poulos and White, 1973 ; Scott, 1973 ; Howarth, 1978 ; Mita *et al.*, 1995). Between the two ruminant species, ram spermatozoa showed higher oxidative activity compared with bull spermatozoa whilst only acetate stimulated oxygen uptake by bull spermatozoa (Scott *et al.*, 1962). Fowl spermatozoa utilised palmitic acid from exogenous substrates to

increase ATP production (Howarth, 1978). Ejaculated ram and bull spermatozoa had the capacity to hydrolyse exogenous phosphatidyl ethanolamine and phosphatidyl inositol whilst the hydrolysis of exogenous phosphatidyl choline was very low (Scott and Dawson, 1968). Poulos and White (1973) working with human spermatozoa, reported that a prolonged aerobic incubation in the presence or absence of glucose produced no significant alteration either in the amount of extractable phospholipid or in phospholipid composition. Mita *et al.* (1995) concluded that in sea-urchin spermatozoa glucose was present at very low levels and that incubation with seawater led to a rapid decrease in triacylglycerol levels with no other lipid changes. It was suggested therefore that such organisms obtained energy through oxidation of fatty acids from triacylglycerol stores in lipid globules within the mid-piece of the spermatozoa tail.

As a consequence of their energy metabolism, a depletion of endogenous lipid in the spermatozoa is possible. However; an ability to replenish lipid reserves by biosynthesis from external substrates has been shown (Mann, 1964b). Thus spermatozoa appear able to utilise carbohydrate not only as a source of energy for motility but also for the replenishment of their lipid reserves (Mann, 1964b), for example fish and human spermatozoa have been shown to incorporate exogenous glucose and acetate for lipid synthesis (Minassian and Terner, 1966).

iii) ACROSOME REACTION

2.6.5 Changes in sperm lipid composition during the acrosome reaction

The mammalian acrosome reaction was observed in the boar (Nikolopoulou *et al.*, 1986). This reaction involves a modification of the plasma membrane followed by vesiculation and release of the sperm plasma membrane. Comparison of released spermatozoa membranes to intact spermatozoa membranes have exhibited the following major characteristics (Nikolopoulou *et al.*, 1986).

i reduced phospholipid/protein (mg/mg)

ii sterol/phospholipid ratio remained the same

iii the major phospholipid was sphingomyelin followed by phosphatidyl ethanolamine and phosphatidyl choline, the opposite found in the membrane of normal fresh spermatozoa .

iv there were also reduced levels of polyunsaturated fatty acids than the membrane would normally exhibit in an intact state.

2.6.6 Role of lipids in capacitation and fertilisation

Before being able to fertilise the ovum spermatozoa must first undergo the sequential series of events of maturation, capacitation (in mammalian species) and the acrosome reaction (Davis, 1981 ; Grogan *et al.*, 1981 ; Bazer *et al.*, 1993 ; Moore, 1995). Spermatozoa capacitation appears to happen in the female reproductive tract and the process of capacitation is associated with lipid changes in the sperm plasma membrane. During capacitation cholesterol becomes depleted at the sperm surface with accompanying changes to the levels of glycosaminoglycans. At the end of capacitation the phospholipid bilayer of the spermatozoa membrane has been destabilised, promoting the acrosomal reaction (Gadella *et al.*, 1994, 1995). The acrosome reaction itself involves a fusion of the sperm plasma membrane with the outer acrosomal membrane followed by extensive vesiculation over the anterior segment of the acrosome (Bearer and Friend, 1982 ; Bazer *et al.*, 1993).

Davis (1981) proposed that capacitation involved a reversible lowering of the cholesterol/phospholipid ratio in the sperm membrane. The effect of cholesterol reduction was to destabilise the plasma membrane. In this study capacitation intervals were observed to range from 1.5 to 7 hours and correlate closely with changes in the cholesterol/phospholipid ratios. Cholesterol was concluded as being a physiologically significant molecular determinant of capacitation. Cholesterol and phospholipid levels in spermatozoa appeared to be inversely correlated and it was suggested that there could be a membrane-sterol barrier to the process of fertilisation, with cholesterol depletion being the molecular mechanism responsible for the capacitation transformation (Davis, 1981) Capacitation in part is believed to result in the formation of specialised areas within the plasma membrane of the acrosomal region that are devoid of intramembrane proteins and

of cholesterol but display high concentrations of anionic phospholipid thought to be cardiolipin (Bearer and Friend, 1982). Clegg (1983) also reported on the detection of cardiolipin in fusogenic areas of the plasma membrane isolated from pig spermatozoa when under appropriate conditions for the acrosome reaction.

The levels of phosphatidyl ethanolamine and phosphatidyl serine were found to increase by approximately 12 % in capacitated spermatozoa and by approximately 24 % in acrosome reacted spermatozoa (Elliott and Higgins, 1983). Boar spermatozoa with intact acrosomes were found to display an apical distribution of glycolipid, it was concluded that this was involved in stabilisation of the plasma membrane and prevention of acrosomal exocytosis (Gadella *et al.*, 1994). During capacitation, the glycolipid was observed to diffuse laterally from the apical to the equatorial segment of the spermatozoa head plasma membrane. The efflux of glycolipid implied that other membrane lipids travelled in the opposite direction. Phosphatidyl choline has been implicated as a possible candidate for the reversed flux and because it is rich in polyunsaturated fatty acids it is believed to increase membrane fluidity and therefore facilitate the acrosome reaction (Gadella *et al.* ,1994, 1995).

2.7 LIPIDS OF THE SEMEN

2.7.1 Total lipid in spermatozoa and seminal plasma

A comparison of total lipid levels reported within spermatozoa and seminal plasma from various species are shown in Table 2.5 As can be seen large differences exist between species both in absolute amounts within spermatozoa and seminal plasma and distribution between the two semen fractions.

Table 2.5. Total lipid levels in spermatozoa and seminal plasma

Species	Spermatozoa mg/10^9	Author
buffalo	1.32	Sarmah *et al.*, 1983
buffalo	1.14	Jain and Anand, 1976 a
bull	0.73	Jain and Anand, 1976 a
ram (testicular sperm)	1.91	Scott, 1973
ram (ejaculated sperm)	1.34	Scott, 1973
domestic fowl (phospholipid)	1.15	Ansah and Buckland, 1982
cockerel	0.90	Howarth, 1981
stallion	1.40	Komarek *et al.*, 1965
	Seminal plasma mg/ml	
buffalo	1.75	Sarmah *et al.*, 1983
buffalo	1.50	Jain and Anand, 1976 a
bull	2.90	Jain and Anand, 1976 a
domestic fowl (phospholipid)	0.55	Ansah and Buckland, 1982
human normospermic	2.75	Sebastian *et al.*, 1987
stallion	2.07	Komarek *et al.*, 1965

2.7.2 Lipid composition of spermatozoa and seminal plasma

The plasma membranes account for in excess of 90 % of total extractable lipid of the spermatozoa. However, distinct differences exist between the major identifiable membranes ; plasma, acrosomal, nuclear and mitochondrial and between different regions of the same membrane (Clegg, 1983). Table 2.6 lists the major sources for information on spermatozoa and semen lipid composition for a wide selection of species.

Phospholipids are the largest class of lipid and account for 59 % to 75 % of the total accompanied by substantial levels of cholesterol with triacylglycerol and diacylglycerol present only in small amounts. Human spermatozoa is the exception to this generalisation (Table 2.7). Distinct variations in the relative proportions of phospholipid and cholesterol are exhibited between species. In contrast with other species in which cholesterol predominates, in the spermatozoa of the monkey the predominant sterol is desmosterol (some 59 % of total) as opposed to cholesterol. Within the spermatozoa desmosterol is present only in the free form, while cholesterol occurs in both the free and esterified forms (Lin *et al.*, 1993).

Table 2.6. Selected references on the lipid composition of semen (adapted from Mann and Lutwak-Mann, 1981c).

Species	Authors
Boar	Komarek *et al.*, (1965) ; Grogan *et al.*, (1966) ; Ahluwalia and Holman, (1969) ; Johnson *et al.*, (1969) ; Darin-Bennett *et al.*, (1973) ; Evans and Setchell, (1978)
Buffalo	Guraya and Sidhu, (1975) ; Jain and Anand, (1976)
Bull	Dietz *et al.*, (1963) ; Komarek *et al.*, (1964) ; Pickett and Komarek, (1966) ; Masaki and Tomizuka, (1966) ; Lavon *et al.*, (1970) ; Neill and Masters, (1972) ; Pursel and Graham, (1973) ; Clegg and Foote, (1973) ; Darin-Bennett *et al.*, (1973) ; Jain and Anand, (1976)
Cockerel	Lake, (1966) ; Darin-Bennett *et al.*, (1974) ; Howarth *et al.*, (1977) ; Ravie and Lake, (1985)
Dog	Darin-Bennett *et al.*, (1974)
Camel	El-Manna *et al.*, (1986)
Goat	Masaki and Tomizuka, (1965)
Honey Bee	Blum *et al.*, (1967)
Man	Ahluwalia and Holman, (1969) ; Peter *et al.*, (1970) ; Poulos and White, (1973) ; White *et al.*, (1976) ; Darin-Bennett *et al.*, (1976) ; Jones *et al.*, (1978 & 1979)
Monkey	Darin-Bennett *et al.*, (1977)
Ram	Scott *et al.*, (1967) ; Darin-Bennett *et al.*, (1973 & 1976) ; Neill and Masters, (1973) ; Poulos *et al.*, (1975) ; Jones and Mann, (1976 & 1977) ; Evans and Setchell, (1978)
Rat	Terner *et al.*, (1975) ; Aveldano *et al.*, (1992)
Stallion	Masaki *et al.*, (1964) ; Komarek *et al.*, (1965)
Sea urchin	Mohri and Horiuchi, (1961) ; Magai and Hoshi, (1975)

Table 2.7. Lipid content and composition of spermatozoa (see Komarek *et al.*,1965 ; Scott, 1973).

		% distribution of lipids			
Species	Total lipid mg/g cells	phospholipids	cholesterol	triacylglycerols	diacylglycerols
bull	25.2	73.4	14.3	3.2	6.4
boar	25.0	74.8	12.8	4.2	5.6
stallion		58.7	13.7	8.0	9.0
stallion	29.1	58.6	23.2	7.9	8.9
human	20.0	66.0	14.0	14.0	2.0
fish (trout)	22.0	75.0	18.0	2.0	1.0

As is the case of the spermatozoa, phospholipids are also the major lipid class in the seminal plasma. Within the seminal plasma lipoprotein complexes have been identified conforming to the physical characteristics of high density lipoproteins and possibly very high density lipoproteins (Blesbois, 1990).

2.7.3 Phospholipids of spermatozoa and seminal plasma

The composition of phospholipids in spermatozoa of different animals are summarised in Table 2.8. In all species phosphatidyl choline is the major phospholipid followed closely by phosphatidyl ethanolamine of which the majority of both are present in the plasmalogen form. The next largest fraction is sphingomyelin, followed by phosphatidyl serine and phosphatidyl inositol with small levels of cardiolipin. However, wide species differences are apparent. Thus the avian species display much higher levels of phosphatidyl serine and phosphatidyl inositol compared to the mammalian species such as the bull, ram and rhesus monkey. The levels of sphingomyelin are higher in the mammalian compared to the avian species.

The seminal plasma displays a significant variation in the phospholipid components between species. Thus phosphatidyl choline, phosphatidyl ethanolamine, phosphatidyl

serine and ethanolamine plasmalogen are all present but at lower levels ranging from 10 - 16 % (Sebastian *et al.*, 1987). Sphingomyelin is also a major phospholipid in boar seminal plasma but with phosphatidyl ethanolamine, phosphatidyl serine, and phosphatidyl choline accounting for 30 %, 13 % and 11 % respectively. Phosphatidyl choline is the main phospholipid class in the seminal plasma of the buffalo.

2.7.4 Fatty acid composition of phospholipids of spermatozoa and seminal plasma

The fatty acid composition of the phospholipid fraction varies considerably from species to species as can be seen in Table 2.9. With respect to the saturated fatty acids 16:0 is quantitatively the most important in mammalian species but 18:0 the most important in the avian species whereas 22:6 (n-3) is quantitatively the most important polyunsaturated fatty acid in man, bull, monkey, ram and boar, however in the dog and rabbit 22:5 (n-6) predominates with 22:4 (n-6) being the major polyunsaturated fatty acid in the avian. The seminal plasma also displays very high concentrations of these respective polyunsaturated fatty acids. High levels of polyunsaturates are therefore a characteristic of the spermatozoa and seminal plasma of all species, as can be seen from Table 2.10 in common with the brain and retina, spermatozoa are unique amongst the major tissues (Anderson *et al.*, 1989 ; Lin *et al.*, 1993).

Table 2.8. Major phospholipid classes within spermatozoa and seminal plasma of various species.

Species	PHOSPHATIDYL -			PHOSPHATIDAL -		SPHINGO-MYELIN	CARDIO-LIPIN	OTHER PHOSPHOLIPIDS	References
	-choline	-ethanolamine	-serine & -inositol	-choline	-ethanolamine				
BOAR									
Spermatozoa				41.4	26.0	15.2	5.5		Scott, 1973
Spermatozoa	45.0	24.0		5.0	5.0	14.0		7.0	Masaki, 1970 *
Seminal plasma	9.0	24.0		2.0	2.0	52.0		11.0	Masaki, 1970 *
BULL									
Spermatozoa				52.8 ***	14.3 ****	12.2			Scott, 1973
Spermatozoa **	305.5	82.5	41.0	665.0	103.5	224.5	25.5		Neill & Masters, 1972
Sperm heads	34.0	6.7	0.4	31.8	3.1	13.2	3.6	7.2	Clegg and Foote, 1973 *
Sperm mid - pieces	28.6	12.8	0.2	32.0	6.8	5.9	9.9	3.8	Clegg and Foote, 1973 *
Sperm tails	25.9	5.4	0.9	28.0	5.0	15.9	6.5	12.4	Clegg and Foote, 1973 *
Seminal plasma	26.3	13.4	3.6	17.6	6.1	13.2	8.8	11.0	Clegg and Foote, 1973 *
BUFFALO									
spermatozoa	28.0	9.3	8.1		5.7	17.4	4.9	2.7	Sarmah *et al.*,1983
seminal plasma	34.1	10.8	6.1		4.9	13.8	3.5	2.6	Sarmah *et al.*, 1983
DOG									
Spermatozoa	27.5	20.1	6.3	3.6	15.3	18.3	3.0	5.9	Darin-Bennett *et al.*, 1974
MAN									
Spermatozoa	38.3	29.7	3.7	2.1	10.1	12.4	2.4	1.3	Jones *et al.*, 1979 c *
Spermatozoa	28.8	21.6	6.6	2.7	9.4	21.4	1.6	7.9	Poulos and White, 1973
Seminal plasma	7.8	8.5	12.9	0.8	12.3	44.0	0.7	13.0	Poulos and White, 1973
RAM									
Spermatozoa	10.8	3.8		42.6	5.9	10.8	6.6		Scott, 1973
Spermatozoa	17.9	6.0	2.1	45.7	7.6	9.3	3.6	7.8	Jones and Mann, 1976 *
Spermatozoa	16.5	5.9	1.5	48.8	6.7	15.8	4.1	0.7	Neill and Masters, 1973
Spermatozoa **	131	52.0	17.0	500.0	84.0	140.0			Neill and Masters, 1973
Spermatozoa	15.8	3.3	2.5	40.0	5.0	12.7	5.9	14.9	Poulos *et al.*, 1975 *
Testicular sperm	13.4	6.2	8.7	24.6	10.3	9.3	11.4	16.1	Poulos *et al.*, 1975 *
MONKEY									
Spermatozoa	33.0	25.0	2.6	6.9	16.1	8.1	4.5	3.8	Darin-Bennett *et al.*, 1977*
Seminal plasma	21.8	15.1	3.7	3.5	24.0	16.9	1.3	13.7	Darin-Bennett *et al.*, 1977*
TURKEY									
Spermatozoa	34.15	15.61	13.6	7.14	11.34	9.91	8.22		Howarth *et al.*, 1977
COCKEREL									
Spermatozoa	38.2	12.6	16.3	9.04	8.04	8.3	7.5		Howarth *et al.*, 1977
Spermatozoa	39.6	8.5	10.5	7.8	5.2	1.1	1.7	12.6	Darin-Bennett *et al.*, 1974
FISH									
Spermatozoa	30.6	20.						5.3	Scott, 1973

* Cited by Mann and Lutwak - Mann, (1981 b).** Expressed Total lipid/10^9 cells***Percentage includes phosphatidyl choline****Percentage includes phosphatidyl ethanolamine.

Table 2.9. Major fatty acids of the phospholipid of spermatozoa and seminal plasma from various animal species.

Species	Fatty acids									References
	Myristic 14:0	Palmitic 16:0	Stearic 18:0	Oleic 18:1	Linoleic 18:2 (n-6)	Arachidonic 20:4 (n-6)	Docosatetraenoic 22:4 (n-6)	Docosapentenoic 22:5 (n-6)	Docosahexaenoic 22:6 (n-3)	
BOAR										
Spermatozoa	3.0	13.0	5.1	2.6	2.1	3.2	1.6	27.9	37.7	Poulos *et al.*, 1973 a
Testicular sperm		17.2	7.7	1.5	1.9	4.5		24.2	38.1	Evans and Setchell, 1978*
BULL										
Spermatozoa	3.9	16.5	6.0	3.9	3.0	3.3		6.9	55.4	Neill and Masters, 1972
Spermatozoa	2.0	14.1	5.3	5.7	3.9	3.5			61.3	Poulos *et al.*, 1973 a
COCKEREL										
Spermatozoa		17.1	23.0	14.2	1.8	6.2	19.2		2.3	Ravie and Lake, 1985
TURKEY										
Spermatozoa		13.0	21.8	9.2	2.8	9.2	10.9		1.8	Ravie and Lake, 1985
DOG										
Spermatozoa	1.0	27.2	13.2	11.8	3.2	6.6		28.4	3.9	Darin-Bennett *et al.*, 1974
MAN										
Spermatozoa		36.8	19.0	20.1	1.8				58.7	Nissen & Kreysel, 1983
Spermatozoa		26.1	10.0	6.9	5.0	3.9			47.5	Jones *et al.*, 1979c *
Spermatozoa		24.8	11.3	8.1	4.0	5.1			35.2	Poulos et al., 1973 a
RAM										
Spermatozoa						5.5			65.1	Neill & Masters, 1973
Spermatozoa	5.2	14.7	5.1	3.8	1.7	4.5			61.4	Poulos *et al.*, 1973 a
spermatozoa	1.0	17.4	8.5	7.1	2.6	3.9			56.2	Poulos *et al.*, 1975 *
Spermatozoa	3.1	14.8	6.2	3.9	3.1	5.0			62.5	Jones and Mann, 1976 *
Acrosomes & membranes	0.9	15.8	8.2	3.7	0.9	2.4			67.8	Jones and Mann, 1977*
Sperm heads	1.0	7.4	3.8	1.5		1.8			84.5	Jones and Mann, 1977*
Mid-piece tails	1.2	4.7	7.2	3.0		8.4			73.1	Jones and Mann, 1977*
Testicular spermatozoa		18.9	8.7	3.5	1.8	8.5			55.2	Evans and Setchell, 1978a*
RHESUS MONKEY										
Spermatozoa		28.2	8.6	10.9	5.4	9.1		0.5	23.9	Lin *et al.*, 1993
Spermatozoa		29.2	11.2	14.8	3.8	8.9		2.8	25.2	Darin-Bennett *et al.*, 1977*
Seminal plasma	1.5	38.2	18.3	14.1	1.6	8.9			10.2	Darin-Bennett *et al.*, 1977*
RABBIT	1.5	24.2	18.7	9.1	4.8			39.0		Poulos *et al.*, 1973 a
Spermatozoa										

*** Cited by Mann and Lutwak - Mann, (1981 b).**

Table 2.10. The fatty acids of mammalian spermatozoa, brain and retina (see Lin *et al.*, 1993).

Fatty acid (% w/w of total fatty acid)	Spermatozoa	Brain	Retina
16:0	22.7	6.3	3.1
18:0	12.8	31.4	23.8
18:1	3.6	7.1	5.9
20:4 (n-6)	9.8	12.4	16.0
22:4 (n-6)	trace	9.4	2.4
22:5 (n-6)	1.9	1.4	0.7
22:6 (n-3)	30.4	22.3	36.4

2.7.5 Lipid composition of the testes

Specific differences exist in the composition of testicular fatty acids between species (Lake, 1971). The significance of this in relation to any differences in reproductive physiology is unclear. Most species contain high levels of C22 polyunsaturated fatty acids in the testes reflecting the proportion that is detectable within the spermatozoa. The avian testes unlike those of mammals contain neither 22:5 (n-3) or 22:6 (n-3) but a large amount of 22:4 (n-6) is present (Ravie and Lake, 1985). It is possible that the appropriate desaturase enzyme is unavailable to complete the n-3 fatty acid synthesis or the appropriate n-3 precursor is unavailable. Aveldano *et al.*, (1992) reported in the rat that there was a predominance of n-9 fatty acids as opposed to the fatty acids of either the n-3 or n-6 series even in well fed, healthy laboratory animals.

22:6 (n-3) is the principal polyunsaturated fatty acid of the human testes (Grogan *et al.*, 1981) whereas in the mouse and guinea pig comparable levels of C22:5 (n-6) and C22:6 (n-3) exist together with appreciable levels of C20:4 (n-6).

2.7.6 Lipid and fatty acid composition during spermatogenesis

Specific variations have been observed to occur in fatty acid distribution during progressive stages of spermatogenesis. Grogan *et al.* (1981) reported that in the mouse, a large variation existed in the levels of 20:4 (n-6) and 22:5 (n-6) with the level of 20:4 (n-6) decreasing and 22:5 (n-6) increasing as spermatozoa differentiation progressed. During this study the level of 22:6 (n-3), which is the most important polyunsaturated fatty acid in mature mammalian spermatozoa, showed no significant changes with respect to any spermatozoa cell type. The decrease in 20:4 (n-6) concomitant with increasing 22:5 (n-6) may reflect conversion of 20:4 (n-6) to 22:5 (n-6). This 22:5 (n-6) was predominantly located in triacylglycerols in the later stages of spermatogenesis and condensing spermatid, whereas 20.4 (n-6) and 22:6 (n-3) were found mainly in the phospholipid of all sperm cell types. It was concluded that the accumulation of 22:5 (n-6)-rich triacylglycerols in condensing spermatids may be the result of a cessation of net incorporation into phospholipid at the round spermatid stage (Grogan *et al.*, 1981).

2.7.7 Lipid and fatty acid composition during maturation

Nikolopoulou *et al.* (1985) measured changes in the lipid content of the boar sperm plasma membrane during epididymal maturation. Phospholipids and sterols were the two major lipid components in spermatozoa suspensions harvested from all tracts of the epididymis. The phospholipid/protein and cholesterol/phospholipid ratios remained constant throughout epididymal maturation. An increase in the levels of phosphatidyl choline, sphingomyelin and polyphosphoinositides were accompanied by decreases in the levels of phosphatidyl ethanolamine, phosphatidyl serine and phosphatidyl inositol classes during epididymal maturation of the boar sperm plasma membrane (Nikolopoulou *et al.*, 1985). In this study, during epididymal maturation, the degree of fatty acid unsaturation in the total lipid of boar sperm membrane did not change. The only change observed was an increase in the 22:5 and 22:6 levels accompanied by a compensatory decrease in the levels of 18:1 and 20:4. During epididymal maturation from caput to cauda epididymis, the fatty acid composition of phosphatidyl choline changed with levels of 16:0, 18:1 and 20:4 decreasing and those of 18:0, 22:5 and 22:6 increasing. The fatty

acids of phosphatidyl ethanolamine displayed similar changes during maturation (Nikolopoulou *et al.*, 1985). These researchers concluded that such changes may be involved in reduced resistance to cold shock and fluidity during spermatozoa transit. In further studies changes were observed within the sterol fraction during epididymal transit. In these studies cholesterol levels decreased and desmosterol and cholesterol sulphate levels increased in ram and bull spermatozoa during epididymal transit (Scott *et al.*, 1967 ; Poulos *et al.*, 1973b).

Poulos *et al.* (1973b) observed that ram, boar and bull spermatozoa lost between 25 and 50 % of their phospholipid content during epididymal transit. Analysis of the individual phospholipid in testicular and ejaculated spermatozoa showed these changes in phospholipid content were due mainly to a marked decrease in the concentration of phosphatidyl serine, phosphatidyl ethanolamine and cardiolipin. The concentration of choline plasmalogen was not significantly different between various spermatozoa cell types. A comparison of the percentages of phospholipid classes has shown that choline plasmalogen levels in ram and bull spermatozoa markedly increased during epididymal transit (Scott *et al.*, 1967 ; Poulos *et al.*, 1973b). In contrast the levels of a number of phospholipid components including phosphatidyl inositol, phosphatidyl serine and phosphatidyl ethanolamine decreased. A decrease in the concentration of selected phospholipids together with a loss in acyl esters and a reduction in chain length of the phospholipid-linked fatty acids strongly suggested that lipids served as energy substrates for ram and bull spermatozoa within the epididymis.

2.7.8. Spermatozoa fatty acid changes within the reproductive tract

Analysis of lipid classes in testicular and ejaculated spermatozoa revealed that the phospholipid content fell after ejaculation and that only choline plasmalogen had been spared (Poulos *et al.*, 1973b). However, this loss of lipid upon passage through the bovine genital tract was not mirrored by the level of 22:6 (n-3) where the level within the spermatozoa remained unchanged as a result of enhancement of its levels within the appropriate phospholipid class (Poulos *et al.*, 1973b). Scott and Dawson (1968) observed that washed testicular and ejaculated ram spermatozoa readily metabolised

added phospholipid and fatty acids on incubation. In another report phosphatidyl inositol, when added, was readily metabolised by the spermatozoa isolated from any section of the rams genital tract, which probably explains the marked decrease of phosphatidyl inositol in spermatozoa during their journey through the epididymis (Scott *et al.*, 1967).

A feature of spermatozoa maturation is the source of phospholipid from which spermatozoa can draw energy for survival. It has been suggested that alterations in the phospholipid and phospholipid-linked fatty acids of the sperm membrane may be an important aspect of spermatozoa maturation and a vital element in the process of preparation of the spermatozoa for ova contact (Schlegal *et al.*, 1986). Some of the changes observed in phospholipid concentration may be due to the loss of the cytoplasmic droplet as the phospholipid component is utilised as an energy source from the cytoplasmic material when other oxidisable substrates become limiting (Schlegal *et al.*, 1986).

2.7.9. Semen lipids and fertility

Work by Sebastian *et al.* (1987), demonstrated that total lipid concentration was elevated in the seminal plasma of oligospermic and azoospermic men (see Table 2.11). The cholesterol content was also higher in the seminal plasma of azoospermic men than other groups. In general in this study, infertility was observed to be associated with increased seminal concentrations for most of the neutral lipids classes. Total phospholipid and most phospholipid classes were diminished in the seminal plasma of oligospermic and azoospermic men. Sebastian *et al.* also reported positive correlations between seminal phospholipid and fertility and a negative correlation between seminal neutral lipids and fertility. Thus, it was concluded that infertility may be associated with altered lipid metabolism in the semen with lipids playing a vitally important metabolic role. Oligospermia has been shown to be associated with a reduced plasma membrane fluidity and in an altered molecular structure of the sperm plasma membrane (Sinha *et al.*,1994). Such a change could account for the altered biochemical behaviour of oligospermic spermatozoa with a resultant incompetence in gamete interaction

There have been reports made in the past that n-3 fatty acids are important in the reproductive system. Research has also suggested a relatable decline in spermatozoa density of humans since the 1950's, with a possible association to changes in the diet involving a decline in n-3 fatty acids. Nissen *et al.* (1981) and Nissen and Kreysel (1983) have shown levels of 22-6 (n-3) in human spermatozoa to be correlated with terato-, astheno-, oligo- and azoospermia. In a normal population of males between 20 and 35 years the spermatozoa density, number of motile spermatozoa and spermatozoa motility correlated with 22:6 (n-3) levels in the semen. Knapp (1990) concluded that fish oil supplementation to human patients led to only small increases in n-3 fatty acid levels within the spermatozoa phospholipids.

Table 2.11. Lipid composition of spermatozoa and seminal plasma in fertile and infertile men (Sebastian *et al.*, 1987).

Samples	Total lipids	Total cholesterol	Total glycerides	Total phospholipid
seminal plasma (mg/100ml)				
Normospermic	275.10	92.88	81.95	73.48
Oligospermic	360.54***	94.36	117.67***	61.61*
Azoospermic	509.29***	152.25***	270.41***	54.40***
spermatozoa (mg/g net weight)				
Normospermic	57.90	17.76	12.89	17.10
Oligospermic	69.02*	17.91	31.07***	12.83***

***P < 0.05 : ***P < 0.001, compared to values for normospermic males.**

Normospermic (40 x 10 6/ml with 60 - 70 % motility) : Oligospermic (< 20 x 10 6/ml with 30 - 40 % motility) : Azoospermic (no spermatozoa present).

Rats normally possess high 22:5 (n-6) levels in their testes and lower levels of 22:6 (n-3) (Ayala *et al.*, 1977). However, these proportions were reversed when the weaning rats

were fed fish oil at 9 weeks of age. Fish oil fed rats exhibited greater testicular maturation due to increased spermatogenesis compared to those receiving a sunflower based diet (high in n-6 fatty acids). It also appears from dietary experimentation that 22:6 (n-3) can functionally replace 22:5 (n-6) in the testes of the rat (Bierei *et al.*, 1969).

Under dietary conditions of essential fatty acid deficiency fowl were found to have deficient testicular development (Lake, 1971). Lake also underlined the importance of linoleic acid to the fertilising potential of fowl spermatozoa, as deficiency resulted in a large increase in seminal plasma docosadienoic acid and a large decrease in docosatetraenoic acids, changes which were associated with a decline in the number of fertilised eggs produced after a single insemination. They concluded that a dietary level of linoleic acid of approximately 1 % was required to ensure normal fertility. Davidenko *et al.* (1991) performed an investigation on rams whose diet was supplemented with 18 to 36 g per day of sunflower oil ; both quality and quantity of semen were increased and as a result from a single ejaculate an additional 9 to 11 ewes were able to be inseminated. The semen lipid content was also increased from 10 to 11%, spermatozoa motility from 11.3 to 12.4 % and spermatozoa survival from 11.3 to 12.4 % compared to the control rams.

Chronic alcoholism in men is commonly associated with fertility disorders and it is responsible for a decrease in spermatozoa quality. In such subjects, marked decreases in spermatozoa phospholipid concentration, mainly in phosphatidyl choline, phosphatidyl ethanolamine and sphingomyelin classes have been observed (Gomanthi *et al.*, 1993). The lipid content of the seminal plasma was also reduced together with reductions in the levels of glyceride and free and esterified cholesterol.

2.8 SEMEN BIOCHEMISTRY

2.8.1 General

The energy required for sperm motility is provided by intracellular ATP. Its use appears to be regulated by the endogenous level of cyclic adenosine monophosphate (cAMP). This not only regulates ATP breakdown but also has a direct effect on spermatozoa motility. A detailed overview of semen biochemistry is provided by Mann and Lutwak-Mann. (1981c). Mann (1964b) and Mann and Lutwak-Mann. (1981c) describe the two major biochemical reactions used by spermatozoa to produce energy, namely glycolysis and respiration.

Ram, bull and rabbit spermatozoa have been reported to be highly active under anaerobic conditions when fructose was present and also in the presence of oxygen with or without fructose (Scott, 1973). Cockerel spermatozoa were found to be highly active under both aerobic and anaerobic conditions whereas turkey spermatozoa were only active under aerobic conditions (Wishart, 1981 ; Wishart, 1989).

2.8.2 Glycolysis

Under anaerobic conditions, spermatozoa metabolise mainly carbohydrates to satisfy energy requirements using the standard glycolytic pathway (Mann and Lutwak-Mann., 1981c). During anaerobic incubation, bull semen is characterised by a progressive decline in the content of fructose with a simultaneous accumulation of lactic acid. In man and bull, the index of fructolysis (mg fructose utilised by 1 x 10^9spermatozoa in 1 hour at 37 °C) is significantly correlated with the concentration of motile spermatozoa (Mann, 1964b). Under *in vitro* conditions spermatozoa from different species have been shown to utilise a range of sugars including fructose, glucose and mannose (Mann, 1964b). Pickett and Komarek (1966) reported that the second ejaculate of the bull was higher in fructose, stimulating motility, but lower in spermatozoa number than the first ejaculate. When fructose and glucose are both available as substrates, ram, bull and cockerel spermatozoa appear to display a preference for the utilisation of glucose (Scott

et al., 1967). Under natural conditions, only fructose is present in the seminal plasma of man, bull and ram, and its content ranges from 250 to 600 ng per ml, with the seminal plasma of the bull possessing levels six times higher than those detected for the ram (Scott *et al.*, 1967). However, the concentration of fructose is very low in boar seminal plasma, and it is almost absent in the seminal plasma of the stallion, dog (Garner and Hafez, 1993) and fowl (Lake, 1984). Minassian and Terner (1966) reported that fish were unable to metabolise carbohydrate but could convert glucose to glyceride glycerol at rates adequate for the formation of glycerolipids.

2.8.3 Respiration

Potential exogenous substrates for the respiration of spermatozoa are glycolysable sugars (glucose, fructose and mannose), glycerol, sorbitol, fatty acids and several amino acids (Mann, 1964a). The most important intracellular substrates for respiratory activity of spermatozoa are the phospholipids, mainly the plasmalogen fraction (Mann, 1964a ; Scott, 1973). The catabolism of fatty acids occurs in the mitochondrial matrix, as opposed to synthesis which occurs in the cytosol. Howarth, (1980) showed that when fowl spermatozoa were stored for 24 hours, the lipid content of the spermatozoa decreased from 18 to 9 %. In fowl spermatozoa doubt exists as to the extent of utilisation of endogenous phospholipid as an energy source as no variation in total lipid, phospholipid or neutral lipid was detected in spermatozoa stored for 24 hours at 41 °C (Howarth, 1981 ; Lake, 1984). Ram spermatozoa has been shown to display oxidative activity for 14:0 and 16:0 fatty acids derived from plasmalogen (Hartree and Mann, 1961). Under aerobic incubation ram spermatozoa also utilised endogenous phospholipids, but in contrast bull spermatozoa did not have the same activity and the phospholipid content of bull spermatozoa did not decrease during incubation (Scott and Dawson, 1968). This behavioural difference may be a reflection of the far higher amounts of fructose available in the bull seminal plasma for energy, as it has been shown in the ram that the addition of fructose suppresses phospholipid breakdown (Scott and Dawson, 1968).

2.8.4 Fatty acid metabolism

The mechanism proposed for the oxidative metabolism of endogenous lipid in mammalian spermatozoa entails the hydrolysis of a fatty acid residue from phospholipid. Carnitine facilitates the passage of fatty acid to the site of beta-oxidation within the mitochondria, where they are believed to be catabolised in a similar way to the liver and other animal tissues. Carnitine, of which large quantities are secreted by certain segments of the epididymis, is readily available for acylation by the spermatozoa which possess their own carnitine acyltransferase, (Mann and Lutwak-Mann., 1981c).

Bull, ram, dog and cockerel spermatozoa are able to utilise short chain (C1 to C8) fatty acids (Scott *et al.*, 1962). In ruminant species, ram spermatozoa possess higher oxidating activity in comparison to the bull (Scott *et al.*, 1962). Ejaculated ram and bull spermatozoa both display the capacity to hydrolyse exogenous ethanolamine phosphoglycerides and phosphatidyl inositol, in contrast the hydrolysis of choline phosphoglycerides is very low (Scott and Dawson, 1968). Fowl spermatozoa have been shown to utilise 16:0 from exogenous substrates to increase ATP production (Howarth, 1978). As a consequence of energy requirements, spermatozoa undergo a depletion of their endogenous lipid content but are able to replenish their lipid reserves by biosynthesis from external substrates such as carbohydrate (Mann, 1964b). Glucose is the utilised substrate in human, fish and bull spermatozoa (Minassian and Terner, 1966) and acetate in buffalo spermatozoa (Sidhu *et al.*, 1989). The incorporation of acetate into the glycolipid fraction of buffalo spermatozoa occurred for at least 7 hours corresponding to the time of maximum capacitation in this species.

2.9 LIPID PEROXIDATION

2.9.1 Peroxidation in male reproductive function

Today there is an ever expanding body of evidence to postulate that a significant factor in the aetiology of male infertility is as the result of oxidative stress (Aitken, 1994). Oxidative stress is a consequence of the generation of reactive oxygen species by the

spermatozoa resulting in peroxidation of polyunsaturated fatty acids in the sperm plasma membrane (Gray, 1931 ; Gottlieb *et al.*, 1988 ; Griveau *et al.*, 1995). Aitken (1994) reported that lipid peroxides showed negative correlations with spermatozoa motility and the capacity for fusion during gamete union at fertilisation. Jones and Mann (1977b) observed damaging effects on spermatozoa of endogenous phospholipid peroxidation brought about by aerobic incubation at 37 °C in the presence of ascorbic acid and ferrous sulphate ; the results included reduced motility, enzyme leakage, acrosomal damage and abolition of frucolytic and respiratory activity. Immobilisation of normal, washed human spermatozoa with spermicidal lipid peroxide resulted in an immediate and irreversible loss of fructolytic activity (Mann and Lutwak-Mann, 1981c). Spermatozoa from cockerels stored *in vitro* in an aerobic environment displayed extensive peroxidation after 24 hours with a considerable reduction in spermatozoa motility (Fujihara and Howarth, 1978).

MacLeod (1943) was the first to describe reactive oxygen species in human spermatozoa and he indirectly suggested that the production of hydrogen peroxide occurred in spermatozoa. He also noted the peroxidative susceptibility of these specialised cells could be counteracted by the presence of catalase and peroxidase enzymes. These findings were later confirmed by Tosic and Walton (1946, 1950) with experiments on bull spermatozoa. Mann (1949) indicated the presence of catalase in ram spermatozoa to be very low whilst in mammalian spermatozoa there was a limited ability to decompose hydrogen peroxide (Tosic and Walton, 1946, 1950). Later work reinforced these early findings (Aitken, 1994). A wide range of observations have been made over recent years identifying the presence of peroxidation processes within spermatozoa. Thus in 1982, Alvarez and Storey identified superoxide and hydrogen peroxide generation in rabbit spermatozoa followed by their presence in spermatozoa of the mouse (Alvarez and Storey, 1984). In 1987, Alvarez *et al.* produced direct evidence for the generation of reactive oxygen species in human spermatozoa with the generation of superoxide ion followed later by possibilities for antioxidant therapeutic protocols (Aitken and Clarkson, 1987). A review of oxygen radicals and reactive oxygen species in spermatozoa is detailed by Riley and Behrman (1991).

Alvarez and Storey (1989) found mammalian spermatozoa lacking in catalase activity but possessing alternate enzymatic defence systems against the superoxide ion and hydrogen peroxide, namely superoxide dismutase and glutathione peroxidase. In this study it was apparent that the glutathione system provided a basal defence without which the superoxide dismutase system in humans would be overwhelmed. Griveau *et al.* (1995) reported two major sources of reactive oxygen species, namely the spermatozoa themselves and leukocytes. The reactive oxygen species were identified as hydrogen peroxide, superoxide and hydroxy radicals and the scavengers as superoxide dismutase, glutathione peroxidase and catalase. It was concluded that a harmonic balance was required between production of reactive oxygen species and antioxidant defence with imbalance in the biological equation leading to cellular damage. A review of reactive oxygen species and enzymatic defence systems in spermatozoa is given by Aitken and Fisher (1994).

In human spermatozoa an enhanced susceptibility to lipid peroxidation was observed to be associated with significant reductions in the percentage of morphologically normal spermatozoa and the percentage of motile spermatozoa (Aitken and West, 1990 ; Aitken *et al.*, 1993a). The morphological abnormalities were found in the structure of the mid-piece but not the head or flagellum of the spermatozoa. A significant negative correlation has also been observed between malondialdehyde generation and sperm-oocyte fusion (Aitken *et al.*, 1993a). Jeulin *et al.* (1989) reported human sperm plasma membranes to be very sensitive to the effect of reactive oxygen species whilst permealisation of the human sperm plasma membrane has been shown to occur at levels lower than those reported for other species (Alvarez *et al.*, 1987). These researchers however confirmed catalase activity in both the spermatozoa and seminal plasma and suggested the use of catalase as a constituent of the semen diluent for cryoprotection in bovine, human and hamster semen.

2.9.2 Superoxide dismutase

The relative activities of superoxide dismutase within the spermatozoa of a range of animal species are listed in Table 2.12. As can be seen the activity levels display a very wide variation and indeed within any particular species (Froman and Thurston, 1981).

Aitken *et al.* (1993b) used the xanthine oxidase system to study the toxic effect of reactive oxygen species in human spermatozoa. The xanthine oxidase system generated a mixture of superoxide and hydrogen peroxide which then secondarily interacted to produce short-lived, highly pernicious radicals such as hydroxy ions, which have a devastating effect on cell function. The scavenging superoxide dismutase and catalase were then used to selectively remove superoxide and hydrogen peroxide from the reaction mixture and thereby determine their relative cytotoxicity towards human spermatozoa. The xanthine oxidase system has the ability to disrupt both the motility of human spermatozoa and their capacity for sperm-oocyte fusion. Since catalase but not superoxide dismutase has a protective effect on spermatozoa function, it has been suggested that it is exclusively hydrogen peroxide that can exert a direct cytotoxic effect on human spermatozoa rather than superoxide. The mechanism by which hydrogen peroxide mediates its cytotoxic effect is currently unknown (Aitken *et al.*, 1993b). Results by Bize *et al.* (1991) suggested that the generation and release of hydrogen peroxide is not merely a means of discarding waste products but that this molecule plays a significant role during spermatozoa capacitation. In this study catalase inhibited the acrosome reaction by scavenging the hydrogen peroxide and it was shown also that direct addition of hydrogen peroxide to capacitation media accelerated the onset of the acrosome reaction.

Table 2.12. The superoxide dismutase activity observed in spermatozoa (units/10^9 cells) and seminal plasma (units/ml) from various animal species.

Species	Spermatozoa u/10^9 cells	Seminal plasma u/ml	Authors
chicken	1.5		Cecil and Bakst, 1993
turkey	0.325		Cecil and Bakst, 1993
cockerel	15.5	9.6	Mennella and Jones, 1980
ram	105	161	Mennella and Jones, 1980
bull	26.0	120	Mennella and Jones, 1980
man	17	149	Mennella and Jones, 1980
donkey	1430	5260	Mennella and Jones, 1980
boar	120	31.7	Mennella and Jones, 1980
stallion	266	1600	Mennella and Jones, 1980
bull	10		Beconi *et al.*, 1993
man	28		Meisel *et al.*, 1993

2.9.3 Glutathione peroxidase

Glutathione peroxidase activity has been found in the semen of the ram, dog, human, goat and bull but not in the boar or rabbit. Kantola *et al.* (1988) revealed its role in the protection of membrane lipids from oxidative damage but found high selenium and glutathione peroxidase in the seminal plasma of bulls but not in the human. It was concluded that selenium dependent glutathione peroxidase was an important component in the protection of bovine spermatozoa against damage caused by oxygen radicals whilst in man the mechanism was defective. Selenium deficiency has been shown to result in impaired reproductive performance in several animal species including the rat and sheep (Pond *et al.*, 1983). These workers suggested that selenium may have another role in reproductive performance namely as a structural component of spermatozoa or as a component of proteins other than glutathione peroxidase. Slaweta *et al.* (1988b) reported a significant correlation between selenium concentration and selenium

dependent glutathione peroxidase activity in the seminal plasma of the bull. After a 3 hour incubation at 37 °C, spermatozoa displayed markedly less selenium and reduced total glutathione peroxidase activity, high concentrations of lipid peroxides and lower motility. Slaweta *et al.* (1988a) reported significant seasonal changes in selenium dependent glutathione peroxidase and levels of lipid peroxide in whole bull semen.

2.9.4 Leukocytic presence

In human semen it has been shown that reactive oxygen species can be produced not only by spermatozoa but also through the presence of leukocytes (Aitken *et al.*, 1992) Leukocytes have been identified in human ejaculates and range in concentration from 400 up to 2.7 x 10^9 cells per ml. In this study the neutrophil predominated and accounted for 16% and T and B lymphocytes were rarely detected. Leukocytes (macrophages and neutrophils) are the main source of reactive oxygen species in human semen (Kessopoulou *et al.*, 1992). Although problems of semen contamination by leukocytes (Wolff and Anderson, 1988 a, b) can occur, it is believed that the spermatozoa are protected by powerful antioxidants present within the seminal plasma. However, contact can occur, for example at ejaculation due to a reproductive tract infection (D'Agata *et al.*, 1990) On removal of seminal plasma, reactive oxygen species from leukocytes can attack spermatozoa and seriously impair their functional ability. For purposes of *in vitro* fertilisation special care must be afforded with respect to the presence of leukocytes (Krausz *et al.*, 1992). Hafez (1993) suggested that fewer leukocytes were present in the complex mucosal folds of cervical crypts where reservoirs of spermatozoa collect with the result that less damage was likely than would occur in the vagina and uterus.

2.9.5 Measured peroxidation products

A major metabolite of lipid peroxidation, (E)-4-hydroxy-2-nonenal (HNE) was found to reduce the motility of ram spermatozoa during incubation at 30 °C (Windsor *et al.*, 1993). The effect was clearly dose dependent and the time taken for motility to decline was also strongly influenced by the concentration of spermatozoa in the incubation

mixture. The effect of HNE on motility was not reversible. HNE did not impair ram spermatozoa fructolysis, but reduced the oxygen uptake. HNE at a concentration of 400 μmol for 1 hour abolished motility and respiration in the spermatozoa, but it did not increase the proportion of eosin stained spermatozoa. This result suggested that the disruption of the spermatozoa plasma membrane by HNE was in fact minimal. It was concluded that the ram spermatozoa membrane is more resistant than erythrocytes to HNE but the responsible mechanism remains to be elucidated (Windsor *et al.*, 1993). When incubated with exogenous HNE, human spermatozoa became totally and irreversibly immobile within minutes, but the presence of seminal plasma appeared to have a protective effect on the survival of the spermatozoa (Selley *et al.*, 1991).

Cecil and Bakst (1993) measured malondialdehyde production in the turkey with increasing levels being found with increased incubation temperatures. In the presence of seminal plasma levels were reduced. Alvarez and Storey (1989) reported on the use of malondialdehyde production as a measure of lipid peroxidation. Aitken *et al.* (1993a) recorded malondialdehyde generation in response to the presence of ferrous ion promoter in infertile human patients. The level was three times higher in spermatozoa exhibiting poorer motility and oxygen uptake. Alvarez *et al.* (1987) reported that 100 nmole malondialdehyde per 10^8 spermatozoa cells correlated to a level of peroxidation that would kill spermatozoa.

Wishart (1984), studying fowl semen, observed the formation of lipid peroxidation during a 5 hour aerobic incubation at 40 °C. Concentrations from 0 up to 8 nmol of malondialdehyde 10^9 cells were observed. Higher concentrations were associated with partial or complete loss of fertilising ability. No association with other characteristics such as motility, morphology or ATP content were recorded. Huszar (1994) reported high levels of lipid peroxidation, as measured by malondialdehyde, in oligospermic compared to normospermic patients. A correlation was also obtained between peroxidation and creatine kinase activity in the oligospermic patients.

2.9.6 Protective agents

Jones and Mann (1977a) showed that egg yolk and milk could protect spermatozoa from the effects of peroxides through the formation of protective coating or an ability to "neutralise" the peroxide toxicity. A suspension of homogenised spermatozoa could also protect motile spermatozoa from a toxic level and thereby reduce the amount available to intact spermatozoa. Blackshaw and Salisbury (1957) also reported the protective effects of egg yolk and lecithin on cold shocked bull spermatozoa whilst in the ram Scott and Dawson (1968) concluded that adding choline plasmalogen and/or cardiolipin to the semen diluent helped to maintain the structural integrity of spermatozoa.

Surai and Ionov (1992b) reported that feeding 40 i.u. per kg of vitamin E resulted in a significant increase in spermatozoa vitamin E concentrations and testicular weight compared to control birds. The fertilising capacity of spermatozoa was also higher in the supplemented ganders. Vitamin E may be considered as a structural compound and a stabiliser of biological membranes.

Govil *et al.* (1992) reported 10 μM of alpha-tocopherol prevented peroxidative damage to goat spermatozoa by ultra violet radiation. Surai (1984) reported that addition of sodium ascorbate to semen promoted lipid peroxidation with a resultant accumulation of malondialdehyde in spermatozoa and accompanying acrosomal membrane damage. Addition of vitamin E to the medium or the diet, alone or in the presence of other vitamins, decreased the rate of lipid peroxidation. The effect of vitamin E was much higher when used as a feed additive than when added to the incubation medium (Blesbois *et al.*; 1993 ; Askari *et al.*, 1994). Mennella and Jones (1980) suggested the addition of free radical scavenging agents to counteract the high concentrations of organic peroxides that were identified in spermatozoa stored at -20 °C for periods of longer than a year. Beconi *et al.* (1993) studied the effect of "natural" antioxidants, vitamin E and sodium ascorbate on semen exposed to thermal stress. These workers concluded that good quality samples, standard cooled in the presence of these antioxidants, displayed higher superoxide dismutase activities, intact acrosomes, mitochondrial coupling and reduced malondialdehyde production. Natural antioxidants seemed to exert a protective effect on

the sperm plasma membrane and metabolic activities of cryopreserved spermatozoa but only in samples of good quality initially. The addition of butylated hydroxytoluene (BHT) or human seminal plasma have also been shown to display protective effects (Jones *et al.*, 1979). Alabi *et al.* (1985) incubated ram spermatozoa with selenium at a range of concentrations and found a significant improvement in spermatozoa motility and oxygen consumption, however, at high concentrations there was an inhibition of spermatozoa function. Hartmen (1994) reported that semen production in older turkeys could be improved by vitamin C supplementation through significant effects on both the volume and concentration of semen. However, fertility did not differ for hens inseminated from the two treatments.

2.9.9 The effect of cryopreservation on spermatozoa lipid composition and viability

Voorst and Lenstra (1995a, b) reported that the successful cryopreservation of spermatozoa is determined by the following factors ; the diluent, the cryoprotectant, freezing and thawing rates, absence of deleterious materials, post-thawing fertilisation requirements, quality of the original semen and optimal freezing conditions.

As pointed out by Wolf (1995) it has been known for more than 200 years that spermatozoa from mammalian species could survive transient exposure to near freezing temperatures. This was first described in 1776 by Spallanzani, for human, stallion and frog spermatozoa which showed recovery of motility after being kept in snow for 30 minutes. In 1866, Mantegazza published some information concerning the benefits of spermatozoa freezing with regards future insemination of domestic animals and humans. However, it was only within the last 50 years or so that the importance of appropriate cryoprotectants has become recognised, particularly the use of glycerol. The successful cryopreservation of human spermatozoa at -78 °C occurred in 1953 and a decade later the use of nitrogen vapour at -196 °C was introduced, a feature which is now current practice in commercial artificial insemination procedures. In general terms it is obviously essential that for successful cryopreservation there is a need to select males displaying sound semen quality and to pay appropriate attention to the routine and practice of semen ejaculation collection procedures (Voorst and Lenstra, 1995a). Beatty *et al.*

(1976) showed that the effect of freezing on spermatozoa fertility differed extensively in the bull, although the period of time spent in the frozen state had no additional effects. McLaughlin *et al.* (1992) also reported changes before and after freezing on human spermatozoa with a wide variation between individuals. Bakst and Sexton (1979) observed differences in fertilising ability, motility and ultrastructural characteristics of fowl and turkey spermatozoa using scanning and electron microscopy where fowl semen survived the freezing process considerably better than turkey.

Compared to semen from control males, Sarmah *et al.* (1984), reported that cold-shocked buffalo semen lost 15.8 % and frozen semen 34.5 % of their total lipids ; values for phospholipid were 6.4 % and 19.1 % respectively. Poor quality semen and cold-shocked semen samples were also shown to be more susceptible to lipid peroxidation than high quality semen. Jones and Stewart (1979) observed a range of structural changes in bovine semen after freeze thawing including acrosomal, mitochondrial and mid-piece disruption. Hinkovska *et al.* (1988) showed that after freezing the concentration of alkenyl-acyl derivative in the plasma membrane of the ovine spermatozoa decreased and the concentration of the diacyl derivatives of phosphatidyl choline and phosphatidyl ethanolamine increased compared to values for fresh spermatozoa. Freezing caused a decrease in the activity of phospholipase A2 but the activity of neutral sphingomylinase was not affected. The incubation of sperm plasma membranes with dipalmitoylphosphatidylcholine was shown to increase membrane rigidity. Nauck (1988) reported that with respect to the freeze-thaw process bull, ram and boar spermatozoa were most at risk and the acrosomal plasma membrane and mitochondria were the structures most damaged by the freeze-thaw process. To prevent peroxidation during freezing of bull, ram and boar semen, the best results were obtained by the addition of sodium triosulphate, epyhide and baclanoside respectively. Antonyuk (1982) working on boar semen, reported cerebroside and ganglioside concentrations increased 12 - 20 fold in the gel and liquid portion of fresh semen after dilution and freezing. No significant fatty acid changes occurred. Quinn and White (1966, 1967) reported that ejaculated ram spermatozoa were less resistant to cold shock than testicular spermatozoa. Moore *et al.* (1976) working on the boar sperm acrosome suggested that the seminal plasma of the boar was detrimental to spermatozoa acrosomal

integrity and cold resistance due to the presence of haemaglutinin, a basic protein of seminal vesicle origin and believed to work by increasing membrane permeability. Picket and Komarek (1966) observed that second ejaculates appeared to be more resistant to freezing and cold shock.

Ochkur *et al.* (1994) suggested cryopreservation reduced glycolysis, the activities of the citric acid cycle and respiratory chain thereby affecting oxidative phosphorylation and inducing injury to the sperm plasma membranes and damage to the motility apparatus. However, the respiratory chain and citric acid cycle retained a high degree of functional integrity. Blackshaw and Salisbury (1957) also observed that aerobic glycolysis and respiration were greatly reduced in cold-shocked spermatozoa. Graham and Hammerstedt (1992) reported that irreversible cellular damage could occur in semen maintained at 0-5 °C due to phospholipid leakage and altered spermatozoa metabolism.

De Leeuw *et al.* (1990) demonstrated that upon cooling to 0 °C a redistribution of intramembraneous particles occurred in the plasma membranes of the head and principle piece of boar and bull spermatozoa, this phenomenon being attributed to lateral phase separation of membrane lipids. Lateral redistribution of intramembranous particles was demonstrated in the post-acrosomal and acrosomal regions of the sperm head and in the principal piece of the tail. The effects were shown to be reversible after thawing. In addition freezing also led to a breakdown in the semi-permeable properties of the plasma membrane not only by primary damage to the plasma membrane but also to injury of other sensitive organelles within the spermatozoa and resultant secondary damage. Buhr *et al.* (1994) reported that the plasma membrane of the cryopreserved boar spermatozoa had less sphingomyelin and more phosphatidyl choline than membranes from fresh spermatozoa. Cryopreserved spermatozoa displayed extensive fatty acid changes that included reduced levels of 18:0 in phophatidyl serine and phosphatidyl ethanolamine, increased levels of 22:4 (n-6) and 20:4 (n-6) in sphingomyelin and phosphatidyl inositol and increased levels of 22:6 (n-3) in phosphatidyl ethanolamine. It was concluded that these alterations changed the basic fluidity characteristics, the lower concentration of saturated fatty acids resulting in increased membrane disorganisation and elimination of the Ca^{2+} sensitivity of the spermatozoa head plasma membrane required for the acrosome

reaction. The polyunsaturated:saturated ratio of sperm phospholipid has been reviewed frequently in respect to freezing responses (Poulos *et al.*, 1973a ; Darin-Bennett *et al.*, 1974 ; Ravie and Lake, 1985).

2.10 THE PRESENT STUDY

It is clear from the present study that a vast amount of literature exists with regards the detailed lipid composition of spermatozoa and seminal plasma in a wide variety of animal species as well as the observations made in such species on the lipid changes with regards spermatozoa storage and spermatogenesis. The use of antioxidants in semen protection during storage has also been reported. However, the literature fails to cover the effects of animal ageing on semen quality, associated lipid properties and major antioxidant systems even though it is known that normal cell ageing is associated with a loss of antioxidant protection accompanied by a reduction in the presence of important long chain polyunsaturated fatty acids. A comparison of these changes to animals of known poorer reproductive performance could then establish any link between loss of fertility either due to natural ageing or inherent below average semen quality.

Supplementation of animal species with various dietary fatty acid precursors and antioxidants has been reported in the literature, however again no reference to the consequences for spermatozoa and seminal plasma lipid composition and fertility were considered in any detail. The present study was therefore undertaken to evaluate the effects of animal ageing, poor semen quality parameters and the incorporation of dietary n-3 fatty acids and antioxidants on domestic avian and mammalian spermatozoa, observing any changes in lipid composition, associated antioxidant systems, spermatozoa quality and ultimate fertility.

CHAPTER 3

MATERIALS AND METHODS

3.1 SEMEN COLLECTION AND TREATMENT

Semen samples were collected into graduated glass conical tubes, for cockerels by abdominal massage, see Figure 3.1 and for the bulls by artificial vagina, see Figures 3.2a, b. All cockerel semen samples were 'pooled' to obtain necessary volumes for subsequent analysis ; the bull semen samples were collected and analysed individually.

Each semen sample was diluted 1:1 with 0.85 % (w/v) sodium chloride and centrifuged for 20 minutes at 2500 rpm at 4°C. This procedure was carried out within 20 minutes of semen collection to avoid any peroxidative destruction or damage to the spermatozoa cells and seminal plasma. The resulting aqueous top layer of seminal plasma was transferred to a fresh tube and the remaining spermatozoa cell pellet washed again with 0.85% (w/v) sodium chloride and re-centrifuged for 20 minutes at 2500 rpm at 4°C. The resulting upper aqueous layer was added to the previously removed seminal plasma and the spermatozoa cells resuspended in an equal volume of 0.85 % (w/v) sodium chloride. Both the spermatozoa and the seminal plasma fractions were immediately transferred into solvents for lipid extraction as detailed in Section 3.7.

Figure 3.1. **Collection of cockerel semen by abdominal massage (Lake and Stewart, 1978).**

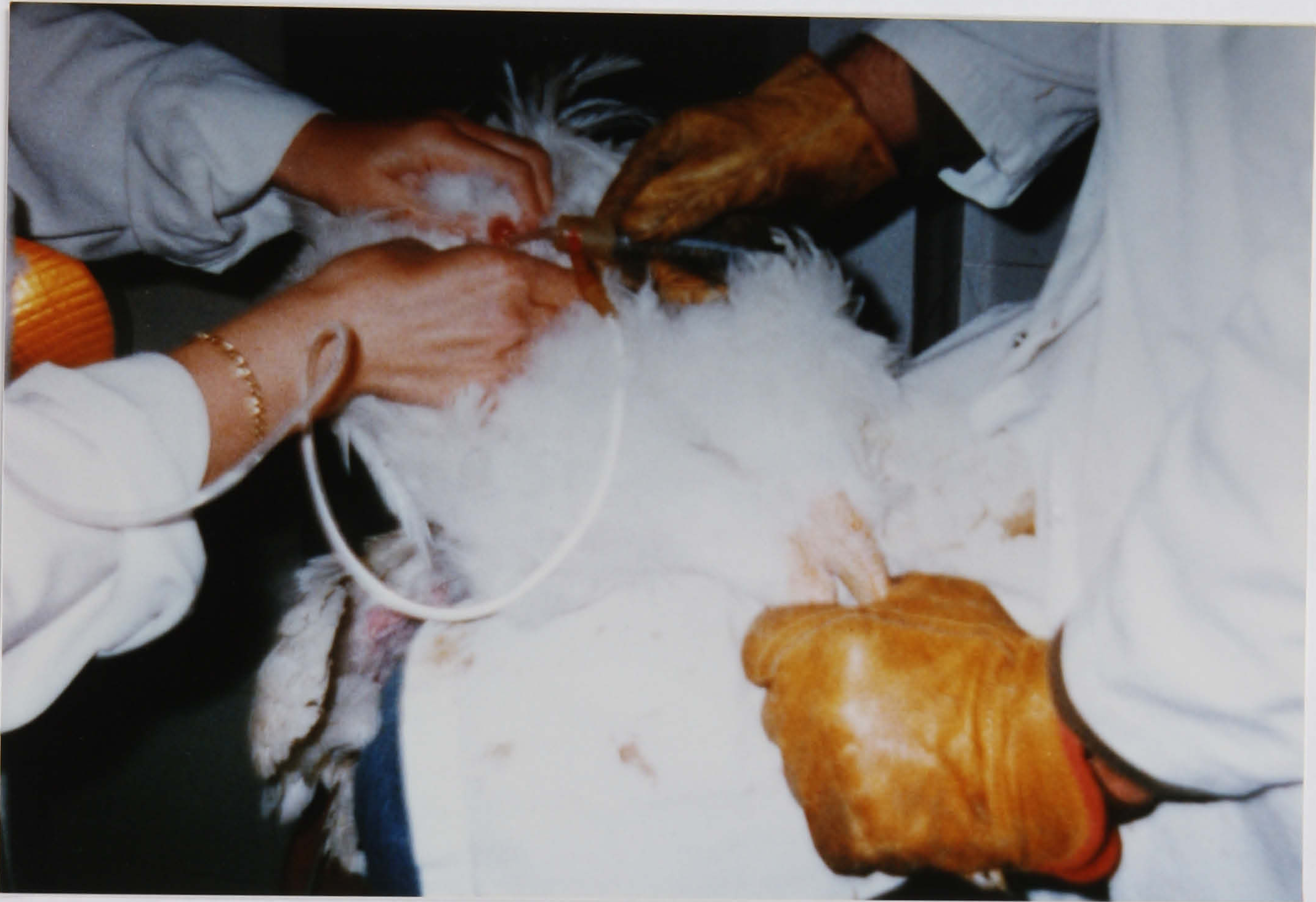


Figure 3.2. Semen collection in the bull. a) teasing the bull prior to semen collection ; b) semen collection by artificial vagina.

3.1.1 Quantification of spermatozoa cell number (non-computerised)

Cockerel semen

Spermatozoa numbers were calculated using a haemocytometer chamber and a regression equation established. 10 μl of fresh semen was diluted in 10 ml of 3.0 % (w/v) sodium chloride. 10 μl of this diluted solution was then used to prepare a haemocytometer chamber. All counting of the spermatozoa was performed after a period of 10 minutes which was sufficient to allow the spermatozoa to settle. Following the introduction by a Gilson pipette of 10 μl of the diluted sample into the chamber, the upper surface of the haemocytometer slide was covered by a fresh coverslip. A light microscope (x 40 magnification) was used to count the spermatozoa within the four corner squares of the large central grid as well as the centre square. This was repeated for each side of the haemocytometer slide. The average of these two counts was calculated. The number of billion spermatozoa cells per ml in the original semen sample was calculated by dividing the average value by 40.

10 μl of the semen was then diluted with 2 ml of 0.85 % (w/v) sodium chloride and following mixing the optical density measured at 535 nm wavelength. A regression equation was calculated between the haemocytometer count and the optical density measurements of forty semen samples according to the method of Brillard and McDaniel (1985) as follows :

$$\mathbf{y = 0.1763 + 2.5094x}$$

Using the regression equation the optical density (x) was converted into spermatozoa cell concentration (y, 10^9 cells per ml semen). All measurements were performed in duplicate.

Bull

For bull semen the concentration was measured spectrophotometrically using an I.M.V.(Instruments Medicines Veterinaire, L'Aigle, France) photometer using 20 μl of spermatozoa in 4.98 ml of 0.85 % (w/v) sodium chloride. All *in vitro* assessments of bull spermatozoa were in line with routine protocols undertaken at Scottish Livestock Services, Scone, Perth.

3.1.2 Computer Software analyses

This involved the use of the CellSoft™ Automated Semen Analyser (Version 3.0, Cryoresources Ltd., New York, USA). The CellSoft™ hardware arrangement consisted of the following major components as shown in Figure 3.3. An IBM AT/PC/XT or CellSoft™ compatible computer with printer, high resolution video monitors and a black and white video camera, Olympus phase contrast microscope.

A live or videotaped image of the semen was fed into the computer and was analysed, where applicable, for semen concentration, spermatozoa motility and forward motility data.

The analyser enabled recognition of spermatozoa and was able to distinguish them from other semen constituents based on size, luminosity, and motion. These three factors together enabled the system to provide accurate and objective data. Spermatozoa cell sizing was performed through the use of filters thereby enabling a distinction to be made between the spermatozoa and any seminal plasma debris. Illumination of the spermatozoa head identified spermatozoa cells on the basis of their luminosity. Since all the analyses was performed using a digitised, grey level image, those "non-spermatozoa" cells which did not display luminosity through phase contrast microscopy were not "seen" by the computer. By setting upper and lower limits for cellular motion a further parameter of spermatozoa identification was enabled. Throughout all analyses, the use of a chamber of 10 micron depth ensured that only one layer of spermatozoa would be present and that all the cells would remain in focus throughout the period of analysis.

Figure 3.3. **The CellSoft Semen Analyser. Comprised of microscope, video camera and computerised integration (facility provided by kind permission of the University of Milan, Italy).**

3.1.3 Estimation of motility in cockerels using electro-optical semen evaluation

Fresh semen was diluted 5:1 with 0.85 % (w/v) sodium chloride and gently mixed. Diluted semen was evaluated using a fresh capillary slide placed into the Sperm Quality Analyser (SQA™, Medical Electronic Systems Ltd., Migdal Haemek, Israel) to give an SMI rating (Sperm Motility Index) which was converted to percentage motility using manufacturers reference data provided.

3.1.4 Subjective estimation of motility

Bull

Using the method of Logue and Greg (1987) each semen sample was assessed for motility at x 100 magnification and scored visually on a scale of 0 - 5 by the same operator. The samples were assessed at 37 °C by the use of a heated slide on a warm stage.

3.1.5 Quantification of live/dead spermatozoa

Live and dead spermatozoa were quantified using the method of El Jack and Lake (1966). A solution of nigrosin and eosin was prepared using 0.16 g of oesin and 0.6 g of nigrosin per 10 ml of 3.6 % (w/v) sodium citrate. The citrate solution was placed in a beaker and the nigrosin was added with constant stirring at a temperature of 15 °C for approximately 20 minutes to dissolve the nigrosin completely. The eosin was then added and the solution mixed thoroughly

Cockerel semen

20 μl of fresh semen was diluted with 0.5 ml of eosin/nigrosin solution in 1.5 ml plastic microtubes, mixed thoroughly and placed in an ice cold water bath for 10 minutes. Using a Gilson pipette 10 μl of the stained semen solution was then smeared onto a clean microscope slide, uniformly drawn along the slide by a clean slide edge, dried to room temperature and covered with a cover slip. Slides were prepared in duplicate for all ejaculates. Using a light microscope at x 100 magnification 300 spermatozoa in total were counted from each slide beginning at the left hand side and working towards the right hand side of the slide. A hand held cell counter was used to count the

spermatozoa. Dead spermatozoa were counted as those which had become stained pink due to dye penetration by non-intact sperm plasma membranes ; live, intact spermatozoa display no signs of such staining. From these results the percentage of live to dead spermatozoa was calculated.

Bull semen

Routine morphology of bull spermatozoa was conducted using oesin/nigrosin staining as described above and to include an assessment of acrosomal integrity. Only bulls with less than 25 % total abnormal spermatozoa and less than 10 % of any one other major abnormality were used for routine semen straw preparation.

3.1.6 Post-freeze assessment of bull semen

Semen was thawed at 37 °C for 1 minute and the motility scored between 0 and 5 as described in Section 3.1.4. The percentage of progressively motile (PPM) spermatozoa was assessed visually on a scale between 0 and 40 % ; assessment of greater than 40 % was still scored at a maximum of 40 %. Only samples with motility scores between 3.5 and 5 and with PPM greater than 35 % were considered acceptable in line with commercial practice. Progressively motile spermatozoa (PPM) were classified as the percentage of spermatozoa in the sample which moved in a forward, uniform pattern.

Citrate analysis test

0.25 ml of semen (i.e. 1 semen straw) was added to 0.75 ml of 3 % (w/v) sodium citrate and warmed to 37 °C. The preparation was left for 20 to 30 minutes and then scored as described in Section 3.1.4. Acrosomal integrity was then assessed after staining with oesin/nigrosin by Differential Interference Contrast (D.I.C.) microscopy. To be considered satisfactory for commercial purposes a score of 70 % normal spermatozoa and a fully intact acrosomal membrane had to be attained. The morphology of the spermatozoa was also assessed by oesin/nigrosin staining as detailed in Section 3.1.5.

3.1.7 Bovine semen dilution and straw processing

Bovine semen was cooled and held for 45 minutes at 5 °C in line with EC Directive 88/407. The straws used were 0.25 ml French straws (I.M.V., L'Aigle, France). The

semen, after full assessment for concentration, motility etc., were diluted from their original concentration, for example typically approximately. 1.2 x 10^9 spermatozoa per ml, to a concentration of approximately 8 x 10^7 spermatozoa per ml using either skimmed milk buffer or Biosophus Plus (Instruments Medicines Veterinaire, L'Aigle, France) as the diluent depending on future processing requirements. Each straw containing 0.25ml of semen was frozen in an I.M.V. Digicool (Instruments Medicines Veterinaire, L'Aigle, France) freezing system using a standard bovine semen freezing curve. In all cases from collection to freezing took less than 7 hours.

3.1.8 Formazan reduction test

Cockerel

The method used was as described by Chaudhuri and Wishart (1988) with minor modifications. This method provided an objective estimation of the metabolic activity of the spermatozoa and also the degree of integrity of the oxido-reductase enzyme activities through the reduction of colourless 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to coloured formazan. The assay components for the test are detailed in Table 3.1.

Table 3.1. Assay components for formazan dye reduction test of cockerel semen.

Assay solutions
150mM NaCl (w/v) + 20 mM TES*
100 mM glucose
100 mM $CaCl_2$
100 mM KCN
4.0 mM INT
0.33 mM PMS**

***TES N-tris (hydroxymethyl) methyl, 2-aminoethane sulphonic acid : **PMS (phenazine methosulphate)**

All assay solutions were kept at 5 °C for a maximum of three weeks. On the day of the assay the solutions were warmed to room temperature and appropriate volumes of all solutions, except INT and PMS, were mixed thoroughly in a test tube with 10 µl of

fresh semen. The mixture was allowed to equilibrate in a water bath at 20 °C for 10 minutes, then the INT and PMS solutions were added and mixed. After 3 hours the reaction was stopped by the addition of 200 µl of 5 % (v/v) Triton X-100 containing 0.38 mM hydrochloric acid. The solution was allowed to stand for 5 minutes at room temperature before centrifugation at 2500 rpm for 10 minutes. The supernatant was carefully removed by pipette and added to cuvettes for spectrophotometric measurement of optical density at 520 nm. The assay blank was treated similarly except that Triton/hydrochloric acid solution was added immediately after INT and PMS addition.

A standard curve was drawn up for solutions containing between 10 and 100 nmols per ml using a stock solution of 0.1 µmols per ml prepared from 16.77 µg formazan in 500 ml Triton/hydrochloric acid.

3.2 ANTIOXIDANT ASSAYS

3.2.1 Superoxide dismutase (SOD) activity

Analysis was performed using a commercial Ransod assay kit (Randox Ltd., Crumlin) and based on the degree of inhibition exhibited by superoxide dismutase on the generation of superoxide radicals in the reaction between xanthine and xanthine oxidase with 2-(4-iodophenyl)-3-(4-nitrophenol)-5-phenyltetrazolium chloride (INT) to form a red formazan dye. Units of superoxide dismutase activity are defined as the amount of superoxide dismutase required to inhibit the rate of dye formation by 50 % under the conditions specified by the kit manufacturers.

Estimations were made on 250 µl of seminal plasma following dilution with 2 ml of water and on an appropriate aliquot of spermatozoa, equivalent to 250 µl of plasma, following their resuspension in 0.85 % (w/v) of sodium chloride.

All solutions were prepared according to the manufacturers instructions. Spectrophotometric absorbance measurements of the blanks, standards and samples were made at 505 nm at 37 °C.

3.2.2 Glutathione peroxidase (GPX) activity

Analysis was performed using a commercial Ransel assay kit (Ransod Ltd., Crumlin) and based on the evolution of $NADP^+$ following the oxidation of NADPH via glutathione peroxidase catalysed activation of glutathione by cumene hydroperoxide. Units of glutathione peroxidase activity are defined as µmol NADPH oxidised/minute.

Estimations on both seminal plasma and spermatozoa were performed as for superoxide dismutase but with spectrophotometric absorbance measurements at 340 nm at 37 °C.

3.3 QUANTIFICATION OF MALONDIALDEHYDE (MDA)

0.1 ml of semen in pyrex tubes was added to 0.1 ml of 1 mM ferrous sulphate and the volume made up to 1 ml with 0.8 ml of distilled water. Samples were then mixed and incubated for 30 minutes at 37 °C in a water bath. After cooling, 1.5 ml of 20% acetic acid, adjusted to pH 3.5 with 60 % (w/v) sodium hydroxide was added together with 1.5 ml of 0.8 % (w/v) 2-thiobarbituric acid (TBA) dissolved in a 1.1 % (w/v) solution of sodium dodecyl sulphate. Following mixing the samples were placed in a dry-block at 95 °C for 1 hour. After cooling 5ml of n-butanol was added to each sample and mixed thoroughly before centrifugation at 3500 rpm for 15 minutes. The upper layer was then pipetted into macrocuvettes and read spectrophotometrically at 532 nm.

A calibration graph of absorbance readings at 532 nm against 1,1,3,3-tetramethoxypropane was prepared to give a response factor per unit volume of semen.

3.4 SAMPLES

Analyses was performed on the following :

diets following grinding to a fine powder with a mortar and pestle ; whole semen within 30 minutes of collection ; spermatozoa and seminal plasma following separation by low speed centrifugation (2 500 rpm) at 4 °C ; blood plasma following separation by low speed centrifugation (2 500 rpm) at 4 °C ; major tissues e.g. liver either fresh or following storage at -20 °C.

3.5 TRACE ELEMENT DETERMINATION

Suitable sample aliquots were prepared for analysis by dry ashing and mineralising in dilute hydrochloric acid and trace elements were measured using a Plasma 100 Inductively Coupled Plasma Emission Spectrophotometer (Thermoelectron, Warrington, England). For measurement of selenium, samples were first digested with an acid mixture comprised of nitric, sulphuric acid and perchloric acids on a heating block (Perstorp Ltd., Bristol, England) and analysis was performed using a 251 Atomic Absorption Spectrometer (Thermoelectron, Warrington, England) by means of a 440 Hydride Generator.

3.6 DETERMINATION OF VITAMIN E

The method of alpha-tocopherol analysis was derived from McMurray *et al.* (1980). Samples were saponified in aqueous medium in order to hydrolyse and convert alpha-tocopherol acetate to the non-acetylated form thereby precluding the problem of both forms of tocopherol giving rise to two peaks in the chromatogram. Pyrogallol in ethanol was added as an antioxidant and alpha-tocopherol was extracted into hexane prior to determination by reverse-phase high performance liquid chromatography, as outlined below.

To a suitable sample aliquot in a 50 ml screw-cap polyethylene centrifuge bottle 10 ml of a 10 % (w/v) solution of 1, 2, 3 - trihydroxybenzene (pyrogallol) in ethanol was added, following shaking, the tube was placed in a waterbath at 70 °C for five minutes to allow temperature equilibrium. 2.5 ml of 60 % (w/v) aqueous potassium hydroxide was then added and the contents heated for 30 minutes at 70 °C for complete saponification.

Approximately 20 ml of deionised water together with 10 ml of hexane were added and the bottles were shaken vigorously to allow extraction of alpha-tocopherol into the hexane. The aqueous and hexane phases were allowed to separate over a suitable period or were centrifuged as necessary. A suitable aliquot of the hexane layer was transferred to a 5 ml test-tube and evaporated to dryness at 50 °C under a stream of oxygen-free nitrogen. Immediately after drying, the residue was redissolved in 600 µl of methanol, transferred to fresh microvials and sealed.

Alpha-tocopherol was separated from other components by reversed-phase HPLC and quantified by fluorimetry. The HPLC and fluorimeter conditions are listed in Table 3.2

Table 3.2. HPLC and fluorimeter conditions used for alpha-tocopherol separation.

HPLC	**Column**	15cm x 4 mm 'Superspher' 4 µm RP18 with 'Lichrocart' 4-4 guard column (Merck ltd., Lutterworth, England)
	Eluant	methanol : water (98.04 : 1.96 v/v)
	Pump	dual-piston Spectra System P1000 (Thermo Separation Products, Stone, England) isocratic pump ; flow rate : 1.5 ml/min
	Injection	manual injection via Rheodyne valve with 20 µl injection loop ; sample volume : 150 µl.
Fluorimeter	**Type**	JASCO 821 - FP Spectrofluorometer (Mettler-Toledo Ltd., Beaumont Leys, England)
	Wavelength	excitation : 295 nm emission : 330 nm
Integrator	**Type**	SP 4400 Datajet Integrator (Thermo Separation Products, Stone, England)

The eluant was degassed using helium. A standard alpha-tocopherol (Eastman Kodak, Deeside, Wales) solution in methanol, containing 105 ppm alpha-tocopherol, was injected until consistent peak areas were obtained. The concentration of the alpha-tocopherol standard was verified by measuring its absorbance at 292 nm in a SP8 - 500 UV/Visible Spectrophotometer (A.T.I. Unicam, Cambridge, England). and comparing the results with standards quoted in the literature. Comparison between results obtainable from the literature and the standard prepared was as follows :

Standard from literature : $A^{100\ \mu g/ml}$ at 292 nm = 0.75 in ethanol

Prepared standard : $A^{46.6\ \mu g/ml}$ at 292 nm = 0.337

= 0.72 (for 100 µg/ml)

The prepared standard therefore compared favourably with the given figures in the literature for absorbance. Additionally, the same concentration of alpha-tocopherol was

prepared in methanol in order to determine whether this solvent caused a difference in the spectrophotometric absorbance. The result was as follows :

$$A^{46.6\ \mu g/ml} \text{ at } 292 \text{ nm} = 0.354 \text{ in ethanol}$$
$$= 0.71 \text{ (for } 100\ \mu g/ml)$$

Hence, the effect of methanol on the absorbance was negligible. The mean alpha-tocopherol standard peak was used as an external standard for the calculation of vitamin E content.

3.7 DETERMINATION OF LIPID AND FATTY ACID COMPOSITION

3.7.1. Extraction of lipid

Body fluids and tissues

The total lipid associated with each sample was extracted with chloroform : methanol, 2:1 (v/v) according to the method of Folch *et al.* (1957). The addition of a suitable excess of methanol, and subsequently chloroform to the sample and following complete homogenisation, the extract was filtered using a fluted Whatman's Number 41 filter paper. To the filtrate was added 12 ml of 0.88 % (w/v) potassium chloride and the solution shaken vigorously. After allowing separation of the aqueous and organic phases for 16 hours, the upper aqueous layer was siphoned off and discarded. The lower organic phase was freed of organic solvent by evaporation under vacuum using a rotary film evaporator Following dissolution in chloroform, the residual was transferred quantitatively to a 10 ml screw-cap vial which was then sealed under oxygen-free nitrogen and stored at 20 °C until analysis.

Feed

Samples of the diets in pelleted form were finely ground in a mortar and pestle and approximately 2 g were measured into 100 ml round-bottomed flasks. 20 ml of methanol were added and the contents refluxed for 20 minutes ; this was followed by the addition of 40 ml of chloroform and the contents were refluxed for a further 20 minutes. Glass beads were added to prevent excessive 'bumping'. After cooling, the mixture was filtered via Whatman Number 41 filter paper into a measuring cylinder and the residue

washed well with chloroform : methanol, 2:1 (v/v). To the filtrate was added 20 % by volume of a 0.88 % (w/v) solution of potassium chloride and the mixture vigorously shaken. Subsequent steps were performed as described above.

3.7.2 Separation of major lipid classes

Lipids were fractionated into their major lipid classes on thin layer chromatoplates (20 cm x 20 cm) of silica gel G (Merck Ltd., Lutterworth, England), thickness 0.25 mm, using a solvent system of hexane : diethyl ether : formic acid, 80:20:1 (v/v/v). Using a glass syringe a suitable aliquot of the lipid was applied as a band to the origin. Following separation of the major lipid fractions, the plates were allowed to air dry and were then sprayed with a 0.1 % (w/v) solution of 2, 7-dichlorofluorescein in methanol. Bands corresponding to phospholipid, free cholesterol, free fatty acid, triacylglycerol and cholesterol ester were visualised under ultraviolet light and scraped from the plate into centrifuge tubes. The phospholipid fraction was eluted from the silica gel by two washings with a suitable volume of methanol ; all other fractions were eluted by washing with diethyl ether In each case the tubes were centrifuged to sediment the silica gel and the lipid-containing solvent was transferred to a 50 ml round-bottomed flask by decantation. All lipid fractions were identified by reference to co-chromatography with pure standards.

3.8 DETERMINATION OF FATTY ACID COMPOSITION

3.8.1 Transmethylation of fatty acids

Fatty acid methyl esters were generated by refluxing the lipid fractions with dry methanolic sulphuric acid. In all cases a pentadecanoic acid (15:0) internal standard in methanol was added to flasks containing the lipid fractions, 0.322 mg pentadecanoic acid being added to phospholipid and 0.0322 mg of pentadecanoic acid added to all other fractions to increase the accuracy of the quantification. Following the addition of the internal standard, lipid fractions were freed of solvent by rotary evaporation and 4 ml of a mixture of dried methanol : toluene : concentrated sulphuric acid, 20:10:1 (v/v/v), added and the mixture refluxed for 30 minutes. After refluxing, the flasks were allowed to cool and 10 ml each of deionised water and hexane were added. The flasks were then

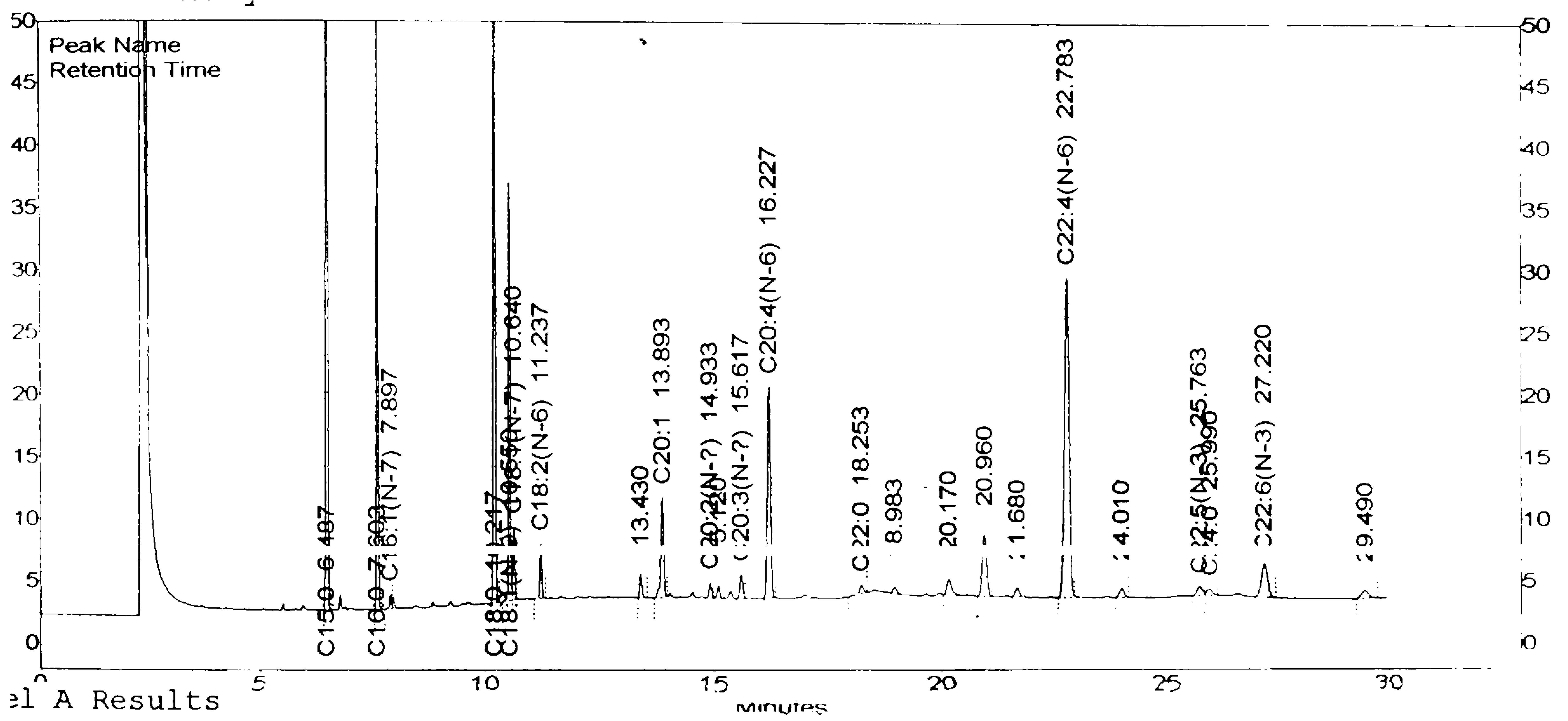
shaken vigorously to allow full extraction of fatty acid methyl esters into the hexane phase and the contents were transferred to a suitable test-tube and allowed to separate into two phases. The upper (hexane) layer was transferred to a further test-tube and residual water removed by the addition of approximately 10 g anhydrous sodium sulphate : sodium hydrogen carbonate, 4:1 (w/w). The methyl ester-containing hexane was then transferred into small test-tube and dried at 50 °C under a stream of nitrogen gas. The methyl esters were then taken up in volumes of hexane appropriate to their amount present.

3.8.2 Determination of fatty acids by gas-liquid chromatography

The resultant fatty acid methyl esters were analysed by 1 µl injection via CP 9010 Autosampler (Chrompack, London, UK), to a Gas-liquid Chromatographer using a 30m x 0.25 mm diameter, 0.25 µm film thickness Carbowax capillary column (Econo-Cap, Alltech UK Ltd., Camforth, UK). Integration of the peaks using an "EZ-Chrom" Data Handling System (Speck Analytical, Alloa, UK) enabled the derivation of the fatty acid composition (% w/w of total fatty acid). Figure 3.5 shows a typical gas-liquid chromatographic separation obtained under such conditions,. The systemic and common names of the fatty acids determined have been detailed previously (see Section 2.5.5).

Absolute amounts of phospholipid, free fatty acid, triacylglycerol and cholesterol ester were quantified by relating the total amount of fatty acids to the amount of the internal standard according to the method of Christie *et al.* (1970). The weights of the lipid classes were obtained as the product of the weight of total fatty acids in each class and a factor calculated by dividing the molecular weight of the pentadecanoic acid derivative of the lipid class by the molecular weight of methyl pentadecanoate.

Figure 3.4. Gas-liquid chromatographic trace of major fatty acids present in cockerel spermatozoa. Conditions of separation were as designated in Section 3.7.2. Pentadecanoic acid standard = 15:0 ; all other abbreviations as per Table 2.4, Section 2.5.5.



3.9 DETERMINATION OF FREE CHOLESTEROL

Following the separation of free cholesterol from other lipid components by thin layer chromatography as described in Section 3.7.2., the diethyl ether was removed by evaporation under a flow of nitrogen and the free cholesterol redissolved in 250 µl propan-2-ol and was quantified using a standard Cholesterol Test Kit (Boehringer Mannheim, Lewes, England) according to the approved protocol as described. The method is based on the colorimetric determination of the yellow lutidine dye, 3, 5 - diacetyl - 1, 4 - dihydrolutidine, which is stoichiometric with respect to the amount of cholesterol present. A calibration curve of free cholesterol was prepared as follows.

Table 3.3 Preparation of calibration curve for the determination of free cholesterol.

cholesterol (mg/ml)	volume (ml) of 1.0 mg/ml cholesterol standard	volume propan-2-ol (ml)
1.00	no dilution	
0.80	0.40	0.10
0.60	0.30	0.20
0.50	0.20	0.30
0.20	0.10	0.40
0.10	0.10	0.50

In all cases, duplicates of standards and samples were added to the cells of a 96 cell MR500 microplate reader (Dynatech Laboratories Ltd., Billingshurst, England) and absorbance measured at 405 nm.. The standard curve was plotted (typically $r^2 = 0.999$) and free cholesterol was expressed either in absolute amounts or in conjunction with the other lipid fractions in relative terms per unit weight of lipid. The final cholesterol results for spermatozoa and seminal plasma were divided by 4 as samples would normally be diluted with 1.0 ml of propan-2-ol according to the kit manufacturers instruction, however to make levels more easily detectable from the small levels of sample available this was reduced.

3.10 HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY (HPTLC)

Separation of lipid classes by HPTLC was performed according to the method of Olsen and Henderson (1989) and their quantification by scanning densitometry. HPTLC plates (silica gel 60, 10 x 10 cm, Whatman International Ltd., Maidstone, England) were pre-developed in hexane : diethyl ether, 1:1 (v/v) and "activated" at 110 °C in the oven for 1 hour. Following the application of suitable aliquots of lipid extracts in chloroform as discrete spots the plates were developed in a solvent mixture of methyl acetate : isopropanol : methanol : 0.25 % (w/v) potassium chloride, 10:10:10:4:3.6 (v/v). The plates were then removed and following air-drying were sprayed with a mixture of 3 % (w/v) cupric acetate in 8 % (v/v) orthophosphoric acid and charred at 160 °C for 20 minutes. The separated lipid moieties were then photometrically scanned using a Shimadzu CS-9001 PC dual wavelength scanner (Dyson Instruments, Houghton-le-Spring, England) linked to a data recorder. The data was transposed into either relative percentage compositional terms or absolute values by appropriate integration. Identification of the separated lipid fractions was by reference to known standards.

3.11 HIGH PERFORMANCE LIQUID CHROMATOGRAPHY (HPLC)

This was undertaken according to the method of Christie (1988) for the separation of the major phospholipid classes. Manual integration was performed using a 10 μl loop with a split ratio of 9:1. All solvents were of HPLC grade and were de-gassed with helium prior to and during use to remove any dissolved oxygen. The mobile phase flow ratio was 1.7 ml/minute. The mobile phase consisted of a ternary solvent system of increasing polarity and delivered at a rate described below. Prior to the application of the sample for analysis the system was purged with the most polar solvent (solvent C) and ending with the least polar solvent (solvent A) as detailed in Table 3.4.

Table 3.4. Ternary solvent system and delivery rates of increasing polarity.

Time (minutes)	hexane : tetrahydroforum - 99:1 (v/v)	isopropanol : chloroform - 4 :1 (v/v)	isopropanol : water - 1 : 1 (v/v)
0	100	0	0
1	100	0	0
5	80	20	0
5.1	42	52	6
25	32	52	16
25.1	30	70	0
30	100	0	0
35	100	0	0

The pump was a SP8800 Ternary HPLC pump (Thermo Separation Products, Stone, England). The column was a 10 cm x 5 mm, 3 μ silica Spherisorb ™ (Phase Separation Ltd., Clwyd, Wales) fitted with a 2 μ column (Alltech Associates Applied Science Ltd., Camforth, England).

Detection of the solutes was via an Evaporative Light Scattering Detector Model IIA (Varex Inc., Burtonsville, Maryland, USA). For detection and measurement of the separated solutes, the detector requires the vaporisation of solvent in a heated stream of gas followed by measurement of the degree of light scattering caused by non-volatile solute. The nebuliser gas was supplied via a Jun-Air Oil-less Compressor (Norresundby, Denmark) and was passed through a desiccant prior to entry into the detector. By inspection, an optimal detector tube temperature and nebuliser gas flow were found ; an optimum signal/noise ratio and highest peak ratio occurred at a tube temperature of 90 °C and a gas flow of 1.7 ml/min.

In order to determine the relative amounts of each fraction as well as the fatty acid composition of the phospholipids, the phospholipid classes were collected manually for methylation and gas-liquid chromatographic analysis. To achieve this a Nupro stream-splitter (P.S. Instruments ltd., Sevenoaks, England) was positioned between the column and light-scattering detector. By altering the resistance to flow through the action of a needle-valve, the stream-splitter was adjusted to give a satisfactory split ratio between the light-scattering detector and the eluant collection point. The action of a micrometer screw gauge allowed adjustment of the needle valve to give consistent and accurate split

ratios. The phospholipid classes were collected as designated from the moment the integrator plot showed the start of a peak until the start of the next significant peak. Verification of synchronisation between the time at which the detector responded and the time at which the fraction was collected was undertaken by inspection.

3.11.1 Gas-liquid chromatographic analysis of the eluted phospholipid

In all cases the phospholipid classes were methylated according to the methodology described above (see Section 3.8.1) in the presence of an appropriate amount of pentadecanoic fatty acid standard. Gas-liquid chromatographic analysis of each class was performed as described in Section 3.8.2.

3.12 THIN LAYER SEPARATION OF PHOSPHOLIPIDS

This was undertaken according to the method of Christie (1982) using 0.25 mm plates of silica gel D (Cameg Ltd., Mutteuz, Switzerland) and 1 mM sodium carbonate and developed in chloroform : methanol : acetic acid : water, 50:30:8:4 (v/v). The separated classes were eluted as described previously (see Section 3.7.2, 3.8) and their proportions and fatty acid compositions determined by gas-liquid chromatography (see Sections 3.7.2, 3.8). All classes were identified by reference to appropriate standards.

3.13 SOLVENTS, REAGENTS AND GASES

Where necessary, all reagents, chemicals and gases were of the highest purity obtainable. In all cases, they were obtained from reputable sources. The major suppliers were :

Solvents

Rathburn Chemicals ltd., Walkerburn, Scotland
Fisons Scientific Equipment, Loughborough, England
BDH, Lutterworth, England
Hayman Ltd., Witham, England

Reagents

Sigma Chemical, Poole, England

Gases

BOC, Glasgow, Scotland

3.14 STATISTICS

All results are presented as means ± S.E. of measurements as indicated throughout. Students *t-test* was used for statistical comparison of the data between two experimental groups. Statistical comparison and correlation coefficients were calculated using Microsoft Excel Version 6.0 (Microsoft Corporation).

CHAPTER 4

AGE RELATED CHANGES IN THE LIPID AND ANTIOXIDANT CAPACITIES OF COCKEREL AND BULL SEMEN

4.1 INTRODUCTION

There is little literature available on the lipid and antioxidant changes which occur during the reproductive period in either mammalian or avian species. There have been only a few investigations on changes and abnormalities exhibited by spermatozoa during ageing in bulls (Gray, 1931 ; Bishop, 1970 ; Rao and Bane, 1985) that include theories on spermatozoa senescence. Early work (Gray, 1931) had already suggested the possibility that toxic by-products produced by ageing spermatozoa may suppress normal spermatozoa metabolism and function. Bishop (1970) reported a decrease during ageing in daily spermatozoa production from 6×10^9 cells/ml at 3 to 4 years of age to only 4×10^9 cells/ml between the ages of 6 to 13 years ; there was an accompanying decrease in non-return rates from 65 to 54 % in bulls from 3 to 12 years of age. In the majority of instances lipid analyses performed on spermatozoa and seminal plasma have been reported without reference to age.

The present study was carried out to investigate the following features of cockerel and bull semen ;

- i normal lipid levels in spermatozoa and seminal plasma for young, healthy animals.
- ii changes in semen characteristics associated with the progression of the reproductive period.
- iii changes in the amounts and proportions of the major lipid classes, particularly phospholipid classes, associated with ageing.
- iv changes in the fatty acids of the major phospholipid classes with age.
- v the identification of major antioxidant enzyme systems and their quantitative changes with age.
- vi vitamin and trace element compositions within the spermatozoa and seminal plasma.
- vii comparative observations between two breeds of bulls, the Holstein/Fresian and the Belgian Blue.

4.2 MATERIALS AND METHODS

Full details of all procedures undertaken during the investigations are detailed in the relevant sections of Chapter 3. Further relevant details are as follows.

4.2.1 The animals used

Cockerel

Semen was collected from two groups of cockerels, each containing 12 Naked Neck broiler breeder cockerels. The first group were selected randomly from a larger group of cockerels of approximately 26 weeks of age, that is at the beginning of their reproductive period and the second group from a selection of cockerels of approximately 60 weeks of age, that is towards the end of their reproductive period. The birds were obtained from the standard flock maintained by the Poultry Science Department, SAC, Auchincruive, Ayr.

Bull

Normal healthy bulls were selected to provide four groups each containing four bulls. All the selected bulls were involved in the commercial collection of semen for artificial insemination at Scottish Livestock Services, Scone, Perth. Constraints were placed as to the number of bulls available during this study due to cost of housing and removing animals from commercial operation. Appropriate semen samples were collected to cover the beginning (2 to 3 years), middle (5 to 6 years) and end (greater than 9 years) of the reproductive period of the commercial bull.

4.2.2 Diets

Cockerel

The cockerels were housed in individual cages and were fed 120g per day of a commercial broiler breeder diet with a crude protein content of 16 %, a lipid content of 4.5 % and a metabolisable energy level of 11.5 MJ/kg ; the fatty acid composition of the lipid is given in Table 4.1a. Vitamin E was supplied at a rate of 32 iu/kg of feed. The hours of light supplied per day were 11 at 29 weeks of age which was increased by 0.5 hours per week to reach 15 hours per day at 37 weeks of age which was then maintained

throughout the remainder of the reproductive period. The environmental temperature was maintained at 18 °C throughout.

Bull

The bulls were fed a standard diet comprising of a mixture of commercial bull pellets with a crude protein content of 16 %, a lipid content of 6 % and a metabolisable energy level of 12.5 MJ/kg and had access *ad libitum* to an average to good quality silage. Young bulls received 3 kg of the pellets twice per day and older bulls 5 kg twice per day. Vitamin E was supplied at a rate of 15 iu/kg of feed. All bulls were housed in a controlled environment during the semen collection period from October until May, before being returned to grass. The fatty acid composition of the lipid of the diet is given in Table 4.1b.

Table 4.1a. Fatty acid composition of the standard commercial poultry diet.

Major fatty acids	% w/w of total fatty acid
14:0	1.0
16:0	25.0
18:0	3.0
18:1 (n-9)	25.0
18:2 (n-6)	42.0
18:3 (n-3)	5.0

All fatty acids of less than 1 % of total have been omitted.

Table 4.1b. Fatty acid composition of the commercial bull pellets.

Major fatty acids	% w/w of total fatty acid
14:0	1.0
16:0	20.0
18:0	4.0
18:1(n-9)	30.0
18:2 (n-6)	40.0
18:3 (n-3)	3.0
22:6 (n-3)	1.0

All fatty acids of less than 1 % of total have been omitted.

Feed samples for the cockerels and bulls were checked regularly throughout the experimental period for consistency of lipid and fatty acid composition. All feeds were stored in air tight containers to avoid peroxidative deterioration.

4.2.3 Collection of semen samples

Cockerel

All males were trained for semen collection for approximately 3 weeks prior to sample collection for experimental purposes. Collection from the cockerels took place three times per week during the experimental period with sufficient surplus semen to allow for any *in vitro* analysis. Outwith the experimental collection period, semen was collected from all cockerels regularly twice a week to maintain desirable and representative semen quality.

Bull

All semen was collected routinely every week during the experimental period and otherwise once every one to two weeks to maintain desirable and representative semen quality.

4.2.4 Evaluation of semen

All measurements were performed as described previously (see Sections 3.1, 3.2, 3.5, 3.6)

In the case of the cockerels enzyme analysis was performed only on whole semen as the ability to purify or store semen efficiently had not been adequately established. In the case of the bulls the spermatozoa and seminal plasma were separated by centrifugation at 3 000 rpm for 10 minutes for analysis of seminal plasma. Seminal plasma analysis was performed on the bull semen samples as this is where the majority of antioxidant enzyme activity is reported to occur (Mennella and Jones, 1980), indeed on analysis of the spermatozoa from these animals negligible levels of antioxidant activity were detectable.

4.2.5 Lipid analysis of spermatozoa and seminal plasma

All techniques were carried according to the procedures detailed previously (see Sections 3.7, 3.8, 3.9, 3.10, 3.11).

4.2.6 Statistical analysis

All antioxidant characteristics and metabolic activities were specified to two decimal places to emphasise the values obtained which were quite low and/or expressed very small standard errors, all other lipid measurements were specified to only one decimal place. Levels of vitamins and trace elements varied considerably and were expressed to appropriate decimal places.

Cockerel

The two cockerel groups were analysed by students *t-test.* Lipid analyses were performed on samples from 12 cockerels and 36 ejaculates (3 ejaculates per cockerel) for non-lipid measurements.

Bull

All observations were compared to the young Holstein/Fresian group by students *t-test.* Four ejaculates were used for lipid analyses and 8 ejaculates (2 ejaculates per bull) for non-lipid measurements.

4.3 RESULTS

4.3.1 Semen characteristics

The characteristics of the semen samples of both species are shown in Tables 4.2 and 4.3.

Cockerel

The concentration of spermatozoa in the samples decreased significantly with age. The proportion of live spermatozoa and the associated metabolic activity also decreased with age, in the latter case by a factor of four.

In the old cockerel a ten fold decrease in spermatozoa of glutathione peroxidase activity was recorded although no significant difference was observed in the level of superoxide dismutase.

Bull

The concentration of spermatozoa in the semen decreased with age. The young Belgian Blue bulls also showed significantly lower concentrations of spermatozoa when compared to the young Holstein/Fresian bulls of the same age. The motility of the spermatozoa also decreased steadily with age. Spermatozoa motility in the young Belgian Blue bulls were comparable only to the old Holstein/Fresian bulls.

For the Holstein/Fresian bulls the activities of both glutathione peroxidase and superoxide dismutase decreased during ageing. The activities of both antioxidant enzymes were lower in the young Belgian Blue bulls when compared to the levels present in the young Holstein/Fresian bulls.

4.3.2 Lipid compositions of the spermatozoa and seminal plasma

The proportions of the major lipid classes present within the spermatozoa and seminal plasma are shown in Table 4.4 for the cockerel and Tables 4.5a and 4.5b for the bull.

Cockerel

The total lipid within the spermatozoa and the seminal plasma increased with age and varied greatly between the young and older cockerels with the greatest variation in total lipid levels being displayed by the older cockerels. Phospholipids were the predominant lipid class in the spermatozoa of the cockerel accompanied by high levels of free cholesterol. Levels of phospholipid increased significantly in the spermatozoa with age in the cockerel but decreased within the seminal plasma. The major lipid classes within the seminal plasma were also phospholipid and free cholesterol although the cockerel exhibited significant levels of free fatty acid, triacylglycerol and cholesterol ester, the latter class, cholesterol ester being significantly higher in the older birds.

Bull

The levels of total lipid in the spermatozoa exhibited by the Holstein/Fresian bulls at all ages and by the young Belgian Blue bulls were similar. Within the seminal plasma there was a significant decrease in total lipid during ageing and within the young Belgian Blue bulls. Phospholipids were the predominant lipid class in the spermatozoa of the bull accompanied by high levels of free cholesterol. The proportion of phospholipid within spermatozoa decreased markedly in the old Holstein/Fresian bulls. However, phospholipid appeared to increase within the seminal plasma during ageing and was also higher in the young Belgian Blue bulls compared to the young Holstein/Fresians. The major classes within seminal plasma were again phospholipid and free cholesterol with low levels of free fatty acid, triacylglycerol and cholesterol ester in both the spermatozoa and seminal plasma.

4.3.3 The fatty acid composition of total phospholipid of the spermatozoa and seminal plasma

The fatty acid profiles of the major phospholipid fraction in the spermatozoa and seminal plasma are shown in Table 4.6, 4.7a and 4.7b.

Cockerel

The major saturated fatty acids present within the total phospholipid of both the spermatozoa and seminal plasma were 16:0 and 18:0, with 18:1 (n-9) being the major monunsaturate. The major polyunsaturated fatty acids were 20:4 (n-6) and 22:4 (n-6) in

both the spermatozoa and seminal plasma. The levels of 22:6 (n-3) in the spermatozoa and seminal plasma were low although older cockerels displayed significantly higher levels of 22:6 (n-3) within the spermatozoa. In the seminal plasma of the old cockerels there were significantly lower levels of 16:0, 18:2 (n-6) and 20:4 (n-6) compared to the young cockerels.

Bull

The major saturated fatty acids present within the total phospholipid of the spermatozoa and seminal plasma of the bull were 14:0, 16:0 and 18:0. The most striking feature of the fatty acid composition was the exceptionally high proportion of 22:6 (n-3) in the phospholipid of both the spermatozoa and seminal plasma, and the negligible levels of 20:4 (n-6) and 22:4 (n-6). No significant differences were observed in the fatty acid composition of spermatozoa and seminal plasma in groups of bulls studied.

4.3.4 The phospholipid classes of the spermatozoa and seminal plasma

Substantial effects of age were observed on the phospholipid profile of both the spermatozoa and seminal plasma. Results are shown in Tables 4.8, 4.9a and 4.9b.

Cockerel

Phosphatidyl choline and phosphatidyl ethanolamine were the major classes in both the spermatozoa and seminal plasma. In the spermatozoa phosphatidyl ethanolamine decreased with age and was accompanied by a concomitant increase in phosphatidyl choline. A similar pattern of change occurred within the seminal plasma. Cardiolipin and phosphatidyl inositol increased significantly and phosphatidyl serine decreased with age in the spermatozoa and the seminal plasma. Sphingomyelin decreased significantly in the seminal plasma of older cockerels.

Bull

Phosphatidyl choline and phosphatidyl ethanolamine were the major classes in spermatozoa and seminal plasma. In the spermatozoa phosphatidyl ethanolamine decreased with age and was accompanied by an increase in phosphatidyl choline, the levels in the Belgian Blue bulls being similar to those displayed by the old Holstein/Fresian bulls. Phosphatidyl inositol decreased with age in the spermatozoa. A

similar pattern of phospholipid compositional change occurred within the seminal plasma, however the Belgian Blue bulls resembled the young Holstein/Fresian bulls in this instance.

4.3.5 Fatty acid composition of the phosphatidyl ethanolamine and phosphatidyl choline

Major effects of age were observed on the fatty acid compositions of both phosphatidyl ethanolamine and phosphatidyl choline fractions of the spermatozoa, particularly with respect to the levels of C20 and C22 polyunsaturates (Tables 4.10 and 4.11a and 4.11b).

Cockerel

The phosphatidyl ethanolamine class of the cockerel spermatozoa contained high levels of 20:4 (n-6) and 22:4 (n-6), the levels being reduced by some 50% and 35% respectively in the samples of the older cockerels. The phosphatidyl choline in the spermatozoa contained much higher levels of saturates compared with phosphatidyl ethanolamine and the proportions of both 20:4 (n-6) and 22:4 (n-6) remained relatively unaffected with age.

Bull

In the Holstein/Fresian bulls there were significant reductions in the levels of 20:4 (n-6) and 22:6 (n-3) with age within phosphatidyl ethanolamine and phosphatidyl choline. The young Belgian Blue bulls displayed higher levels of saturated fatty acids but comparable levels of 20:4 (n-6) and 22:6 (n-3) within the phosphatidyl ethanolamine compared to the young Holstein/Fresian bulls. The phosphatidyl choline fraction was more polyunsaturated than phosphatidyl ethanolamine; however there was a significant decrease in the level of 22:6 (n-3) with age. The level of 22:6 (n-3) was also significantly lower in the young Belgian Blue bulls compared to young Holstein/Fresian.

4.3.6 Vitamin A and E and trace elements levels within the spermatozoa and seminal plasma

The levels of vitamins A and E and trace elements present within the spermatozoa and seminal plasma are given in Tables 4.12 and 4.13 for the cockerel and bull respectively

Cockerel

Whereas vitamin E level remained unchanged in the spermatozoa its level increased in the seminal plasma of the older cockerels. Vitamin A was almost undetectable at both ages. Calcium levels increased with age and decreased in the seminal plasma. Phosphorous levels remained unchanged. Levels of magnesium were negligible. Levels of potassium were very high in the seminal plasma and low in the spermatozoa. Copper, zinc and iron levels in the spermatozoa all increased with age ; in the seminal plasma the level of zinc reduced with age and that of iron increased. Manganese levels were high in the older cockerels. Selenium levels were similar at both ages.

Bull

Vitamin E levels were very low in the spermatozoa and seminal plasma at both ages, as for the cockerel, although very high levels were detectable within the seminal plasma of Belgian Blue bulls Vitamin A was almost undetectable. With age there was a decrease in the calcium level in the spermatozoa but an increase in the seminal plasma. Phosphorous showed a decrease with age. Levels of magnesium also decreased. Levels of copper, zinc and iron in spermatozoa increased with age ; similarly in the seminal plasma the level of iron also increased but that of zinc decreased. The Belgian Blue displayed much higher levels of copper, zinc and iron in both the spermatozoa and seminal plasma compared to the Holstein/Fresian. The levels of manganese and selenium in both the spermatozoa and seminal plasma were similar for both breeds and remained unchanged with age.

Table 4.2. Semen characteristics of cockerels.

		Age (weeks)		
		26	60	
Concentration	10^9 cells/ml	2.1 ± 0.1	1.7 ± 0.1	a
Live cells	%	91.2 ± 0.7	86.2 ± 1.1	a
Metabolic activity	nmol reduced dye/10^9 cells/min	0.24 ± 0.03	0.06 ± 0.01	a
Glutathione peroxidase	units/10^9 cells	2.53 ± 0.09	0.23 ± 0.05	a
Superoxide dismutase	units/10^9 cells	0.24 ± 0.05	0.17 ± 0.02	

Values are means ± S.E. of 12 observations.
Significant differences between groups are illustrated with character ^{a}P<0.001.

Table 4.3. Semen characteristics of Holstein/Fresian and Belgian Blue bulls.

		Holstein/Fresian					B. Blue	
		Age (years)						
		2 -3	5 - 6		greater than 9		2 - 3	
Concentration	10^9 cells/ml	1.4 ± 0.1	1.0 ± 0.1	b	0.9 ± 0.1	b	0.8 ± 0.2	a
Motility	(0 - 5)	5	4		2		2	
Glutathione peroxidase	units/ml seminal plasma	17.63 ± 1.55	10.73 ± 2.10	c	5.38 ± 2.53	b	13.13 ± 0.68	c
Superoxide dismutase	units/ml seminal plasma	4.79 ± 0.63	3.52 ± 0.43		3.27 ± 0.16	c	1.99 ± 0.40	b

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with characters ^{a}P<0.001, ^{b}P<0.01, ^{c}P<0.05.

Table 4.4. Lipid composition of spermatozoa and seminal plasma of the cockerels.

	Spermatozoa			Seminal plasma		
	26	60	Age (weeks)	26	60	
	Total lipid µg/10^9 cells			Total lipid µg/ml plasma		
	574.6 ± 55.7	1122.4 ± 92.5	a	142.8 ± 15.0	1104.0 ± 233.5	b
Lipid class (% w/w of total lipid)						
Phospholipid	66.2 ± 2.0	72.1 ± 1.6	c	34.2 ± 2.4	28.4 ± 1.7	
Free cholesterol	18.5 ± 1.3	20.2 ± 1.5		19.9 ± 3.8	16.0 ± 1.4	
Free fatty acid	nd	4.0 ± 0.6	a	13.0 ± 2.6	14.3 ± 1.4	
Triacylglycerol	9.7 ± 1.6	2.2 ± 0.6	a	22.0 ± 3.8	20.1 ± 3.2	
Cholesterol ester	5.6 ± 1.0	2.4 ± 0.4	c	11.0 ± 2.1	20.8 ± 3.5	c

Values are means ± S.E. of 12 observations.
Significant differences between groups are illustrated with characters [a]P<0.001, [c]P<0.05.
nd represents not detectable.

Table 4.5a. Lipid composition of spermatozoa of the bulls.

	Holstein/Fresian					B. Blue
	Age (years)					
	2 - 3	5 - 6		greater than 9		2 - 3
Total lipid (μg/10^9 cells)	595.5 ± 42.5	654.3 ± 29.9		670.9 ± 99.2		644.9 ± 101.2
Lipid class (% w/w of total lipid)						
Phospholipid	74.3 ± 1.3	74.8 ± 2.2		62.8 ± 1.8	b	71.7 ± 3.2
Free cholesterol	16.7 ± 0.8	21.8 ± 0.2	b	19.6 ± 1.7		18.7 ± 2.4
Free fatty acid	2.4 ± 0.4	2.2 ± 0.5		4.5 ± 1.0		2.5 ± 1.0
Triacylglycerol	2.8 ± 0.6	2.8 ± 0.3		5.9 ± 3.0		2.0 ± 0.3
Cholesterol ester	3.1 ± 0.2	2.9 ± 0.3		3.2 ± 0.5		3.8 ± 0.9

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with character ^{b}P<0.01.

Table 4.5b. Lipid composition of seminal plasma of the bulls.

	Holstein/Fresian					B. Blue	
	Age (years)						
	2 - 3	5 - 6		greater than 9		2 - 3	
Total lipid (μg/ml plasma)	569.9 ± 63.2	333.3 ± 48.9	c	215.3 ± 44.9	b	330.7 ± 32.9	c
Lipid class (% w/w of total lipid)							
Phospholipid	39.2 ± 3.8	44.2 ± 1.2		47.0 ± 0.5		46.3 ± 2.0	
Free cholesterol	35.3 ± 0.6	34.5 ± 0.8		36.2 ± 2.0		35.1 ± 1.0	
Free fatty acid	6.4 ± 0.6	5.9 ± 1.4		5.6 ± 0.7		6.0 ± 0.9	
Triacylglycerol	9.4 ± 1.8	7.0 ± 0.9		4.7 ± 1.2		5.2 ± 0.7	
Cholesterol ester	8.0 ± 2.2	7.2 ± 0.1		5.3 ± 1.7		5.5 ± 0.8	

Values are means ± S.E. of 4 observations.
Significant differences compared to the young groups of bulls are illustrated with characters ^{b}P<0.01, ^{c}P<0.05.

Table 4.6. Major fatty acids of total phospholipid of spermatozoa and seminal plasma of the cockerels.

	Spermatozoa			Seminal plasma		
	26	60	Age (weeks)	26	60	
Fatty acids (% w/w of total fatty acid)						
16:0	13.4 ± 0.2	12.4 ± 0.7		16.5 ± 0.5	11.2 ± 1.2	b
18:0	18.0 ± 0.3	18.4 ± 0.5		17.6 ± 0.5	15.0 ± 1.5	
18:1 (n-7)	1.6 ± 0.1	1.6 ± 0.1		nd	nd	
18:1 (n-9)	10.2 ± 0.4	10.2 ± 0.4		14.6 ± 0.8	13.0 ± 1.4	
20:1 (n-9)	3.4 ± 0.1	3.6 ± 0.1		nd	nd	
18:2 (n-6)	1.9 ± 0.1	1.5 ± 0.2		8.7 ± 0.8	5.4 ± 0.7	b
20:4 (n-6)	11.2 ± 0.1	10.7 ± 0.2		14.9 ± 0.6	9.1 ± 1.0	a
22:4 (n-6)	19.7 ± 0.4	21.0 ± 0.6		9.6 ± 0.4	8.4 ± 1.3	
22:6 (n-3)	2.5 ± 0.2	3.7 ± 0.2	c	2.9 ± 0.6	2.7 ± 0.5	

Values are means ± S.E. of 12 observations.
Significant differences between groups are illustrated with characters [a]P<0.001, [b]P<0.01, [c]P<0.05.
nd represents not detectable.
All fatty acids of less than 1 % total lipid have been omitted.

Table 4.7a. Major fatty acids of total phospholipid of spermatozoa of the bulls.

	Holstein/Fresian			B. Blue
	Age (years)			
	2 - 3	5 - 6	greater than 9	2 - 3
Fatty acid (% w/w of total fatty acid)				
14:0	2.5 ± 0.1	2.5 ± 0.1	2.5 ± 0.6	1.8 ± 0.2
16:0	12.5 ± 0.1	11.7 ± 0.4	11.0 ± 1.7	11.6 ± 0.1
18:0	4.0 ± 0.2	3.9 ± 0.1	3.7 ± 0.4	4.2 ± 0.5
18:1 (n-7)	1.5 ± 0.1	1.4 ± 0.1	1.2 ± 0.1	2.0 ± 0.5
18:1 (n-9)	1.5 ± 0.2	1.1 ± 0.1	1.9 ± 0.8	1.5 ± 0.1
18:2 (n-6)	2.4 ± 0.2	2.7 ± 0.1	2.9 ± 0.9	4.5 ± 0.1
20:2 (n-6)	nd	1.1 ± 0.2	nd	nd
20:4 (n-6)	4.1 ± 0.1	3.6 ± 0.1	4.0 ± 0.2	4.8 ± 0.4
22:5 (n-3)	0.9 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.3 ± 0.2
22:6 (n-3)	69.1 ± 0.6	70.1 ± 1.0	67.3 ±2.3	65.7 ± 3.5

Values are means ± S.E. of 4 observations.
nd represents not detectable.
All fatty acids of less than 1 % total lipid have been omitted.

Table 4.7b. Major fatty acids of total phospholipid of seminal plasma of the bulls.

	Holstein/Fresian			B. Blue
	Age (years)			
	2 - 3	5 - 6	greater than 9	2 - 3
Fatty acid(% w/w of total fatty acid)				
14:0	2.3 ± 0.5	1.4 ± 0.2	2.2 ± 0.3	3.2 ± 1.1
16:0	20.2 ± 1.1	15.0 ± 1.0	20.1 ± 0.8	13.6 ± 0.2
18:0	4.6 ± 0.5	4.6 ± 0.4	6.0 ± 1.4	4.6 ± 0.5
16:1 (n-7)	nd	nd	nd	2.0 ± 0.3
18:1 (n-9)	nd	3.6 ± 0.3	5.9 ± 2.8	2.7 ± 1.1
18:2 (n-6)	4.7 ± 0.2	4.0 ± 0.4	4.9 ± 2.0	5.0 ± 2.1
20:2 (n-6)	nd	nd	nd	1.4 ± 0.1
20:4 (n-6)	1.9 ± 0.3	1.5 ± 0.1	1.6 ± 0.1	1.4 ± 0.2
22:5 (n-3)	1.2 ± 0.1	1.6 ± 0.1	nd	1.5 ± 0.6
22:6 (n-3)	57.9 ± 3.1	64.8 ± 1.6	54.6 ± 7.8	53.1 ± 4.8

Values are means ± S.E. of 4 observations.
nd represents not detectable.
All fatty acids of less than 1 % total lipid have been omitted.

Table 4.8. Major phospholipid classes of spermatozoa and seminal plasma of the cockerels.

	Spermatozoa			Seminal plasma		
	26	60	Age (weeks)	26	60	
Phospholipid class (% w/w of total phospholipid)						
Phosphatidyl ethonalomine	30.3 ± 0.9	17.2 ± 2.4	a	52.6 ± 2.0	34.6 ± 1.1	b
Phosphatidyl choline	31.7 ± 1.6	46.5 ± 2.0	a	9.8 ± 0.9	21.3 ± 2.5	b
Phosphatidyl serine	24.0 ± 1.8	5.9 ± 1.7	a	18.2 ± 1.8	14.7 ± 2.3	
Cardiolipin	nd	10.7 ± 1.9	a	nd	11.3 ± 1.0	a
Phosphatidyl inositol	3.6 ± 0.4	10.0 ± 1.8	b	nd	10.6 ± 1.8	b
Sphingomyelin	10.3 ± 1.3	9.6 ± 1.5		19.4 ± 1.7	7.5 ± 3.0	c

Values are means ± S.E. of 12 observations.
Significant differences between groups are illustrated with characters [a]P<0.001, [b]P<0.01, [c]P<0.05.
nd represents not detectable.

Table 4.9a. Major phospholipid classes of spermatozoa of the bulls.

	Holstein/Fresian			B. Blue
	Age (years)			
	2 - 3	5 - 6	greater than 9	2 - 3
Phospholipid class (% w/w of total phospholipid)				
Phosphatidyl choline	41.9 ± 1.3	48.9 ± 1.8 c	57.0 ± 0.4 a	53.4 ± 1.5 a
Phosphatidyl ethonalomine	28.6 ± 1.6	28.3 ± 0.7	21.0 ± 1.2 c	22.5 ± 0.6 b
Sphingomyelin	10.2 ± 1.3	13.7 ± 0.8	6.6 ± 3.3	10.9 ± 1.0
Cardiolipin	8.9 ± 0.9	6.9 ± 0.3	11.6 ± 0.4 c	8.3 ± 1.0
Phosphatidyl inositol	4.3 ± 0.5	2.3 ± 0.5 c	2.0 ± 0.5 b	4.1 ± 0.9
Phosphatidyl serine	<1.0	<1.0	<1.0	<1.0

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with characters $^{a}P<0.001$, $^{b}P<0.01$, $^{c}P<0.05$.

Table 4.9b. Major phospholipid classes of seminal plasma of the bulls.

	Holstein/Freisan			B. Blue
	Age (years)			
	2 - 3	5 - 6	greater than 9	2 - 3
Phospholipid class (% w/w of total phospholipid)				
Phosphatidyl choline	44.2 ± 1.3	53.8 ± 0.4 b	51.3 ± 1.6 c	44.2 ± 1.3
Phosphatidyl ethonalomine	28.7 ± 1.0	26.3 ± 0.7	22.5 ± 0.7 b	32.0 ± 1.4
Sphingomyelin	15.3 ± 1.9	13.7 ± 1.9	15.1 ± 0.5	12.9 ± 1.7
Cardiolipin	<1.0	<1.0	1.3 ± 0.6	1.2 ± 0.0
Phosphatidyl inositol	3.0 ± 0.8	2.3 ± 0.5	1.5 ± 0.3	2.8 ± 0.4
Phosphatidyl serine	6.3 ± 0.8	6.3 ± 1.4	8.6 ± 1.1	7.5 ± 0.8

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with characters $^{b}P<0.01$, $^{c}P<0.05$.

Table 4.10. Fatty acid composition of phosphatidyl ethanolamine and phosphatidyl choline in the spermatozoa of the cockerels.

	phosphatidyl ethanolamine			phosphatidyl choline		
	26	60	Age (weeks)	26	60	
Fatty acid (% w/w of total fatty acid)						
16:0	8.1 ± 1.0	9.7 ± 1.3		23.1 ± 0.8	21.4 ± 1.5	
18:0	14.7 ± 0.3	10.5 ± 1.6		32.0 ± 1.1	32.3 ± 1.2	
18:1 (n-9)	8.0 ± 0.2	10.5 ± 1.6		16.8 ± 0.8	13.9 ± 0.8	c
20:1 (n-9)	6.8 ± 0.2	4.2 ± 1.2		4.6 ± 0.2	4.7 ± 0.2	
20:4 (n-6)	28.9 ± 2.4	14.0 ± 0.9	b	9.7 ± 0.4	9.6 ± 0.6	
22:4 (n-6)	33.5 ± 3.0	23.1 ± 0.2	c	12.3 ± 0.2	15.2 ± 1.0	c
20:5 (n-3)	nd	6.2 ± 0.9	b	nd	nd	
22:6 (n-3)	nd	5.8 ± 1.4	c	nd	nd	

Values are means ± S.E. of 12 observations.
Significant differences between groups are illustrated with characters [b]P<0.01, [c]P<0.05.
nd represents not detectable.
all fatty acids of less than 1 % of total lipid have been omitted.

Table 4.11a. Fatty acid composition of phosphatidyl ethanolamine in the spermatozoa of the bulls.

	Holstein/Fresian					B. Blue	
	Age (years)						
	2 - 3	5 - 6		greater than 9		2 - 3	
Fatty acid (% w/w of total fatty acid							
16:0	8.6 ± 1.2	16.5 ± 2.9	c	13.8 ± 0.5	c	18.6 ± 1.4	b
18:0	14.8 ± 1.5	13.9 ± 1.6		12.9 ± 0.9		20.5 ± 0.9	c
18:1 (n-7)	3.4 ± 1.2	< 1		3.7 ± 0.3		nd	
18:1 (n-9)	4.3 ± 0.1	9.5 ± 1.7	c	10.9 ± 1.5	c	nd	
18:2 (n-6)	15.7 ± 1.2	14.0 ± 0.7		15.4 ± 0.4		nd	
20:4 (n-6)	15.0 ± 0.4	10.0 ± 0.8	a	9.9 ± 1.0	b	15.4 ± 1.5	
20:3 (n-3)	nd	nd		3.1 ± 0.1		nd	
22:6 (n-3)	47.3 ± 4.6	39.2 ± 2.4		30.9 ± 1.7	c	44.7 ± 1.4	

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with characters [a]P<0.001, [b]P<0.01, [c]P<0.05.
nd represents not detectable.
all fatty acids of less than 1 % of total lipid have been omitted.

Table 4.11b. Fatty acid composition of phosphatidyl choline in the spermatozoa of the bulls.

	Holstein/Fresian				B. Blue	
	Age (years)					
	2 - 3	5 - 6		greater than 9	2 - 3	
Fatty acid (% w/w of total fatty acid						
16:0	5.5 ± 0.4	8.4 ± 1.0	c	4.8 ± 0.4	13.8 ± 2.4	c
18:0	1.8 ± 0.7	3.7 ± 0.8		3.4 ± 1.4	5.2 ± 1.1	c
18:1 (n-7)	nd	nd		nd	2.2 ± 1.3	
18:1 (n-9)	< 1	5.2 ± 1.3		1.3 ± 0.2	4.3 ± 1.7	
22:5 (n-3)	1.2 ± 0.1	1.3 ± 0.1		1.8 ± 0.1	< 1	
22:6 (n-3)	91.1 ± 1.2	76.0 ± 3.8	b	78.4 ± 10.4	71.3 ± 5.1	c

Values are means ± S.E. of 4 observations.
Significant differences compared to the young group of bulls are illustrated with characters [b]P<0.01, [c]P<0.05.
nd reprents not detectable.
all fatty acids of less than 1 % of total lipid have been omitted.

Table 4.12. Vitamin A and E and trace element levels of spermatozoa and seminal plasma of the cockerels.

Micro element	Spermatozoa		Seminal plasma	
	Age (weeks)			
	26	60	26	60
	μg/10^9 cells		μg/ml	
vit E	0.1	0.1	0.1	0.7
vit A	< 0.001	< 0.001	< 0.001	< 0.001
Ca	9.0	15.5	240.0	163.2
P	367.4	337.5	120.0	120.0
Mg	< 0.001	< 0.001	< 0.001	< 0.001
K	60.2	22.0	1944.5	2208.8
Cu	0.4	0.5	1.4	1.4
Zn	0.3	3.4	23.0	1.4
Fe	1.1	4.1	8.2	19.2
Mn	0.1	0.2	0.7	2.4
Se	2.4	3.3	5.6	5.5

All results are the mean of 12 replicates measurements from a pooled sample (n = 12).

Table 4.13. Vitamin A and E and trace element levels of spermatozoa and seminal plasma of the bulls.

Micro element	Holstein/Fresian			B. Blue	Holstein/Fresian			B. Blue
	Age (years)							
	2 - 3	5 - 6	> 9	2 - 3	2 - 3	5 - 6	> 9	2 - 3
	μg/10^9 cells				μg/ml plasma			
Vitamin E	<0.001	<0.001	<0.002	<0.005	0.01	0.06	0.003	0.76
Vitamin A	<0.01	<0.01	<0.01	<0.01	<0.001	<0.001	<0.001	<0.001
Ca	21.2	18.3	7.1	29.6	7.2	9.4	13.9	2.3
P	257.8	131.1	236.5	202.4	26.4	6.5	10.7	31.6
Mg	10.2	6.7	4.7	9.1	7.0	2.9	2.7	2.8
K	189.7	101.9	52.0	137.7	66.0	54.3	83.3	126.1
Cu	0.2	0.3	0.3	0.3	0.01	0.02	0.02	0.03
Zn	1.9	1.5	2.0	2.7	0.2	0.1	0.1	1.0
Fe	0.7	0.7	1.1	4.9	0.01	0.02	0.04	0.15
Mn	0.1	0.1	0.1	0.1	<0.01	<0.01	<0.01	<0.01
Se	0.1	0.1	0.2	0.2	0.01	0.01	0.01	0.03

All results are a mean of 4 replicate measurements from a pooled sample (n = 4).

> represents greater than.

4.6 DISCUSSION

In general the characteristics, composition and specific lipid and fatty acid features of the spermatozoa and seminal plasma displayed by the young animals were similar to those which have been reported previously for normal, healthy cockerels (Darin-Bennett *et al.*, 1974 ; Ravie and Lake, 1985) and bulls (Pickett and Komarek, 1966 ; Poulos *et al.*, 1973b ; Neill and Masters, 1972). As with other animal species the lipids of spermatozoa and to a large extent those of the seminal plasma were dominated by polyunsaturated phospholipids consisting mainly of phosphatidyl ethanolamine and phosphatidyl choline, with the latter in the bull containing some 70 to 80 % 22:6 (n-3). Most animal species are characterised by the presence of C20 and C22 polyunsaturated fatty acids in spermatozoa and seminal plasma (Poulos *et al.*, 1973a ; Neill and Masters, 1972 ; Ravie and Lake, 1985 ; Darin-Bennett *et al.*, 1974 ; Nissen and Kreysel, 1983 ; Lin *et al.*, 1993). However, the major polyunsaturated fatty acids present in the cockerel were 20:4 (n-6) and 22:4 (n-6), in contrast to the bull where 22:6 (n-3) predominated, despite the presence of similar levels of fatty acid precursors within the lipid fraction of the diets of both species. The only other tissues which express such high levels of polyunsaturated fatty acids are the brain and retina where 22:6 (n-3) has been shown to play an essential role in development and function (Neuringer *et al.*, 1988). Studies on mammalian semen have shown that reductions in spermatozoa number, impaired spermatozoa motility and decreased fertility are associated with reduced levels of 22:6 (n-3) within the total phospholipid of the spermatozoa (Nissen and Kreysel, 1983).

The present results indicate that the proportion of C20 and C22 long chain polyunsaturated fatty acids were significantly reduced in the spermatozoa phospholipid classes as a consequence of ageing. In addition, ageing was associated with a change in the proportions of the different phospholipid classes, a feature that was also associated with the poorer quality Belgian Blue spermatozoa. In particular, the proportion of phosphatidyl ethanolamine, one of the major sources of C20 and C22 polyunsaturated fatty acids in the spermatozoa, was markedly reduced during ageing in the cockerel, being replaced by more highly saturated phosphatidyl choline. In the bull the changes in polyunsaturate levels were associated with the phosphatidyl ethanolamine and phosphatidyl choline of the spermatozoa ; levels of polyunsaturates in the phosphatidyl choline in the bull spermatozoa were equivalent to those of phosphatidyl ethanolamine in

the cockerel. However a similar decrease in phosphatidyl ethanolamine and increase in phosphatidyl choline, the major phospholipid classes, also occurred with ageing in the bull The detailed consequences of such changes in the polyunsaturated fatty acid composition of the phospholipid of the spermatozoa are not known but effects that include alterations in the biophysical properties of the membranes and impairment of leukotriene production, signal transduction and membrane fusion may be envisaged (Bearer and Friend, 1982 ; Oliw and Sprecher, 1989 ; Roldan and Harrison, 1993). The causes of such alterations in lipid composition with age are a matter of speculation. The cockerels and bulls were maintained on their respective diets throughout their reproductive periods and so deficiencies in dietary provision cannot be involved. One possibility is that the testicular enzymatic capacities for the synthesis of C20 and C22 polyunsaturated fatty acids from their shorter chain precursors and/or the incorporation of the fatty acid products into phospholipids during the differentiation of spermatozoa may decline with age and indeed differ between species and between certain breeds of the same species. A significant reduction in long chain fatty acid desaturase activity is known to be a feature of ageing (Brenner, 1989) as well as a reduction in the activity of the major antioxidant glutathione peroxidase (Griveau *et al.*, 1995). Alternatively the lower antioxidant protection capacity of the semen from older animals may result in enhanced peroxidative destruction of the important long chain polyunsaturated fatty acids (Nissen and Kreysel, 1983 : Aitken, 1994). The presence of pro-oxidants such as iron have been shown to enhance levels of peroxidation when added to spermatozoa. In this study this peroxidative effect was related to poorer spermatozoa motility and a reduction in associated oxidative capacities (Aitken *et al.*, 1993a). All older animals in the present study displayed higher levels of this trace element within both spermatozoa and seminal plasma, the presence of this element may, accompanied by the reduced antioxidant protection, enhance the peroxidative effects inflicted upon spermatozoa from such animals leading to poorer motility and loss of polyunsaturated fatty acid characteristics (Aitken, 1994 ; Aitken and Fisher, 1994 ; Griveau *et al.*, 1995).

In addition to reductions in spermatozoa concentration, the spermatozoa from older cockerels displayed very low rates of oxidative metabolism. *In vitro* parameters measured in the bull spermatozoa exhibited reduced motility during ageing in the Holstein/Fresian and in the young Belgian Blue breed. It is possible that changes in membrane fluidity due to a decreased degree of polyunsaturation, accompanied by

membrane damage resulting from an inadequate antioxidant protection, may result in loss of membrane integrity and overall cell function (Bearer and Friend, 1982 ; Sebastian *et al.*, 1987 ; Aitken, 1994). The spermatozoa from the younger cockerels contained neutral lipids, triacylglycerols and cholesterol ester. This potential energy source for the spermatozoa was however present at much lower proportions in the older semen samples despite the fact these older animals had significantly higher levels of total lipid in both their spermatozoa and seminal plasma. Sebastian *et al* (1987) reported higher levels of lipids present in the semen as a characteristic of poor fertility in oligospermic and azoospermic men. In this study the lipids of the seminal plasma contained much higher proportions of neutral lipids. Ageing in the cockerel appeared to reduce the ability of spermatozoa to utilise endogenous and exogenous lipid sources as an energy means for motility and viability, particularly as the amount of fructose is known to be very low in domestic avian semen (Hafez, 1993). In contrast, bull semen possesses high fructose levels for metabolism (Neil and Masters, 1972), reducing the need for lipid metabolism and therefore accounting for the absence of significant changes in spermatozoa lipid levels with age. However, the increasing use of lipid from the bull seminal plasma may indicate glycolytic dysfunction perhaps due to increased peroxidation with age and poor semen quality breeds (Mann and Lutwak-Mann, 1981 ; Gottlieb *et al*, 1995).

An interesting feature of the semen from both the cockerel and the bull was that the fatty acids of the total phospholipid showed no significant differences during the entire reproductive period ; it was only following separation of the total phosholipid into the various classes that differences in the levels of the major long chain polyunsaturated fatty acids were able to be highlighted. In the cockerel this was explained solely by changes within phosphatidyl ethanolamine and phosphatidyl choline, the major phospholipid classes of the phospholipid. In the bull the pattern of change also involved extensive differences within the phosphatidyl ethanolamine and phosphatidyl choline classes but again no significant affect on total phospholipid fatty acid composition. It may be that other changes also took place within other phospholipid classes with age, such as phosphatidyl serine which is known to be very high in long chain C20 to C22 polyunsaturated fatty acids (Salem *et al.*, 1986). Such alterations remain to be established, however changes in other phospholipid class fatty acid compositions may play a significant role in creating a balance of C20 to C22 polyunsaturated fatty acids which allow total phospholipid fatty acid profiles to remain unchanged. The importance

of maximum levels of phospholipid polyunsaturated fatty acids in relation to spermatozoa function and viability have been frequently reviewed (Nissen *et al.*, 1981 ; Nissen and Kreysel, 1983 ; Sebastian *et al.*, 1987).

The present results undoubtedly indicate that in both the spermatozoa and seminal plasma ageing is associated with a marked reduction in the proportions of C20 and C22 polyunsaturated fatty acids and in the antioxidant capacities of the spermatozoa as well as changes in a range of additional lipid compositional parameters. The nature of any causal relationships between these lipid associated changes and impairments of spermatozoa viability and fertility remain to be investigated. It may be envisaged that maintenance of maximum fertility potential would involve a combination of an optimal phospholipid fatty acid composition together with an appropriate sufficiency of antioxidant protection. In this respect it is significant that in the present study the major changes in the phospholipid composition and associated reduction in levels of polyunsaturated fatty acids with age and "poor" breed (Belgian Blue) were accompanied by a marked reduction in the activity of the antioxidant enzyme glutathione peroxidase.

4.7 SUMMARY

A summary of the major similarities and differences exhibited by both cockerel and bull semen are detailed below.

4.7.1 Common features associated with ageing in both species.

1 decreased spermatozoa concentration.
2 decrease in the activity of glutathione peroxidase enzyme.
3 little or no decrease in the activity of superoxide dismutase.
4 no major change in the fatty acid profile of the total phospholipid in the spermatozoa and seminal plasma.
5 decreased phosphatidyl ethanolamine in spermatozoa and seminal plasma.
6 increased phosphatidyl choline in spermatozoa and seminal plasma.
7 increased cardiolipin levels in spermatozoa and seminal plasma.
8 decreased levels of C20 and C22 polyunsaturated fatty acids within the phosphatidyl ethanolamine fraction.

8 decreased levels of C20 and C22 polyunsaturated fatty acids within the phosphatidyl ethanolamine fraction.
9 higher levels of copper, zinc and iron in spermatozoa of older animals.

4.7.2 Differences between cockerels and bull during reproductive period.

1 in the cockerels total lipid content of spermatozoa and seminal plasma increases with age.
2 in the bull lipid levels showed a decrease in seminal plasma with no change within the spermatozoa.
3 in cockerels the major polyunsaturated fatty acid was 22:4 (n-6)
4 in the bull the major polyunsaturated fatty acid was 22:6 (n-3).
5 cockerels exhibit significant levels of phosphatidyl serine which bulls do not.
6 in the cockerel 22:4 (n-6) decreased with age in the phosphatidyl ethanolamine fraction but not the phosphatidyl choline.
7 in the bull 22:6 (n-3) decreased with age in both the phosphatidyl ethanolamine and the phosphatidyl choline phospholipid classes.

CHAPTER 5

COMPARISON OF LIPID COMPOSITION AND ANTIOXIDANT CAPACITY BETWEEN COCKERELS EXHIBITING HIGH AND LOW SEMEN QUALITY PARAMETERS

5.1 INTRODUCTION

The quality of semen from individuals of the same species and even the same breed can vary considerably in terms of spermatozoa concentration, motility and ultimate fertilising ability (Cherms, 1968 ; Ansah and Buckland, 1982 ; Rao and Bane, 1985 ; Van Krey, 1990). These important semen parameters have been reported to be associated with the lipid composition (Sebastian *et al.*, 1987) and antioxidant capacities (McLaughlin *et al.*, 1992 ; Aitken, 1994) of spermatozoa and seminal plasma. Sebastian *et al.* (1987) correlated spermatozoa and seminal plasma lipid levels in the human with spermatozoa concentration and fertility. Earlier work, again in humans, correlated levels of 22:6 (n-3) in spermatozoa with conçentration and motility (Nissen and Kreysel, 1981). The importance of n-6 fatty acids in fowl fertility have also been discussed (Watkins, 1995). However, the literature available has as yet not related detailed lipid parameters in domestic avians to semen quality and overall fertility.

The present investigations were carried out to investigate the following features of cockerels displaying high and low semen quality parameters ;

i. semen characteristics and fertility.

ii differences in lipid composition within spermatozoa and seminal plasma.

iii changes in antioxidant levels in spermatozoa and seminal plasma.

5.2 MATERIALS AND METHODS

Full details of all procedures involved are given in the relevant Sections of Chapter 3. Further relevant details are as follows ;

5.2.1. The cockerels

Two groups of Cobb broiler breeder males each containing 15 cockerels were used. Males were purchased from Morini Brothers, Firenze, Italy. The males were allocated into respective groups on the basis of spermatozoa concentration and motility as assessed by CellSoft (see Section 3.11) from a larger group of cockerels of variable semen quality, when males were 23 weeks of age. The results obtained which allowed

the distinction to be made between cockerels of high and low semen quality were similar to those obtained and reported in Table 5.3a for cockerels at 24 weeks of age.

Cockerels were housed at the University of Milan, Italy and semen characteristics were assessed at this Institute, however all other lipid analyses were performed at SAC, Auchincruive after lipid extraction at the University of Milan and appropriate transportation of the samples.

5.2.2 Diets

Birds were fed 130g per day of a standard commercial broiler breeder diet containing 12.5% crude protein, 9% lipid, 11.5 MJ/kg metabolisable energy and 40 mg/kg of vitamin E. The fatty acid composition of the diet is shown in Table 5.1. The feed was analysed routinely throughout the experiment to ensure the maintenance of the fatty acid composition. All feeds were stored in air tight containers to avoid peroxidative deterioration.

Table 5.1. Fatty acid composition of the diet.

Fatty acids	% (w/w of total fatty acid)
16:0	12.0
18:0	4.0
18:1 (n-9)	23.0
18:2 (n-6)	50.0
18:3 (n-3)	6.0

All fatty acids of less than 1% total lipid have been omitted.

5.2.3 Housing

All cockerels were housed in individual cages with 13 hours of light supplied per day and an environmental temperature maintained at 18 °C.

5.2.4 Semen collection

Cockerels were trained for approximately 3 weeks and then milked regularly from 21 weeks of age twice weekly and thrice weekly during periods of semen collection for analysis. Collection periods were organised to meet peaks of reproductive performance as predetermined according to the data provided by the supplier (Table 5.2).

Table 5.2. Collection periods.

Collection period	Stage of reproductive period	Age (weeks)
1	beginning	23 - 25
2	peak	38 - 40
3	decreasing	53 - 55
4	end	71 - 73

Period 4 was included as fertility of the breed was still considered by the supplier to be relatively high at 50 weeks of age.

5.2.5 Artificial insemination

Pooled samples of three ejaculates, five replicates from each group of cockerels were used to inseminate Warren laying hens intravaginally with a single fixed insemination dose of 70 x 10^7 spermatozoa. Warren laying hens were purchased at the same time and were of the same age as the cockerels and housed under the same environmental and dietary conditions. At each of the four ages studied the hens were randomised to receive semen from either the high or low fertility cockerels. During this study it was not possible to use hens at peak fertility by replacing them for each insemination experiment. The same group of hens was therefore used during the entire experimental period. Fertilised eggs were collected over a 7 day period and incubated. The eggs were incubated at 37 °C and 60 % relative humidity with automatic egg turning. Candling identified the proportion of eggs exhibiting embryonic development, only those containing no embryos were classed as infertile. This procedure was then repeated for another 7 days to establish the fertility of the eggs two weeks after the single fixed insemination.

5.2.6 Spermatozoa evaluation and analysis

Spermatozoa quality parameters were measured at 24, 39, 54 and 72 weeks of age. Pooled samples of 3 ejaculates, 5 replicates per group, were analysed. All measurements were performed as described previously (see Sections 3.1, 3.2, 3.7, 3.8, 3.9, 3.10, 3.11). No antioxidant measurements were performed at 24 weeks of age due to the lower amount of semen available for the analyses. Adequate volumes of semen became available after 24 weeks of age and so it was decided to wait until peak fertility, 39 weeks of age, to perform the measurements for group comparison. The fatty acid profiles of the major phospholipid classes within the seminal plasma of the cockerels was unable to be performed accurately due to the lack of seminal plasma volume after semen separation. It was also felt that these results were less important than those which were involved in the phospholipid structure of the spermatozoa themselves.

5.2.7 Statistical analysis

Students *t-test* was used for statistical comparisons and correlation coefficients were determined according to standard procedures. Data included 5 replicates per group at each collection period for lipid analyses with 15 replicates per group at each collection period for semen volume, concentration and motility. 5 replicates within each week of the egg collections were used to assess fertility rate. Statistical analysis and correlations was undertaken using Microsoft Excel version 5.0.

In this study antioxidant activities are specified to two decimal places to emphasise the values obtained which were quite low and/or displayed small standard errors. All other measurements were specified to only one decimal place for comparison purposes.

5.3 RESULTS

5.3.1 Changes in semen characteristics

The characteristics of the semen samples from the groups of cockerels designated to be of high and low semen quality are shown in Tables 5.3a to 5.3d.

Comparison of high and low semen quality males

The concentration of spermatozoa was greater in the high semen quality cockerels from 54 to 72 weeks of age. The percentage of motile spermatozoa and forward motility were also greater in the high semen quality cockerels. Within the seminal plasma the high semen quality cockerels showed consistently higher levels of glutathione peroxidase activity throughout the reproductive period particularly at 72 weeks of age although the opposite trend occurred within the spermatozoa. Superoxide dismutase activity appeared to be lower in the cockerels displaying high semen quality, only at 72 weeks of age did this trend alter with the high semen quality group displaying higher superoxide dismutase activity within seminal plasma. *In vivo* fertility levels in weeks 1 and 2 were both consistently higher in the high semen quality group throughout the reproductive period, although only significantly so for overall fertility at 24 weeks of age.

The antioxidant enzyme levels recorded during this study differed considerably with those previously reported in Chapter 4. Although such analyses were performed on different breeds of cockerel and at different Institutes all procedures were performed according to the kit manufacturers instructions as specified in Section 3.2. No explanation can be offered although a similar pattern of change during the reproductive period was recorded. Different methods of spermatozoa concentration assessment were also used which may explain the higher levels reported during this study.

5.3.2 Lipid compositions of the spermatozoa and seminal plasma.

The proportions of the major lipid classes present within the spermatozoa are shown in Tables 5.4a to 5.4d and for seminal plasma, Tables 5.5a to 5.5d.

The major lipid class in both groups of cockerels within spermatozoa and seminal plasma was phospholipid followed by substantial levels of free cholesterol and cholesterol ester, this latter lipid class being considerably higher within the seminal plasma. Lower levels of triacylglycerol and free fatty acids were observed in both semen fractions.

Comparison of high and low semen quality males

No significant differences were observed in the lipid compositions of the spermatozoa between the two groups of cockerels. However, within the seminal plasma, total lipid levels were significantly higher at 39 weeks of age in the cockerels of lower semen quality. At 39 weeks this group also exhibited reduced levels of triacylglycerol. At 54 weeks of age the situation was reversed with the cockerels of low semen quality displaying a significant increase in triacylglycerol concentration and a lower level of free fatty acid.

5.3.3 Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma

The fatty acid composition of the total phospholipid for the spermatozoa and seminal plasma of the cockerels are given in Tables 5.6a to 5.6d and Tables 5.7a to 5.7d respectively.

The major saturated fatty acids present within the total phospholipid of both spermatozoa and seminal plasma for both groups of cockerels were 16:0 and 18:0 with 18:1 (n-9) being the major monounsaturated fatty acid. The major polyunsaturated fatty acids were 20:4 (n-6) and 22:4 (n-6). Levels of 22:6 (n-3) were low within both spermatozoa and seminal plasma.

Comparisons of high and low semen quality cockerels

Within the spermatozoa of the high semen quality cockerels the only major difference was a small increase in the level of n-3 fatty acids accompanied by a reduction in the n-6/n-3 ratio, at 54 weeks of age. This was in contrast to the seminal plasma in which more differences were observed from 54 to 72 weeks of age. In this case cockerels of high semen quality displayed lower levels of n-6, 18:2, 20:4 and 22:4, fatty acids with an

associated reduction in the n-6/n-3 ratio at 54 and 72 weeks of age. At 72 weeks of age the high semen quality cockerels showed significantly higher levels of 22:5 (n-3).

5.3.4 Major phospholipid classes of the spermatozoa and seminal plasma

The major phospholipid classes within the spermatozoa and seminal plasma of the cockerels are given in Tables 5.8a to 5.8d and Tables 5.9a to 5.9d respectively.

Phosphatidyl ethanolamine and phosphatidyl choline were the major phospholipid classes within both spermatozoa and seminal plasma for both groups of cockerels with substantial levels of phosphatidyl serine and sphingomyelin. Cardiolipin was present only at lower levels.

Comparison of high and low semen quality cockerels

Within both the spermatozoa and seminal plasma few differences were exhibited between the two groups of cockerels. At 24 weeks of age high semen quality cockerels displayed significantly higher levels of sphingomyelin within both spermatozoa and seminal plasma, however from 54 weeks levels were similar in both groups. Within the seminal plasma of high semen quality cockerels greater levels of cardiolipin were displayed throughout the experimental period, this difference was significant at 54 weeks of age.

5.3.5 Fatty acid composition of the phosphatidyl ethanolamine and phosphatidyl choline of the spermatozoa

The major saturated fatty acids present within phosphatidyl ethanolamine and phosphatidyl choline were 16:0 and 18:0 with 18:1 (n-9) being the major monounsaturate. The major polyunsaturated fatty acids within both phosholipid classes were 20:4 (n-6) and 22:4 (n-6) although phosphatidyl choline displayed a more saturated fatty acid composition with polyunsaturated fatty acids present at less than 25 % within both groups of cockerels.

Comparison of high and low semen quality cockerels

Phosphatidyl ethanolamine

The fatty acid composition of phosphatidyl ethanolamine at 24 and 39 weeks of age displayed few differences between cockerels of high and low semen quality apart from a higher level of 16:0 in high semen quality cockerels at 24 weeks of age. Levels of the major polyunsaturated fatty acids, 20:4 (n-6) and 22:4 (n-6) were very similar between groups at these two collection periods. However, from 54 weeks of age significant differences in almost all fatty acids were observed. High semen quality cockerels displayed lower saturated fatty acids, 16:0 and 18:0, accompanied by significantly higher P/S ratios. The levels of the major polyunsaturated fatty acids 20:4 (n-6) and 22:4 (n-6) were more than double in the high semen quality cockerels at 54 weeks. At 72 weeks levels were still higher although less dramatically so. The levels of n-3 fatty acids at 54 weeks were also considerably higher in the high semen quality cockerels. In particular the level of 22:6 (n-3) was not detectable in the low semen quality cockerels compared to over 7 % in the other group.

Phosphatidyl choline

The fatty acid composition of the phosphatidyl choline displayed only a few differences between the two groups of cockerels. At 39 weeks cockerels of high semen quality showed no detectable levels of minor polyunsaturated fatty acids 20:3 (n-6) and 22:6 (n-3) although small levels were displayed by the other group. At 72 weeks of age small differences in 18:1 (n-9) and 18:2 (n-6) were detected although again no differences in major fatty acid composition was observed.

5.3.6 Correlations between age, lipid parameters and fertility

Correlation coefficients between age, lipid parameters and fertility are shown in Tables 5.12a to 5.12d

As can be seen from Table 5.12a age was negatively correlated with all semen parameters, including antioxidant levels within both spermatozoa and seminal plasma. Glutathione peroxidase was positively correlated with spermatozoa concentration.

whereas superoxide dismutase was correlated with motility, although the correlation was negative for overall and forward motility levels.

From Table 5.12b it can be seen that there was a negative correlation between age and phospholipid levels in spermatozoa and a positive correlation between age and the minor lipid classes of spermatozoa. The total lipid levels in the seminal plasma were positively correlated with age.

Table 5.12c shows that ageing negatively correlates with major semen characteristics, long chain polyunsaturated fatty acid levels and P/S ratios. The long chain polyunsaturated fatty acids within the phospholipid of the spermatozoa phospholipids correlated positively with concentration, motility and overall fertility. There was a negative correlation of these features with levels of saturated fatty acids.

The phospholipid classes and their correlations to semen characteristics are shown in Table 5.12d. Almost all phospholipid classes of spermatozoa and seminal plasma correlate negatively with ageing except for cardiolipin within the seminal plasma which exhibited a positive relationship. Phosphatidyl ethanolamine and phosphatidyl serine show positive relationships with concentration and motility, whereas phosphatidyl choline and sphingomyelin displayed the opposite effect. Phosphatidyl choline levels within the spermatozoa showed a negative correlation with *in vivo* fertility at week 1.

Table 5.3a. Semen characteristics at 24 weeks of age.

		Semen quality high		low
Semen concentration	10^9 cells/ml	4.2 ± 0.3		4.8 ± 0.6
Motile	%	69.1 ± 3.1	a	35.4 ± 3.1
Forward motility	%	1.4 ± 0.1		1.4 ± 0.1
Fertility (overall)	%	69.2 ± 5.8	c	51.6 ± 2.9
Fertility (week 1)	%	51.8 ± 6.2		48.0 ± 4.4
Fertility (week 2)	%	60.5 ± 5.0		49.6 ± 2.6

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between high and low semen quality groups : [a]P< 0.001 ; [c]P<0.05.

Table 5.3b. Semen characteristics at 39 weeks of age.

		Semen quality high	low
Semen concentration	10^9 cells/ml	6.0 ± 0.6	7.2 ± 0.5
Motile	%	61.5 ± 3.9	51.4 ± 4.3
Forward motility	%	1.6 ± 0.1	1.6 ± 0.1
Glutathione peroxidase	units/10^9 cells	0.36 ± 0.09	0.49 ± 0.01
Glutathione peroxidase	units/ml plasma	0.27 ± 0.03	0.24 ± 0.01
Superoxide dismutase	units/10^9 cells	0.80 ± 0.05	0.88 ± 0.06
Superoxide dismutase	units/ml plasma	0.77 ± 0.08	1.11 ± 0.20
Fertility (overall)	%	69.8 ± 7.1	59.8 ± 9.0
Fertility (week 1)	%	80.9 ± 5.6	72.4 ± 12.3
Fertility (week 2)	%	58.7 ± 11.6	47.3 ± 10.5

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group.

Table 5.3c. Semen characteristics at 54 weeks of age.

		Semen quality		
		high		low
Semen concentration	10^9 cells/ml	6.9 ± 0.4		5.9 ± 0.5
Motile	%	40.1 ± 4.0		32.3 ± 3.3
Forward motility	%	1.4 ± 0.1		1.7 ± 0.1
Glutathione peroxidase	units/10^9 cells	0.25 ± 0.04	c	0.40 ± 0.05
Glutathione peroxidase	units/ml plasma	0.24 ± 0.02		0.18 ± 0.03
Superoxide dismutase	units/10^9 cells	0.50 ± 0.04		0.49 ± 0.03
Superoxide dismutase	units/ml plasma	1.45 ± 0.05	c	2.19 ± 0.26
Fertility (overall)	%	60.2 ± 7.3		44.2 ± 8.7
Fertility (week 1)	%	76.8 ± 4.0		61.9 ± 10.3
Fertility (week 2)	%	43.5 ± 9.0		26.5 ± 8.9

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between high and low semen quality groups : [c]$P<0.05$.

Table 5.3d. Semen characteristics at 72 weeks of age.

		Semen quality		
		high		low
Semen concentration	10^9 cells/ml	2.8 ± 0.5	c	1.6 ± 0.3
Motile	%	41.8 ± 2.9		49.6 ± 4.6
Forward motility	%	2.8 ± 0.5	c	1.6 ± 0.3
Glutathione peroxidase	units/10^9 cells	0.19 ± 0.02		0.29 ± 0.05
Glutathione peroxidase	units/ml plasma	0.20 ± 0.05	c	0.08 ± 0.02
Superoxide dismutase	units/10^9 cells	0.40 ± 0.09		0.56 ± 0.13
Superoxide dismutase	units/ml plasma	1.96 ± 0.80		1.43 ± 0.22
Fertility (overall)	%	49.6 ± 9.8		47.4 ± 5.9
Fertility (week 1)	%	65.1 ± 9.2		56.8 ± 8.9
Fertility (week 2)	%	34.0 ± 6.6		38.0 ± 5.8

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between high and low semen quality groups : [c]$P<0.05$.

Table 5.4a. Lipid composition of spermatozoa at 24 weeks of age.

	Semen quality	
	high	low
Total lipid (μg/10 ^{9} cells)	384.5 ± 68.5	243.5 ± 37.7
Lipid class (% w/w of total lipid)		
Phospholipid	70.2 ± 1.2	64.1 ± 4.0
Free cholesterol	18.0 ± 0.8	22.1 ± 3.1
Free fatty acid	4.7 ± 0.6	5.4 ± 0.5
Triacylglycerol	2.7 ± 0.6	2.8 ± 0.8
Cholesterol ester	4.4 ± 1.5	5.6 ± 0.8

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.4b. Lipid composition of spermatozoa at 39 weeks of age.

	Semen quality	
	high	low
Total lipid (μg/10 ^{9} cells)	409.0 ± 65.8	265.4 ± 30.0
Lipid class (% w/w of total lipid)		
Phospholipid	65.2 ± 3.3	68.6 ±2.9
Free cholesterol	13.8 ±2.2	16.2 ± 0.9
Free fatty acid	7.3 ±1.5	5.3 ± 0.9
Triacylglycerol	4.6 ± 1.7	1.8 ±0.4
Cholesterol ester	9.0 ± 2.7	8.0 ±1.4

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.4c. Lipid composition of spermatozoa at 54 weeks of age.

	Semen quality	
	high	low
Total lipid (μg/10 ^{9} cells)	350.5 ± 39.0	300.0 ± 75.7
Lipid class (% w/w of total lipid)		
Phospholipid	58.0 ± 2.0	55.0 ± 4.7
Free cholesterol	18.0 ± 2.2	21.2 ± 0.9
Free fatty acid	7.8 ± 1.2	8.8 ± 1.7
Triacylglycerol	5.9 ± 1.8	4.4 ± 0.9
Cholesterol ester	10.3 ± 2.3	9.1 ± 0.8

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.4d. Lipid composition of spermatozoa at 72 weeks of age.

	Semen quality	
	high	low
Total lipid (μg/10 ^{9} cells)	468.5 ± 73.1	577.1 ± 63.8
Lipid class (% w/w of total lipid)		
Phospholipid	47.6 ± 5.0	52.2 ± 4.6
Free cholesterol	16.5 ± 2.3	18.0 ± 2.4
Free fatty acid	11.9 ± 1.1	10.6 ± 2.7
Triacylglycerol	7.6 ± 2.6	5.3 ± 1.6
Cholesterol ester	16.4 ± 3.8	13.9 ± 2.7

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.5a. Lipid composition of seminal plasma at 24 weeks of age.

	Semen quality high		Semen quality low
Total lipid (μg/ml plasma)	464.5 ± 81.9		474.7 ± 111.9
Lipid class (% w/w of total lipid)			
Phospholipid	28.3 ± 5.7		31.0 ± 3.1
Free cholesterol	18.9 ± 3.2		22.1 ± 1.7
Free fatty acid	21.0 ± 4.6		21.9 ± 1.1
Triacylglycerol	9.3 ± 3.3		3.8 ± 0.8
Cholesterol ester	22.3 ± 2.9		21.1 ± 3.7

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.5b. Lipid composition of seminal plasma at 39 weeks of age.

	Semen quality high		Semen quality low
Total lipid (μg/ml plasma)	581.5 ± 40.3	b	1 000 ± 115.7
Lipid class (% w/w of total lipid)			
Phospholipid	31.1 ±1.9		40.6 ± 4.5
Free cholesterol	24.8 ±2.0		19.4 ± 2.3
Free fatty acid	11.1 ±5.7		13.8 ±1.3
Triacylglycerol	6.0 ±1.0	b	1.5 ± 0.1
Cholesterol ester	27.1 ± 4.1		24.7 ± 6.8

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : $^{b}P<0.01$.

Table 5.5c. Lipid composition of seminal plasma at 54 weeks of age.

	Semen quality		
	high		low
Total lipid (μg/ml plasma)	736.1 ± 187.1		698.4 ± 55.8
Lipid class (% w/w of total lipid)			
Phospholipid	24.2 ± 6.1		31.1 ± 2.5
Free cholesterol	20.5 ± 1.9		17.3 ± 1.2
Free fatty acid	20.7 ± 1.1	b	13.5 ± 1.3
Triacylglycerol	3.9 ± 0.9	b	13.0 ± 1.5
Cholesterol ester	35.8 ± 5.7		25.1 ± 3.0

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : ^{b}P<0.01.

Table 5.5d. Lipid composition of seminal plasma at 72 weeks of age.

	Semen quality	
	high	low
Total lipid (μg/ml plasma)	935.4 ± 244.4	1 231 ± 213.9
Lipid class (% w/w of total lipid)		
Phospholipid	18.1 ± 1.7	24.1 ± 2.2
Free cholesterol	12.2 ± 5.5	9.2 ± 6.5
Free fatty acid	32.3 ± 3.8	27.4 ± 5.9
Triacylglycerol	5.1 ± 1.1	16.2 ± 7.6
Cholesterol ester	32.2 ± 1.1	23.1 ± 4.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.6a. Major fatty acids of the total phospholipid of the spermatozoa at 24 weeks of age.

	Semen quality	
	high	low
Fatty acid (% w/w of total fatty acid)		
16:0	14.5 ± 0.8	14.3 ± 0.5
18:0	18.9 ± 0.9	20.3 ± 6.3
18:1 (n-9)	13.3 ± 0.8	13.7 ± 0.8
18:2 (n-6)	2.4 ± 0.1	2.4 ± 0.1
20:4 (n-6)	11.7 ± 0.6	12.2 ± 0.1
22:4 (n-6)	22.0 ± 1.0	22.8 ± 1.0
18:3 (n-3)	< 1.0	< 1.0
22:5 (n-3)	1.1 ± 0.2	2.6 ± 1.2
22:6 (n-3)	2.2 ± 0.1	2.3 ± 0.1
n-6/n-3	12.2 ± 0.8	9.6 ± 2.0
P/S	1.2 ± 0.1	1.3 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.6b. Major fatty acids of the total phospholipid of the spermatozoa 39 weeks of age.

	Semen quality		
	high		low
Fatty acid (% w/w of total fatty acid)			
16:0	14.6 ± 0.5		13.3 ± 0.4
18:0	19.7 ± 0.1		19.4 ± 0.1
18:1 (n-9)	12.9 ± 0.3		12.7 ± 0.4
18:2 (n-6)	3.3 ± 0.2		3.5 ± 0.2
20:4 (n-6)	12.3 ± 0.1		11.6 ± 0.5
22:4 (n-6)	23.8 ± 0.5		23.6 ± 0.7
18:3 (n-3)	< 1.0		< 1.0
22:5 (n-3)	< 1.0		2.0 ± 0.6
22:6 (n-3)	2.1 ± 0.2		2.0 ± 0.1
n-6/n-3	13.7 ± 1.2	c	9.5 ± 1.2
P/S	1.3 ± 0.2		1.4 ± 0.1

Values are means ± S.E. observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : $^{c}P<0.05$.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.6c. Major fatty acids of the total phospholipid of the spermatozoa at 54 weeks of age.

	Semen quality		
	high		low
Fatty acid (% w/w of total fatty acid)			
16:0	12.8 ± 0.7		16.7 ± 2.7
18:0	19.4 ± 0.7		24.1 ± 2.9
18:1 (n-9)	11.1 ± 0.8	c	15.5 ± 1.1
18:2 (n-6)	5.0 ± 0.7		4.0 ± 0.5
20:4 (n-6)	9.9 ± 1.1		10.2 ± 1.4
22:4 (n-6)	18.8 ± 2.3		18.8 ± 2.8
18:3 (n-3)	1.3 ± 0.7	a	nd
22:5 (n-3)	< 1.0		nd
22:6 (n-3)	2.0 ± 0.2		1.8 ± 0.1
n-6/n-3	10.0 ± 1.8	b	21.3 ± 0.7
P/S	1.1 ± 0.1		1.2 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.6d. Major fatty acids of the total phospholipid of the spermatozoa at 72 weeks of age

	Semen quality	
	high	low
Fatty acid (% w/w of total fatty acid)		
16:0	19.7 ± 2.2	16.5 ± 0.9
18:0	23.1 ± 2.4	20.4 ± 0.4
18:1 (n-9)	18.8 ± 1.6	17.5 ± 1.6
18:2 (n-6)	5.4 ± 1.1	6.5 ± 1.1
20:4 (n-6)	9.5 ± 1.6	11.7 ± 0.8
22:4 (n-6)	14.6 ± 2.4	15.8 ± 2.3
18:3 (n-3)	< 1.0	< 1.0
22:5 (n-3)	< 1.0	< 1.0
22:6 (n-3)	1.5 ± 0.1	1.2 ± 0.4
n-6/n-3	19.8 ± 4.3	15.5 ± 1.0
P/S	0.8 ± 0.2	1.0 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.7a. Major fatty acids of the total phospholipid of the seminal plasma at 24 weeks of age.

	Semen quality	
	high	low
Fatty acid (% w/w of total fatty acid)		
16:0	11.4 ± 0.8	14.1 ± 1.5
18:0	15.1 ± 2.2	19.8 ± 1.2
18:1 (n-9)	10.2 ± 1.6	14.1 ± 1.8
18:2 (n-6)	8.1 ± 1.1	9.8 ± 1.3
20:4 (n-6)	9.0 ± 1.5	11.3 ± 1.3
22:4 (n-6)	8.2 ± 1.1	7.9 ± 2.5
18:3 (n-3)	< 1.0	1.7 ± 1.4
22:5 (n-3)	2.7 ± 1.8	2.7 ± 1.7
22:6 (n-3)	1.1 ± 0.5	1.6 ± 0.4
n-6/n-3	3.1 ± 1.5	5.3 ± 1.9
P/S	1.9 ± 0.5	1.1 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.7b. Major fatty acids of the total phospholipid of the seminal plasma at 39 weeks of age.

	Semen quality	
	high	low
Fatty acid (% w/w of total fatty acid)		
16:0	13.4 ± 0.4	10.8 ± 0.5
18:0	19.7 ± 0.6	18.4 ± 0.4
18:1 (n-9)	11.3 ± 0.7	10.4 ± 0.6
18:2 (n-6)	8.5 ± 1.4	7.6 ± 0.5
20:4 (n-6)	13.3 ± 1.0	11.8 ± 0.6
22:4 (n-6)	11.2 ± 3.1	14.9 ± 0.7
18:3 (n-3)	nd	nd
20:5 (n-3)	< 1.0	< 1.0
22:3 (n-3)	nd	nd
22:5 (n-3)	< 1.0	< 1.0
22:6 (n-3)	2.3 ± 0.3	1.9 ± 0.2
n-6/n-3	11.5 ± 1.3	13.6 ± 4.2
P/S	1.1 ± 0.1	1.0 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.7c. Major fatty acids of the total phospholipid of the seminal plasma at 54 weeks of age.

	Semen quality		
	high		low
Fatty acid (% w/w of total fatty acid)			
16:0	20.6 ± 3.2		16.1 ± 1.0
18:0	27.9 ± 3.8		21.0 ± 1.5
18:1 (n-9)	15.9 ± 2.9		18.0 ± 2.0
18:2 (n-6)	7.1 ± 0.8	c	9.8 ± 0.6
20:4 (n-6)	5.2 ± 0.7	c	8.3 ± 1.0
22:4 (n-6)	3.0 ± 0.8	c	6.9 ± 0.9
18:3 (n-3)	< 1.0		< 1.0
20:5 (n-3)	2.2 ± 1.7		< 1.0
22:6 (n-3)	< 1.0		1.9 ± 0.2
n-6/n-3	4.1 ± 2.2	c	11.1 ± 1.5
P/S	0.5 ± 0.2		0.7 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.7d. Major fatty acids of the total phospholipid of the seminal plasma at 72 weeks of age.

	Semen quality		
	high		low
Fatty acid (% w/w of total fatty acid)			
16:0	21.6 ± 1.6	b	14.2 ± 1.1
18:0	24.5 ± 3.3		20.6 ± 2.3
18:1 (n-9)	23.4 ± 2.1		23.7 ± 2.7
18:2 (n-6)	11.6 ± 2.2		17.4 ± 1.7
20:4 (n-6)	5.1 ± 0.7	c	9.0 ± 1.4
22:4 (n-6)	2.7 ± 0.6		5.3 ± 1.5
18:3 (n-3)	< 1.0		< 1.0
20:5 (n-3)	< 1.0		< 1.0
22:5 (n-3)	4.3 ± 0.4	c	2.6 ± 0.5
22:6 (n-3)	< 1.0		< 1.0
n-6/n-3	4.2 ± 1.0		9.1 ± 2.2
P/S	0.5 ± 0.1	b	1.1 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [b]P<0.01 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.8a. Phospholipid classes of spermatozoa at 24 weeks of age.

	Semen quality		
	high		low
Phospholipid fraction (% w/w of total phospholipid)			
Phosphatidyl ethanolamine	29.1 ± 1.1		33.2 ± 2.0
Phosphatidyl choline	32.5 ± 1.0		32.8 ± 1.6
Phosphatidyl serine	20.1 ± 1.1		19.3 ± 0.1
Sphingomyelin	14.1 ± 0.8	b	9.0 ± 1.0
Cardiolipin	4.2 ± 0.7		5.7 ± 0.8

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds. Significance of difference between high and low semen quality groups : $^{b}P<0.01$.

Table 5.8b. Phospholipid classes of spermatozoa at 39 weeks of age.

	Semen quality	
	high	low
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	30.3 ± 1.1	33.3 ± 1.9
Phosphatidyl choline	28.2 ± 0.7	27.0 ± 1.8
Phosphatidyl serine	26.3 ± 0.7	25.7 ± 0.6
Sphingomyelin	11.0 ± 1.0	8.5 ± 0.9
Cardiolipin	4.2 ± 0.2	5.4 ± 0.7

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.8c. Phospholipid classes of spermatozoa at 54 weeks of age.

	Semen quality	
	high	low
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	33.2 ± 0.6	35.8 ± 1.5
Phosphatidyl choline	32.2 ± 1.0	30.1 ± 2.3
Phosphatidyl serine	20.3 ± 0.4	19.9 ± 1.0
Sphingomyelin	9.1 ± 0.7	8.1 ± 0.2
Cardiolipin	5.2 ± 0.3	6.0 ± 0.4

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.8d. Phospholipid classes of spermatozoa at 72 weeks of age.

	Semen quality	
	high	low
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	29.7 ± 2.0	25.1 ± 2.6
Phosphatidyl choline	38.7 ± 1.8	41.1 ± 2.9
Phosphatidyl serine	12.3 ± 1.4	15.4 ± 2.6
Sphingomyelin	13.7 ± 1.4	14.1 ± 2.3
Cardiolipin	5.6 ± 0.8	4.3 ± 0.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.9a. Phospholipid classes of seminal plasma at 24 weeks of age.

	Semen quality		
	high		low
Phospholipid fraction (% w/w of total phospholipid)			
Phosphatidyl ethanolamine	27.9 ± 0.8		33.9 ± 2.6
Sphingomyelin	31.6 ± 1.3	b	24.1 ± 1.3
Phosphatidyl choline	19.0 ± 0.8		21.0 ± 1.8
Phosphatidyl serine	19.4 ± 0.5		19.6 ± 0.6
Cardiolipin	2.1 ± 0.3		1.5 ± 0.3

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [b]P<0.01.

Table 5.9b. Phospholipid classes of seminal plasma at 39 weeks of age.

	Semen quality	
	high	low
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	33.9 ± 2.1	35.6 ± 1.5
Sphingomyelin	25.4 ± 3.7	25.0 ± 0.9
Phosphatidyl choline	20.7 ± 2.3	19.5 ± 1.1
Phosphatidyl serine	17.2 ± 2.2	17.6 ± 0.9
Cardiolipin	3.6 ± 0.5	2.2 ± 0.6

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.9c. Phospholipid classes of seminal plasma at 54 weeks of age.

	Semen quality		
	high		low
Phospholipid fraction (% w/w of total phospholipid)			
Phosphatidyl ethanolamine	39.4 ± 1.6		38.6 ± 1.8
Sphingomyelin	27.4 ± 1.0		27.2 ± 0.8
Phosphatidyl choline	14.4 ± 1.5		15.7 ± 2.9
Phosphatidyl serine	16.9 ± 0.7		18.5 ± 0.8
Cardiolipin	2.3 ± 0.4	a	nd

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001.

Table 5.9d. Phospholipid classes of seminal plasma at 72 weeks of age.

	Semen quality	
	high	low
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	24.1 ± 1.25	24.6 ± 2.3
Sphingomyelin	32.6 ± 2.0	30.5 ± 1.4
Phosphatidyl choline	22.5 ± 2.9	28.5 ± 3.8
Phosphatidyl serine	14.6 ± 1.8	12.6 ± 2.0
Cardiolipin	6.2 ± 1.5	3.6 ± 0.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 5.10a. Fatty acid composition of phosphatidyl ethanolamine at 24 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	8.2 ± 0.1	c	6.7 ± 0.5
18:0	13.7 ± 0.3		13.8 ± 0.6
18:1 (n-9)	13.9 ± 1.2		11.9 ± 0.9
18:2 (n-6)	3.1 ± 0.2		3.2 ± 0.6
20:3 (n-6)	1.6 ± 0.4		1.4 ± 0.1
20:4 (n-6)	16.8 ± 0.4		18.1 ± 0.9
22:4 (n-6)	28.6 ± 1.2		30.8 ± 1.1
22:5 (n-3)	1.2 ± 0.1		0.7 ± 0.3
22:6 (n-3)	3.6 ± 0.4		3.9 ± 0.3
n-6/n-3	10.1 ± 1.1		12.3 ± 1.4
P/S	2.5 ± 0.2		2.9 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : $^{c}P<0.05$.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.10b. Fatty acid composition of phosphatidyl ethanolamine at 39 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high	low
16:0	8.1 ± 2.1	12.2 ± 0.4
18:0	15.2 ± 4.2	22.1 ± 0.3
18:1 (n-9)	9.0 ± 2.3	12.8 ± 0.5
18:2 (n-6)	7.6 ± 1.6	8.6 ± 0.9
20:4 (n-6)	18.9 ± 2.5	19.5 ± 0.4
22:4 (n-6)	25.3 ± 6.1	24.8 ± 0.1
22:6 (n-3)	< 1	nd
n-6/n-3	12.7 ± 9.6	-
P/S	2.9 ± 0.9	1.5 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.10c. Fatty acid composition of phosphatidyl ethanolamine at 54 weeks of age

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	5.4 ± 1.4	c	13.5 ± 2.3
18:0	13.5 ± 0.7	c	32.4 ± 5.1
18:2 (n-6)	2.2 ± 0.01	c	5.2 ± 1.0
20:4 (n-6)	24.2 ± 1.6	b	11.8 ± 1.5
22:4 (n-6)	39.5 ± 0.9	a	17.0 ± 2.2
22:5 (n-3)	1.4 ± 0.3		< 1.0
22:6 (n-3)	7.2 ± 2.4	a	nd
n-6:n-3	10.3 ± 3.8		-
P/S	4.0 ± 0.5	a	0.7 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.10d. Fatty acid composition of phosphatidyl ethanolamine at 72 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	4.5 ± 0.5	b	7.2 ± 0.6
18:0	10.7 ± 0.3	b	17.3 ± 1.4
18:1 (n-9)	7.8 ± 0.8	a	12.6 ± 0.2
18:2 (n-6)	3.6 ± 0.5	a	7.8 ± 0.5
20:3 (n-6)	1.3 ± 0.1	b	1.8 ± 0.1
20:4 (n-6)	19.4 ± 0.9	c	15.2 ± 1.3
22:4 (n-6)	38.4 ± 1.4	a	27.6 ± 0.6
22:5 (n-3)	< 1.0		< 1.0
22:6 (n-3)	3.6 ± 0.4		3.0 ± 0.9
n-6/n-3	14.4 ± 1.3		15.8 ± 3.8
P/S	4.3 ± 0.3	a	2.3 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.11a. Fatty acid composition phosphatidyl choline at 24 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	22.1 ± 0.8		21.9 ± 1.0
18:0	26.0 ± 0.2		26.2 ± 0.5
18:1 (n-9)	18.7 ± 0.2		20.9 ± 1.0
18:2 (n-6)	2.6 ± 0.2		2.4 ± 0.3
20:3 (n-6)	1.5 ± 0.1		1.5 ± 0.1
20:4 (n-6)	8.3 ± 0.5		7.1 ± 0.8
22:4 (n-6)	11.7 ± 0.6		9.7 ± 0.9
22:5 (n-3)	< 1.0		< 1.0
22:6 (n-3)	< 1.0		< 1.0
n-6/n-3	17.1 ± 0.7		18.1 ± 2.1
P/S	0.5 ± 0.1		0.5 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.11b. Fatty acid composition of phosphatidyl choline at 39 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	29.8 ± 1.5		26.9 ± 0.6
18:0	28.5 ± 3.3		27.3 ± 0.8
18:1 (n-9)	20.5 ± 0.6		20.7 ± 1.9
18:2 (n-6)	4.4 ± 0.9		3.8 ± 0.1
20:3 (n-6)	nd	**a**	1.6 ± 0.1
20:4 (n-6)	5.0 ± 1.1		5.7 ± 0.6
22:4 (n-6)	4.3 ± 1.1		6.3 ± 0.8
22:6 (n-3)	nd	**a**	1.0 ± 0.2
n-6/n-3	-		17.6 ± 0.6
P/S	0.3 ± 0.1		0.4 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [a]P<0.001.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.11c. Fatty acid composition of phosphatidyl choline at 54 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	22.8 ± 0.1		24.5 ± 1.8
18:0	26.5 ± 0.5		34.3 ± 4.5
18:1 (n-9)	15.0 ± 0.9		18.3 ± 1.7
18:2 (n-6)	3.7 ± 0.4		3.1 ± 0.1
20:3 (n-6)	1.7 ± 0.3		< 1.0
20:4 (n-6)	9.6 ± 0.6		6.2 ± 1.8
22:4 (n-6)	11.7 ± 2.2		7.8 ± 0.6
22:5 (n-3)	nd		nd
n-6/n-3	-		-
P/S	0.5 ± .0.2		0.3 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 5.11d. Fatty acid composition of phosphatidyl choline at 72 weeks of age.

Fatty acid (% w/w total fatty acid)	Semen quality high		low
16:0	18.1 ± 0.8		18.9 ± 1.7
18:0	22.6 ± 0.8		25.4 ± 1.1
18:1 (n-9)	16.7 ± 0.3	c	18.4 ± 0.5
18:2 (n-6)	9.2 ± 1.3	c	5.1 ± 1.0
20:3 (n-6)	1.5 ± 0.1		1.7 ± 0.1
20:4 (n-6)	9.4 ± 0.5		8.6 ± 0.9
22:4 (n-6)	13.1 ± 0.8		10.6 ± 1.4
22:5 (n-3)	nd		nd
22:6 (n-3)	< 1.0		nd
n-6/n-3	44.3 ± 7.1		-
P/S	0.9 ± 0.1	b	0.6 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between high and low semen quality groups : [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.

Table 5.12a. Correlation coefficients between semen parameters and fertility.

	age	conc 10^9 cells/ml	motility	forward motility	SOD sperm	SOD plasma	Fertility week 1	Fertility week 2
conc 10^9 cells/ml	-0.77^a	ns	ns	ns	ns	ns	ns	ns
motility %	-0.48^c	ns	ns	ns	ns	ns	ns	ns
forward motility	ns	ns	ns	ns	ns	ns	ns	ns
SOD sperm	-0.64^b	ns	0.46^c	-0.50^c	ns	ns	ns	ns
SOD plasma	ns	ns	-0.49^c	ns	ns	ns	ns	ns
GPX sperm	-0.49^c	ns	ns	ns	0.45^c	ns	ns	ns
GPX plasma	-0.66^a	0.54^b	ns	ns	0.44^c	ns	ns	ns
Fertility week 1	ns	ns	ns	ns	ns	-0.45^c	ns	ns
Fertility week 2	ns	ns	0.44^c	ns	ns	-0.67^a	0.65^a	ns
Overall fertility	ns	ns	ns	ns	ns	-0.62^a	0.90^a	0.91^a

5.12b. **Correlation coefficients between semen lipid parameters and fertility.**

	age	conc 10^9 cells/ml	motility	forward motility	fertility week 1	fertility week 2	phopsholipid	free fatty acid	cholesterol ester	phospholipid	free cholesterol
							Spermatozoa			Seminal plasma	
age	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
conc 10^9 cells/ml	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
motility	-0.49^{b}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
forward motility	0.58^{b}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 2	ns	ns	ns	ns	0.61^{a}	ns	ns	ns	ns	ns	ns
overall fertility	ns	ns	ns	ns	0.90^{a}	0.89^{a}	ns	ns	ns	ns	ns
Spermatozoa											
phospholipid	-0.71^{a}	ns	0.53^{b}	ns	ns	ns	ns	ns	ns	ns	ns
free cholesterol	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
free fatty acid	0.64^{a}	-0.44^{c}	ns	ns	ns	ns	-0.65^{a}	ns	ns	ns	ns
triacylglycerol	0.41^{c}	ns	-0.40^{c}	ns	ns	ns	-0.44^{c}	ns	ns	ns	ns
cholesterol ester	0.57^{b}	ns	ns	ns	ns	ns	0.68a	ns	ns	ns	ns
Seminal plasma											
phospholipid	ns	ns	ns	ns	ns	ns	0.44^{c}	-0.41^{c}	ns	ns	ns
free cholecsterol	ns	ns	ns	ns	ns	ns	ns	-0.46^{c}	ns	ns	ns
free fatty acid	ns	ns	ns	0.55^{b}	ns	ns	ns	ns	ns	-0.61^{a}	-0.44^{c}
triacylglycerol	ns	ns	ns	ns	ns	ns	ns	0.41^{c}	ns	ns	-0.58^{b}
cholesterol ester	ns	ns	ns	ns	ns	ns	ns	ns	ns	-0.71^{a}	ns
total lipid	0.51^{b}	ns	ns	ns	ns	ns	-0.45^{c}	0.49^{b}	0.42^{c}	ns	-0.46^{c}

5.12c. **Correlation coefficients between phospholipid fatty acids of spermatozoa and fertility.**

	age	conc 10^9 cells/ml	motility	forward motility	fertility week 1	fertity week 2	overall	16:0	18:0	18:1 (n-9)	20:4 (n-6)	22:4 (n-6)	22:5 (n-3)	22:6 (n-3)	P/S
age	ns	-0.37[c]	-0.51[b]	0.43[b]	ns	-0.36[c]	ns	0.42[b]	ns	0.52[a]	ns	-0.60[a]	-0.36[c]	-0.60[a]	-0.58[a]
conc 10^9 cells/ml	-0.37[c]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
motility	-0.51[b]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
forward motility	0.43[b]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 1	ns	ns	ns	ns	0.57[a]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 2	-0.36[c]	ns	0.35[c]	ns	0.88[a]	0.89[a]	ns	ns	ns	ns	ns	ns	ns	ns	ns
overall fertility	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
16:0	0.42[b]	-0.53[a]	-0.35[c]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
18:0	ns	ns	-0.41[c]	ns	ns	ns	ns	0.79[a]	ns	ns	ns	ns	ns	ns	ns
18:1 (n-9)	0.52[a]	-0.69[a]	-0.33[c]	ns	ns	ns	ns	0.74[a]	0.48[b]	ns	ns	ns	ns	ns	ns
20:4 (n-6)	ns	ns	ns	ns	ns	0.33[c]	ns	-0.41[c]	-0.50[b]	ns	ns	ns	ns	ns	ns
22:4 (n-6)	-0.60[a]	0.50[a]	0.40[c]	ns	ns	0.41[c]	0.33[c]	-0.63[a]	-0.48[b]	-0.58[a]	0.79[a]	ns	ns	ns	ns
22:5 (n-3)	-0.36[c]	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
22:6 (n-3)	-0.60[a]	0.45[b]	ns	ns	ns	ns	ns	-0.44[b]	ns	-0.58[a]	0.36[c]	0.69[a]	ns	ns	ns
P/S	-0.58[a]	ns	0.56[a]	ns	ns	ns	ns	-0.88[a]	-0.80[a]	-0.66[a]	0.58[a]	0.72[a]	0.58[a]	0.47[b]	ns

5.12d. Correlation coefficients between phospholipid classes of spermatozoa and seminal plasma and fertility.

	age	conc	mot	fert 1	fert 2	PE	PS	PC	SPH	PE/PC	PS/PC	PE	CL	PS
								Spermatozoa					Seminal plasma	
age	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
conc 10^{9} cells/ml	-0.46^{c}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
motility	-0.49^{b}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
forward motility	0.44^{c}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 1	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
fertility week 2	ns	ns	ns	0.39^{c}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
overall fertility	ns	ns	ns	0.84^{a}	0.82^{a}	ns	ns	ns	ns	ns	ns	ns	ns	ns
Spermatozoa														
PE	ns	0.62^{a}	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
CL	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PS	-0.53^{b}	0.72^{a}	0.42^{c}	ns	ns	0. 45^{c}	ns	ns	ns	ns	ns	ns	ns	ns
PC	0.57^{b}	-0.76^{a}	-0.40^{c}	-0.39^{c}	ns	-0.80^{a}	-0.80^{a}	ns	ns	ns	ns	ns	ns	ns
SPH	ns	-0.63^{a}	ns	ns	ns	-0.72^{a}	-0.67^{a}	0.61^{a}	ns	ns	ns	ns	ns	ns
PE/PC	-0.46^{c}	0.72^{a}	ns	ns	ns	0.92^{a}	0.66^{a}	-0.94^{a}	-0.67^{a}	ns	ns	ns	ns	ns
PS/PC	-0.51^{b}	0.75^{a}	0.44^{c}	ns	ns	0.55^{b}	0.96^{a}	-0.89^{a}	-0.64^{a}	0.79^{a}	ns	ns	ns	ns
Seminal plasma	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
PE	-0.41^{c}	0.71^{a}	ns	ns	ns	0.54^{b}	0.55^{b}	-0.56^{b}	-0.70^{a}	0.55^{b}	0.54^{b}	ns	ns	ns
CL	0.60^{a}	ns	ns	ns	ns	ns	-0.48^{c}	ns	ns	ns	-0.40^{b}	-0.55^{b}	ns	ns
PS	-0.69^{a}	0.56^{b}	ns	ns	ns	0.44^{c}	0.64^{a}	-0.62^{a}	-0.50^{b}	0.49^{b}	0.58^{b}	0.41^{c}	-0.53^{b}	ns
PC	ns	-0.54^{b}	ns	ns	ns	-0.51^{b}	-0.38^{c}	0.51^{b}	0.50^{b}	-0.45^{b}	ns	-0.71^{a}	0.39^{c}	-0.49^{b}
SPH	ns	-0.52^{b}	ns	ns	ns	ns	-0.52^{b}	0.44^{c}	0.51^{b}	-0.43^{b}	-0.52^{b}	-0.50^{b}	ns	-0.46^{c}

5.4 DISCUSSION

The observations made during this study confirm and enhance those already obtained in Chapter 4 throughout the reproductive period of the domestic cockerel. In particular, the present data enabled the estimation of the degree of correlation between spermatozoa concentration, motility, fertility and the various lipid parameters of the spermatozoa and seminal plasma.

The differences in lipid composition and associated parameters between cockerels of high and low semen quality also highlighted a number of important factors. Cockerels of high semen quality displayed better spermatozoa motility and fertility compared to the low semen quality cockerels. Although few differences were observed in the lipid classes within the two groups of males, low semen quality cockerels exhibited elevated levels of total lipid within the seminal plasma at 39 weeks of age or peak fertility. These elevated levels of total lipid could indicate limited utilisation of this exogenous seminal plasma lipid as a potential energy source for spermatozoa motility. This is already known to occur in spermatozoa from humans displaying below average semen quality and infertility (Sebastian *et al.*, 1987). In the present study such differences in lipid composition were also associated with decreased levels of C20 and C22 polyunsaturated fatty acids of the major phospholipid class, phosphatidyl ethanolamine. It has been reported that such losses may lead to impairment in the physical and/or signal transduction properties of the sperm membrane. For example, in the brain and retina, major phospholipid classes, like spermatozoa, are highly enriched in these C20 and C22 polyunsaturated fatty acids (Neuringer *et al.*, 1988) and it has been suggested that such long chain fatty acids play a major role in regulating membrane fluidity, ionic interactions, membrane excitation and also interact with and regulate a range of membrane proteins and enzymes (Salem *et al.*, 1986). Furthermore, it has been proposed that the interaction of Ca^{2+} with sperm membrane phospholipid fatty acids may trigger phase transitions which initiate membrane fusion events similar to those of the acrosome reaction necessitating the presence of these fatty acids for normal spermatozoa function and viability (Cullis and Hope, 1991).

In association with these reductions in the major C20 and C22 long chain polyunsaturates in cockerels of low semen quality parameters was a reduced level of major antioxidants, particularly glutathione peroxidase at 54 and 72 weeks in the cockerels displaying low semen quality parameters. Such changes in antioxidant protection and loss of spermatozoa polyunsaturated fatty acid content suggest peroxidative damage to spermatozoa. In the absence of adequate glutathione peroxidase activity the superoxide dismutase system of spermatozoa becomes overwhelmed leading to accumulation of peroxidation products (Griveau *et al.*, 1995). Peroxidation has been frequently reported to be associated with significant reductions in normal morphology and spermatozoa motility as well as being detrimental to spermatozoon-oocyte fusion events at fertilisation (Fujihara and Howarth, 1978 ; Aitken and West, 1990 ; Aitken *et al.*, 1993a).

The decrease in the proportions of the major polyunsaturated fatty acids 20:4 (n-6) and 22:4 (n-6) in spermatozoa phospholipids cannot be explained by changes in dietary provision since the cockerels were supplied with the same diet throughout the experimental period. The importance of these n-6 fatty acids have been reported to be essential in the correct form and at sufficient levels to maximise fowl fertility levels (Watkins, 1995). It would appear that with varying semen quality and fertility such changes may arise from a reduced ability to synthesise C20 to C22 polyunsaturated fatty acids from their dietary C18 precursors and/or to incorporate such fatty acids into important phospholipid moieties such as phosphatidyl ethanolamine which has been shown to correlate positively with major semen characteristics and fertility in the present study. It is of interest that the proportions of 20:4 (n-6) and 22:4 (n-6) in spermatozoa showed significant correlations with the week 2 fertility levels but not with the week 1 fertility results. This suggests that these polyunsaturates may also play a key role in maintaining survival of the spermatozoa in the female reproductive tract prior to interaction with the oocyte.

It may be concluded that differences displayed in fertility between groups of cockerels were associated with distinctive differences in the fatty acid composition of the major phospholipid classes, in particular those of phosphatidyl ethanolamine involving the major polyunsaturates 20:4 (n-6) and 22:4 (n-6). There is a great deal of evidence to suggest that the lipid composition of spermatozoa membranes play a key role in

determining a range of features associated with overall fertility both in fresh and stored ejaculates (Darin-Bennett *et al*, 1974 ; Wishart, 1994), a high level relating to desirable membrane fluidity characteristics (Nissen *et al.*, 1981 ; Nissen and Kreysel, 1983 ; Hammerstedt, 1993), the ultimate acrosome reaction and membrane fusion events at fertilisation. It is evident that the achievement and maintenance of maximum semen quality and fertility requires a combination of optimum levels of long chain polyunsaturates and a combination of optimum levels of the appropriate antioxidant protection. It remains to be established if the semen quality of the domestic broiler breeder cockerel can be manipulated by dietary means to enhance and/or maintain the necessary levels of long chain polyunsaturated fatty acids required for good spermatozoa characteristics and fertility throughout the reproductive period.

CHAPTER 6

THE EFFECT OF 18:3 (N-3) DIETARY SUPPLEMENTATION ON THE LIPID COMPOSITION, SEMEN QUALITY AND FERTILITY OF COCKERELS

6.1 INTRODUCTION

As already discussed (see Chapter 4), the fatty acid composition of domestic avian spermatozoa is characterised by high levels of n-6 fatty acids (Darin-Bennett *et al.*, 1974 ; Howarth *et al.*, 1977 ; Ravie and Lake, 1985), in contrast to the mammal where n-3 fatty acids predominate (Neill and Masters, 1972 ; Poulos and White, 1973 ; Scott, 1973 ; Jain and Anand 1976; Lin *et al.*, 1993). For obvious reasons data previously available for avians is largely confined to commercial poultry with little information available for birds living under wild conditions. The question therefore arises as to whether the spermatozoa fatty acid profiles represent a true phylogenic difference between Aves and Mammalia or whether the observed absence of any substantial levels of n-3 fatty acids in the former is simply a reflection of the commercially manufactured diets currently in use for poultry production in which n-6 fatty acids overidingly predominate (Harfoot, 1981)

It is well established that animals which are deprived a source of n-3 fatty acids at critical stages of development display greatly reduced levels of 22:6 (n-3) in their neuronal and retinal phospholipids and, by way of compensation, accumulate high proportions of long chain n-6 fatty acids such as 22:4 (n-6) and 22:5 (n-6) (Salem *et al.*, 1986 ; Neuringer *et al.*, 1988 ; Anderson *et al.*, 1989). Thus the characteristic fatty acid profiles of poultry spermatozoa may be said to be more typical of that found under conditions of dietary n-3 in sufficiency rather than a balance of polyunsaturated fatty acids in the diet. The typical fatty acid composition currently observed for poultry semen may be simply an aberration due to an imbalance of n-3 versus n-6 fatty acids, the possibility arises that the fertility of poultry may be amenable to improvement via appropriate dietary manipulation. An experiment was therefore performed in an attempt to address these points positively.

The investigation was carried out to cover the following features following dietary supplementation with 18:3 (n-3) ;

i composition of the spermatozoa and seminal plasma lipids.

ii *in vitro* parameters of semen quality.

iii fertility of the cockerels

iv antioxidant levels in spermatozoa and seminal plasma.

6.2 MATERIALS AND METHODS

Full details of all procedures involved are given in the relevant sections of Chapter 3. Further relevant details are as follows ;

6.2.1. The cockerels

Two groups of Cobb broiler breeder males each containing 15 cockerels were used. Males were purchased from Morini Brothers, Firenze, Italy. The males were allocated into their respective groups on the basis of spermatozoa concentration and motility as assessed by CellSoft (see Section 3.11) from a larger group of cockerels of variable semen quality, when males were 23 weeks of age. Cockerels were housed at the University of Milan, Italy and semen characteristics we assessed at this Institute, however all other lipid analyses were performed at SAC, Auchincruive after lipid extraction and appropriate transportation of the samples. Cockerels were grouped to achieve close semen characteristics as seen in Table 6.1.

Table 6.1. Semen quality criteria of the cockerels at 23 weeks of age.

n-3 supplementation	+	-
Spermatozoa quality	**Average**	**Average**
Concentration 10^9 cells/ml	4.65 ± 0.81	4.89 ± 1.21
Motile %	48.4 ± 3.4	50.5 ± 3.4

Mean ± S.E. of 15 ejaculates per group.

6.2.2 Diets

Birds were fed 130g per day of the allocated diet, both diets containing 12.5 % crude protein, 9 % lipid and 11.5 MJ/kg metabolisable energy and 40 mg/kg of vitamin E. The 18:3 (n-3) enriched diet was produced by supplementation with linseed oil (Crippsar, Milan, Italy) at 6% (w/w of total feed). The fatty acid composition of each diet is detailed in Table 6.2. Appropriate adjustments were made to the diets in order to ensure a comparability of energy content. The feeds were analysed routinely throughout the

experiment to ensure the maintenance of fatty acid composition. All feeds were stored in air tight containers to avoid peroxidative deterioration.

Table 6.2. Fatty acid composition of the diets.

Fatty acids	control	n-3 supplemented
	% w/w of total fatty acid	
16:0	12	10
18:0	4	4
18:1 (n-9)	23	21
18:2 (n-6)	50	30
18:3 (n-3)	6	34

all fatty acids of less than 1% total lipid have been omitted.

All cockerels were weighed before and after the experimental period to observe any effect of dietary differences. However, no differences were detected between groups fed the standard diet and those the 18:3 (n-3) supplemented diet.

6.2.3 Housing

All males were housed in individual cages with 13 hours of light supplied per day and an environmental temperature maintained at 18 °C.

6.2.4 Semen collection

Cockerels were trained for approximately 3 weeks and then milked regularly from 21 weeks of age twice weekly and thrice weekly during periods of semen collection. Collection periods were organised to meet peaks of reproductive performance as predetermined according to the data provided by the supplier (Table 6.3).

Table 6.3. Collection periods.

Collection period	Stage of reproductive period	Age (weeks)
1	beginning	23 - 25
2	peak	38 - 40
3	decreasing	53 - 55
4	end	71 - 73

Period 4 was included as fertility of the breed was still considered by the supplier to be relatively high at 50 weeks of age.

6.2.5 Artificial insemination

Pooled samples of three ejaculates, five replicates, from each group of cockerels were used to inseminate Warren laying hens intravaginally with a single fixed insemination dose of 70 x 10^7. Warren laying hens were purchased at the same time and age as the cockerels and housed under the same environmental and dietary conditions. At each of the four ages studied the hens were randomised to receive semen from either the high or low fertility cockerels. During this study it was not possible to use hens at peak fertiity by replacing them for each insemination experiment. The same group of hens was therefore used during the entire experimental period. Fertilised eggs were collected over a 7 day period and incubated. The eggs were incubated at 37 °C and 60 % relative humidity with automatic egg turning. Candling identified the proportion exhibiting embryonic development, only those containing no embryos were classed as infertile. This procedure was then repeated for another 7days to establish the fertility of the eggs two weeks after the single fixed insemination.

6.2.6 Spermatozoa evaluation and analysis

Spermatozoa quality parameters were measured at 24, 39, 54 and 72 weeks of age. Pooled samples of 3 ejaculates, 5 replicates per group, were analysed. All measurements were performed as described previously (see Sections 3.1, 3.2, 3.7, 3.8, 3.9, 3.10, 3.11).

No antioxidant measurements were performed at 24 weeks of age due to the lower amount of semen available for the analyses. Adequate volumes of semen became available after 24 weeks of age and so it was decided to wait until peak fertility, 39 weeks of age, to perform the measurements for group comparison. The absence of fatty acid profiles of the major phospholipid classes within the seminal plasma of the cockerels was unable to be performed acurately due to the lack of seminal plasma volume after semen separation. It was also felt that these results were less important than those which were involved in the phospholipid structure of the spermatozoa themselves.

6.2.7 Statistical analysis

Students *t-test* was used for statistical comparisons of the means. Data included 5 replicates per group at each collection period for lipid analyses with 15 replicates per group at each collection period for semen volume, concentration and motility. 5 replicates within each week of the egg collection for each collection period were used to assess fertility rate. Statistical analysis was undertaken using Microsoft Excel version 5.0.

In this study antioxidant activities are specified to two decimal places to emphasise the values obtained which wee quite low and/or displayed small standard errors. All other measurements were specified to only one decimal place for comparison purposes.

6.3 RESULTS

6.3.1 Changes in semen characteristics

The characteristics of the semen samples from n-3 supplemented and unsupplemented cockerels during the reproductive period are shown in Tables 6.4a to 6.4d.

Comparison of n-3 supplemented and unsupplemented cockerels

The supplemented cockerels displayed higher spermatozoa concentration than those values displayed by the unsupplemented cockerels. Such differences between groups were significant at 54 weeks of age. The percentage of motile spermatozoa displayed a similar pattern to that of spermatozoa concentration with significantly higher values for supplemented cockerels at 54 weeks, however at 72 weeks of age this group displayed lower motile spermatozoa although not significantly so.

The glutathione peroxidase activity displayed no significant differences between the two groups of cockerels within both spermatozoa and seminal plasma. The activity of superoxide dismutase throughout the experimental period also showed no differences between the two groups of cockerels, apart from a significantly higher superoxide dismutase activity in supplemented cockerels within spermatozoa at 39 weeks of age.

Fertility levels between the two experimental groups showed a significantly higher level of week 2 fertility at 24 weeks of age and week 1 fertility levels in supplemented cockerels at 39 weeks of age. At 72 weeks of age this group also displayed higher fertility parameters compared to unsupplemented cockerels although such differences were not significant.

The antioxidant enzyme levels recorded during this study differed considerably with those previously reported in Chapter 4. Although such analyses were performed on different breeds of cockerel and at different Institutes all procedures were performed according to the kit manufacturers instructions as specified in Section 3.2. No explanation can be offered although a similar pattern of change during the reproductive period was recorded. Results for this study were in general agreement with those reported in Chapter 5 also measured at the Univerisity of Milan, Italy. Different methods

of spermatozoa concentration assessment were also used in Chapter 4 which may explain the higher levels reported during this study.

6.3.2 Lipid composition of the spermatozoa and seminal plasma.

The proportions of the lipid classes present within the spermatozoa are detailed in Tables 6.5a to 6.5d and for seminal plasma, Tables 6.6a to 6.6d.

The major lipid class in both groups of cockerels within spermatozoa and seminal plasma was phospholipid followed by substantial levels of free cholesterol and cholesterol ester, this latter lipid class being considerably higher within the seminal plasma. Lower levels of triacylglycerol and free fatty acids were observed in both semen fractions.

Comparison between supplemented and unsupplemented cockerels

Within spermatozoa supplemented cockerels exhibited higher phospholipid levels at 24 weeks of age, however after this period no differences were observed. Levels of free cholesterol differed during 39 and 72 weeks where supplemented cockerels displayed higher levels of this lipid class than unsupplemented cockerels. No other major differences were observed within spermatozoa. Within the seminal plasma phospholipid levels were lower at 24 weeks and 39 weeks of age in supplemented cockerels, however throughout the reproductive period cholesterol ester levels of this group were higher. Total lipid levels within seminal plasma were higher in the unsupplemented cockerels at 39 and 54 weeks of age. The only significant differences were observed at 54 weeks of age with higher triacylglycerol levels in supplemented compared with unsupplemented cockerels.

6.3.3 Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma

The fatty acid composition of the total phospholipid for the spermatozoa and seminal plasma are shown in Tables 6.7a to 6.7d and Tables 6.8a to 6.8d respectively.

The major saturated fatty acids present within the total phospholipid of both spermatozoa and seminal plasma for both groups of cockerels were 16:0 and 18:0 with

18:1 (n-9) being the major monounsaturated fatty acid. The major polyunsaturated fatty acids were 20:4 (n-6) and 22:4 (n-6). Levels of 22:6 (n-3) were low within both spermatozoa and seminal plasma.

Comparison between supplemented and control cockerels

Within spermatozoa supplemented cockerels displayed significantly higher levels of n-3 fatty acids accompanied by lower n-6/n-3 ratios at 39 and 54 weeks of age. At 72 weeks of age supplemented cockerels also displayed significantly higher levels of the major saturated fatty acids, 16:0 and 18:0 accompanied by a significant decrease in 18:2 (n-6) levels and n-6/n-3 ratios. Despite these differences both groups of cockerels exhibited similar fatty acid profiles throughout the experimental period with no increase in 22:6 (n-3) observed in supplemented cockerels.

Within the seminal plasma at 39, 54 and 72 weeks the supplemented cockerels displayed significantly higher n-3 fatty acid levels accompanied by lower n-6/n-3 ratios. This pattern was particularly significant at 39 weeks of age. Again, despite these differences the fatty acid profiles of the two groups of cockerels displayed similar patterns with no increase in 22:6 (n-3) levels observed in supplemented cockerels.

6.3.4 Major phospholipid classes of the spermatozoa and seminal plasma

The major phospholipid classes of the spermatozoa and seminal plasma are shown in Tables 6.7a to 6.7d and Tables 6.8a to 6.8d respectively.

Phosphatidyl ethanolamine and phosphatidyl choline were the major phospholipid classes within both spermatozoa and seminal plasma for both groups of cockerels with substantial levels of phosphatidyl serine and sphingomyelin. Cardiolipin was present only at lower levels.

Comparison between supplemented and unsupplemented cockerels

There were no significant differences with the spermatozoa despite a decrease in cardiolipin in supplemented cockerels at 54 weeks of age. Within the seminal plasma the level of phosphatidyl ethanolamine was higher in the unsupplemented cockerels at 24 weeks, these levels equalised at 39 weeks accompanied by a reduced level of

phosphatidyl choline in supplemented cockerels. At 54 weeks of age supplemented cockerels displayed higher levels of phosphatidyl ethanolamine, sphingomyelin and cardiolipin accompanied by lower levels of phosphatidyl choline and phosphatidyl serine at 54 weeks of age, this trend continued to 72 weeks of age.

6.3.5 Fatty acid composition of the phosphatidyl ethanolamine and phosphatidyl choline of the spermatozoa

The major saturated fatty acids present within phosphatidyl ethanolamine and phosphatidyl choline were 16:0 and 18:0 with 18:1 (n-9) being the major monounsaturated fatty acid. The major polyunsaturated fatty acids within both phospholipid classes were 20:4 (n-6) and 22:4 (n-6) although phosphatidyl choline displayed a more saturated fatty acid composition with polyunsaturated fatty acids present at less than 25 % within both groups of cockerels.

Comparison between supplemented and control cockerels

Phosphatidyl ethanolamine

Throughout the experimental period supplemented cockerels displayed higher levels of n-3 fatty acids including 22:6 (n-3) and from 39 weeks of age an accompanied reduction in the level of n-6 fatty acids was observed. These differences in n-6 fatty acids were not displayed at 24 weeks of age, indeed levels of n-6 fatty acids were significantly higher in supplemented cockerels at this time. At this age there was also a significant reduction in the level of 16:0, 18:0 and 18:1 (n-9) in the supplemented group.

Phosphatidyl choline

At 24 and 39 weeks of age both groups of cockerels displayed very similar patterns of fatty acids with an absence of 22:6 (n-3). However, at 54 weeks of age a number of differences were observed between groups, in particular at 54 weeks of age. At this time there was a reduction in the level of 22:4 (n-6) and a higher level of 22:5 (n-3) in supplemented cockerels, although again no 22:6 (n-3) was detected in either group. At 72 weeks the levels of 20:4 (n-6) and 22:4 (n-6) were significantly higher in supplemented cockerels as was the level of 22:5 (n-3). At this stage equal levels of 22:6 (n-3) were observed in both groups of cockerels

Table 6.4a. Semen characteristics at 24 weeks of age.

n-3 supplementation		+		-
Concentration	10^9 cells/ml	5.0 ± 0.6		4.7 ± 0.5
Motile	%	56.5 ± 4.4		56.4 ± 4.1
Forward motility	%	1.6 ± 0.1		1.7 ± 0.1
Fertility (overall)	%	60.5 ± 9.9		56.6 ± 5.5
Fertility (week 1)	%	69.5 ± 9.1		68.3 ± 4.9
Fertility (week 2)	%	67.9 ± 6.6	c	45.0 ± 7.1

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between supplemented and unsupplemented groups : [c]P<0.05.

Table 6.4b. Semen characteristics at 39 weeks of age.

n-3 supplementation		+		-
Concentration	10^9 cells/ml	7.6 ± 0.6		7.7 ± 0.6
Motile	%	62.5 ± 5.1		54.5 ± 3.8
Forward motility	%	1.7 ± 0.1		1.7 ± 0.1
Glutathione peroxidase	units/10^9 cells	0.55 ± 0.08		0.48 ± 0.04
Glutathione peroxidase	units/ml plasma	0.23 ± 0.02		0.25 ± 0.01
Superoxide dismutase	units/10^9 cells	1.10 ± 0.04	b	0.58 ± 0.09
Superoxide dismutase	units/ml plasma	0.90 ± 0.05		1.21 ± 0.14
Fertility (overall)	%	70.7 ± 9.9		72.3 ± 4.6
Fertility (week 1)	%	96.8 ± 3.0	c	82.8 ± 4.9
Fertility (week 2)	%	57.5 ± 7.3		61.7 ± 4.7

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between supplemented and unsupplemented groups : [b]P<0.01 ; [c]P<0.05.

Table 6.4c. Semen characteristics at 54 weeks of age.

n-3 supplementation		+		-
Concentration	10^9 cells/ml	6.8 ± 0.7	c	5.1 ± 0.7
Motile	%	53.9 ± 4.7	b	33.8 ± 3.9
Forward motility	%	1.7 ± 0.1		1.5 ± 0.1
Glutathione peroxidase	units/10^9 cells	0.38 ± 0.06		0.59 ± 0.12
Glutathione peroxidase	units/ml plasma	0.18 ± 0.02		0.24 ± 0.02
Superoxide dismutase	units/10^9 cells	0.57 ± 0.01		0.59 ± 0.05
Superoxide dismutase	units/ml plasma	1.38 ± 0.22		1.73 ± 0.10
Fertility (overall)	%	65.7 ± 5.0		61.1 ± 5.1
Fertility (week 1)	%	76.8 ± 3.8		74.4 ± 4.6
Fertility (week 2)	%	54.6 ± 8.4		47.9 ± 6.4

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group. Significance of difference between supplemented and unsupplemented groups : [b]P<0.01 ; [c]P<0.05.

Table 6.4d. Semen characteristics at 72 weeks of age.

n-3 supplementation		+	-
Concentration	10^9 cells/ml	2.1 ± 0.3	1.8 ± 0.3
Motile	%	47.2 ± 5.4	59.3 ± 5.1
Forward motility	%	2.1 ± 0.3	1.8 ± 0.3
Glutathione peroxidase	units/10^9 cells	0.24 ± 0.07	0.34 ± 0.08
Glutathione peroxidase	units/ml plasma	0.16 ± 0.03	0.19 ± 0.02
Superoxide dismutase	units/10^9 cells	0.40 ± 0.09	0.50 ± 0.06
Superoxide dismutase	units/ml plasma	1.26 ± 0.24	0.92 ± 0.06
Fertility (overall)	%	57.7 ± 5.3	45.2 ± 11.9
Fertility (week 1)	%	77.0 ± 8.0	59.8 ± 12.1
Fertility (week 2)	%	38.4 ± 2.9	26.1 ± 9.5

Values are means ± S.E. of observations from 3 lots of 5 pooled ejaculates (15 samples) per group.

Table 6.5a. Lipid composition of spermatozoa from of cockerels at 24 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/10 ^{9} cells)	261.2 ± 12.1	290.0 ± 27.6
Lipid class (% w/w of total lipid)		
Phospholipid	68.3 ± 4.4	60.1 ± 2.3
Free cholesterol	12.1 ± 2.6	12.6 ± 1.4
Free fatty acid	5.9 ± 1.0	4.9 ± 1.6
Triacylglycerol	5.3 ± 2.8	9.3 ± 3.1
Cholesterol ester	8.4 ± 3.9	13.1 ± 3.6

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.5b. Lipid composition of spermatozoa from of cockerels at 39 weeks of age.

n-3 supplementation	+		-
Total lipid (μg/10 ^{9} cells)	316.0 ± 44.0		274.9 ± 16.9
Lipid class (% w/w of total lipid)			
Phospholipid	68.5 ± 1.6		69.2 ±2.1
Free cholesterol	17.8 ±0.9	b	12.9 ±0.6
Free fatty acid	5.3 ±0.5		6.9 ±1.2
Triacylglycerol	3.2 ±2.1		3.8 ±1.1
Cholesterol ester	5.2 ± 0.8		7.2 ±1.3

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : ^{b}P<0.01.

Table 6.5c. Lipid composition of spermatozoa from cockerels at 54 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/10 ^{9} cells)	427.7 ± 84.1	364.4 ± 75.3
Lipid class (% w/w of total lipid)		
Phospholipid	57.4 ± 3.4	57.7 ± 1.4
Free cholesterol	23.8 ± 1.8	24.8 ± 1.8
Free fatty acid	4.9 ± 1.4	9.0 ± 1.8
Triacylglycerol	4.7 ± 1.3	3.1 ± 0.7
Cholesterol ester	9.2 ± 2.2	8.5 ± 0.6

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.5d. Lipid composition of spermatozoa from cockerels at 72 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/10 ^{9} cells)	657.3 ± 122.4	783.3 ± 94.5
Lipid class (% w/w of total lipid)		
Phospholipid	48.3 ± 4.8	47.8 ± 5.8
Free cholesterol	21.8 ± 3.4	14.6 ± 3.3
Free fatty acid	10.7 ± 1.5	13.5 ± 3.2
Triacylglycerol	3.2 ± 0.9	5.0 ± 2.7
Cholesterol ester	15.9 ± 4.5	19.0 ± 4.9

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.6a. Lipid composition of seminal plasma from cockerels at 24 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/ml plasma)	631.0 ± 264.1	280.4 ± 109.7
Lipid class (% w/w of total lipid)		
Phospholipid	19.5 ± 4.4	29.2 ± 3.2
Free cholesterol	15.1 ± 1.9	17.4 ± 1.9
Free fatty acid	32.7 ± 4.4	22.4 ± 7.4
Triacylglycerol	9.7 ± 2.0	12.6 ± 0.2
Cholesterol ester	23.0 ± 8.8	18.4 ± 5.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.6b. Lipid composition of seminal plasma of cockerels at 39 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/ml plasma)	649.8 ± 24.4	1 009 ± 159.9
Lipid class (% w/w of total lipid)		
Phospholipid	32.6 ± 5.5	44.4 ± 4.0
Free cholesterol	20.2 ± 2.4	16.3 ± 2.1
Free fatty acid	14.7 ± 0.7	12.8 ± 1.6
Triacylglycerol	8.6 ± 4.7	6.2 ± 2.6
Cholesterol ester	23.8 ± 5.4	20.3 ± 2.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.6c. Lipid composition of seminal plasma of cockerels at 54 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/ml plasma)	770.9 ± 184.4	1 120 ± 261.4
Lipid class (% w/w of total lipid)		
Phospholipid	33.1 ± 6.8	32.0 ± 6.6
Free cholesterol	24.1 ± 2.4	18.2 ± 2.5
Free fatty acid	12.7 ± 3.3	19.0 ± 2.4
Triacylglycerol	11.2 ± 2.1	[c] 4.3 ± 1.0
Cholesterol ester	43.4 ± 10.8	29.7 ± 3.7

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [c]P<0.05.

Table 6.6d. Lipid composition of seminal plasma of cockerels at 72 weeks of age.

n-3 supplementation	+	-
Total lipid (μg/ml plasma)	887.3 ± 142.2	860.4 ± 134.7
Lipid class (% w/w of total lipid)		
Phospholipid	30.6 ± 3.8	26.1 ± 2.8
Free cholesterol	13.5 ± 4.6	13.2 ± 3.4
Free fatty acid	22.0 ± 1.7	21.2 ± 3.5
Triacylglycerol	5.5 ± 1.5	11.8 ± 2.8
Cholesterol ester	28.4 ± 3.8	27.6 ± 0.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.7a. Major fatty acids of total phospholipid of spermatozoa at 24 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	13.9 ± 0.3		13.8 ± 0.4
18:0	20.3 ± 0.3		20.0 ± 0.3
18:1 (n-9)	13.0 ± 0.6		13.6 ± 0.3
18:2 (n-6)	2.4 ± 0.1		2.7 ± 0.2
20:4 (n-6)	13.1 ± 0.4		12.5 ± 0.4
22:4 (n-6)	23.0 ± 0.8		22.8 ± 1.1
18:3 (n-3)	1.2 ± 0.7		< 1.0
22:5 (n-3)	< 1.0		< 1.0
22:6 (n-3)	2.4 ± 0.1		2.2 ± 0.1
n-6/n-3	10.1 ± 1.7		10.7 ± 1.8
P/S	1.3 ± 0.1		1.3 ± 0.3

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.7b. Major fatty acids of total phospholipid of spermatozoa at 39 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	14.1 ± 0.4		14.1 ± 0.2
18:0	20.3 ± 0.2		19.5 ± 0.4
18:1 (n-9)	13.1 ± 0.6		12.2 ± 0.2
18:2 (n-6)	2.3 ± 0.1		3.4 ± 0.5
20:4 (n-6)	12.1 ± 0.5		11.8 ± 0.4
22:4 (n-6)	19.9 ± 0.8		22.9 ± 1.0
18:3 (n-3)	< 1.0		< 1.0
22:5 (n-3)	5.3 ± 0.9	a	< 1.0
22:6 (n-3)	2.3 ± 0.1		2.5 ± 0.2
n-6/n-3	4.5 ± 0.5	a	9.8 ± 0.8
P/S	1.3 ± 0.1		1.2 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.7c. Major fatty acids of total phospholipid of spermatozoa at 54 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	13.9 ± 0.4		13.9 ± 0.6
18:0	20.2 ± 0.2		20.1 ± 0.2
18:1 (n-9)	14.7 ± 0.8		14.0 ± 0.5
18:2 (n-6)	3.7 ± 0.3		4.7 ± 0.4
20:4 (n-6)	11.7 ± 0.2		11.9 ± 0.2
22:4 (n-6)	19.2 ± 0.6		21.7 ± 1.5
18:3 (n-3)	< 1.0		< 1.0
22:5 (n-3)	3.4 ± 0.1	a	< 1.0
22:6 (n-3)	2.4 ± 0.1		1.9 ± 0.1
n-6/n-3	5.9 ± 0.2	a	14.5 ± 0.6
P/S	1.3 ± 0.2		1.3 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds. Significance of difference between supplemented and unsupplemented groups : [a]P<0.001. Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.7d. Major fatty acids of total phospholipid of spermatozoa at 72 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	23.1 ± 3.1	c	15.4 ± 0.8
18:0	31.0 ± 3.5	c	20.4 ± 0.3
18:1 (n-9)	16.9 ± 1.0		16.2 ± 1.1
18:2 (n-6)	2.5 ± 0.8	c	6.0 ± 0.8
20:4 (n-6)	5.8 ± 2.4		11.0 ± 0.4
22:4 (n-6)	9.4 ± 3.8		17.8 ± 1.7
18:3 (n-3)	< 1.0		< 1.0
22:5 (n-3)	< 1.0		< 1.0
22:6 (n-3)	1.5 ± 0.5		1.7 ± 0.1
n-6/n-3	8.7 ± 1.4	b	18.9 ± 1.8
P/S	0.6 ± 0.2		1.0 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds. Significance of difference between supplemented and unsupplemented groups : [b]P<0.01 ; [c]P<0.05. Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.8a. Major fatty acids of total phospholipid of seminal plasma at 24 weeks of age.

n-3 supplementation	+	-
Fatty acid (% w/w of total fatty acid)		
16:0	14.1 ± 1.3	14.6 ± 0.6
18:0	18.9 ± 1.3	22.9 ± 1.4
18:1 (n-9)	12.6 ± 0.9	14.3 ± 0.8
18:2 (n-6)	8.7 ± 1.2	9.2 ± 3.1
20:4 (n-6)	11.8 ± 1.6	13.7 ± 0.4
22:4 (n-6)	9.2 ± 3.3	13.4 ± 3.3
18:3 (n-3)	6.5 ± 2.9	5.3 ± 1.3
22:5 (n-3)	1.1 ± 0.5	1.3 ± 0.9
22:6 (n-3)	1.4 ± 0.6	1.8 ± 0.9
n-6/n-3	3.8 ± 1.0	5.1 ± 1.2
P/S	1.1 ± 0.1	1.2 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds. Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.8b. Major fatty acids of total phospholipid of seminal plasma at 39 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	11.8 ± 1.2		12.7 ± 0.6
18:0	18.4 ± 1.2		19.2 ± 0.6
18:1 (n-9)	11.5 ± 0.8		11.0 ± 0.4
18:2 (n-6)	6.1 ± 0.6		7.3 ± 0.9
20:4 (n-6)	10.6 ± 0.6		12.5 ± 0.7
22:4 (n-6)	8.7 ± 1.3		13.3 ± 2.3
18:3 (n-3)	1.1 ± 0.2	a	nd
20:5 (n-3)	3.0 ± 0.3	a	< 1.0
22:3 (n-3)	1.7 ± 0.8	a	nd
22:5 (n-3)	2.1 ± 0.2	a	< 1.0
22:6 (n-3)	2.7 ± 0.4		2.8 ± 0.2
n-6/n-3	2.8 ± 0.4	b	7.4 ± 1.0
P/S	1.0 ± 0.4		1.0 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds. Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [b]P<0.01. nd represents not detectable. Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.8c. Major fatty acids of total phospholipid of seminal plasma at 54 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	15.3 ± 0.7		14.3 ± 1.1
18:0	20.5 ± 0.4		18.1 ± 1.0
18:1 (n-9)	24.3 ± 1.5		21.3 ± 2.5
18:2 (n-6)	12.5 ± 1.3		12.1 ± 1.8
20:4 (n-6)	9.5 ± 0.7		10.5 ± 1.3
22:4 (n-6)	7.3 ± 1.0		10.0 ± 1.7
18:3 (n-3)	< 1.0		< 1.0
20:5 (n-3)	2.2 ± 0.9	a	< 1.0
22:6 (n-3)	< 1.0		1.6 ± 0.4
n-6/n-3	7.4 ± 1.2	b	15.7 ± 1.2
P/S	0.9 ± 0.1		1.0 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [b]P<0.01.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.8d. Major fatty acids of total phospholipid of seminal plasma at 72 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w of total fatty acid)			
16:0	13.2 ± 1.0		15.2 ± 0.6
18:0	18.9 ± 0.9		18.8 ± 1.0
18:1 (n-9)	23.1 ± 1.4		25.7 ± 3.4
18:2 (n-6)	13.1 ± 1.4		16.6 ± 1.6
20:4 (n-6)	8.2 ± 0.8		8.3 ± 2.0
22:4 (n-6)	5.9 ± 1.5		4.8 ± 1.7
18:3 (n-3)	1.3 ± 0.2		< 1.0
20:5 (n-3)	1.5 ± 0.1		< 1.0
22:5 (n-3)	2.7 ± 0.2	a	< 1.0
22:6 (n-3)	1.7 ± 0.6		1.1 ± 0.5
n-6/n-3	4.2 ± 0.5	c	21.2 ± 5.7
P/S	1.1 ± 0.1		0.9 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.9a. Phospholipid classes of spermatozoa at 24 weeks of age.

n-supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	31.7 ± 1.2	33.1 ± 1.6
Phosphatidyl choline	33.4 ± 1.1	32.3 ± 1.5
Phosphatidyl serine	18.9 ± 0.7	19.2 ± 2.4
Sphingomyelin	11.9 ± 1.3	10.5 ± 1.1
Cardiolipin	4.1 ± 0.5	4.9 ± 0.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.9b. Phospholipid classes of spermatozoa at 39 weeks of age.

n-3 supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	32.9 ± 0.8	33.8 ± 1.2
Phosphatidyl choline	26.6 ± 1.3	25.4 ± 1.6
Phosphatidyl serine	22.3 ± 0.8	24.4 ± 0.7
Sphingomyelin	12.9 ± 2.9	11.5 ± 0.9
Cardiolipin	5.2 ± 0.8	4.8 ± 0.4

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.9c. Phospholipid classes of spermatozoa at 54 weeks of age.

n-3 supplementation	+		-
Phospholipid fraction (% w/w of total phospholipid)			
Phosphatidyl ethanolamine	32.9 ± 0.9		31.1 ± 0.4
Phosphatidyl choline	32.4 ± 1.1		34.7 ± 1.2
Phosphatidyl serine	21.6 ± 0.5		20.9 ± 0.8
Sphingomyelin	9.4 ± 0.5		8.4 ± 0.5
Cardiolipin	3.7 ± 0.2	c	4.9 ± 0.3

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [c]P<0.05.

Table 6.9d. Phospholipid classes of spermatozoa at 72 weeks of age.

n-3 supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	30.1 ± 3.2	27.6 ± 2.0
Phosphatidyl choline	39.1 ± 1.4	39.3 ± 2.6
Phosphatidyl serine	14.7 ± 1.0	13.3 ± 1.3
Sphingomyelin	12.1 ± 1.6	13.3 ± 1.7
Cardiolipin	4.0 ± 0.6	6.4 ± 2.0

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.10a. Phospholipid classes of seminal plasma at 24 weeks of age.

n-3 supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	30.6 ± 0.7	37.0 ± 2.7
Sphingomyelin	27.5 ± 2.4	24.1 ± 3.1
Phosphatidyl choline	21.3 ± 1.3	21.5 ± 3.3
Phosphatidyl serine	17.6 ± 2.6	16.0 ± 1.2
Cardiolipin	3.0 ± 1.5	2.3 ± 0.7

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.10b. Phospholipid classes of seminal plasma at 39 weeks of age.

n-3 supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	40.0 ± 2.2	37.3 ± 1.2
Sphingomyelin	26.1 ± 2.1	25.4 ± 2.7
Phosphatidyl choline	12.9 ± 1.2	18.4 ± 3.4
Phosphatidyl serine	17.6 ± 0.9	17.2 ± 1.1
Cardiolipin	3.5 ± 1.4	2.1 ± 0.5

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.10c. Phospholipid classes of seminal plasma at 54 weeks of age.

n-3 supplementation	+		-
Phospholipid fraction (% w/w of total phospholipid)			
Phosphatidyl ethanolamine	38.1 ± 2.7		33.8 ± 1.8
Sphingomyelin	30.0 ± 1.4		23.5 ± 3.1
Phosphatidyl choline	14.2 ± 1.0	c	19.5 ± 1.7
Phosphatidyl serine	15.6 ± 1.4	c	22.8 ± 1.9
Cardiolipin	2.0 ± 0.3	a	nd

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [c]P<0.05.
nd represents not detectable.

Table 6.10d. Phospholipid classes of seminal plasma at 72 weeks of age.

n-3 supplementation	+	-
Phospholipid fraction (% w/w of total phospholipid)		
Phosphatidyl ethanolamine	32.4 ± 3.8	28.0 ± 2.9
Sphingomyelin	29.9 ± 2.7	25.6 ± 2.0
Phospahtidyl choline	20.5 ± 2.2	25.9 ± 3.8
Phosphatidyl serine	10.5 ± 2.1	13.3 ± 2.6
Cardiolipin	6.7 ± 1.2	7.1 ± 0.4

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.

Table 6.11a. Fatty acid composition of phosphatidyl ethanolamine at 24 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	7.1 ± 0.5	b	11.7 ± 0.7
18:0	15.0 ± 0.7	c	18.6 ± 1.3
18:1 (n-9)	11.8 ± 1.6	b	18.3 ± 0.5
18:2 (n-6)	2.6 ± 0.1		3.5 ± 0.8
20:3 (n-6)	1.1 ± 0.3		1.3 ± 0.6
20:4 (n-6)	18.4 ± 0.9	c	15.2 ± 0.6
22:4 (n-6)	30.0 ± 1.3	b	22.2 ± 1.3
22:5 (n-3)	1.4 ± 0.2		< 1.0
22:6 (n-3)	3.6 ± 0.3	c	2.3 ± 0.3
n-6/n-3	10.2 ± 0.4		16.6 ± 3.6
P/S	2.7 ± 0.3	b	1.5 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [b]P<0.01 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.11b. Fatty acid composition of phosphatidyl ethanolamine at 39 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	11.5 ± 2.7		9.7 ± 2.4
18:0	20.8 ± 2.4		25.8 ± 0.2
18:1 (n-9)	12.9 ± 2.3		13.2 ± 1.9
18:2 (n-6)	5.1 ± 0.9		6.6 ± 0.1
20:4 (n-6)	18.9 ± 2.6		20.7 ± 2.2
22:4 (n-6)	20.1 ± 3.0		23.8 ± 2.4
22:6 (n-3)	2.2 ± 1.1	a	nd
n-6/n-3	8.4 ± 1.5	a	-
P/S	1.6 ± 0.5		1.5 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.11c. Fatty acid composition of phosphatidyl ethanolamine at 54 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	15.2 ± 1.5		14.9 ± 2.2
18:0	13.8 ± 1.1		13.9 ± 0.7
18:1 (n-9)	18.6 ± 2.4	c	8.3 ± 1.3
18:2 (n-6)	3.9 ± 1.4		5.0 ± 1.2
20:4 (n-6)	21.0 ± 3.0		25.3 ± 0.8
22:4 (n-6)	32.4 ± 9.6		34.7 ± 1.8
22:5 (n-3)	< 1.0	a	nd
22:6 (n-3)	3.1 ± 1.0	a	nd
n-6/n-3	12.4 ± 6.0	a	-
P/S	3.7 ± 1.5		4.5 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.11d. Fatty acid composition of phosphatidyl ethanolamine at 72 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	9.0 ± 1.3		7.7 ± 1.1
18:0	16.9 ± 1.2		17.6 ± 1.5
18:1 (n-9)	15.9 ± 1.7		12.4 ± 0.8
18:2 (n-6)	9.7 ± 2.1	c	4.7 ± 0.1
20:3 (n-6)	1.6 ± 0.2		1.7 ± 0.3
20:4 (n-6)	11.9 ± 1.6		13.1 ± 1.4
22:4 (n-6)	21.3 ± 2.1	c	31.9 ± 3.3
22:5 (n-3)	2.2 ± 0.2	a	< 1.0
22:6 (n-3)	3.6 ± 0.4		3.4 ± 0.4
n-6/n-3	7.7 ± 0.5	a	13.1 ± 0.7
P/S	1.9 ± 0.1		2.3 ± 0.3

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.12a. Fatty acid composition of phosphatidyl choline at 24 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	30.1 ± 2.7	c	18.8 ± 1.0
18:0	29.7 ± 2.1		24.0 ± 2.0
18:1 (n-9)	19.2 ± 1.7		30.2 ± 6.9
18:2 (n-6)	2.5 ± 0.5		1.9 ± 0.2
20:3 (n-6)	1.2 ± 0.2		1.7 ± 0.4
20:4 (n-6)	3.4 ± 1.4		6.5 ± 0.1
22:4 (n-6)	4.1 ± 1.5		8.5 ± 1.8
n-6/n-3	13.3 ± 7.0		16.9 ± 2.6
P/S	0.2 ± 0.1		0.4 ± 0.2

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : ^{c}P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.12b. Fatty acid composition of phosphatidyl choline at 39 weeks of age.

n-3 supplementation	+	-
Fatty acid (% w/w total fatty acid)		
16:0	30.5 ± 0.9	30.8 ± 1.0
18:0	28.1 ± 1.7	29.0 ± 1.5
18:1 (n-9)	22.6 ± 2.3	20.9 ± 1.0
18:2 (n-6)	3.3 ± 0.3	3.6 ± 0.1
20:3 (n-6)	1.5 ± 0.2	< 1.0
20:4 (n-6)	4.5 ± 0.03	4.2 ± 0.2
22:4 (n-6)	3.9 ± 0.6	4.7 ± 0.2
n-6/n-3	-	-
P/S	0.2 ± 0.1	0.2 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.12c. Fatty acid composition of phosphatidyl choline at 54 weeks of age.

n-3 supplementation	+		-
(% w/w total fatty acid)			
16:0	21.3 ± 2.2		18.4 ± 0.9
18:0	28.0 ± 0.6		29.8 ± 1.5
18:1 (n-9)	18.0 ± 0.4	b	16.0 ± 0.2
18:2 (n-6)	2.9 ± 0.2		3.0 ± 0.4
20:3 (n-6)	1.9 ± 0.1		1.5 ± 0.1
20:4 (n-6)	8.6 ± 0.4		9.0 ± 1.5
22:4 (n-6)	9.3 ± 1.7	c	14.2 ± 0.1
22:5 (n-3)	2.3 ± 0.1	a	nd
n-6/n-3	8.2 ± 1.2	a	-
P/S	0.5 ± 0.1		0.6 ± 0.1

Values are means ± S.E. of individual observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 6.12d. Fatty acid composition of phosphatidyl choline at 72 weeks of age.

n-3 supplementation	+		-
Fatty acid (% w/w total fatty acid)			
16:0	17.4 ± 1.0	c	23.6 ± 1.2
18:0	25.5 ± 0.1		23.6 ± 1.8
18:1 (n-9)	17.3 ± 0.9		15.3 ± 1.4
18:2 (n-6)	4.1 ± 0.4		4.0 ± 0.5
20:3 (n-6)	2.0 ± 0.03		1.8 ± 0.2
20:4 (n-6)	10.0 ± 0.6	b	6.2 ± 0.6
22:4 (n-6)	12.3 ± 0.3	c	8.7 ± 1.0
22:5 (n-3)	1.7 ± 0.1	a	nd
22:6 (n-3)	1.3 ± 0.1		1.3 ± 0.4
n-6/n-3	9.4 ± 0.4		21.2 ± 7.7
P/S	0.8 ± 0.2	a	0.4 ± 0.1

Values are means ± S.E. of observations from 5 pooled ejaculates each of 3 birds.
Significance of difference between supplemented and unsupplemented groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

6.4 DISCUSSION

The fatty acid compositions of the tissue lipids of animals maintained in captivity should be interpreted with some caution since the commercial diets provided may differ greatly in their fatty acyl profiles from the natural diets available in the wild. In several cases, it has been demonstrated that an excessive proportion of n-6 fatty acids in commercial diets, to the detriment of n-3 fatty acid levels, can produce deleterious effects on the development and viability of the animal (Noble *et al.*, 1993 and 1996 ; Speake *et al.*, 1994). An example of the potential for confusion implicit in data from captive animals is provided by the laboratory guinea pig, which, in contrast to most mammals, is characterised by low proportions of 22:6 (n-3) and high proportions of 22:5 (n-6) in the lipids of the brain and retina (Neuringer *et al.*, 1988). Although this discrepancy was originally interpreted as a true species difference, recent work has demonstrated that provision of the developing guinea pigs with a diet enriched in n-3 fatty acids, as opposed to commercial chow, resulted in the elevation of the tissue 22:6 (n-3) content to typical mammalian levels concomitant with the disappearance of 22:5 (n-6) (Weisinger *et al.*, 1995). Of greater pertinence to the present study, the low levels of 22:6 (n-3) and correspondingly high levels of 22:5 (n-6) typical of spermatozoa lipids of the laboratory rat (in contrast to the usual pattern observed for mammalian spermatozoa) were completely reversed by replacing the laboratory chow diet with one supplemented with fish oil ; most notably, the resultant enhanced levels of 22:6 (n-3) in the rat spermatozoa were associated with an improvement in testes maturation and by greater spermatogenesis (Salem *et al.*, 1986).

The aims of the present work were two-fold. Firstly, to investigate whether the reported fatty acid profiles for avian spermatozoa represent an aberration due to the feeding of commercial diets ; by supplementation of the diet of cockerels with 18:3 (n-3) in an attempt to increase the proportion of 22:6 (n-3) and simultaneously reduce the proportion of 20:4 (n-6) and 22:4 (n-6). The second aim represented an attempt to improve the fertility of spermatozoa via these dietary changes. A major conclusion of this study is that the fatty acid profile of cockerel spermatozoa showed a distinct resistance to dietary modification. In spite of the provision of an 18:3 (n-3) enriched diet to the cockerels for a total of some 49 weeks, no increase was observed in the proportion of 22:6 (n-3) in the spermatozoa phospholipids. The only positive effect on

the spermatozoa of the dietary supplementation of n-3 fatty acids was the detection of small amounts of 22:5 (n-3) after 15 and 31 weeks of supplementation (i.e. at 39 and 54 weeks of age) in total phospholipid and similar fatty acid changes in the major phospholipid classes throughout the reproductive period. This result suggests that the desaturation/elongation of 18:3 (n-3) to its C20 and C22 derivatives, and/or the incorporation of these derivatives into phospholipids is extremely inefficient in this system. Traditionally, it was believed that the synthesis of n-6 and n-3 C22 polyunsaturates was elaborated via the sequential action of delta-6, delta-5 and delta-4 desaturases plus the intervening elongation steps (Brenner, 1989). However, recent work using a range of mammalian cell and tissue preparations suggests that the direct conversion of 22:4 (n-6) to 22:5 (n-6) and 22:5 (n-3) to 22:6 (n-3) via the action of delta-4 desaturase does not take place. Instead, it appears that this conversion may occur through a more circuitous route involving microsomal elongation to C24 polyunsaturates followed by retroconversion of the intermediates to 22:5 (n-6) and 22:6 (n-3) via peroxisomal beta-oxidation (Sprecher *et al.*, 1995 ; Moore *et al.*, 1995).

The present data, indicating that dietary supplementation of cockerels with 18:3 (n-3) results in the appearance of an albeit low level of 22:5 (n-3) in the spermatozoa phospholipid but with no increase in the proportion of 22:6 (n-3), suggests that the microsomal elongation of 22:5 (n-3) and the subsequent peroxisomal retroconversion pathway does not occur in this system. This conclusion is supported by the fact that, in cockerel spermatozoa 22:4 (n-6) appears to be the end product of the desaturation/elongation of 18:2 (n-6) with no further conversion to 22:5 (n-6).

The significance of the failure of 18:3 (n-3) supplementation to enhance the 22:6 (n-3) levels in spermatozoa, in terms of delineating the "natural" fatty acid profile of avian spermatozoa, can be interpreted in two ways. On the one hand the current results may be taken to support the view that the predominance of C20 to C22 polyunsaturates of the n-6 series represents a true phylogenic distinction between avians and mammals and is not simply a displacement from the norm brought about by feeding commercial diets. Alternatively, it is feasible that the free-living cockerel under natural conditions would consume a diet rich in pre-formed 22:6 (n-3) (e.g. from invertebrates) as opposed to the precursor 18:3 (n-3) and, as a consequence, would have no need to express the synthetic pathway for the formation of n-3 C20 to C22 polyunsaturates. For example, certain

carnivores including the cat family do not express delta-6 and delta-5 desaturase activity presumably because an adequate supply of C20 to C22 polyunsaturates is ensured by the meat-rich diet (Brenner, 1989 ; Salem *et al.*, 1986).

As regards the second aim of this study, dietary supplementation with 18:3 (n-3) did indeed result in significant improvements in fertility and associated parameters. In the light of the small effects of such a dietary regime on the fatty acid composition of the spermatozoa, the mechanism of these improvements on semen quality and fertility are obscure. It should however be noted that the diet-induced changes in the proportions of the individual fatty acids, although subtle, did result in marked decreases in the n-6/n-3 ratio of total phospholipid at 39, 54 and 72 weeks of age. The possible beneficial effects of such changes on the properties of the spermatozoa membranes or on eicosanoid formation remain to be established.

CHAPTER 7

THE EFFECT OF DIETARY SUPPLEMENTATION OF 22:6 (N-3) AND VITAMIN E ON THE LIPID COMPOSITION, SEMEN QUALITY AND FERTILITY OF THE COCKEREL

7.1 INTRODUCTION

Fish oils have been incorporated into animal feeds for many years and are efficiently utilised in poultry to permit growth (Karrick, 1990). Despite this knowledge no previous work has been undertaken to establish the detailed effects of dietary 22:6 (n-3) supplementation on the semen quality and fertility of domestic poultry.

The use of fish oils containing high levels of polyunsaturated fatty acids also require the addition of an appropriate antioxidant, such as vitamin E. Previous results obtained with turkey, cockerel and gander semen showed that vitamin E was located mainly in the spermatozoa and that dietary supplementation by this vitamin increased its levels in the semen and as a result increased resistance of spermatozoa to different factors including lipid peroxidation, spermatozoa dilution, storage and deep freezing (Surai and Ionov, 1992 a, b). Dietary vitamin E requirements in relation to polyunsaturated fatty acid intake have been reviewed by Bassler (1991).

The investigation was carried out to investigate the following features following dietary supplementation with 22:6 (n-3) and vitamin E ;

i. composition of spermatozoa and seminal plasma lipids.
ii *in vitro* parameters of semen quality.
iii fertility of the cockerels.
iv fatty acid composition of total phospholipid of testes and liver.

7.2 MATERIALS AND METHODS

Full details of each procedure are given in the relevant sections of Chapter 3 Further relevant details are as follows ;

7.2.1. The cockerels

Three groups of Ross broiler breeder males each containing four cockerels were used cockerels were purchased from Ross Breeders Limited, Newbridge, Midlothian, and randomly allocated into their groups at 10 weeks of age. Dietary supplementation began

from initial housing at 10 weeks of age to observe the effects of dietary supplementation on semen production before the beginning of spermatogenesis (Lake, 1984 ; Hocking, 1990).

7.2.2 Diets

The first group of cockerels, the control group, were maintained on a standard commercial broiler breeder diet with 3% (w/w of total feed) maize oil supplementation and the other cockerels were maintained on a diet supplemented with 3% (w/w of total feed) tuna orbital oil (oils provided by Scotia Pharmaceuticals, Carlisle, Scotland) giving a final oil content for both diets of 6 %. One group of the cockerels given the 22:6 (n-3) supplemented diet also had additional vitamin E supplementation to approximately 4 times the normal level offered to the other groups, i.e. 160 mg/kg (% w/w feed) compared to 40 mg/kg. All cockerels were fed according to body weight from 10 weeks of age up to 130 g/day. The diets comprised 12 % crude protein and 11.5 MJ/day of metabolisable energy. The fatty acid composition of the diets are given in Table 7.1. The feed was analysed throughout the experimental period to ensure the maintenance of fatty acid composition. All feeds were stored in air tight containers to avoid peroxidative deterioration.

Table 7.1. Fatty acid composition of the diets.

Fatty acids % w/w of total fatty acid	**Control diet**	**22:6 (n-3) diet**
14:0	< 1	3
16:0	16	22
18:0	4	6
18:1 (n-9)	25	19
18:2 (n-6)	48	20
18:3 (n-3)	3	3
22:6 (n-3)	< 1	14

All other fatty acids of less than a 1% of total lipid have been omitted.

All cockerels were weighed before and after the experimental period to observe any effect of dietary difference. However, no differences were detected between groups.

7.2.3 Housing

All males were housed in individual cages with 11 hours of light at 29 weeks of age increasing by 0.5 hours per day to reach 15 hours by 37 weeks of age with the environmental temperature maintained at 18°C.

7.2.4 Semen collection

Cockerels were trained for approximately 3 weeks from 20 weeks of age and then milked regularly from 21 weeks of age twice weekly and thrice weekly during periods of semen collection for lipid analysis and *in vitro* semen parameters. Collection periods were organised to meet the recognised highlights in the reproductive lives of the breed as predetermined according to the data provided by the supplier (Table 7.2) to give three final collection periods.

Table 7.2. Collection periods.

Collection period	Stage of reproduction	age/weeks
1	beginning	23 - 25
2	middle	39 - 41
3	end	57 - 59

7.2.5 Artificial insemination

Pooled samples of three ejaculates, four replicates, from each group of cockerels were used to inseminate Ross hens with a single fixed insemination dose of 70 x 10^7 spermatozoa. Fertilised eggs were collected over a 7 day period and incubated. At each stage of the ages studied the hens were randomised to receive semen from either the high or low fertility cockerels. The eggs were incubated at 37 °C and 60 % relative humidity with automatic egg turning. Candling identified the proportion of eggs exhibiting

embryonic development, only those containing no embryos were classed as infertile. This procedure was then repeated for another 7 days to establish the fertility of the eggs two weeks after the single insemination.

The hens used were replaced at each insemination to ensure they were at peak fertility, 38 weeks. No artifical insemination was performed at 24 weeks of age due to the absence of adequate volumes of semen.

7.2.6 Spermatozoa evaluation and analysis

Spermatozoa quality was measured at 24, 40 and 58 weeks of age. Pooled samples of 3 ejaculates, 4 replicates per group, were analysed. Basic spermatozoa measurements were performed within 20 minutes of collection. all measurements performed as described previously (see Sections 3.1, 3.2, 3.7, 3.8, 3.8, 3.10, 3.11). The absence of fatty acid profiles of the major phospholipid classes within the seminal plasma of the cockerels was due to the lack of plasma volume after semen preparation. It was also felt that these results were less important than those involved in the phospholipid structure of the spermatozoa themselves.

All cockerels were slaughtered at approximately 60 weeks of age for post-mortem assessment of the testes and liver. The aim was to observe any differences in testes weight and associated vitamin E and fatty acid compositions. Liver dissection was assessed as this organ plays a special role in lipid metabolism.

7.2.7 Statistical analysis

The three cockerel groups were analysed by students *t-test* by comparison of means and standard errors. Comparison of both supplemented groups to unsupplemented cockerels and also a comparison between the two supplemented groups of differing vitamin E content was performed. Lipid analyses were performed on four replicates per group for each collection period with twelve replicates per group for non-lipid measurements. Four replicates within each week of the egg collection for each collection period were used to assess fertility rates. Statistical analysis was undertaken using Microsoft Excel version 5.0.

7.3 RESULTS

7.3.1 Changes in semen characteristics

The *in vitro* semen characteristics of the semen samples from supplemented and unsupplemented cockerels reproductive period are detailed in Tables 7.3a to 7.3c.

Comparison between supplemented and unsupplemented cockerels

Semen concentration was consistently higher within the supplemented groups with the highest concentration for all three collection periods being recorded in the n-3 and vitamin E supplemented cockerels. The motility measurements were highest in the n-3 and vitamin E supplemented males at 24 weeks of age although after this time no significant differences were observed. The n-3 supplemented cockerels with no additional vitamin E displayed significantly lower motility measurements at 24 weeks than those cockerels supplemented with 22:6 (n-3) and additional vitamin E.

The fertility results obtained at 40 weeks of age were higher in both groups of supplemented cockerels for week 1, week 2 and overall fertility. At 58 weeks of age significant improvements in week 1 fertility were shown by n-3 supplemented cockerels. Those cockerels with additional vitamin E displayed significant improvements in both week 2 and overall fertility levels at this last collection period.

The spermatozoa concentrations measured in this study were in agreement with those previously reported (see Chapter 4). Both studies measured concentration using method 3.11 compared to the higher results obtained inChapters5 and 6 assessed using method 3.1.2. Differences in analysis techniques may explain the differences in values obtained.

7.3.2 Lipid composition of the spermatozoa and seminal plasma.

The proportions of the major lipid classes present within the spermatozoa and seminal plasma are detailed in Tables 7.4a to 7.4c.

The major lipid classes present within both spermatozoa and seminal plasma was phospholipid, followed by free cholesterol with only minor lipid classes present at less than 5%.

Comparison between supplemented and unsupplemented cockerels

Both groups of supplemented cockerels displayed higher levels of phospholipid and lower levels of triacylglycerol within spermatozoa than unsupplemented cockerels at 24 weeks of age. No differences were observed within the seminal plasma at this stage. However, at 40 weeks the cockerels supplemented with only n-3 fatty acids displayed significantly higher levels of phospholipid and lower levels of free cholesterol and cholesterol ester compared to other groups of cockerels. Significant differences were also observed within the seminal plasma with both groups of supplemented cockerels exhibiting lower levels of total lipid, higher levels of phospholipid and lower free cholesterol. At the final collection period few differences were observed in the lipid classes of spermatozoa and seminal plasma. However, supplemented cockerels without additional vitamin E displayed higher levels of spermatozoa and seminal plasma total lipid levels compared to unsupplemented cockerels and those supplied with additional vitamin E.

7.3.3 Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma.

The fatty acids present during the reproductive period from all groups of cockerels are detailed for spermatozoa and seminal plasma in Tables 7.5a to 7.5c.

Comparison between supplemented and unsupplemented cockerels

Throughout each period of semen analysis both supplemented groups exhibited significantly higher levels of n-3 fatty acids and lower levels of n-6 fatty acids. In particular, both groups of supplemented cockerels showed highly significant increases in the level of 22:6 (n-3) within spermatozoa and seminal plasma. These changes were accompanied by significant decreases in the n-6/n-3 ratios present within both semen fractions as well as lower P/S ratios throughout the reproductive period

7.3.4 Fatty acid composition of the free fatty acid, triacylglycerol and cholesterol ester lipid classes of the spermatozoa and seminal plasma.

The fatty acids present within the free fatty acid, triacylglycerol and cholesterol ester classes of the spermatozoa and seminal plasma are detailed in Tables 7.6a to 7.6c, 7.7a to 7.7c and 7.8a to 7.8c respectively (see Appendix Tables).

Comparison between supplemented and unsupplemented cockerels

Within the free fatty acid class supplemented cockerels exhibited significantly higher levels of 22:6 (n-3) within both spermatozoa and seminal plasma at 24 weeks of age, accompanied by lower levels of n-6 fatty acids and n-6/n-3 ratios. This pattern continued at 40 weeks although fewer significant differences were observed. At 58 weeks of age spermatozoa of supplemented groups displayed significantly higher levels of n-3 fatty acids and lower n-6/n-3 ratios. However, within the seminal plasma few polyunsaturates were detected within all groups of cockerels at this stage.

Within the triacylglycerol class levels of 22:6 (n-3) were higher in supplemented compared to unsupplemented cockerels, this was particularly clear within the seminal plasma results at 24 and 40 weeks of age. At 58 weeks there were significantly higher levels of 22:6 (n-3) in supplemented groups, however within the seminal plasma no long chain polyunsaturates greater than 18:2 (n-6) were detected within any group throughout the entire reproductive period, apart from small but significant levels of 22:6 (n-3) at 24 and 40 weeks.

Within the cholesterol ester class a similar pattern occurred with elevated levels of 22:6 (n-3) and reduced levels of n-6 fatty acids in supplemented compared to unsupplemented cockerels. Within the seminal plasma at 40 and 58 weeks of age 20:4 (n-6) and 22:4 (n-6) were not detectable in any group of cockerels. At 58 weeks within both semen fractions no long chain polyunsaturates beyond 18:2 (n-6) were detected within all groups of cockerels.

7.3.5 Major phospholipid classes of spermatozoa and seminal plasma

The major phospholipid classes detected within the spermatozoa and seminal plasma from all groups of cockerels are detailed in Tables 7.9a to 7.9c.

Comparison between supplemented and unsupplemented cockerels

The major phospholipid classes present within both spermatozoa and seminal plasma for all groups of cockerels were phosphatidyl ethanolamine and phosphatidyl choline with substantial levels of phosphatidyl serine and lower levels of sphingomyelin and cardiolipin. No phosphatidyl inositol was detected within either semen fraction.

At 24 weeks of age the spermatozoa of supplemented cockerels showed higher levels of phosphatidyl ethanolamine, although these differences did not become significant until 58 weeks of age. Phosphatidyl choline levels also decreased significantly in supplemented males at 58 weeks of age, although supplemented cockerels with no added vitamin E showed significantly lower levels of phosphatidyl ethanolamine and higher levels of phosphatidyl choline within the seminal plasma. Again at 58 weeks of age the level of phosphatidyl serine increased in supplemented cockerels without additional vitamin E with a decrease in cardiolipin within spermatozoa accompanied by a decrease in this class within the seminal plasma. Cockerels supplemented with additional vitamin E exhibited no changes in phosphatidyl serine, however a significant increase in cardiolipin within the spermatozoa of this group was shown.

7.3.6 Major fatty acids within the phospholipid classes of spermatozoa.

The fatty acid profiles of the phospholipid class, phosphatidyl ethanolamine are detailed in Tables 7.10a to 7.10c ; phosphatidyl choline in Tables 7.11a to 7.11c ; phosphatidyl inositol/serine in Tables 7.12a to 7.12c ; sphingomyelin Tables 7.13a to 7.13b ; cardiolipin in Tables 7.14a to 7.14c.

Comparison between supplemented and unsupplemented cockerels

Phosphatidyl ethanolamine

Both groups of supplemented cockerels displayed significantly lower levels of n-6 fatty acids and higher levels of n-3 fatty acids accompanied by reduced n-6/n-3 ratios throughout the reproductive period. The increase in the level of 22:6 (n-3) was less dramatic in those cockerels supplemented only with 22:6 (n-3).

Phosphatidyl choline

Within this class supplemented cockerels again displayed significantly lower levels of n-6 fatty acids with smaller but significant increases in 22:6 (n-3) accompanied by reduced n-6 /n-3 ratios. Cockerels supplemented with 22:6 (n-3) alone showed significantly smaller increases in this polyunsaturated fatty acid at 24 and 40 weeks of age.

Phosphatidyl inositol/serine

Supplemented cockerels displayed significantly elevated levels of 22:6 (n-3) and all other n-3 fatty acids as well as a reduction in n-6 fatty acids. This trend continued throughout the reproductive period, however increases in 22:6 (n-3) levels in both supplemented groups of cockerels became less dramatic at 58 weeks of age.

Sphingomyelin

At 24 weeks of age supplemented cockerels showed significantly increased levels of 22:6 (n-3), although the increase was smaller in those cockerels supplemented only with 22:6 (n-3) who expressed significantly lower levels compared to cockerels with additional vitamin E. At 40 weeks of age, this pattern altered so that the unsupplemented cockerels displayed significantly higher levels of 22:6 (n-3) compared to the supplemented cockerels in particular those supplemented with additional vitamin E. At the end of the reproductive period at 58 weeks there was a complete loss of n-6 fatty acids in all groups with no significant differences observed in the levels of n-3 fatty acids, although the cockerels supplemented with 22:6 (n-3) and vitamin E showed smaller levels of 22:6 (n-3) compared to both other groups of cockerels.

Cardiolipin

At 24 weeks of age supplemented cockerels displayed increases in n-3 fatty acids and decreases in n-6 fatty acids, this trend continued at 40 weeks although the level of 22:6 (n-3) measured was some ten per cent lower than previously recorded in supplemented cockerels. At 58 weeks of age the pattern was again similar to the 40 week collection period, however levels of n-6 fatty acids had decreased in unsupplemented cockerels had fallen from around 40 - 50% to around 10%, these losses being replaced by more saturated fatty acids. Both supplemented groups of cockerels continued to display significantly higher levels of 22:6 (n-3) at this collection period.

7.3.7 The vitamin E content of sperm, testes and liver as well as testicular weight and spermatozoa peroxidation levels taken at the end of the reproductive period.

The final post mortem analysis of spermatozoa, testes and liver characteristics are detailed in Table 7.15.

Testicular weight

The supplemented cockerels displayed higher testicular weight than unsupplemented cockerels. This was highly significant within those cockerels suppplemented with 22:6 (n-3) and vitamin E.

Vitamin E levels of spermatozoa, testes and liver

The tissues of n-3 and vitamin E supplemented cockerels displayed significantly higher levels of vitamin E in all tissues examined. Those cockerels supplemented with 22:6 (n-3) alone showed significant differences in the testes vitamin E levels only although all assessments of vitamin E for this group were significantly lower than those of cockerels in the other supplemented group.

Malondialdehyde levels within semen as an assessment of peroxidation

Peroxidation levels were lowest within the cockerels given additional vitamin E, however levels were highest within cockerels supplemented with 22:6 (n-3) polyunsaturates without vitamin E addition. Unsupplemented cockerels also showed significantly lower levels of peroxidation than cockerels supplemented with only 22:6 (n-3).

7.3.8 Major fatty acids within the total phospholipid of testes and liver.

The major fatty acids of the total phospholipid within testes and liver taken at the end of the reproductive period post-mortem are detailed in Tables 7.16 for testes and 7.17 for liver.

In both tissues examined supplemented cockerels exhibited significantly higher levels of n-3 fatty acids associated with reduced levels of n-6 fatty acids. This was reflected in the reduced n-6/n-3 ratios within both supplemented groups. The supplemented cockerels without additional vitamin E also displayed lower levels of n-3 and higher n-6 fatty acids compared to the groups with 22:6 (n-3) and vitamin E.

Table 7.3a. Semen characteristics at 24 weeks of age.

		22:6 (n-3) supplementation			
		-	+		+/vit E
Concentration	10^9 cells/ml	2.1 ± 0.1	2.2 ± 0.2		2.6 ± 0.3
Motile	%	48.2 ± 3.1	42.4 ± 2.1	**ns/b**	53.8 ± 3.2

Values are means ± S.E. of observations from 3 lots of 4 pooled ejaculates (12 samples) per group.
Significance of difference between groups : [b]P<0.01.
ns represents not significant.

Table 7.3b. Semen characteristics and fertility at 40 weeks.

		22:6 (n-3) supplementation		
		-	+	+/vit E
Concentration	10^9 cells/ml	2.1 ± 0.1	2.1 ± 0.1	2.2 ± 0.1
Motile	%	59.5 ± 2.1	55.6 ± 2.2	58.7 ± 2.4
Fertility (overall)	%	39.0 ± 4.4	43.9 ± 7.8	46.1 ± 3.3
Fertility (week 1)	%	40.5 ± 6.6	55.4 ± 4.2	52.2 ± 2.6
Fertility (week 2)	%	37.4 ± 6.7	32.4 ± 12.3	40.1 ± 4.3

Values are means ± S.E. of observations from 3 lots of 4 pooled ejaculates (12 samples) per group.

Table 7.3c. Semen characteristics and fertility at 58 weeks of age.

		22:6 (n-3) supplementation			
		-	+		+/vit E
Concentration	10^9 cells/ml	2.2 ± 0.2	2.4 ± 0.1		2.6 ± 0.1
Motile	%	58.3 ± 2.5	58.6 ± 1.2		57.3 ± 2.4
Fertility (overall)	%	39.0 ± 5.3	50.0 ± 9.2		55.2 ± 4.8 **c**
Fertility (week 1)	%	54.7 ± 2.3	68.5 ± 4.9	**c/ns**	67.0 ± 12.5
Fertility (week 2)	%	35.0 ± 4.6	44.0 ± 9.7		51.3 ± 3.8 **c**

Values are means ± S.E. of observations from 3 lots of 4 pooled ejaculates (12 samples) per group.
Significance of difference between groups : [c]P<0.05.
ns represents not significant.

Table 7.4a. Lipid characteristics of spermatozoa and seminal plasma at 24 weeks of age.

	Spermatozoa				Seminal plasma		
22:6 (n-3) supplementation	-	+		+/vit E	-	+	+/vit E
Total lipid ($\mu g/10^9$ cells/ml)	674.1 ± 26.0	745.1 ± 20.1		660.6 ± 29.6	280.9 ± 24.8	340.1 ± 33.3	281.9 ± 44.8
Lipid class (%w/w of total lipid)							
Phospholipid	70.1 ± 1.3	74.6 ± 0.7	**c/ns**	73.2 ± 1.9	41.5 ± 2.3	40.7 ± 4.0	40.5 ± 0.5
Free cholesterol	20.4 ± 0.9	20.4 ± 0.4		20.3 ± 0.7	38.8 ± 1.2	38.0 ± 2.4	36.3 ± 0.7
Free fatty acid	3.3 ± 0.7	1.8 ± 0.2		2.2 ± 0.3	6.1 ± 1.0	5.5 ± 0.7	6.9 ± 0.4
Triacylglycerol	4.1 ± 0.8	1.7 ± 0.3	**c/ns**	1.9 ± 0.6	4.2 ± 0.6	3.9 ± 0.8	3.9 ± 0.9
Cholesterol ester	2.0 ± 0.5	1.6 ± 0.3		2.5 ± 1.0	8.3 ± 2.0	12.5 ± 1.8	11.4 ± 0.3

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [c]$P<0.05$.
ns represents not significant.

Table 7.4b. Lipid composition of spermatozoa and seminal plasma at 40 weeks of age.

	Spermatozoa					Seminal plasma			
22:6 (n-3) supplementation	-	+		+/vit E	-	+		+/vit E	
Total lipid (μg/10^9 cells/ml)	1002.8 ± 129.4	945.2 ± 78.0		823.2 ± 105.7	517.1 ± 16.2	325.1 ± 35.5	b/ns	404.3 ± 84.5	
Lipid class (%w/w of total lipid)									
Phospholipid	69.1 ± 2.6	75.6 ± 1.3	c/c	66.3 ± 2.4	30.2 ± 0.9	41.9 ± 2.2	b/ns	44.3 ± 4.6	c
Free cholesterol	24.5 ± 1.9	21.3 ± 1.2	ns/c	28.7 ± 1.8	59.5 ± 1.7	47.8 ± 2.6	bns	43.6 ± 3.7	b
Free fatty acid	1.8 ± 0.1	1.6 ± 0.1		2.5 ± 0.5	5.5 ± 0.4	6.3 ± 0.8		6.1 ± 0.5	
Triacylglycerol	2.6 ± 1.1	0.7 ± 0.05		1.3 ± 0.3	2.3 ± 0.7	1.8 ± 0.6		3.8 ± 1.1	
Cholesterol ester	2.0 ± 0.4	0.8 ± 0.2	c/ns	1.1 ± 0.3	2.5 ± 0.6	2.1 ± 0.8		2.1 ± 0.9	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant

Table 7.4c. Lipid characteristics of spermatozoa and seminal plasma at 58 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Total lipid (μg/10^9 cells/ml)	797.8 ± 37.8	859.9 ± 39.43	**ns/b**	608.5 ± 21.8	**b**	665.5 ± 51.3	1160.6 ± 123.2	**c/c**	567.2 ± 113.3	
Lipid class (%w/w of total lipid)										
Phospholipid	68.9 ± 3.0	65.8 ± 2.4		63.5 ± 2.2		39.3 ± 4.5	45.2 ± 0.9		42.8 ± 4.3	
Free cholesterol	25.9 ± 2.2	27.5 ± 0.8		31.6 ± 1.9		37.3 ± 3.8	25.4 ± 1.1	**c/ns**	28.9 ± 1.2	
Free fatty acid	2.4 ± 0.4	3.1 ± 0.8		2.2 ± 0.2		11.1 ± 1.5	15.0 ± 2.2		8.5 ± 1.1	
Triacylglycerol	1.7 ± 0.3	1.7 ± 0.5		1.1 ± 0.3		7.7 ± 1.8	7.5 ± 3.3		6.6 ± 1.3	
Cholesterol ester	1.1 ± 0.4	0.3 ± 0.1		1.4 ± 0.3		4.7 ± 0.6	8.0 ± 1.6		10.3 ± 0.6	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant

Table 7.5a. **Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma at 24 weeks of age.**

	Spermatozoa					Seminal plasma					
22:6 (n-3) supplementation	-	+		**+/vit E**		-	+		**+/vit E**	
Fatty acid (%w/w total fatty acid)										
16:0	14.3 ± 0.4	15.6 ± 1.0		15.6 ± 0.4		13.3 ± 0.4	14.4 ± 0.7		14.7 ± 1.8	
18:0	19.8 ± 0.3	20.2 ± 0.1		19.8 ± 0.4		19.4 ± 0.1	19.4 ± 1.0		17.0 ± 0.3	**a**
18:1 (n-9)	14.4 ± 0.2	16.7 ± 0.2	**a/ns**	16.4 ± 0.5	**b**	16.6 ± 0.8	20.0 ± 1.3		19.2 ± 0.2	**c**
18:2 (n-6)	1.7 ± 0.2	1.1 ± 0.1		1.1 ± 0.2		8.1 ± 1.1	3.7 ± 0.3	**b/ns**	3.6 ± 0.6	**c**
20:4 (n-6)	13.0 ± 0.1	9.2 ± 0.3	**a/ns**	8.9 ± 0.1	**a**	12.6 ± 0.3	8.3 ± 0.7	**b/ns**	7.7 ± 0.6	**a**
22:4 (n-6)	19.5 ± 0.8	8.3 ± 0.7	**a/ns**	8.5 ± 0.4	**a**	9.0 ± 0.8	5.0 ± 0.7	**c/ns**	4.4 ± 0.9	**b**
20:5 (n-3)	nd	< 1		< 1		1.1 ± 0.1	3.4 ± 0.3	**a/ns**	3.7 ± 0.4	**a**
22:5 (n-3)	1.7 ± 0.1	4.0 ± 0.2	**a/ns**	4.5 ± 0.2	**a**	1.3 ± 0.1	2.6 ± 0.2	**a/ns**	2.7 ± 0.1	**a**
22:6 (n-3)	4.7 ± 0.1	13.1 ± 0.5	**a/ns**	13.3 ± 0.5	**a**	5.1 ± 0.2	10.8 ± 0.5	**a/ns**	11.1 ± 0.6	**a**
n-6/n-3	5.7 ± 0.1	1.2 ± 0.1	**a/ns**	1.1 ± 0.1	**a**	4.6 ± 0.2	1.2 ± 0.1	**a/ns**	1.2 ± 0.1	**a**
P/S	1.2 ± 0.2	1.0 ± 0.1	**c/ns**	1.1 ± 0.1		1.2 ± 0.1	1.1 ± 0.1		1.1 ± 0.1	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.5b. Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma at 40 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		**+/vit E**		-	+		**+/vit E**	
Fatty acid (%w/w total fatty acid)										
16:0	13.7 ± 0.4	14.0 ± 0.2		13.4 ± 0.3		12.6 ± 0.8	12.8 ± 0.6		14.4 ± 1.3	
18:0	20.9 ± 0.6	20.9 ± 0.2		20.4 ± 0.3		20.5 ± 0.6	22.3 ± 0.9		23.3 ± 1.3	
18:1 (n-9)	14.4 ± 0.2	17.2 ± 0.3	**a/ns**	16.3 ± 0.7	**c**	15.7 ± 0.5	19.8 ± 0.6	**b/ns**	22.4 ± 3.5	
18:2 (n-6)	2.5 ± 0.4	1.1 ± 0.02	**c/ns**	1.1 ± 0.1	**c**	4.7 ± 0.04	2.3 ± 0.4	**a/ns**	1.8 ± 0.2	**a**
20:4 (n-6)	11.4 ± 0.3	8.6 ± 0.2	**a/ns**	8.5 ± 0.1	**a**	14.5 ± 0.4	10.1 ± 0.3	**a/ns**	9.0 ± 0.4	**a**
22:4 (n-6)	21.7 ± 0.2	14.9 ± 0.4	**a/ns**	15.0 ± 0.6	**a**	13.3 ± 0.5	11.2 ± 0.9		11.6 ± 1.2	
20:5 (n-3)	nd	1.7 ± 0.03		1.9 ± 0.1		< 1.0	2.8 ± 0.7		1.8 ± 0.1	
22:5 (n-3)	nd	< 1.0		< 1.0		2.9 ± 0.1	2.4 ± 0.3		1.7 ± 0.2	
22:6 (n-3)	3.8 ± 1.1	9.8 ± 0.3	**a/ns**	10.1 ± 0.2	**a**	2.5 ± 0.1	3.2 ± 0.9		5.0 ± 2.5	
n-6/n-3	13.5 ± 4.1	2.1 ± 0.1	**c/ns**	2.0 ± 0.1	**c**	5.8 ± 0.3	2.9 ± 0.3	**a/ns**	2.9 ± 0.6	**b**
P/S	1.1 ± 0.2	1.0 ± 0.1	**c/ns**	1.0 ± 0.2	**c**	1.1 ± 0.1	0.9 ± 0.1		0.8 ± 0.2	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : ^{a}P<0.001 ; ^{b}P<0.01 ; ^{c}P<0.05.
nd represents not detectable.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.5c. **Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma at 58 weeks of age.**

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid (%w/w total fatty acid)										
16:0	13.2 ± 0.5	14.0 ± 0.5		13.4 ± 0.2		21.9 ± 4.4	16.2 ± 0.3	**ns/c**	17.5 ± 0.2	
18:0	20.7 ± 0.4	20.7 ± 0.2		20.9 ± 0.2		21.4 ± 0.6	22.1 ± 0.2	**ns/b**	23.9 ± 0.3	**b**
18:1 (n-9)	16.0 ± 0.2	18.6 ± 0.3	**a/c**	16.6 ± 0.5		19.6 ± 1.6	19.6 ± 0.4		19.3 ± 0.5	
18:2 (n-6)	2.3 ± 0.2	1.3 ± 0.8		1.2 ± 0.1	**b**	3.8 ± 0.6	1.8 ± 0.1	**a/a**	< 1	**a**
20:4 (n-6)	10.7 ± 0.2	8.3 ± 0.2	**a/ns**	8.7 ± 0.2	**a**	8.9 ± 1.0	8.0 ± 0.2		8.8 ± 0.4	
22:4 (n-6)	18.2 ± 0.4	12.4 ± 0.5	**a/ns**	13.7 ± 0.5	**a**	10.1 ± 1.4	9.1 ± 0.5		10.4 ± 0.3	
20:5 (n-3)	< 1	1.0 ± 0.2		1.1 ± 0.1	**b**	nd	2.1 ± 0.2	**a/a**	nd	
22:5 (n-3)	1.9 ± 0.03	3.1 ± 0.1	**a/ns**	3.1 ± 0.2	**a**	2.6 ± 0.3	3.7 ± 0.2	**c/ns**	3.5 ± 0.2	**c**
22:6 (n-3)	5.1 ± 0.2	9.1 ± 0.3	**a/ns**	9.6 ± 0.2	**a**	3.7 ± 0.4	7.6 ± 0.2	**a/ns**	7.5 ± 0.4	**a**
n-6/n-3	4.5 ± 0.1	1.8 ± 0.2	**a/ns**	1.8 ± 0.1	**a**	4.1 ± 0.7	1.5 ± 0.1	**b/c**	1.8 ± 0.1	**c**
P/S	1.2 ± 0.1	1.0 ± 0.3		1.1 ± 0.2		0.7 ± 0.1	0.9 ± 0.1		0.7 ± 0.2	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.9a. Phospholipid composition of spermatozoa and seminal plasma at 24 weeks of age.

	Spermatozoa			Seminal plasma			
22:6 (n-3) supplementation	-	+	+/vit E	-	+		+/vit E
Phospholipid class (% w/w of total phospholipid)							
Phosphatidyl ethanolamine	31.8 ± 1.7	35.8 ± 0.6	32.7 ± 1.4	46.0 ± 1.0	41.3 ± 1.3	c/ns	44.7 ± 2.7
Phosphatidyl choline	31.7 ± 1.4	30.0 ± 1.1	31.9 ± 0.3	13.3 ± 1.5	15.2 ± 0.6		17.2 ± 1.0
Phosphatidyl serine	22.2 ± 0.9	20.9 ± 0.3	21.8 ± 1.1	20.0 ± 0.6	21.7 ± 1.1		20.0 ± 0.7
Sphingomyelin	9.4 ± 0.7	8.4 ± 0.3	8.1 ± 0.5	19.9 ± 0.9	21.4 ± 1.1		18.6 ± 0.4
Cardiolipin	4.9 ± 0.3	4.8 ± 0.1	5.4 ± 1.1	< 1.0	< 1.0		1.3 ± 0.1

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.

Table 7.9b. Phospholipid composition of spermatozoa and seminal plasma at 40 weeks of age.

	Spermatozoa			Seminal plasma		
22:6 (n-3) supplementation	-	+	**+/vit E**	-	+	**+/vit E**
Phospholipid class (% w/w of total phospholipid)						
Phosphatidyl ethanolamine	30.1 ± 0.9	29.6 ± 1.3	31.2 ± 1.8	45.9 ± 0.4	44.2 ± 1.8	45.8 ± 1.2
Phosphatidyl choline	33.9 ± 0.3	34.8 ± 1.9	32.9 ± 1.0	13.7 ± 0.4	14.3 ± 1.0	13.4 ± 0.5
Phosphatidyl serine	19.5 ± 1.2	20.3 ± 0.5	20.0 ± 1.2	18.2 ± 0.3	18.8 ± 1.0	17.5 ± 1.0
Sphingomyelin	10.9 ± 0.3	10.5 ± 0.7	11.6 ± 0.3	21.2 ± 1.3	22.6 ± 1.1	24.8 ± 1.1
Cardiolipin	4.6 ± 0.6	4.1 ± 0.1	3.7 ± 0.3	< 1.0	< 1.0	< 1.0

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.

Table 7.9c. Phospholipid composition of spermatozoa and seminal plasma at 58 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Phospholipid class (% w/w of total phospholipid)										
Phosphatidyl ethanolamine	21.8 ± 1.0	27.8 ± 0.9	**b/ns**	28.6 ± 1.3	**b**	24.4 ± 0.6	23.6 ± 0.4	**ns/c**	25.6 ± 0.5	
Phosphatidyl choline	37.1 ± 1.7	32.3 ± 0.6	**c/ns**	31.8 ± 0.6	**c**	35.3 ± 0.7	37.0 ± 0.8	**ns/a**	31.5 ± 0.3	**b**
Phosphatidyl serine	21.8 ± 1.8	24.8 ± 1.3	**ns/c**	20.7 ± 0.3		13.8 ± 1.8	14.0 ± 0.5		16.6 ± 1.1	
Sphingomyelin	16.3 ± 1.1	12.8 ± 1.6		13.5 ± 1.1		23.4 ± 1.3	24.3 ± 1.0		23.3 ± 0.8	
Cardiolipin	3.1 ± 0.3	2.8 ± 0.2	**ns/a**	4.6 ± 0.2	**b**	2.2 ± 1.3	1.3 ± 0.3		2.0 ± 0.4	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant

Table 7.10a. The fatty acid composition of spermatozoa phosphatidyl ethanolamine at 24 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(%w/w of total fatty acid)					
16:0	6.3 ± 1.0	5.8 ± 0.5		5.3 ± 1.0	
18:0	15.4 ± 2.0	18.2 ± 0.4	**ns/a**	11.3 ± 0.5	
18:1 (n-9)	13.7 ± 0.6	16.7 ± 1.2	**ns/b**	11.0 ± 0.9	
18:2 (n-6)	3.9 ± 1.4	1.7 ± 0.2		1.4 ± 0.2	
20:4 (n-6)	12.3 ± 1.2	10.9 ± 1.2		12.7 ± 0.5	
22:4 (n-6)	29.7 ± 2.8	13.1 ± 1.2	**b/ns**	14.0 ± 1.0	**b**
20:5 (n-3)	3.33 ± 1.7	4.0 ± 3.1		2.0 ± 0.4	
22:5 (n-3)	2.0 ± 0.1	6.2 ± 0.5	**a/ns**	8.0 ± 0.6	**a**
22:6 (n-3)	6.7 ± 0.7	15.9 ± 1.5	**b/c**	22.6 ± 1.4	**a**
n-6/n-3	4.8 ± 1.1	1.1 ± 0.1	**c/ns**	< 1.0	c

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : $^{a}P<0.001$; $^{b}P<0.01$; $^{c}P<0.05$.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.10b. The fatty acid composition of spermatozoa phosphatidyl ethanolamine at 40 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	3.4 ± 1.0	3.3 ± 0.4		3.6 ± 0.1	
18:0	13.3 ± 1.9	11.8 ± 1.0		11.0 ± 0.7	
18:1 (n-9)	8.3 ± 1.0	9.2 ± 0.5		9.2 ± 1.3	
18:2 (n-6)	1.9 ± 0.4	1.2 ± 0.1		1.3 ± 0.2	
20:4 (n-6)	15.6 ± 1.9	13.6 ± 0.2		12.7 ± 0.8	
22:4 (n-6)	34.8 ± 1.0	22.2 ± 1.1	**a/ns**	21.8 ± 0.6	**a**
20:5 (n-3)	1.4 ± 0.7	8.0 ± 0.3	**a/ns**	10.3 ± 1.8	**b**
22:5 (n-3)	1.3 ± 0.1	3.5 ± 0.01	**a/ns**	4.0 ± 0.6	**b**
22:6 (n-3)	6.1 ± 1.2	16.1 ± 0.1	**a/ns**	16.4 ± 1.0	**a**
n-6/n-3	5.5 ± 0.4	1.3 ± 0.1	**a/ns**	1.2 ± 0.1	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.10c. The fatty acid composition of spermatozoa phosphatidyl ethanolamine at 58 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	2.4 ± 0.2	3.4 ± 0.2	**c/ns**	4.3 ± 0.7	**c**
18:0	11.8 ± 0.4	14.1 ± 0.7	**c/ns**	16.0 ± 2.8	
18:1 (n-9)	8.3 ± 0.2	13.3 ± 0.5	**a/ns**	13.2 ± 2.0	**c**
20:1 (n-9)	4.4 ± 0.2	4.5 ± 0.2		4.7 ± 0.2	
18:2 (n-6)	2.2 ± 0.1	1.5 ± 0.1	**a/ns**	1.5 ± 0.2	**c**
20:4 (n-6)	18.5 ± 0.5	14.2 ± 0.4	**a/ns**	12.9 ± 1.0	**b**
22:4 (n-6)	30.8 ± 0.5	20.5 ± 0.8	**a/ns**	19.9 ± 1.3	**a**
20:5 (n-3)	< 1	2.2 ± 0.2	**b/ns**	2.0 ± 0.5	**c**
22:5 (n-3)	3.1 ± 0.1	5.2 ± 0.3	**a/ns**	5.1 ± 0.6	**c**
22:6 (n-3)	9.4 ± 0.2	15.8 ± 0.8	**a/ns**	14.9 ± 1.1	**b**
n-6/n-3	4.0 ± 0.1	1.6 ± 0.1	**a/ns**	1.7 ± 0.1	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.11a. The fatty acid composition of spermatozoa phosphatidyl choline at 24 weeks of age.

Fatty acid (% w/w of total fatty acid)	22:6 (n-3) supplementation -	+		+/vit E	
16:0	22.6 ± 0.7	25.9 ± 1.6		21.5 ± 1.0	
18:0	25.3 ± 0.9	29.1 ± 2.2		24.6 ± 0.5	
18:1 (n-9)	19.5 ± 0.9	23.1 ± 0.7		20.6 ± 0.5	
18:2 (n-6)	2.0 ± 0.4	1.0 ± 0.1		2.2 ± 0.1	
20:4 (n-6)	8.4 ± 1.1	4.1 ± 0.5	c/ns	6.4 ± 0.2	
22:4 (n-6)	10.7 ± 2.2	3.5 ± 0.3	c/ns	5.4 ± 0.4	
22:5 (n-3)	1.0 ± 0.2	1.3 ± 0.2		2.4 ± 0.1	a
22:6 (n-3)	1.7 ± 0.3	3.3 ± 0.4	c/a	7.0 ± 0.3	a
n-6/n-3	8.3 ± 0.4	2.2 ± 0.1	a/ns	1.5 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.11b. The fatty acid composition of spermatozoa phosphatidyl choline at 40 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	21.5 ± 0.7	20.8 ± 0.4	ns/c	17.8 ± 0.9	c
18:0	28.5 ± 0.5	27.4 ± 0.2		27.0 ± 0.5	
18:1 (n-9)	21.5 ± 0.7	22.4 ± 0.9		20.9 ± 0.5	
18:2 (n-6)	2.3 ± 0.3	< 1.0		< 1.0	
20:4 (n-6)	6.1 ± 0.2	5.2 ± 0.4		5.6 ± 0.2	
22:4 (n-6)	9.9 ± 0.4	7.7 ± 0.5	c/c	9.4 ± 0.1	
22:5 (n-3)	< 1	< 1		1.3 ± 0.1	
22:6 (n-3)	1.1 ± 0.3	4.1 ± 0.2	a/c	5.2 ± 0.4	a
n-6/n-3	21.3 ± 8.9	3.1 ± 0.2		2.4 ± 0.1	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [c]P<0.05.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.11c. The fatty acid composition of spermatozoa phosphatidyl choline at 58 weeks of age.

Fatty acid (% w/w of total fatty acid)	22:6 (n-3) supplementation -	+		+/vit E	
16:0	19.0 ± 2.0	22.1 ± 1.3		21.9 ± 3.0	
18:0	26.9 ± 0.2	28.1 ± 1.0		27.0 ± 2.7	
18:1 (n-9)	21.0 ± 0.2	24.7 ± 0.5	a/b	21.0 ± 0.6	
20:1 (n-9)	4.1 ± 0.3	4.5 ± 0.2		4.9 ± 0.4	
18:2 (n-6)	2.2 ± 0.2	1.0 ± 0.1	b/ns	1.0 ± 0.1	b
20:4 (n-6)	7.0 ± 0.3	4.0 ± 0.1	a/ns	6.1 ± 2.2	
22:4 (n-6)	10.2 ± 0.8	5.7 ± 0.6	b/ns	9.3 ± 3.2	
22:5 (n-3)	1.1 ± 0.1	1.1 ± 0.1		1.7 ± 0.6	
22:6 (n-3)	2.6 ± 0.2	3.2 ± 0.3		5.7 ± 2.7	
n-6/n-3	5.7 ± 0.5	2.7 ± 0.1	b/ns	2.7 ± 0.4	b

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.12a. The fatty acid composition of spermatozoa phosphatidyl serine at 24 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	7.9 ± 0.7	11.1 ± 1.0	c/ns	15.4 ± 0.7	a
18:0	19.8 ± 0.7	21.1 ± 0.6	ns/c	14.3 ± 1.7	c
18:1 (n-9)	14.0 ± 1.3	17.5 ± 1.3		15.6 ± 0.6	
18:2 (n-6)	2.1 ± 0.3	1.2 ± 0.1	c/ns	3.2 ± 0.1	c
20:4 (n-6)	15.5 ± 1.4	10.7 ± 0.6	c/ns	9.0 ± 0.8	b
22:4 (n-6)	20.1 ± 2.2	9.3 ± 0.4	b/c	6.8 ± 0.7	b
20:5 (n-3)	3.5 ± 1.4	1.0 ± 0.3	ns/b	13.3 ± 2.1	c
22:5 (n-3)	2.2 ± 0.2	4.1 ± 0.4	b/ns	4.1 ± 0.1	a
22:6 (n-3)	6.1 ± 0.9	13.9 ± 1.9	b/ns	20.5 ± 2.0	a
n-6/n-3	3.9 ± 0.7	1.3 ± 0.2	c/c	0.6 ± 0.1	b

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.12b. The fatty acid composition of spermatozoa phosphatidyl serine at 40 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	8.1 ± 0.4	6.7 ± 0.1	**c/ns**	7.6 ± 1.4	
18:0	21.3 ± 0.7	19.2 ± 1.0		16.0 ± 1.1	**b**
18:1 (n-9)	13.9 ± 0.3	15.0 ± 1.4		14.8 ± 1.7	
18:2 (n-6)	2.0 ± 0.3	1.0 ± 0.1	**c/ns**	1.5 ± 0.5	
20:4 (n-6)	15.2 ± 0.3	13.2 ± 1.3		13.3 ± 1.2	
22:4 (n-6)	23.1 ± 0.8	17.3 ± 0.4	**a/ns**	17.3 ± 0.7	**a**
20:5 (n-3)	2.0 ± 0.1	3.0 ± 0.2	**b/c**	5.6 ± 1.0	**c**
22:5 (n-3)	1.4 ± 0.1	2.9 ± 0.1	**a/ns**	2.9 ± 0.1	**a**
22:6 (n-3)	5.6 ± 0.4	15.3 ± 0.8	**a/ns**	17.5 ± 1.1	**a**
n-6/n-3	4.6 ± 0.3	1.5 ± 0.2	**a/ns**	1.2 ± 0.1	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.12c. The fatty acid composition of spermatozoa phosphatidyl serine at 58 weeks.

Fatty acid	22:6 (n-3) supplementation				
	-	**+**		**+/vit E**	
(% w/w of total fatty acid)					
16:0	7.6 ± 1.4	7.4 ± 0.2		9.0 ± 0.7	
18:0	23.2 ± 1.2	22.4 ± 1.0		27.0 ± 5.9	
18:1 (n-9)	16.6 ± 1.3	19.1 ± 0.6		20.9 ± 2.1	
20:1 (n-9)	3.4 ± 0.2	4.2 ± 0.3		5.7 ± 1.0	
18:2 (n-6)	2.8 ± 0.2	1.0 ± 0.1	**a/a**	nd	**a**
20:4 (n-6)	13.8 ± 1.5	10.4 ± 0.4		8.7 ± 1.7	
22:4 (n-6)	21.2 ± 1.2	14.6 ± 0.5	**b/ns**	12.9 ± 1.4	**b**
20:5 (n-3)	nd	< 1	**a/ns**	< 1	**a**
22:5 (n-3)	1.9 ± 0.1	3.1 ± 0.2	**a/ns**	3.0 ± 0.1	**b**
22:6 (n-3)	7.1 ± 0.7	13.1 ± 0.7	**a/ns**	10.0 ± 1.4	
n-6/n-3	4.7 ± 0.3	1.7 ± 0.1	**a/ns**	2.0 ± 0.2	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.13a. The fatty acid composition of spermatozoa sphingomyelin at 24 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	32.3 ± 1.7	34.9 ± 4.2		29.4 ± 1.6	
18:0	25.4 ± 2.5	26.9 ± 1.1		22.5 ± 1.9	
18:1 (n-9)	13.6 ± 3.6	9.3 ± 1.7		11.0 ± 2.3	
18:2 (n-6)	2.5 ± 0.8	1.6 ± 0.2		2.2 ± 0.1	
20:4 (n-6)	3.5 ± 0.9	2.1 ± 0.8		< 1.0	
22:4 (n-6)	2.4 ± 0.5	2.6 ± 0.9		< 1.0	
20:5 (n-3)	1.6 ± 0.6	3.9 ± 0.1		9.9 ± 1.9	c
22:6 (n-3)	4.8 ± 2.6	8.3 ± 1.7	ns/c	16.4 ± 2.3	c
n-6/n-3	1.8 ± 0.8	0.5 ± 0.2		0.3 ± 0.2	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.13b. The fatty acid composition of spermatozoa sphingomyelin at 40 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	23.4 ± 1.8	30.9 ± 1.2	c/ns	37.9 ± 4.6	c
18:0	19.8 ± 1.2	22.6 ± 2.7		26.9 ± 2.0	c
18:1 (n-9)	4.9 ± 1.3	6.2 ± 0.9		7.2 ± 1.6	
18:2 (n-6)	2.2 ± 0.2	nd	a/ns	nd	a
20:4 (n-6)	7.2 ± 1.7	9.5 ± 1.8		9.4 ± 2.0	
22:4 (n-6)	6.3 ± 1.0	6.3 ± 1.1		8.5 ± 1.8	
20:5 (n-3)	< 1.0	2.7 ± 1.7		< 1.0	
22:5 (n-3)	8.1 ± 1.6	7.7 ± 1.6		5.3 ± 4.1	
22:6 (n-3)	25.5 ± 3.6	11.2 ± 3.6	c/ns	6.3 ± 1.8	b
n-6/n-3	0.5 ± 0.2	0.8 ± 0.2		0.6 ± 0.3	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]$P<0.001$; [b]$P<0.01$; [c]$P<0.05$.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.13c. The fatty acid composition of spermatozoa sphingomyelin at 58 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	25.1 ± 3.3	26.3 ± 2.6		27.9 ± 2.5	
18:0	21.0 ± 1.4	23.4 ± 3.0		25.9 ± 0.2	c
18:1 (n-9)	6.9 ± 2.6	7.3 ± 0.5	ns/c	5.5 ± 0.1	
18:2 (n-6)	nd	nd		nd	
20:4 (n-6)	nd	nd		nd	
22:4 (n-6)	nd	nd		nd	
20:5 (n-3)	5.9 ± 0.9	5.6 ± 0.3		7.8 ± 1.3	
22:5 (n-3)	10.1 ± 1.7	11.1 ± 1.0		13.3 ± 2.9	
22:6 (n-3)	14.4 ± 2.2	17.7 ± 2.3		6.4 ± 2.9	
n-6/n-3	-	-		-	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [c]P<0.05.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.14a. The fatty acid composition of spermatozoa cardiolipin at 24 weeks of age.

Fatty acid (% w/w of total fatty acid)	22:6 (n-3) supplementation				
	-	+		+/vit E	
16:0	4.6 ± 0.3	5.2 ± 0.3	**ns/a**	7.2 ± 0.1	**a**
18:0	9.4 ± 0.2	9.0 ± 0.1		10.8 ± 0.6	
18:1 (n-9)	6.8 ± 0.4	8.2 ± 0.2	**c/ns**	7.5 ± 0.8	
18:2 (n-6)	2.3 ± 0.3	2.0 ± 0.3	**ns/c**	3.6 ± 0.3	**c**
20:4 (n-6)	19.3 ± 0.4	14.0 ± 0.2	**a/ns**	13.4 ± 1.2	**b**
22:4 (n-6)	31.4 ± 1.9	13.4 ± 0.8	**b/ns**	13.7 ± 0.9	**a**
22:5 (n-3)	2.2 ± 0.1	7.0 ± 0.4	**a/ns**	6.0 ± 0.6	**a**
22:6 (n-3)	9.2 ± 0.2	24.2 ± 0.7	**a/ns**	23.5 ± 1.2	**a**
n-6/n-3	4.5 ± 0.3	1.0 ± 0.1	**a/ns**	1.0 ± 0.1	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.14b. The fatty acid composition of spermatozoa cardiolipin at 40 weeks of age.

Fatty acid	22:6 (n-3)-supplementation				
	-	**+**		**+/vit E**	
(% w/w of total fatty acid)					
16:0	12.8 ± 1.9	9.2 ± 1.3		10.9 ± 1.8	
18:0	11.5 ± 1.3	11.1 ± 0.6		9.8 ± 1.3	
18:1 (n-9)	9.0 ± 1.2	8.4 ± 1.2		10.7 ± 1.3	
18:2 (n-6)	5.3 ± 0.6	2.6 ± 0.5	**c/ns**	3.3 ± 0.5	**c**
20:4 (n-6)	11.3 ± 1.4	10.9 ± 0.4		8.2 ± 1.0	
22:4 (n-6)	25.9 ± 2.6	19.8 ± 1.3		17.4 ± 1.9	**c**
20:5 (n-3)	5.3 ± 0.6	7.9 ± 0.2	**b/ns**	7.8 ± 0.4	**c**
22:5 (n-3)	1.0 ± 0.1	3.1 ± 0.3	**a/ns**	2.9 ± 0.4	**b**
22:6 (n-3)	5.3 ± 0.8	14.0 ± 1.8	**b/ns**	12.1 ± 0.9	**b**
n-6/n-3	3.5 ± 0.1	1.2 ± 0.1	**a/ns**	1.3 ± 0.1	**a**

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.14c. The fatty acid composition of spermatozoa cardiolipin at 58 weeks of age.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	15.5 ± 3.8	7.8 ± 2.2		8.3 ± 3.0	
18:0	16.1 ± 2.0	11.4 ± 2.5		12.0 ± 2.4	
18:1 (n-9)	17.2 ± 3.0	11.1 ± 2.5		12.0 ± 2.5	
20:1 (n-9)	2.0 ± 0.3	3.9 ± 0.8		3.0 ± 0.5	
18:2 (n-6)	4.6 ± 0.5	2.0 ± 0.2		2.2 ± 0.4	
20:4 (n-6)	3.6 ± 0.9	10.1 ± 0.4	**b/ns**	7.3 ± 1.3	
22:4 (n-6)	6.5 ± 1.9	14.4 ± 1.7	**c/ns**	11.3 ± 1.5	
20:5 (n-3)	6.3 ± 2.5	3.5 ± 2.4		2.3 ± 0.2	
22:5 (n-3)	1.8 ± 0.3	4.6 ± 0.6	**c/ns**	2.8 ± 0.5	
22:6 (n-3)	2.7 ± 0.3	14.3 ± 1.6	**b/ns**	9.8 ± 1.0	**b**
n-6/n-3	2.7 ± 0.4	1.8 ± 0.2	**c/ns**	2.1 ± 0.4	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.15. Spermatozoa, testes and liver characteristics taken at the end of experimental period.

	22:6 (n-3) supplementation				
	-	+		+/vit E	
Weight testes g	14.0 ± 0.6	17.7 ± 2.4		19.1 ± 0.9	b
Vitamin E µg/10^9 cells	0.1 ± 0.1	0.1 ± 0.1	ns/b	0.4 ± 0.3	c
Vitamin E µg/g testes	7.2 ± 0.6	5.1 ± 0.2	c/c	14.7 ± 2.2	c
Vitamin E µg/g liver	24.5 ± 4.1	25.0 ± 4.1	ns/b	147.9 ± 22.6	b
MDA nmoles/ml semen	131.4 ± 19.7	260.7 ± 27.8	b/a	69.3 ± 5.6	c

Values are means ± S.E. of observations from 4 pooled samples each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.16. Fatty acid composition of the total phospholipid of the testes.

Fatty acid	22:6 (n-3) supplementation				
(% w/w of total fatty acid)	-	+		+/vit E	
16:0	23.0 ± 0.3	23.8 ± 0.3		24.2 ± 0.2	c
18:0	16.1 ± 0.1	16.7 ± 0.2	c/ns	16.8 ± 0.2	b
18:1 (n-9)	11.0 ± 0.1	13.4 ± 0.4	a/ns	12.5 ± 0.3	b
20:1 (n-9)	3.4 ± 0.1	3.4 ± 0.2		3.7 ± 0.1	c
18:2 (n-6)	3.1 ± 0.2	1.6 ± 0.1	a/c	1.4 ± 0.1	a
20:4 (n-6)	15.8 ± 0.3	11.7 ± 0.1	a/ns	11.3 ± 0.2	a
22:4 (n-6)	14.1 ± 0.3	9.4 ± 0.4	a/ns	9.7 ± 0.5	a
20:5 (n-3)	< 1.0	2.5 ± 0.1	a/c	2.9 ± 0.1	a
22:5 (n-3)	1.1 ± 0.2	2.0 ± 0.1	a/ns	2.1 ± 0.1	a
22:6 (n-3)	2.5 ± 0.4	6.0 ± 0.2	a/c	6.9 ± 0.3	a
n-6/n-3	7.9 ± 0.2	2.3 ± 0.1	a/c	2.0 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled samples each of 3 birds.
Significance of difference between groups : $^{a}P<0.001$; $^{b}P<0.01$; $^{c}P<0.05$.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.17. Fatty acid composition of the total phospholipid of liver.

Fatty acid	22:6 (n-3) supplementation				
	-	+		+/vit E	
(% w/w of total fatty acid)					
16:0	21.1 ± 0.3	24.7 ± 0.3	a/ns	24.8 ± 0.7	b
18:0	23.9 ± 0.3	21.5 ± 0.5	b/ns	20.9 ± 0.4	a
18:1 (n-9)	4.0 ± 0.1	5.6 ± 1.1		6.5 ± 1.8	
18:2 (n-6)	21.6 ± 0.4	8.6 ± 0.2	a/b	7.5 ± 0.2	a
20:4 (n-6)	7.5 ± 0.3	4.8 ± 0.3	a/ns	3.7 ± 0.3	a
20:5 (n-3)	2.4 ± 0.1	3.4 ± 0.3	c/ns	2.7 ± 0.1	c
22:5 (n-3)	1.4 ± 0.1	1.7 ± 0.2		1.7 ± 0.1	
22:6 (n-3)	13.0 ± 0.2	24.9 ± 0.5	a/ns	25.7 ± 1.1	a
n-6/n-3	1.9 ± 0.2	0.6 ± 0.1	a/a	0.5 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled samples each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

7.4 DISCUSSION

It appears that although domestic avian species seem unable to utilise 18:3 (n-3) as a precursor for the eventual synthesis of 22:6 (n-3), as discussed in Chapter 6, they seem amenable to direct dietary supplementation of 22:6 (n-3) which is readily and efficiently incorporated into the liver, testes, spermatozoa and seminal plasma of the cockerels. Such dietary increases of 22:6 (n-3) which led to these changes also appear to require the addition of an appropriate antioxidant, such as vitamin E, to avoid peroxidative damage and ultimate loss of these long chain polyunsaturated fatty acids (Bassler, 1991 ; Aitken, 1994).

The results observed were similar to those previously observed in laboratory animals where fish oil feeding led to a decrease in standard 22:5 (n-6) levels of spermatozoa lipids in rats fed a standard laboratory feed and an increase in 22:6 (n-3) associated with an improvement in testes maturation and enhanced spermatogenesis (Salem *et al.*, 1986 and 1989). The importance of 22:6 (n-3) in male farm animal fertility has been frequently reviewed. Nissen *et al.* (1981) and Nissen and Kreysel (1983) reported significant positive correlations between the level of 22:6 (n-3) in human spermatozoa and corresponding values for spermatozoa concentration and motility. Indeed these workers reported lower levels of 22:6 (n-3) within the spermatozoa of men suffering from fertility problems. 22:6 (n-3) may play a vital role in membrane composition and fluidity by contributing to the plasma membrane structure. Polyunsaturated fatty acids of this type are known to introduce a "kink" into the fatty acid sidechain encouraging a more open and mobile membrane phospholipid conformation, this flexibility being reported to play a key role in ultimate spermatozoa viability (Cossins, 1983 ; Holt and North, 1985 ; Gottlieb and Bygdeman, 1988 ; Knapp, 1990 ; Watkins, 1991).

The changes which took place within the fatty acid profiles of the individual phospholipid classes displayed several features. The major phospholipid classes, phosphatidyl ethanolamine and phosphatidyl choline displayed standard patterns of increased n-3, decreased n-6 and n-6/n-3 ratios. These changes were less dramatic in phosphatidyl choline which has been previously reported as being much more saturated (Chapter 4, 5 and 6). However, fatty acid changes within the phospholipid classes as a result of 22:6 (n-3) feeding showed great variation for example increased levels of 22:6

(n-3) within phosphatidyl ethanolamine with reduced levels in the sphingomyelin class. It has been frequently reported that such changes in spermatozoa phospholipid levels as well as alterations in the fatty acid composition of phospholipid are associated with differences in semen quality (Nissen and Kreysel, 1983 ; Holt and North, 1985 ; Sebastian *et al.*, 1987). The mechanism by which these phospholipid class-specific changes in 22:6 (n-3) content are accomplished is unclear but could reflect changes in the relative affinity of the respective acyltransferases for docosahexaenyl CoA under conditions of increasing concentrations of this substrate. Indeed the net effect of such a change is to almost cancel out the other major increases in 22:6 (n-3) levels within all other phospholipid classes in order to achieve the correct balance of polyunsaturates of n-6 and n-3 series for maximum membrane function, motility and fertilising potential.

The results clearly demonstrate that a considerable increase in the level of 22:6 (n-3) in the lipids of cockerel spermatozoa can be readily achieved by dietary supplementation with an appropriate source of this fatty acid. In this respect feeding with preformed 22:6 (n-3) is more successful than simply providing the birds with the 18:3 (n-3) precursor since spermatozoa appear unable to convert the latter to the former (see Chapter 6). It is however important to note that the proportions of 22:6 (n-3) that are attained in the phospholipid of the cockerel spermatozoa as a result of fish oil feeding fell far short of that which is routinely observed for mammals on standard, non-22:6 (n-3) supplemented diets. Feeding large amounts of 22:6 (n-3) to cockerels only succeeded in raising the proportion of this fatty acid in the cockerel spermatozoa to approximately 10 % compared to approximately 60 % in the bull and ram (Poulos *et al.*, 1973a). Moreover, although the spermatozoa levels of 20:4 (n-6) and 22:4 (n-6) in cockerel were reduced by fish oil feeding, the resultant proportion of these n-6 polyunsaturated fatty acids were still far higher than are found in mammalian spermatozoa. Thus, a major conclusion of this work is that dietary supplementation of birds with n-3 fatty acids does not result in a dramatic conversion of the characteristic fatty acid profile of avian spermatozoa into that typical of mammals. Rather it induces a more limited displacement in that direction to produce a somewhat hybrid composition. In fact, it could be said that cockerel spermatozoa displays a remarkable resilience to changes in fatty acid composition in the face of a high and sustained dietary input of n-3 fatty acids. The obvious implication of such findings is that the typical predominance of n-6 polyunsaturated fatty acids in poultry spermatozoa probably reflects a true difference between avian and mammalian

species and is not an artefact consequential of the feeding of the relatively n-3 deficient commercial diets. A clear resolution of this question could be provided by analysis of semen obtained from free-living birds in the wild.

What is the functional significant of this n-6/n-3 dichotomy between avian and mammalian spermatozoa ? A possible suggestion may be that the difference in fatty acid profiles could represent an adaptation to temperature. The higher body temperature of birds compared to mammals (41 °C compared to 37 °C) (Romijn and Lokhorst, 1966) coupled with the fact that mammalian but not avian testes are usually maintained at a temperature which is cooler than the rest of the body due to externalisation from the body cavity, indicates that avian spermatozoa must develop and function in an environment which may be up to 6 °C warmer than that experienced by their mammalian counterparts. Since the major polyunsaturated fatty acid of avian spermatozoa, 22:4 (n-6) displays the same chain length but 2 fewer double bonds than 22:6 (n-3) which characterises mammalian spermatozoa, the difference in the degree of polyunsaturation could represent a means of maintaining the appropriate biophysical properties of the spermatozoa membranes at the two different temperatures. Indeed there is a great variation in spermatozoa morphology between the two species, the spear-like shape of the cockerel spermatoon and the bulbous headed mammalian spermatozoon could indicate a need for different membrane compositional properties (Hafez, 1993 ; Austin, 1995).

In the light of the above considerations, the improvement of fertility associated with the higher proportion of n-3 fatty acids in the spermatozoa phospholipids may seem surprising. It is possible that although the n-6 predominance may represent the natural situation for avian spermatozoa, the additional presence of levels of 22:6 (n-3) which are higher than those currently attained by commercial feeding regimes may provide a more appropriate balance between these two classes of polyunsaturated fatty acids for the promotion of maximal fertilising ability. The investigation of such speculations awaits further study. Clearly the results obtained illustrated a dramatic increase in the level of n-3 fatty acids within supplemented cockerels in a much more efficient manner than by providing the precursor 18:3 (n-3). The mechanism of chain elongation and desaturation allowing this process to occur naturally would appear to be absent from domestic avian species as in the wild the access to a diet rich in preformed 22:6 (n-3) would be far

greater. The use of this dietary manipulation in the commercial broiler breeder industry could have importance for the future, particularly if it could be proved that improvements in *in vivo* fertility on a large scale were feasible.

CHAPTER 8

THE EFFECT OF DIETARY 22:6 (N-3) SUPPLEMENTATION ON THE FRESH AND FROZEN SEMEN QUALITY OF THE BULL

8.1. INTRODUCTION

It is well recognised that the major polyunsaturated fatty acid consumed by grazing animals is 18:3 (n-3) in contrast to the predominance of 18:2 (n-6) in grain-based and manufactured feeds (Harfoot, 1981). Ashes *et al.* (1992) concluded that 20:5 (n-3) and 22:6 (n-3) were not biohydrogenated to any significant extent by rumen micro-organisms, in contrast to the extensive biohydrogenation of C18 fatty acids. In this study feeding fish oil to sheep and cattle resulted in a 12 - 17 % increase in 20:5 (n-3) and a 5 - 7 % increase in 22:6 (n-3) accompanied by a decrease in 18:0 and 18:2 (n-6) within the blood serum lipids of both animal species. However, there have been no previous studies on the effects of dietary supplementation of ruminant animals with C20 (n-3) fatty acids on the composition and quality of fresh and frozen semen.

Thus this experiment was carried out to investigate the following points ;

i the effect of 22:6 (n-3) supplementation on the fresh and frozen semen characteristics of commercial Holstein/Fresian bulls.

ii effects of supplementation on the lipid composition of spermatozoa and seminal plasma.

iii to assess any long term beneficial effects of 22:6 (n-3) on both fresh and frozen semen quality.

8.2. MATERIALS AND METHODS

Full experimental methods for each procedure are outlined in the relevant Sections of Chapter 3. Further details are as follows ;

8.2.1. The bulls

Semen samples were obtained from three groups, each containing three Holstein/Fresian bulls. There was a restriction on the number of bulls which could be, for the purposes of this study, withdrawn from commercial practice from Scottish Livestock Services, Scone, Perth, therefore only nine bulls were permitted for study. It was decided that to include three groups of bulls of varying semen quality parameters would offer the best

opportunity to observe changes in bulls of high and low semen quality as a result of 22:6 (n-3) supplementation and to use average semen quality bulls as the unsupplemented control group. Bulls were selected from a larger group from between the ages of 2 to 3 years, to obtain the three distinct groups of bulls with varying *in vitro* semen parameters, classified as high, average and low as discussed below. Semen was collected twice from each bull prior to collection for lipid and *in vitro* measurements to ensure semen production was representative. Thereafter semen was collected on a weekly basis.

Bulls placed in group A were classified as average bulls and were selected to represent the unsupplemented control group, group B were selected to represent those of below average semen quality and group C as bulls possessing above average semen quality parameters, as detailed in Table 8.1. For the purpose of the experiment the control bulls were fed Diet 1 and all other bulls, groups B and C were fed the 22:6(n-3) supplemented Diet 2, as outlined in Table 8.2.

All diets were milled by North Eastern Farmers Ltd., Bannermill, Aberdeen, according to instruction using 3 % (w/w of feed) soyabean oil, high in 18:2 (n-6) for the control diet (Diet 1), and 3% (w/w of feed) tuna orbital oil, high in 22:6 (n-3) (Scotia Pharmaceuticals, Carlisle, Scotland) for the supplemented diet to give a final oil content of 5.5 %. All other nutritional parameters were identical, with 15 % crude protein and 40 mg/kg of vitamin E. Both diets were prepared to ensure energy levels were equal and gave a final metabolisable energy of 12.5 MJ/kg. A full ration analysis for both diets was provided by the feed manufacturer and all feed batches were stored in air tight containers and routinely analysed to ensure uniformity of their fatty acid composition.

Table 8.1. Classification of the bulls used in the experiment from all previous *in vitro* assessments made on the individual bulls at Scottish Livestock Services, Perth.

Bull group	Date of birth	Motility (0-5)	Conc. 10^9/ml	Post-freeze parameters Motility	PPM
A	28.08.92	4	1.16	3.5	36
A	9.09.92	4	1.86	4	38
A	28.04.93	4	1.3	3.5	35
B	5.03.92	3	1.09	3.5	36
B	12.12.93	3	0.75	3.5	36
B	13.03.93	2	0.90	4	30
C	22.06.93	5	1.70	4	37
C	4.05.93	5	1.30	4	37
C	5.08.92	5	1.40	4	36

Table 8.2. Fatty acids of the diets.

Fatty acids % (w/w total)	Diet 1 Control	Diet 2 22:6 (n-3) supplemented
14:0		2
16:0	15	20
18:0	3	4
18:1 (n-9)	17	16
18:2 (n-6)	52	32
18:3 (n-3)	6	4
20:5 (n-3)	nd	2
22:6 (n-3)	1	11

All minor fatty acids comprising less than 1% of total lipid have been omitted.
nd presents not detectable.

8.2.2. Spermatozoa evaluation and analysis

Semen measurements were performed as described previously (see Sections 3.1, 3.7, 3.8, 3.9, 3.10, 3.12). For each bull *in vitro* semen parameters were measured regularly every

2 weeks during the experimental period to allow comparison of both fresh and frozen semen measurements.

There was no semen available to perform lipid analysis on the fatty acid composition of the phospholipid classes before supplementation, therefore statistical comparisons were made only on post-supplementation results from spermatozoa. Using the method for phospholipid separation (see Section 3.12) it was not possible to separate phosphatidyl inositol and phosphatidyl serine so fatty acids of these phospholipid classes were considered together.

8.2.3. Statistical analysis

Student *t-test* was used for statistical comparison of each group of bulls, before and after the supplementation period versus the post-supplementation period results of group A. Statistical significance would therefore be represented as e.g. c versus a (c/a). Results included 6 samples per group (2 ejaculates per bull) for lipid and *in vitro* semen parameters. Statistical analysis was undertaken using Microsoft Excel version 5.0.

8.3. RESULTS

8.3.1 Changes in semen characteristics.

The *in vitro* semen parameters measured both before and after dietary supplementation are detailed in Table 8.3.

The semen characteristics of the unsupplemented bulls at the beginning of the experimental period until the end showed no significant differences within all semen parameters. In comparison the supplemented bulls of group B showed significant improvements in spermatozoa concentration, post-freeze PPM (% progressively motile spermatozoa) and acrosomal integrity compared to those measurements made in group B at the beginning of the experimental period. Group C also showed improvements in post-freeze motility and acrosomal integrity after the supplementation period. At the beginning of the experiment group B bulls exhibited significantly lower levels of concentration, motility and acrosomal integrity when compared to the control bulls, however after dietary supplementation no such differences were detected. When comparing groups B and C after prolonged supplementation fresh semen parameters were still better in group C in terms of concentration and motility but no significant differences were observed in post-freeze semen parameters.

8.3.2 Fatty acid composition of the blood plasma.

The changes observed in the fatty acid composition of the blood plasma both before and after dietary supplementation are presented in Table 8.4.

The level of 18:2 (n-6) accounted for approximately 30 % of total fatty acids with 18:3 (n-3) present at less than 10 %. There were very low levels of 20:3 (n-6), 20:4 (n-6) and the long chain polyunsaturated fatty acids of n-3 series prior to supplementation. However, levels of 18:3 (n-3) and 22:6 (n-3) in supplemented bulls were significantly higher compared to pre-supplementation levels. The control bulls of group A showed only a small increase in 18:2 (n-6) levels after the experimental period as did bulls of group C, however the control bulls showed no differences in n-3 fatty acid levels.

8.3.3 Lipid composition of the spermatozoa and seminal plasma.

The proportions of the major lipid classes present within spermatozoa and seminal plasma are detailed in Tables 8.5a and 8.5b respectively.

The major lipid class within both semen fractions was phospholipid, followed by free cholesterol. All other lipid classes were present at levels of less than 5%. Total lipid levels did not vary between groups of bulls ; however supplemented bulls of groups B and C displayed lower levels of phospholipid and increased levels of free cholesterol within the spermatozoa compared to control bulls. Within the seminal plasma supplemented bulls displayed lower levels of phospholipid, free cholesterol and elevated free fatty acids compared to the control bulls. Groups B and C also displayed a decrease in the level of triacylglycerol and cholesterol ester after the supplementation period within spermatozoa and seminal plasma.

8.3.4. Fatty acid composition of the total phospholipid of the spermatozoa and seminal plasma.

The fatty acids present within the total phospholipid of spermatozoa and seminal plasma are detailed in Tables 8.6a and 8.6b respectively.

The major fatty acids present within the total phospholipid were 16:0 and 22:6 (n-3) for both semen fractions. Within the spermatozoa no significant differences were observed between groups of bulls. Within the seminal plasma supplemented bulls had significantly higher levels of 22:6 (n-3) after supplementation. Despite these differences in fatty acid composition no significant differences in the n-3/n-6 ratios were observed between bulls.

8.3.5 Fatty acid composition of the minor lipid classes of the spermatozoa and seminal plasma

All other lipid fractions, namely free fatty acids, triacylglycerols and cholesterol ester showed no differences in the fatty acid composition between control and pre-supplementation levels.

8.3.6 Major phospholipid classes of the spermatozoa and seminal plasma

The major phospholipid classes of spermatozoa and seminal plasma are presented in Tables 8.7a and 8.7b respectively.

The major phospholipid classes within both semen fractions were phosphatidyl ethanolamine and phosphatidyl choline. Within spermatozoa this was followed by cardiolipin and sphingomyelin with smaller levels of phosphatidyl inositol and phosphatidyl serine. The seminal plasma displayed negligible levels of cardiolipin with phosphatidyl inositol with phosphatidyl serine present at levels of approximately 5% followed by sphingomyelin at levels equivalent to those found in spermatozoa (10%).

Within spermatozoa a significant increase was observed in the level of phosphatidyl ethanolamine in groups B and C with decreased levels of phosphatidyl inositol and phosphatidyl choline. Group C also displayed higher levels of phosphatidyl serine. Within the seminal plasma supplemented bulls showed an increase in phosphatidyl choline and a decrease in phosphatidyl serine and cardiolipin.

8.3.7 Fatty acid composition of the phospholipid classes of the spermatozoa

The fatty acids present within each phospholipid class after the supplementation period are presented in Tables 8.8a to 8.8e for phosphatidyl ethanolamine, phosphatidyl choline, sphingomyelin, cardiolipin and phosphatidyl inositol/serine respectively.

Phosphatidyl ethanolamine

The level of 18:0 decreased in supplemented bulls, accompanied by a decrease in 20:4 (n-6) in bulls of group B. The level of 22:6 (n-3) increased significantly in groups B and C compared to those levels detected within the unsupplemented bulls although the value of 22:6 (n-3) was higher in bulls of group B. There was an accompanying increase in the n-3/n-6 ratio with significantly higher P/S ratios within this phospholipid class in supplemented bulls.

Phosphatidyl choline

There were few significant differences in B and C bulls with only a higher level of 16:0 being detected in Group B compared to the control as well as significantly lower levels of 22:6 (n-3) and n-3/n-6 ratio within the spermatozoa of supplemented bulls. This loss of 22:6 (n-3) was accompanied by a significant reduction in n-3/n-6 and P/S ratios.

Sphingomyelin

Supplemented bulls displayed higher levels of 14:0, 16:0 and 18:0 with a significant reduction in the level of 22:6 (n-3). This loss of 22:6 (n-3) within this phospholipid class was approximately 50 % in supplemented bulls. In addition the n-3/n-6 and P/S ratios were significantly lower compared to the unsupplemented group of bulls.

Cardiolipin

Extensive differences were observed in this phospholipid class with supplemented groups displaying similar trends. Both groups displayed lower levels of 14:0, increased levels of 18:0, with significant decreases in 18:1 (n-9) and 18:2 (n-6). The largest increases were observed in 20:4 (n-6) and in particular in the level of 22:6 (n-3) with a corresponding increase in n-3/n-6 ratios in supplemented compared to unsupplemented bulls of group A.

Phosphatidyl inositol/phosphatidyl serine

No significant differences were observed in this phospholipid class between groups. Most notably these combined phospholipid classes had more than 80% 22:6 (n-3) with only negligible amounts of 18:0, 18:1 (n-9) and 22:5 (n-3) within all groups of bulls examined.

Table 8.3. Fresh and frozen semen characteristics.

	Fresh semen		Frozen semen					
	conc.	motility	standard drop		citrate		acrosomes	
Bull groups	10^9cells /ml	0 - 5	PPM %	motility	PPM %	motility	NIA %	IA %
A (pre)	1.3 ± 0.1	5.0 ± 0.1	31.7 ± 0.3	3.5 ± 0.3	35.7 ± 0.3	3.7 ± 0.3	22.7 ± 1.8	77.3 ± 1.8
A (post)	1.1 ± 0.1	3.7 ± 0.7	32.0 ± 3.5	3.7 ± 0.3	34.0 ± 2.0	4.0 ± 0.1	22.7 ± 2.7	77.3 ± 2.7
Mean A	1.2 ± 0.1	4.3 ± 0.4	31.8 ± 2.2	3.6 ± 0.6	34.8 ± 1.0	3.8 ± 0.1	22.7 ± 1.4	77.3 ± 1.4
B (pre)	0.6 ± 0.1	3.2 ± 0.2	21.7 ± 4.4	3.5 ± 0.3	26.7 ± 4.4	3.7 ± 0.2	35.0 ± 3.8	65.0 ± 3.8
B (post)	0.9 ± 0.1	3.5 ± 0.5	36.0 ± 0.6	3.5 ± 0.1	35.3 ± 0.3	3.7 ± 0.2	16.7 ± 2.4	83.3 ± 2.4
Sig. diff.	c		c				c	c
C (pre)	1.7 ± 0.3	4.7 ± 0.2	23.3 ± 7.3	3.3 ± 0.2	34.3 ± 2.2	3.7 ± 0.3	30.0 ± 1.2	70.0 ± 1.2
C (post)	1.6 ± 0.2	4.8 ± 0.2	37.3 ± 1.3	4.0 ± 0.1	36.0 ± 0.6	4.0 ± 0.1	10.0 ± 2.0	90.0 ± 2.0
Sig. diff.				c			a	a
	Comparison of all groups before and after supplementation and with the mean of the unsupplemented group of bulls (A)							
Pre B/A	b	c	ns	ns	ns	ns	c	c
Post B/A	ns	ns	ns	ns	ns	ns	ns	ns
Pre C/A	ns	ns	ns	ns	ns	ns	b	b
Post C/A	c	ns	c	ns	ns	ns	a	a
Pre C/Pre B	c	b	ns	ns	ns	ns	ns	ns
Pre C/Post B	c	c	ns	ns	ns	ns	b	b
Post C/Post B	b	c	ns	ns	ns	ns	ns	ns

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group at the beginning of the experiment and after a 2 month period.
Significant difference between groups are illustrated with characters : a P<0.001 ; b P<0.01 ; c P<0.05.
ns represents not significant.
Pre- represents pre supplementation and post- represents post supplementation.

Table 8.4. The n-3 and n-6 fatty acids detected within blood plasma of bulls.

Fatty acid	A		B		C	
(% w/w of total lipid)	Pre	Post	Pre	Post	Pre	Post-feed
n-6 fatty acids						
18:2 (n-6)	29.2 ± 1.8	35.2 ± 1.6c	28.4 ± 2.9	28.0 ± 2.1	22.5 ± 2.6	32.5 ± 2.9 **c/ns**
20:3 (n-6)	1.0 ± 0.1	0.7 ± 0.1	1.4 ± 0.4	1.4 ± 0.2	1.1 ± 0.1	1.0 ± 0.1
20:4 (n-6)	2.4 ± 0.2	2.6 ± 0.1	2.1 ± 0.2	3.0 ± 0.5	2.0 ± 0.3	3.2 ± 0.1 **c/b**
n-3 fatty acids						
18:3 (n-3)	6.7 ± 1.1	8.2 ± 0.6	5.3 ± 0.3	7.3 ± 0.8**c/ns**	5.6 ± 0.7	8.4 ± 0.6 **c/ns**
20:5 (n-3)	1.0 ± 0.1	1.2 ± 0.1	nd	1.7 ± 0.3	nd	1.7 ± 0.2
22:5 (n-3)	0.8 ± 0.1	0.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	0.8 ± 0.2	0.9 ± 0.1
22:6 (n-3)	0.9 ± 0.2	1.0 ± 0.1	0.7 ± 0.2	1.5 ± 0.2**c/ns**	0.7 ± 0.1	1.7 ± 0.1**a/ns**

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a $P<0.001$; b $P<0.01$; c $P<0.05$.
Significance compares pre and post-supplementation versus group A post-supplementation period results.
ns represents not significant.
nd represents not detectable.

Table 8.5a. Lipid composition of spermatozoa.

	Spermatozoa							
	A		B			C		
	Pre	Post	Pre		Post	Pre		Post
Total lipid, µg/109 cells	535.89 ± 80.3	499.79 ± 89.4	420.96 ± 33.6		545.4 ± 77.7	469.77 ± 11.0		595.71 ± 62.3
Lipid class (% w/w of total lipid)								
Phospholipid	69.7 ± 3.6	72.5 ± 1.4	67.8 ± 1.7	**ns/c**	66.1 ± 1.5	67.3 ± 1.3	**ns/c**	66.2 ± 1.2
Free cholesterol	24.1 ± 0.8	22.4 ± 1.3	21.3 ± 2.9	**ns/c**	27.2 ± 1.1	24.5 ± 1.7	**ns/c**	28.6 ± 0.6
Free fatty acid	1.5 ± 0.5	2.5 ± 0.7	2.7 ± 0.3		2.8 ± 0.4	2.1 ± 1.1		3.3 ± 0.5
Triacylglycerol	3.5 ± 1.6	2.4 ± 0.4	3.7 ± 1.3		2.4 ± 0.4	3.4 ± 0.2	**b/ns**	1.9 ± 0.3
Cholesterol ester	1.9 ± 0.9	1.2 ± 0.4	4.4 ± 1.0	**c/ns**	1.8 ± 0.2	2.6 ± 0.5	**c/ns**	1.2 ± 0.2

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : b $P<0.01$; c $P<0.05$. Significance compares pre and post supplementation versus post-supplementation period group A results. ns represents not significant.

Table 8.5b. Lipid composition of seminal plasma.

	Seminal plasma						
	A		B			C	
	Pre	Post	Pre		Post	Pre	Post
Total lipid, μg/ml plasma	687.42 ± 76.5	612.78 ± 54.8	520.80 ± 118.6		617.63 ± 74.9	467.33 ± 89.5	578.89 ± 103.8
Lipid class (% w/w of total lipid)							
Phospholipid	51.2 ± 3.3	53.4 ± 3.0	46.9 ± 3.4	**ns/c**	44.1 ± 1.5	45.2 ± 2.7	49.6 ± 2.7
Free cholesterol	41.1 ± 1.8	37.0 ± 1.8	33.1 ± 5.0	**ns/c**	44.4 ± 2.5	41.4 ± 2.1	40.5 ± 3.9
Free fatty acid	3.0 ± 0.6	2.0 ± 0.2	3.6 ± 1.6	**ns/c**	3.7 ± 0.7	4.6 ± 2.4	3.3 ± 0.6
Triacylglycerol	2.6 ± 0.9	1.9 ± 0.3	11.4 ± 3.0	**c/ns**	3.4 ± 0.7	5.9 ± 3.4	3.4 ± 0.7
Cholesterol ester	2.3 ± 0.5	2.8 ± 1.7	5.0 ± 1.7		3.7 ± 2.1	2.8 ± 1.2	3.7 ± 2.3

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant **differences between groups are illustrated with characters : c P<0.05.** Significance **compares pre and post-supplementation supplementation versus post-supplementation period group A results. ns represents not significant.**

Table 8.6a. Fatty acids of total phospholipid of the spermatozoa.

	Spermatozoa					
	A		B		C	
	Pre	Post	Pre	Post	Pre	Post
Fatty acid (% w/w total)						
16:0	16.4 ± 1.9	12.1 ± 0.7	13.0 ± 0.7	13.3 ± 0.7	14.5 ± 0.5	13.7 ± 0.6
18:0	4.8 ± 0.9	3.5 ± 0.2	3.7 ± 0.3	3.9 ± 0.2	4.0 ± 0.3	3.7 ± 0.1
18:1 (n-9)	2.8 ± 0.8	1.3 ± 0.2	1.4 ± 0.2	1.5 ± 0.2	1.9 ± 0.2	1.4 ± 0.1
18:2 (n-6)	3.9 ± 1.3	2.4 ± 0.2	2.8 ± 0.3	2.9 ± 0.2	2.0 ± 0.2	2.2 ± 0.1
20:4 (n-6)	3.8 ± 0.1	3.9 ± 0.1	4.2 ± 0.1	3.9 ± 0.1	3.7 ± 0.2	3.7 ± 0.2
22:6 (n-3)	64.8 ± 1.0	67.1 ± 0.9	67.6 ± 2.5	65.4 ± 1.4	66.5 ± 1.6	66.5 ± 2.0
n-3/n-6	13.2 ± 3.4	9.4 ± 0.6	9.3 ± 0.6	9.0 ± 0.6	12.0 ± 1.0	10.3 ± 0.8
P/S	3.4 ± 0.1	3.7 ± 0.1	4.0 ± 0.3	3.5 ± 0.3	3.5 ± 0.3	3.5 ± 0.4

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 8.6b. Major fatty acids of total phospholipid of the seminal plasma.

	Seminal plasma							
	A		B			C		
Fatty acid (% w/w total)	Pre	Post	Pre		Post	Pre		Post
16:0	21.8 ± 1.7	18.0 ± 1.0	18.1 ± 0.1		19.0 ± 0.8	21.3 241 0.9		18.0 ± 1.1
18:0	5.8 ± 0.6	5.1 ± 0.4	5.9 ± 1.0		5.1 ± 1.0	4.7 241 0.2		4.2 ± 0.3
18:1 (n-9)	5.7 ± 1.3	3.7 ± 0.4	3.4 ± 1.2		4.4 ± 1.2	3.9 241 0.7		3.0 ± 0.4
18:2 (n-6)	6.9 ±1.4	6.6 ± 0.6	5.0 ± 1.4		5.1 ± 1.4	3.5 241 0.4		3.5 ± 0.3
20:4 (n-6)	2.4 ± 0.9	2.1 ± 0.2	1.7 ± 0.3		1.8 ± 0.2	1.8 241 0.3		1.8 ± 0.2
22:5 (n-3)	< 1.0	1.2 ± 0.03	1.4 ± 0.2		1.3 ± 0.1	1.0 ± 0.1		1.1 ± 0.2
22:6 (n-3)	54.5 ± 1.7	56.1 ± 2.1	53.7 ± 1.6	**b/c**	63.3 ± 1.3	56.7 ± 0.3	**c/ns**	60.0 ± 1.3
n-3/n-6	5.2 ± 1.4	5.3 ± 0.6	11.4 ± 3.8		9.8 ± 1.2	9.3 ± 0.7		9.2 ± 0.8
P/S	2.3 ± 0.4	2.7 ± 0.2	2.3 ± 0.2		2.6 ± 0.2	2.4 ± 0.1		2.5 ± 0.1

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : b $P<0.01$; c $P<0.05$. Significance compares pre and post-supplementation supplementation versus post-supplementation period group A results.
Only major fatty acids and n-6 and n-3 fatty acids have been included. ns represents not significant.

Table 8.7a. Phospholipid classes of spermatozoa.

Phospholipid class (% w/w of total phospholipid)	A Pre	A Post	B Pre		B Post	C Pre		C Post
Phosphatidyl ethanolamine	26.4 ± 0.8	29.7 ± 1.2	26.4 ± 1.6	**c/ns**	30.7 ± 0.7	21.6 ± 1.3	**b/ns**	31.4 ± 2.2
Phosphatidyl choline	51.6 ± 1.9	48.9 ± 1.4	46.9 ± 1.4		45.1 ± 1.2	52.1 ± 1.0	**c/ns**	43.9 ± 2.6
Sphingomyelin	10.2 ± 1.2	8.7 ± 0.5	9.9 ± 1.0		7.7 ± 0.9	12.1 ± 1.4		10.0 ± 1.2
Cardiolipin	9.1 ± 0.4	10.0 ± 0.6	11.6 ± 0.7		11.9 ± 1.4	10.2 ± 1.3	**ns/c**	12.0 ± 0.5
Phosphatidyl inositol	2.6 ± 0.6	2.4 ± 0.4	1.4 ± 0.3	**ns/c**	1.1 ± 0.3	1.9 ± 0.6	**c/a**	0.2 ± 0.1
Phosphatidyl serine	< 1.0	< 1.0	3.6 ± 1.6	**ns/c**	3.1 ± 0.7	< 1.0	**c/ns**	2.3 ± 1.0

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a $P<0.001$; b $P<0.01$; c $P<0.05$. Significance compares pre and post-supplementation supplementation versus post-supplementation period group A results.
ns represents not significant

Table 8.7b. Phospholipid classes of seminal plasma.

	Seminal plasma							
	A		B			C		
Phospholipid class (% w/w of total phospholipid)	Pre	Post	Pre		Post	Pre		Post
Phospahtidyl ethanolamine	32.2 ± 1.2	33.1 ± 4.0	29.4 ± 1.9		27.0 ± 0.2	34.0 ± 2.1		34.5 ± 3.1
Phosphatidyl choline	45.0 ± 0.7	44.0 ± 2.6	39.5 ± 1.1	**a/c**	52.1 ± 0.3	41.0 ± 3.4		48.2 ± 6.2
Sphingomyelin	11.7 ± 2.9	11.2 ± 2.6	13.3 ± 0.8		14.0 ± 0.3	11.3 ± 2.1		10.2 ± 1.1
Cardiolipin	2.3 ± 0.4	1.8 ± 0.2	< 1.0	**ns/b**	< 1.0	1.9 ± 0.2	**ns/c**	1.1 ± 0.3
Phosphatidyl inositol	4.4 ± 1.2	5.9 ± 0.3	4.5 ± 0.8		3.9 ± 1.0	4.1 ± 1.3		6.6 ± 1.8
Phospahtidyl serine	4.2 ± 0.9	3.8 ± 0.7	10.9 ± 1.1	**a/ns**	2.3 ± 0.1	7.6 ± 0.9		5.4 ± 3.9

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a $P<0.001$; b $P<0.01$; c $P<0.05$. Significance compares pre and post-supplementation supplementation versus post-supplementation period group A results.
ns represents not significant.

Table 8.8a. Fatty acids of phosphatidyl ethanolamine within spermatozoa ; post-supplementation.

Fatty acid (% w/w of total fatty acid)	Group of bulls				
	A	B		C	
14:0	4.5 ± 1.8	4.3 ± 1.8		3.6 ± 0.7	
16:0	20.9 ± 2.6	18.1 ± 3.3		20.8 ± 0.5	
18:0	20.8 ± 2.8	12.1 ± 3.3		11.0 ± 0.3	c
18:1 (n-9)	4.7 ± 0.9	< 1.0		2.4 ± 0.4	
18:2 (n-6)	4.1 ± 1.1	3.5 ± 1.8		< 1.0	
20:3 (n-6)	1.2 ± 0.6	nd		nd	
20:4 (n-6)	9.9 ± 0.5	2.7 ± 1.4	a	10.6 ± 0.9	
22:6 (n-3)	33.3 ± 1.0	60.6 ± 0.7	a	42.5 ± 0.7	a
n-3/n-6	2.6 ± 0.3	5.5 ± 3.3		4.1 ± 0.4	c
P/S	1.1 ± 0.1	2.3 ± 0.2	b	2.4 ± 0.5	c

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a P<0.001 ; b P<0.01 ; c P<0.05.
Significance compared to post-supplementation control (A) results.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 8.8b. Fatty acids of phosphatidyl choline of spermatozoa ; post-supplementation.

Fatty acid (% w/w of total fatty acid)	Bulls				
	A	B		C	
14:0	5.1 ± 1.1	9.2 ± 3.4		7.9 ± 1.0	
16:0	9.9 ± 2.1	18.7 ± 1.1	b	10.1 ± 0.8	
18:0	1.4 ± 0.3	2.8 ± 1.8		3.6 ± 1.4	
18:1 (n-9)	< 1.0	2.4 ± 1.4		4.7 ± 1.6	
18:2 (n-6)	< 1.0	6.0 ± 2.6		12.5 ± 4.7	
20:4 (n-6)	< 1.0	nd		nd	
20:5 (n-3)	nd	< 1.0		3.2 ± 2.9	
22:5 (n-3)	1.0 ± 0.1	nd		nd	
22:6 (n-3)	77.9 ± 1.9	63.0 ± 3.8	c	58.0 ± 0.7	a
n-3/n-6	91.8 ± 8.6	5.5 ± 0.6	a	4.7 ± 2.8	a
P/S	5.3 ± 0.7	2.4 ± 0.6	c	4.6 ± 0.8	

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a P<0.001 ; b P<0.01 ; c P<0.05.
Significance compared to post-supplementation control (A) results.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 8.8c. Fatty acids of sphingomyelin ; post-supplementation.

	Groups of bulls				
Fatty acid (% w/w of total fatty acid)	A	B		C	
14:0	6.3 ± 1.5	23.8 ± 2.1	b	26.1 ± 7.3	c
16:0	18.7 ± 1.1	22.7 ± 1.1	c	23.8 ± 1.9	c
18:0	5.3 ± 0.9	9.1 ± 0.7	b	7.6 ± 0.5	c
18:1 (n-9)	5.4 ± 0.9	5.6 ± 0.6		4.2 ± 0.4	
18:2 (n-6)	5.9 ± 3.5	12.7 ± 0.4		8.7 ± 0.6	
20:3 (n-6)	nd	1.6 ± 0.1		1.5 ± 0.2	
20:4 (n-6)	nd	4.0 ± 0.8		4.9 ± 1.1	
20:5 (n-3)	nd	< 1.0		2.0 ± 0.9	
22:6 (n-3)	59.4 ± 4.7	9.9 ± 2.6	a	14.4 ± 3.4	a
n-3/n-6	11.8 ± 2.6	0.5 ± 0.1	b	1.1 ± 0.1	b
P/S	2.5 ± 0.3	0.6 ± 0.1	a	0.6 ± 0.2	b

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a P<0.001 ; b P<0.01 ; c P<0.05.
Significance compared to post-supplementation control (A) results.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 8.8d. Fatty acids of cardiolipin within spermatozoa ; post-supplementation.

	Group of bulls				
Fatty acid (% w/w of total fatty acid)	A	B		C	
14:0	15.4 ± 1.4	2.1 ± 0.3	a	4.5 ± 1.2	a
16:0	24.9 ± 0.3	27.5 ± 1.8		29.2 ± 3.2	
18:0	8.4 ± 0.1	10.6 ± 0.4	a	11.1 ± 0.5	a
18:1 (n-9)	3.7 ± 0.2	2.6 ± 0.2	b	2.4 ± 0.2	b
18:2 (n-6)	7.7 ± 0.5	2.1 ± 0.5	a	2.2 ± 0.2	a
20:3 (n-6)	1.3 ± 0.1	1.4 ± 0.1		1.2 ± 0.1	
20:4 (n-6)	8.6 ± 0.2	12.8 ± 0.4	c	11.5 ± 0.8	c
22:6 (n-3)	22.0 ± 1.7	37.5 ± 1.6	a	33.9 ± 3.1	b
n-3/n-6	1.3 ± 0.1	2.3 ± 0.1	a	2.4 ± 0.2	a
P/S	0.8 ± 0.1	1.4 ± 0.1	a	1.1 ± 0.2	

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
Significant differences between groups are illustrated with characters : a P<0.001 ; b P<0.01 ; c P<0.05.
Significance compared to post-supplementation control (A) results.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 8.8e. Fatty acids of phosphatidyl inositol/phosphatidyl serine of spermatozoa ; post-supplementation.

	Group of bulls		
Fatty acid (% w/w of total fatty acid)	A	B	C
14:0	5.5 ± 0.5	3.5 ± 1.2	3.7 ± 1.4
16:0	4.8 ± 0.9	7.1 ± 0.7	6.2 ± 1.0
18:0	1.1 ± 0.1	1.2 ± 0.2	1.1 ± 0.1
18:1 (n-9)	nd	1.1 ± 0.1	< 1.0
22:5 (n-3)	< 1.0	1.1 ± 0.1	1.0 ± 0.1
22:6 (n-3)	85.2 ± 2.5	81.8 ± 2.0	84.2 ± 2.4
n-3/n-6	90.9 ± 14.0	85.7 ± 16.6	117.9 ± 7.7
P/S	8.4 ± 1.1	8.4 ± 1.6	9.6 ± 1.6

Values are means ± S.E. of observations from 6 samples (2 ejaculates from each of 3 bulls) in each group.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

8.5 DISCUSSION

In general the characteristics, composition and specific lipid and fatty acid features of the spermatozoa and seminal plasma of the unsupplemented bulls were similar to those reported previously for normal, healthy bulls (Pickett and Komarek, 1966 ; Poulos *et al.*, 1973b ; Neill and Masters, 1972) and those already presented in Chapter 4.

Dietary supplementation of the bulls with fish oil resulted in small but significant increases in the proportion of 22:6 (n-3) in the total lipids of the blood plasma. This result indicates that at least some of the dietary 22:6 (n-3) was resistant to biohydrogenation in the rumen and was able to enter the circulation for distribution to the tissues, as previously demonstrated in ruminant species by Ashes *et al.* (1992). The much higher levels of 22:6 (n-3) reported in the study by Ashes may have been due to the "protection" of the lipid component of the diet from biohydrogenation. However, in spite of this increase in the amount of 22:6 (n-3), in the blood plasma of the bulls studied at this time, potentially available for the uptake by the developing spermatozoa, the proportion in the total phospholipid of the spermatozoa was not elevated by dietary supplementation. Thus on this basis it would appear that the proportion of 22:6 (n-3) in the spermatozoa phospholipid of the bull is somehow resistant to dietary manipulation. It should be noted however that the levels of 22:6 (n-3) within total phospholipid are already extremely high (64 - 68%) in the absence of additional 22:6 (n-3) supplementation, in agreement with previous reports (Picket and Komarek, 1966 ; Neill and Masters, 1972), thus possibly limiting the flexibility for any further increase. In a previous study by Poulos *et al.* (1973b) these workers concluded that the level of 22:6 (n-3) remained unchanged during ejaculation of bull sperm despite a loss of total lipid. In this study it was concluded that somehow such vital polyunsaturated fatty acids were "spared" to maintain the correct plasma membrane composition for spermatozoa function. However, further investigations revealed that the apparent constancy of the fatty acid profiles of the total phospholipid in response to fish oil feeding in the present study were in fact masking a complex array of underlying changes in the 22:6 (n-3) levels in the individual phospholipid classes. Thus the proportion of 22:6 (n-3) in the phosphatidyl ethanolamine class was enhanced markedly by dietary supplementation whereas the levels within phosphatidyl choline changed in the opposite direction. Dietary-induced changes in the 22:6 (n-3) levels in the more minor phospholipid classes

were also observed with fish oil feeding resulting in a dramatic decrease in the proportion of 22:6 (n-3) in the sphingomyelin but causing a significant increase in the proportion of this fatty acid in cardiolipin. The very high proportion (85 %) of 22:6 (n-3) in the jointly reported phosphatidyl inositol and phosphatidyl serine classes were unchanged by the fish oil diet. This phenomenon has already been observed to occur in human spermatozoa following fish oil supplementation where an appropriate balance of n-6 and n-3 fatty acids was retained (Knapp, 1990). The importance of phosphatidyl serine has been reported in relation to brain and retinal function and it has been suggested that the high level of polyunsaturates present within this phospholipid class play a major role in regulating the fluidity of the cell membrane as well as regulating a range of membrane proteins (Salem *et al.*, 1986 ; Neuringer *et al.*, 1988). In terms of spermatozoa function such levels of C22 polyunsaturates within important phospholipid classes may reflect the optimum properties required for correct membrane function and characteristics required for motility (Buhr *et al.*, 1994 ; Gadella *et al.*, 1994). The mechanism by which these phospholipid class-specific changes in 22:6 (n-3) content are accomplished is unclear. It appears that the net effect of these phospholipid class specific-changes in 22:6 (n-3) content on the overall proportion of this fatty acid in total phospholipid is to cancel each other out. This may be to due to a mechanism responsible for maintaining the correct balance of polyunsaturates within the spermatozoa membranes for optimal viability (Nissen and Kreysel, 1983 ; Sebastian *et al.*, 1987 ; Oliw and Sprecher, 1989 ; Sinha *et al.*, 1994).

Supplementation with 22:6 (n-3) also led to increased levels of free cholesterol within the spermatozoa of bulls during this study. The presence of cholesterol is known to improve membrane integrity (Ansah and Buckland, 1982). A reduction in the level of cholesterol before the acrosome reaction is also believed to destabilise the sperm plasma membrane promoting ultimate fusion with the underlying female gamete during fertilisation (Bearer and Friend, 1982). The trend observed by the supplemented bulls may indicate a more beneficial cholesterol/phospholipid ratio with regards membrane stability and ultimate viability of the spermatozoa after freezing. The freezing process has been frequently reported to reduce the level of saturated fatty acids within the spermatozoa plasma membrane resulting in an increase in the proportion of polyunsaturated fatty acids and thereby influencing the polyunsaturated/saturated fatty acid ratio of the phospholipid component of the sperm plasma membrane. The correct

balance of such fatty acids has been frequently reported to influence the freezing capacities of spermatozoa (Darin-Bennett *et al.*, 1974 ; Buhr *et al.*, 1994). As observed during the supplementation period a reduction in the 22:6 (n-3) polyunsaturate within the major phospholipid phosphatidyl choline may have a positive effect on membrane stability and fluidity during the freezing process, particularly as it is believed to be situated on the external surface of the spermatozoa membrane where it is more exposed to environmental factors (Yeagle, 1987 ; Mann and Lutwak-Mann, 1981c ; Alberts *et al.*, 1983).

The effects of fish oil feeding on the semen quality parameters were generally beneficial, resulting particularly in improved acrosomal integrity after freeze-storing. Whether this is due to the dietary-induced changes in the levels of 22:6 (n-3) in the individual phospholipid classes or changes in the relative proportions of 22:6 (n-3) within these phospholipid classes or to some other factor such as altered eicosanoid production, as reported by Knapp (1990), cannot be stated at present. Clearly although fish oil feeding to ruminants results in alteration of the blood plasma fatty acid profiles this does not necessarily indicate that this will in turn lead to elevated levels of 22:6 (n-3) within spermatozoa phospholipids. In order to verify such changes of spermatozoa quality in relation to fish oil feeding a large scale supplementation experiment would be required which also incorporated an assessment of *in vivo* fertility.

CHAPTER 9

THE EFFECT OF ADDITION OF VITAMIN E IN THE FORM OF ALPHA-TOCOPHEROL ACETATE TO BULL SEMEN IN ORDER TO ASSESS ANY IMPROVEMENTS IN FRESH OR FROZEN SEMEN QUALITY PARAMETERS

9.1 INTRODUCTION

Antioxidant systems present in the spermatozoa include fat-soluble antioxidants such as vitamin E and ubiquinones as well as water-soluble antioxidants such as vitamin C and enzymes such as glutathione peroxidase, superoxide dismutase and catalase (Riley and Behrman, 1991). Among these inherent systems, vitamin E is known to play a key role in the protection of spermatozoa against lipid peroxidation, where it acts within the sperm plasma membrane to prevent chain reactive oxidation (Niki, 1993). The role of vitamin E in membrane integrity is associated with direct stabilisation of the membrane by interaction with phospholipid. A review of Vitamin E, its function and structure is reported by Burton (1994).

There have been several attempts to improve spermatozoa characteristics, in particular freeze survival capacity, by different means :

i) to include antioxidants (mainly vitamin E) into diluents used for semen storage and cryopreservation.

ii) to increase the level of vitamin E in the spermatozoa by means of vitamin supplementation to the diet with the aim of enhancing semen quality, including increasing spermatozoa resistance to lipid peroxidation during storage and deep freezing.

These objectives have been investigated in a number of cases. The post-thaw motility of human spermatozoa was shown to be increased by inclusion of vitamin E into the freezing media. The improvement was significant but very small (Askari *et al.*, 1994). Similar but more pronounced effects of vitamin E inclusion into the diluent were found in fowl semen stored for 24 hours at 4 °C (Blesbois *et al.*, 1993) ; in this case the fertilising ability of the fowl semen increased significantly. In goat semen the addition of vitamin E in the form of alpha-tocopherol to semen exposed to ultraviolet radiation prevented spermatozoa peroxidation (Govil *et al.*, 1992). Natural antioxidants have been shown to exert a positive effect on the membrane of cryopreserved bull spermatozoa producing increases in superoxide dismutase activity, percentage of intact acrosomes and mitochondrial coupling in spermatozoa after vitamin E inclusion into the diluent (Beconi *et al.*, 1993).

A positive effect of vitamin E inclusion directly into the diet was confirmed in the ram. After thawing, semen of supplemented rams had a significantly lower percentage of abnormal acrosomes compared to the control batch (Gokcen *et al.*, 1990). Another study concluded that additional vitamin E supplementation in the stallion above the minimal requirement was unnecessary (Rich *et al.*, 1984).

Differences in the effect of vitamin E inclusion into semen diluents may be explained to some extent by the inefficiency of the addition of the fat-soluble vitamin E into the water phase of semen diluents as well as the use, as in early research work, of vitamin E in the form of tocopherol-acetate which did not posses the desired antioxidant properties unlike the active alpha-tocopherol form.

Taking into account the results illustrated it is possible to explain such anomalies by differences in the initial level of vitamin E in the animal diets and differing efficiency of its assimilation, especially in ruminant animals, where vitamin changes take place during rumen digestion. The main task of this work was to study different ways of vitamin E inclusion into the diluent of bull semen and its effect on lipid peroxidation in the semen after deep freezing at -196 °C as well as to highlight some physiological semen characteristics.

9.2 MATERIALS AND METHODS

9.2.1 Bull details

Bulls of known reproductive performance were selected, one of the Holstein/Fresian and one of the Belgian Blue breed. Both bulls chosen were known to exhibit problems with routine freezing of their semen, particularly post-freeze survival of spermatozoa and maintenance of acrosomal integrity. Bulls were approximately 5 to 6 years of age and fed and housed in the same manner as the bulls described for the experiment detailed in Chapter 4. The limits imposed in terms of housing space, financial and commercial restrictions at Scottish Livestock Services, Scone, Perth meant that only one bull of each breed was available for assessment during this study.

9.2.2 The addition of vitamin E in the form of alpha-tocopherol acetate to semen diluents pre and post-freeze.

Addition of alpha-tocopherol to the semen was performed to avoid deviation from the normal semen preparation protocol used at Scottish Livestock Services, Scone, Perth to eliminate variation between control samples and those with vitamin E addition. All treatments used in the experiment are detailed in Table 9.1.

Table 9.1. Treatments prepared for addition to the semen diluent.

Treatment code	Vitamin E added	Semen diluent used
A	0 (control)	skimmed milk
B	10mg/ml	skimmed milk
C	1mg/ml	skimmed milk
D	10mg/ml	egg yolk/biosophus plus
E	1mg/ml	egg yolk/biosophus plus
F	10mg/ml	egg yolk/0.85 % saline (w/v)
G	1mg/ml	egg yolk/0.85 % saline (w/v)

9.3 ADDITIVE PREPARATION

9.3.1 Alpha-tocopherol in milk buffer

To prepare the diluent, 500 μg of DL-alpha-tocopherol acetate (Sigma Chemicals, Poole, Dorset) was carefully weighed into a fresh test-tube. Immediately afterwards 5 ml of fresh skimmed milk buffer made freshly at Scottish Livestock Services, Scone, Perth, was added and the preparation mixed thoroughly. To disperse the vitamin in the milk the preparation was homogenised thoroughly for 30 to 40 seconds to ensure

complete solubilisation of the vitamin until a clean, milky texture was obtained. The contents of the test tube were then carefully poured into a darkened glass vial and stoppered. The vial was stored immediately at 4 °C and out of any direct sunlight to keep the vitamin and milk in the best condition for addition to the semen.

For the 1 mg/ml preparation, 500 µl of the above vial contents were pipetted, carefully avoiding any 'froth' which may appear at the top of the preparation caused by homogenisation, into a fresh darkened glass vial and then 4.5 ml of the fresh milk buffer was added by pipette. The contents were sealed immediately as described above and gently shaken before being stored at 4 °C as usual.

9.3.2 Alpha-tocopherol in egg yolk

Fresh egg yolk was used to aid in solubilisation of the lipid-soluble vitamin, egg yolk being a normal addition to the semen diluent buffers used by commercial artificial insemination companies. A stock solution of Biosophus Plus (IMV, L' aigle, Cedex, France) 1:4 (v/v) in distilled water was prepared and mixed thoroughly by manual inversion. A few drops of egg yolk were placed in the bottom of a clean test-tube. 500 µg of alpha-tocopherol was carefully weighed ; the drops were placed directly onto the egg yolk. The resultant mixture was then diluted with 5.0 ml of the Biosophus Plus solution and homogenised and stored as described in Section 9.3.1. Subsequent dilutions were also prepared as described in Section 9.3.1.

9.3.3 Alpha-tocopherol in saline buffer

This preparation was made up in the same way as others already described only dilution was made with 5.0 ml of physiological saline 0.85 % (w/v).

9.3.4 Protocol for diluent addition

Fresh semen from each bull was placed in a water bath at 37°C and treated individually in the same way as routine semen preparation at Scottish Livestock Services, Scone, Perth. Each ejaculate was then split equally into the required number of aliquots for addition of

the additives, namely seven. 100μl of each additive was added to each 1ml of fresh semen.

Final prepared semen straws contained 200 μl of semen in its appropriate diluent (treatments A to G) with a concentration in each of approximately 25 x 10^6 spermatozoa; for each treatment 10 straws were prepared, half were used for *in vitro* pre-freeze analysis and the rest stored at - 196 °C for post-freeze analysis one week later.

The results obtained were the average results from four collection periods per bull.

9.3.5 Spermatozoa evaluation and analysis

Spermatozoa was assessed as described previously (see Sections 3.1, 3.3, 3.6). Analyses for vitamin E and malondialdehyde determination were performed on all prepared straws from both bulls studied to allow enough "pooled" semen for replicate analysis.

9.3.6 Artificial insemination experiment using Belgian Blue semen

It was decided jointly that the Belgian Blue semen would be most useful to use in live artificial insemination due to the problems which exhibit normally in this breed and also because the opportunity to perform insemination using this breed became available. The batch of semen processed using 10 mg/ml milk for the Belgian Blue bull, which showed the best *in vitro* parameters, was used together with the control batch from the bull for a split insemination experiment at the Scottish Agricultural College, Edinburgh.

50 Limousine and Fresian cross heifers were synchronised with the Eazi-breed CIDR prostaglandin programme (Animal Reproductive Technologies Ltd., Leominster, England). Intravaginal CIDRs were inserted on day 0. Prostaglandin injections were administered on day 9 (Estrumate : Mallinckrodt Veterinary Ltd., Harefield, Uxbridge, Middlesex). CIDR devices were removed on day 12 and heifers were artificially inseminated at 50 hours after device removal. 25 heifers received the control Belgian Blue semen and 25 heifers the treated semen contained 10 mg/ml (treatment B) of alpha-tocopherol acetate in milk buffer. The two groups of heifers inseminated were then

blood progesterone assayed at 21 days at SAC, Auchincruive Veterinary Services. using the standard ELISA technique.

9.3.7 Statistical analysis

Correlation coefficients were undertaken using Microsoft Excel version 5.0.

9.4 RESULTS

9.4.1 The effect of alpha-tocopherol on semen characteristics before and after freezing.

The effect of alpha-tocopherol addition on pre-freeze semen characteristics from both bulls are detailed in Table 9.2 for the Belgian Blue bull and Table 9.3 for the Holstein/Fresian bull.

The control spermatozoa from both bulls exhibited *in vitro* parameters similar to those which would be expected for these breeds. A number of the treatments used showed improvements in spermatozoa motility both in citrate and standard drop analyses as well as other, equal or greater spermatozoa parameters compared to the control samples, in particular treatment B showed improvement in both bulls for all semen characteristics.

The post-freeze semen characteristics for the Belgian Blue bull are shown in Table 9.4 and Table 9.5 for the Holstein/Fresian bull.

The quality of the Belgian Blue semen was not acceptable for commercial use, however some, small improvements were assessed in treated semen. The differences within the Holstein/Fresian bull were much more marked with improvements in post-thaw motility and acrosomal integrity observed in particular, within the semen using treatment B.

Figure 9.1 illustrates the levels of vitamin E in the form of alpha-tocopherol determined in the semen treated with the various additives after freezing at -196 °C.

The control group of semen displayed very low levels of vitamin E as no vitamin E was added routinely during commercial semen preparation, these levels increased dramatically in semen treated with 10 mg/ml of vitamin E (treatment B) with lower levels detected within semen treated with only 1 mg/ml vitamin E.

Figure 9.2 details the malondialdehyde levels detected within semen after freezing at -196 °C.

Semen containing 10 mg/ml of vitamin E, treatments B and D, exhibited the lowest levels of malondialdehyde production. The highest levels of malondialdehyde were detected within the untreated semen samples and the other treatments used. A strong negative correlation was calculated between the vitamin E and malondialdehyde results of the experiment suggested a reduced peroxidation level in samples with enhanced levels of vitamin E in the form of alpha-tocopherol acetate.

The results of the insemination of experiment using treated and untreated semen from the Belgian Blue bull are shown in Table 9.6.

Despite the poor results obtained for the post-freeze parameters of the Belgian Blue bull, the straws of semen treated with 10mg/ml in milk (B) were used in an assessment of *in vivo* fertility with positive results. After progesterone assays 14 out of 25 heifers gave blood progesterone indicating pregnancy from the control semen group and 16 out of 25 gave blood progesterone indicating pregnancy from the treated semen group.

Table 9.2. Pre-freeze semen characteristics of the Belgian Blue bull.

Treatment	*In vitro* semen parameters			
	Citrate test		Standard drop	
	motility	PPM	motility	PPM
A	3	28	4	39
B	**4**	**38**	4	**40**
C	3.5	**35**	4	38
D	3	**30**	4	37
E	3	27	4	35
F	3	15	3.5	34
G	3	18	3.5	36

Those parameters greater than those of the control have been highlighted in bold

Table 9.3. Pre-freeze semen characteristics of the Holstein/Fresian bull.

Treatment	*In vitro* semen parameters			
	Citrate test		Standard drop	
	motility	PPM	motility	PPM
A	3.5	36	3.5	35
B	**4**	**37**	**4**	**38**
C	**4**	35	**4**	**36**
D	3	36	3.5	**36**
E	3.5	35	3.5	34
F	**4**	**38**	3.5	35
G	3.5	36	**4**	**37**

Those parameters greater than those of the control have been highlighted in bold

Table 9.4. Post-freeze semen characteristics of the Belgian Blue bull.

Treatment	*In vitro* semen parameters						
	Citrate test		Standard drop		Acrosomal integrity %		
	motility	PPM	motility	PPM	abnormal	non intact	intact
A	3	22	3	10	31	28	72
B	3	20	2.5	**13**	37	29	71
C	3	15	2.5	**13**	33	29	71
D	3.5	15	2	7	30	28	72
E	2	17	2.5	**12**	37	39	61
F	2.5	20	3	8	**14**	34	66
G	2	**25**	3	10	**14**	56	44

Those parameters greater than those of the control have been highlighted in bold

Table 9.5. Post-freeze semen characteristics of the Holstein/Fresian bull.

Treatment	*In vitro* semen parameters						
	Citrate test		Standard drop		Acrosomal integrity %		
	motility	PPM	motility	PPM	abnormal	non intact	intact
A	3.5	34	3	23	16	18	82
B	**4**	**35**	**3.5**	**29**	**13**	**14**	**86**
C	3.5	30	**3.5**	22	**14**	**17**	**83**
D	3.5	30	3	18	**10**	18	82
E	3	25	3	20	16	36	64
F	3	10	2.5	15	**10**	28	72
G	3.5	31	3	14	**13**	18	82

Those parameters greater than those of the control have been highlighted in bold

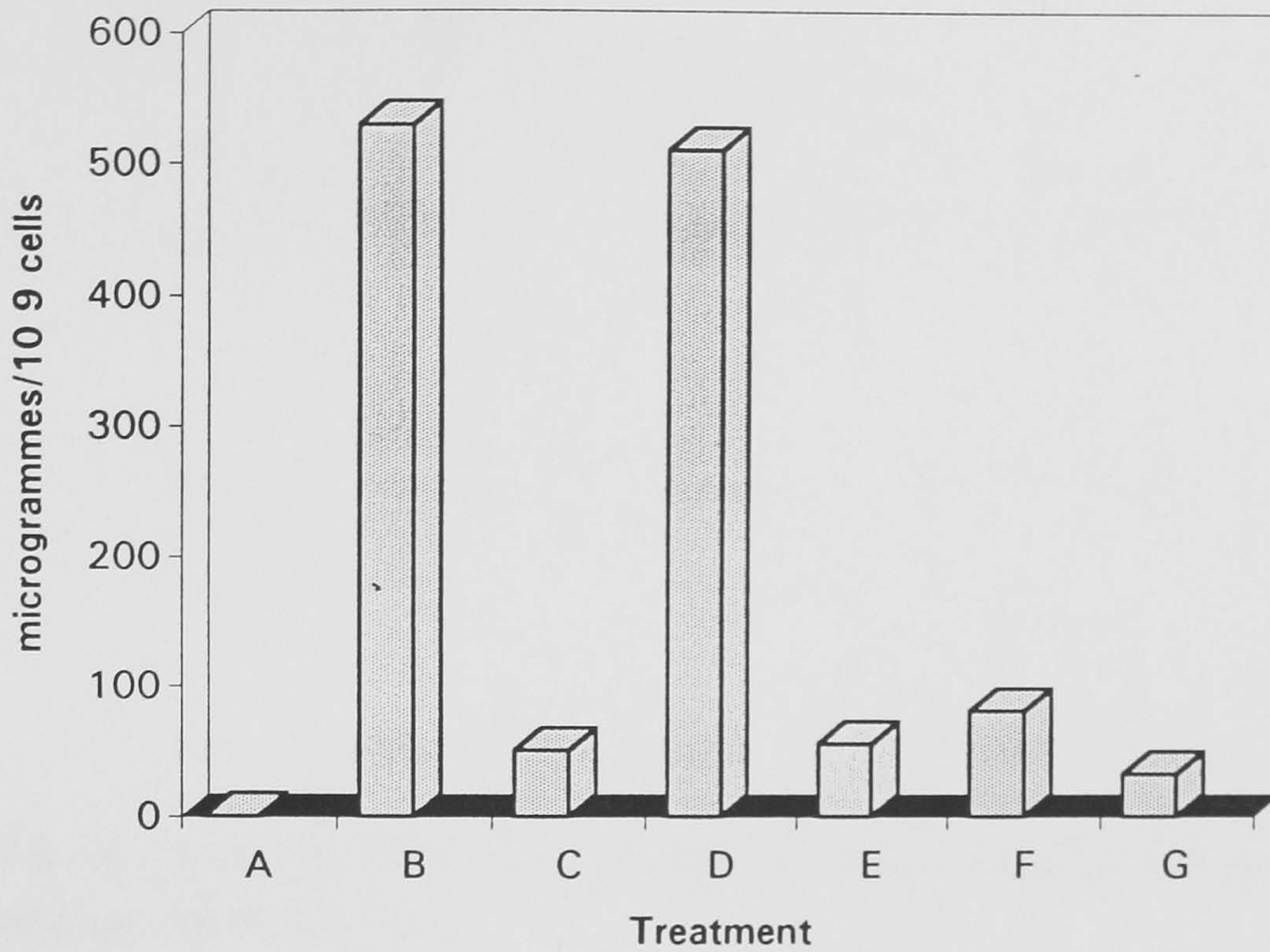


Figure 9.1. Vitamin E levels in semen with various treatments.

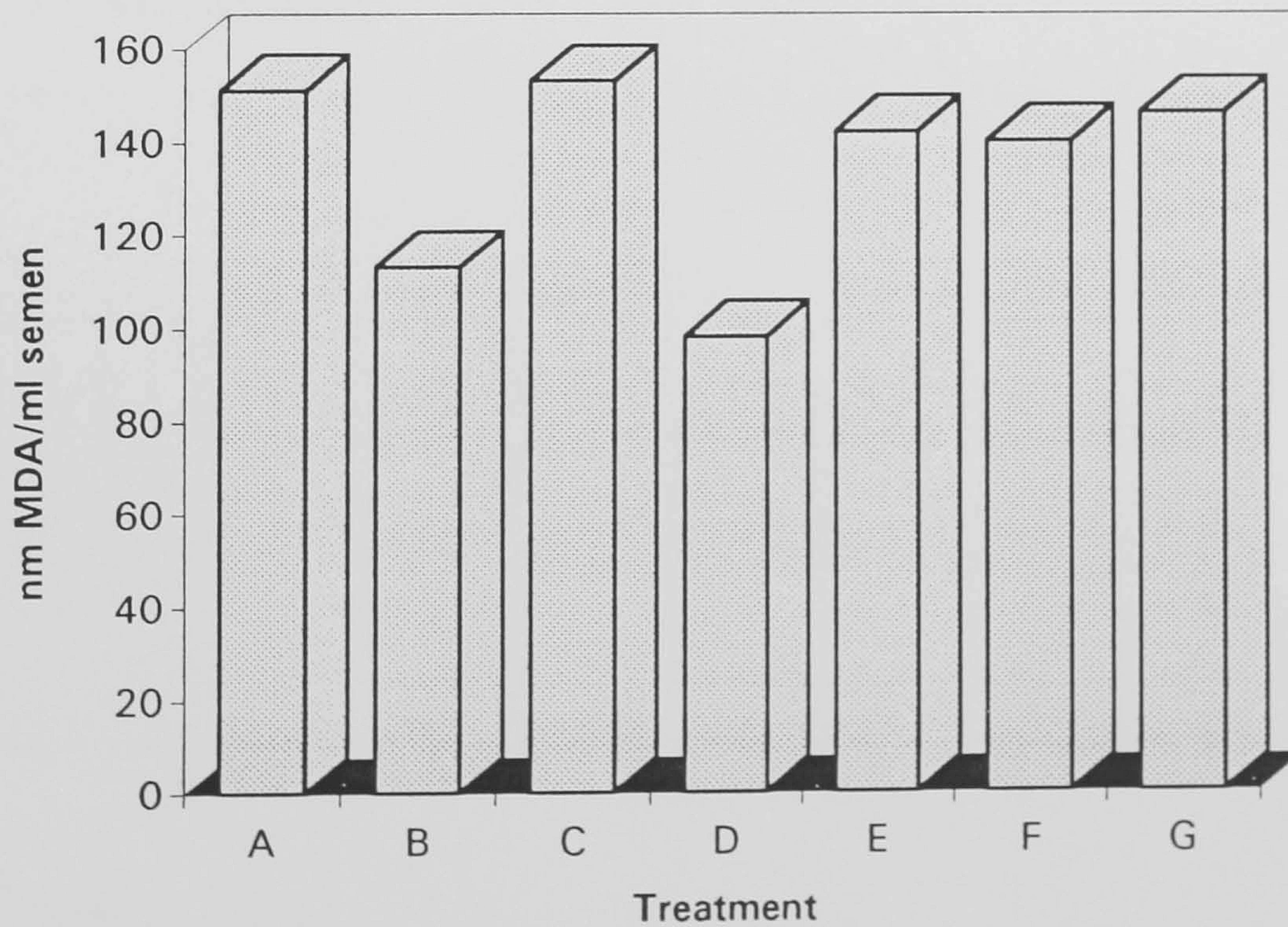


Figure 9.2. Malondialdehyde in semen with various vitamin E treatments.

correlation coefficient between vitamin E and malondialdehyde ; $r^2 = -0.96$ ([a]$P<0.001$).

Table 9.6. ***in vivo*** **inseminations performed on synchronised heifers using a control and a treated batch of semen.**

Treatment used	A Control (untreated)	B 10 mg/ml milk
No. heifers pregnant out of 25	14	16
% heifers pregnant	56	64

9.5 DISCUSSION

Improvements in fresh semen quality were observed in both bulls examined. In both breeds an improvement in fresh semen analysis above commercial recommendations with high levels of vitamin E in the form of alpha-tocopherol acetate were achieved. In the Belgian Blue bull control measurements were relatively poor, although fresh semen quality parameters were found to exhibit desirable semen quality paramaeters after addition of high levels of alpha-tocopherol acetate.

Post-freeze survival of Holstein/Fresian bull semen at -196 °C again showed improvements using treatment B to meet commercial requirements under all test conditions. The Belgian Blue bull however exhibited continually poor *in vitro* semen quality parameters under control circumstances as was expected for this breed. Indeed with addition of the diluent additives few improvements were observed with more abnormal spermatozoa and non-intact acrosomes being present. These particularly poor post-freeze results may be a result of the great variation and unpredictability known to occur in this breed from one collection to another. Beatty *et al.* (1976) reported that deep freezing at -196 °C depressed fertility of sperm of different bulls unequally. Indeed variation of freeze curves is commonplace in commercial AI Centres to help establish more reliable and representative routine semen quality (Parkinson and Whitfield, 1987). It has been frequently reported that freezing causes alteration of the phospholipid species and their associated fatty acid composition ultimately altering important membrane properties such as fluidity and fertilising ability (Beatty *et al.*, 1976 ; Sarmah *et al.*, 1984 ; Wolf, 1995). Buhr *et al.* (1994) reported that changes in the boar spermatozoa head as a result of cryopreservation altered the plasma membrane fluidity, these worker concluded that these changes also altered the interaction of spermatozoa with Ca^{2+}, a vital requisite for the exocytotic changes during the acrosome reaction.

A strong negative correlation was obtained between vitamin E and malondialdehyde in semen, the latter frequently used as an assessment of lipid peroxidation. Lipid peroxidation is known to increase as a result of freezing (Mennella and Jones, 1980 ; Aitken and Fisher, 1994). Indeed vitamin E has been previously reported to exert a positive effect on spermatozoa membranes of good quality semen with increased acrosomal integrity and enhanced respiratory activity, the latter being a good indicator of

membrane integrity and mitochondrial function. However, initially low quality semen did not express the same beneficial parameters (Beconi *et al.*, 1993). This latter statement may help to explain the poor post-freeze results obtained in the Belgian Blue semen. Low quality semen may already possess pre-existing, irreparable alterations in spermatozoa membrane integrity.

Although frequently described as detrimental, a degree of lipid peroxidation is believed to be desirable for correct spermatozoa function (Bize *et al.*, 1991 ; MacPherson, 1994 ; Oehringer *et al.*, 1995), however at higher than required levels these workers observed deleterious effects on spermatozoa plasma membranes and intracellular homeostasis. Vitamin E helps provides membrane protection from lipid peroxidation particularly as bull seminal plasma contains only superoxide dismutase and glutathione peroxidase enzyme systems which are considerably diluted during semen preparation. Griveau *et al.* (1995) treated human spermatozoa with hydrogen peroxide, during this study it was shown to inactivate several enzymic activities involved in natural antioxidant defences of spermatozoa, namely superoxide dismutase and glutathione peroxidase. Beconi *et al.* (1993) also reported that both good and bad quality semen exhibited reduced superoxide dismutase activity associated with increased malondialdehyde levels, with the opposite situation occurring in good quality semen samples with vitamin E added before freezing. Clearly to obtain optimal membrane properties for spermatozoa function these specialised cells must be protected against peroxidation by the addition of an appropriate antioxidant such as vitamin E.

The most promising results overall were obtained using vitamin E dissolved in a lipid based diluent and clearly water-based diluents routinely used in commercial AI practices do not allow maximum function of vitamin E and its protective benefits. The economic viability of this addition of vitamin E however could prove to be highly desirable. Semen, frozen commercially, contains no added antioxidants and it would therefore seem desirable to adopt such methods routinely to enhance spermatozoa survival both pre and post-freeze at least in bulls who already possess semen of commercially acceptable standards.

It has been estimated (Redpath, 1995) that in modern dairy herds a 10 % rise in non-return rates would mean in an average herd of 70 cows, approximately 5 extra cows in

calf for a 365 day calving interval. This represents a saving of £100 per cow in milk revenue, £500 per herd with 2 300 herds giving a national saving per annum of £1 150 000 to the dairy industry in Scotland alone. In beef cattle the total savings are more difficult to quantify but on 100 cow suckler herd a 10 % increase in non-return rates means a saving of £100 in semen charges. This ignores all other savings with better production. To the artificial insemination industry, if we produce semen from bulls that have been difficult to process, the value to the AI companies could be substantial. For example, a modest 5% increase in batches of semen used in Belgian Blue bulls alone could amount to another 5 000 to 10 000 straws in an average stud farm. If this were mirrored in other breeds the time taken to reach desired stock levels would be less and would assist in meeting market demands for top quality genetic bulls, thereby allowing greater usage of bulls and a potentially quicker rate of genetic gain to the cattle industry

Clearly benefits exist in adopting the use of vitamin E in routine semen preparation, particularly as a positive fertility result was obtained even for the poor quality semen of the Belgian Blue bull assessed during this experiment. Although it would require some 450 inseminations to establish any significance, certainly no detrimental effect on the inseminations and resulting progesterone pregnancy assays were observed.

CHAPTER 10

GENERAL DISCUSSION

Current animal industries rely upon a regular supply of good quality semen for natural service or artificial insemination to meet the increasing demands of modern agricultural practices. An increasing proportion of males of many species are now failing to meet these requirements demanded upon them. A further complicating feature arising from commercial interests is the overriding tendency to breed animals for their growth and feed conversion characteristics at the detriment of their sexual performance and fertility. Lipids and antioxidants are known to play a variety of key roles in spermatozoa structure, function and ultimate viability. However, despite the large amounts of available literature on the spermatozoa lipid composition of most animal species, as well as investigations into the action of antioxidant systems and lipid components within semen, no study has yet brought together these two major areas of interest in detail and attempted to manipulate these components by dietary or other means.

The aim of the present series of experiments was to identify the major lipid and antioxidant changes within spermatozoa and seminal plasma of ageing animals and also in those displaying different parameters of semen quality and fertility. In addition, the consequences of n-3 fatty acids and natural antioxidant supplementation on semen quality and ultimate fertility were investigated in both cockerels and bulls.

In general terms the spermatozoa and seminal plasma characteristics derived from the present series of experiments concluded that the lipid composition and fatty acid features displayed by the young, unsupplemented animals were similar to those which have been reported previously for normal healthy cockerels (Darin-Bennett *et al.*, 1974 ; Ravie and Lake, 1985) and bulls (Pickett and Komarek, 1966 ; Neil and Masters, 1972 ; Poulos *et al.*, 1973b).

The results of this study also concluded that the phospholipid fatty acids of the normal commercially fed bull were dominated by n-3 fatty acids in particular 22:6 (n-3) ; the cockerels in contrast were found to be dominated by n-6 fatty acids, namely 20:4 (n-6) and 22:4 (n-6). This striking and distinctive difference between these two species of domestic farm animals was observed in spite of the inclusion of similar levels of n-3 fatty acid precursors within the lipid component of the standard commercial diet used by both animal industries. Such high levels of these polyunsaturated fatty acids have also been reported in previous studies in relation to other important body tissues such as the brain

and retina, which possess very high levels of C20 to C22 polyunsaturated fatty acids. The presence of these components within the cell membranes have been reported to be vital for normal development and function (Neuringer *et al*., 1988). Levels of C20 to C22 polyunsaturated fatty acids have also previously been reported to be positively correlated with normal spermatozoa function, with an associated lack of appropriate and desirable levels being associated with spermatozoa dysfunction and reduced fertility (Nissen *et al*., 1981 ; Nissen and Kreysel, 1983). Long chain polyunsaturated fatty acids comprise a major component of the phospholipid plasma membrane structure of the spermatozoa, contributing to both membrane integrity and fluidity characteristics (Buhr *et al*., 1994 ; Sinha *et al*., 1994). It is apparent that adequate and appropriate levels of these fatty acids are essential for gamete development and function as they are in embryo development (Noble and Cocchi, 1989, 1990; Noble *et al*., 1993) leading to the accomplishment of the spermatozoon-egg interaction. Detailed reports on spermatozoa phospholipid fatty acids, have implicated their further role in interactions with Ca^{2+}, a vital prerequisite for sperm head exocytosis, the acrosome reaction and subsequent membrane associated changes required for successful fertilisation (Cullis and Hope, 1991 ; Gadella *et al*., 1994, 1995). There is considerable evidence from many animal species that the lipid composition of the spermatozoon membrane may play a key role in determining a range of features associated with overall fertility, both in fresh ejaculates through an effect on motility and stored ejaculates through an effect on temperature sensitivity, (Darin-Bennett *et al*., 1974 ; Nissen *et al*, 1981 ; Nissen and Kreysel, 1983 Ravie and Lake, 1985). Such functional roles of highly polyunsaturated phospholipids have implicated other specific roles in membrane fusion events, (Bearer and Friend, 1982), leukotriene production, (Oliw and Sprecher, 1989) and membrane signal transduction during sperm-egg recognition, (Roldan and Harrison, 1993 ; Roldan, 1994) although all such theories require further investigation.

In this study, age related decreases in semen quality were associated with marked reductions in the proportion of C20 to C22 polyunsaturated fatty acids in spermatozoa phospholipids. In addition, ageing was accompanied by a loss of semen antioxidant capacity particularly in the activity of glutathione peroxidase. It is known that without adequate levels of the major enzyme glutathione peroxidase, the activity of superoxide dismutase becomes overwhelmed leading to an accumulation of damaging products of peroxidation which are detrimental to spermatozoa function (Alvarez and Storey, 1989)

The observations of lipid and antioxidant changes in the present study were also associated with reduced spermatozoa concentration, motility and in the bull poorer resistance to freezing, as has already been reported in spermatozoa as a result of peroxidative damage (Aitken and West, 1990 ; Aitken, 1994). Associated with a loss of antioxidant protection is the loss of desaturase activity, enhanced peroxidation and free radical production during the natural process of ageing within cells (Brenner, 1989 ; Bunker, 1992). A combination of such factors may lead to the ultimate loss of vital C20 to C22 polyunsaturated fatty acids with detrimental effects on the correct balance of polyunsaturates to saturates within the membrane for correct fluidity characteristics and indeed the loss of potential energy reserves for motility (Scott, 1973 ; Hammerstedt, 1993). The major part of the age related decreases in the level of these polyunsaturated fatty acids in both species was associated in particular with one of the major phospholipid classes, phosphatidyl ethanolamine. The proportion of this phospholipid class was also shown to decrease with age accompanied by an increase in phosphatidyl choline, where each of these classes was shown to correlate positively and negatively respectively with semen characteristics and fertility. Results suggests that phosphatidyl ethanolamine may play a key role in spermatozoa structure and/or optimum metabolic function in normal, healthy spermatozoa from young animals.

The amount of total lipid within both semen fractions of the cockerels was shown to increase with age and was also shown to be higher in those cockerels displaying below average semen quality parameters. The utilisation of lipids for energy by spermatozoa has been frequently reported (Scott, 1973 ; Sebastian *et al.*, 1987 ; Hafez, 1993) Cockerels are known to possess very low fructose levels within their semen compared with bulls and in the presence of fructose spermatozoa have been shown to maintain their lipid content during *in vitro* incubation ; however, in its absence lipid has been shown to be utilised as energy for spermatozoa function. Elevated lipid associated with ageing and poor semen quality in cockerels may indicate reduced metabolic activity as previously reported by Sebastian *et al.* (1987). In addition the long chain fatty acids of phosphatidyl choline have been specifically reported to become oxidised as an energy source for spermatozoa metabolism (Hartree and Mann, 1951, 1961). An increase in the level of this phospholipid class with age and poor sperm quality, may suggest lower metabolic activity as less lipid is utilised as 'fuel' for spermatozoa motility. This may also help to explain the lower levels of spermatozoa motility associated with ageing in

this study. Bulls, however displayed a different pattern of lipid loss during ageing compared to the cockerels, particularly as this species displays high levels of semen fructose (Hafez, 1993) The loss of lipid in bull semen during ageing may suggest dysfunction of normal pathways for fructose metabolism possibly caused by peroxidative damage (Mann and Lutwak-Mann, 1981c ; Griveau *et al.*, 1995). A similar pattern was also observed in the young Belgian Blue bulls which are known to display particular problems of uniform semen production, quality and freezability.

Standard commercial diets possess high levels of n-6 fatty acids which may lead to the appearance of unnaturally high levels of n-6 fatty acids within the tissues and possibly also in semen (Salem *et al.*, 1986 ; Weisinger *et al.*, 1995). Cockerels exhibited a high degree of resistance to changes in spermatozoa fatty acid composition following the addition of 18:3 (n-3) to the diet. Most dramatically no 22:6 (n-3) increase was observed in phospholipid although the appearance of modest levels of 22:5 (n-3) led to some reduction in the ratio of n-6 to n-3 fatty acids. These results suggest that, in the cockerel the ability of developing spermatozoa to convert 18:3 (n-3) to 22:6 (n-3) may be very limited. There is currently a lack of information on the mechanisms involved in the delivery of fatty acyl components from the circulation to the developing spermatozoa and in the selective incorporation of specific fatty acids in the spermatozoa phospholipids. It is clear from the present study that the desaturation/elongation of 18:3 (n-3) to its C20 to 22 derivatives, and/or the incorporation of these derivatives into phospholipids is inefficient in the avian system. Traditionally it was believed that the synthesis of n-6 and n-3 C22 polyunsaturated fatty acids from other C18 precursors was elaborated via the action of a variety of desaturase enzymes accompanied by a series of chain elongation steps (Brenner, 1989). However, recent work has suggested that the direct conversion of 22:4 (n-6) to 22:5 (n-6) and 22:5 (n-3) to 22:6 (n-3) via the specific action of delta-4 desaturase does not take place and that the action of chain elongation and desaturation instead involves microsomal elongation of C24 polyunsaturates followed by eventual synthesis of 22:5 (n-6) and 22:6 (n-3). It has been reported that this final step occurs via peroxisomal beta-oxidation (Sprecher *et al.*, 1995 ; Moore *et al.*, 1995). The present data indicating that dietary supplementation with 18:3 (n-3) results in the appearance of albeit low levels of 22:5 (n-3) in the spermatozoa but with no increase in the proportion of 22:6 (n-3), suggests that this reported microsomal elongation of 22:5 (n-3) and subsequent pathways do not occur in the avian system

This view is further supported by the fact that, in cockerel spermatozoa, 22:4 (n-6) appears to be the end product of the desaturation/elongation of 18:2 (n-6), with no further conversion to 22:5 (n-6). Despite the small changes observed during 18:3 (n-3) supplementation an increase in fertility and associated semen quality parameters were recorded. This suggests a subtle but beneficial effect, possibly on spermatozoa membrane fluidity or eicosanoid formation (Bearer and Friend, 1982 ; Oliw and Sprecher, 1989 ; Roldan and Harrison, 1993). It has also been reported that n-3 fatty acid supplementation produces distinct alteration in prostaglandin formation which could alter spermatozoa function through an effect on spermatozoa motility (Knapp, 1990). In contrast to 18:3 (n-3) supplementation dietary supplementation of cockerels with 22:6 (n-3) produced a significant increase in the level of this fatty acid in the spermatozoa and seminal plasma. However, the level of 22:6 (n-3) achieved by this supplementation fell way short of those levels normally detected within the spermatozoa and seminal plasma of non-supplemented mammals. This was also reflected in the n-6 levels of the cockerels, which although reduced by n-3 supplementation, were still considerably higher than those recorded for the bull. A major conclusion of this work is that n-3 supplementation does not result in a dramatic conversion of characteristics of avian spermatozoa to those of the mammal and that cockerel spermatozoa appear to exhibit a resilience to dietary manipulation. The implication is such that the n-6 fatty acid predominance of cockerel spermatozoa does indeed reflect a true species difference and is not an artefact of feeding the low n-3 commercial diet. Such differences may be explained by the internal position of avian testes which are some degrees higher than those of externally located mammalian testes. The higher body temperature of birds compared to mammals (41 °C compared to 37 °C) (Romijn and Lokhurst, 1966) coupled with the fact that only mammalian testes are usually maintained at a temperature cooler than the rest of the body in the scrotum, indicates that avian spermatozoa must develop and function in an environment which may be several degrees warmer than that experience by their mammalian counterparts. Since the major polyunsaturate of avian spermatozoa 22:4 (n-6) displays the same chain length but fewer double bonds than 22:6 (n-3), characteristic of mammalian spermatozoa, the difference in the degree of polyunsaturation could represent a means of maintaining the appropriate biophysical properties of the spermatozoon plasma membrane at two difference temperatures and may go some way to explaining the differences in *in vivo* survival of spermatozoa within the female as cockerel sperm survive for some weeks compared to only days in the

mammal. The presence of n-6 fatty acids in this context requires further investigation taking into account the additional and vital role of the female reproductive tract. Both species also display considerable differences in spermatozoa morphology, (Hafez, 1993), the pointed avian spermatozoon head may necessitate the presence of n-6 polyunsaturated fatty acids as well as more saturated phospholipid classes such as phosphatidyl choline. This is in contrast to the bull spermatozoon head which is bulbous and possesses large levels of n-3 fatty acids in all the major phospholipid classes. It may be that n-3 fatty acids contribute more to the formation of enhanced membrane fluidity required to establish properties of a larger and fuller membrane structure such as that displayed by the bull and most other mammals.

The supplementation of 22:6 (n-3) to the bulls had a similar effect to cockerels fed with 18:3 (n-3) in that spermatozoa seemed quite resistant to change despite small increases in blood plasma n-3 levels as previously reported by Ashes *et al.* (1992). Instead a rearrangement in the distribution of this fatty acid between different phospholipid classes was observed. The overall effect of these changes was to "cancel out" any increases while appearing to maintain desirable and necessary levels within each phospholipid class. An increase in the 22:6 (n-3) content of phosphatidyl ethanolamine, a phospholipid class already correlated in this study with good semen quality, was observed accompanied by a reduction in the level of 22:6 (n-3) within phosphatidyl choline, a class shown to increase with age and which is associated with poorer semen quality. The level of 22:6 (n-3) within phosphatidyl inositol/phosphatidyl serine was in agreement with previous reports of fish oil supplementation (Knapp, 1990). Such "hidden" alterations within phospholipid classes may reflect changes in the relative affinity of acyltransferases for docosahexaenyl CoA under conditions of increased concentrations of 22:6 (n-3). Such changes were generally beneficial particularly in post-freeze survival and in the maintenance of membrane integrity indicating that a more appropriate balance between the levels of 22:6 (n-3) and other fatty acids may promote maximum conditions for spermatozoa function and indeed, that maintenance of levels of 22:6 (n-3) in certain classes such as phosphatidyl serine are vital to regulate fluidity as well as membrane interaction criteria as previously reported (Salem *et al.*, 1986).

The benefits of vitamin E as a dietary supplement or as a diluent additive were also confirmed. The addition of vitamin E was shown to enhance and protect high

polyunsaturated fatty acid levels within spermatozoa and seminal plasma and reduce peroxidation effects associated with their presence. Such results substantiate previous reports that long chain polyunsaturates require the addition of an appropriate and adequate antioxidant system to avoid peroxidative damage even in the presence of endogenous antioxidants (Bassler, 1991). The routine use of vitamin E may provide improvements, as reported by other workers, in spermatozoa metabolism, motility and acrosomal integrity, all vital to create the optimal environment for fertilisation (Beconi *et al.*, 1993 ; Blesbois *et al.*, 1993)

The study has revealed a complexity of relationships between spermatozoa lipid composition and semen quality, in particular the possibilities of improving fertility by appropriate dietary manipulation of semen lipid parameters may be envisaged. Further investigation of the effects of n-3 fatty acids on sperm plasma membrane fluidity, spermatozoa prostaglandin production and gamete interaction remain to be established. In addition comparison of the commercial semen lipid composition compared to those of free-living animals would clarify the "natural" semen fatty acid composition of avian species.

In conclusion the main features observed in this study were ;

1) changes in semen lipid and antioxidant levels occurring during the ageing process.
2) similarities and differences in lipid and antioxidant levels displayed between domestic avian and mammalian species during ageing and between animals of high and low semen quality.
3) true species differences displayed in major fatty acids present within the semen of domestic avian and mammalian species.
4) a considerable degree of resilience of spermatozoa to changes in dietary fatty acid composition in order to maintain the correct balance of fatty acids for optimal spermatozoa function.
5) the importance of an adequate antioxidant protection for normal spermatozoa function.

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APPENDIX TABLES

Table 7.6a. Fatty acid composition of the free fatty acid of the spermatozoa and seminal plasma at 24 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid (%w/w of total fatty acid)										
16:0	15.9 ± 2.8	18.6 ± 0.9	ns/c	22.0 ± 0.7		17.5 ± 1.4	23.9 ± 1.9	c/ns	24.6 ± 3.7	
18:0	23.7 ± 2.8	29.6 ± 1.2		25.2 ± 1.2		20.2 ± 2.6	33.4 ± 1.6	b/ns	23.3 ± 4.6	
18:1 (n-9)	24.7 ± 0.4	29.2 ± 0.6	a/a	26.9 ± 0.7		33.3 ± 2.6	37.2 ± 1.0		34.4 ± 1.7	
18:2 (n-6)	7.3 ± 3.2	3.2 ± 0.1		4.0 ± 0.5		10.3 ± 2.9	nd	a/a	7.3 ± 1.9	
20:4 (n-6)	4.5 ± 1.0	4.6 ± 0.4		4.1 ± 0.3		4.3 ± 1.1	nd	a/ns	< 1.0	a
22:4 (n-6)	5.1 ± 1.3	3.6 ± 0.4		3.9 ± 0.3		2.8 ± 0.5	nd	a/ns	< 1.0	a
22:6 (n-3)	1.4 ± 0.4	4.5 ± 0.4	b/ns	4.1 ± 0.5	b	1.2 ± 0.2	11.1 ± 4.9		6.7 ± 3.8	
n-6/n-3	5.3 ± 1.8	2.2 ± 0.1		2.5 ± 0.3		9.4 ± 2.0	-	a/ns	2.1 ± 0.2	c

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.6b. **Fatty acid composition of the free fatty acid of the spermatozoa and seminal plasma at 40 weeks of age.**

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid										
(%w/w of total fatty acid)	18.4 ± 1.8	17.8 ± 1.2		21.9 ± 0.9		20.9 ± 2.5	22.6 ± 1.5		23.7 ± 1.4	
18:0	24.7 ± 2.1	25.5 ± 1.0		24.2 ± 0.8		24.4 ± 0.5	28.8 ± 1.7	c	26.9 ± 3.0	
18:1 (n-9)	24.6 ± 0.9	26.9 ± 1.0		26.3 ± 1.7		30.7 ± 1.0	31.1 ± 2.1		36.0 ± 3.1	
18:2 (n-6)	6.3 ± 0.9	2.5 ± 0.2	b/b	4.8 ± 0.5		6.5 ± 0.2	3.2 ± 0.4	a/ns	5.8 ± 1.6	
20:4 (n-6)	5.4 ± 0.7	5.2 ± 0.6	ns/c	3.4 ± 0.2	c	4.4 ± 0.7	3.8 ± 0.2	ns/a	nd	a
22:4 (n-6)	7.5 ± 0.6	6.9 ± 1.1		5.2 ± 0.3	c	3.8 ± 0.3	3.8 ± 0.4		4.1 ± 0.3	
22:6 (n-3)	2.7 ± 0.5	3.7 ± 1.0		2.1 ± 0.9		1.8 ± 0.2	3.2 ± 0.5	c	4.3 ± 0.9	c
n-6/n-3	8.1 ± 1.7	3.2 ± 0.5	c/ns	6.2 ± 1.8		4.9 ± 0.3	3.4 ± 0.4	c/b	1.8 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.6c. Fatty acid composition of the free fatty acid of the spermatozoa and seminal plasma at 58 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid (%w/w of total fatty acid)										
16:0	17.1 ± 0.5	16.9 ± 1.8		20.3 ± 2.4		35.2 ± 1.7	32.6 ± 5.1		39.3 ± 3.2	
18:0	21.9 ± 3.4	26.5 ± 1.2		26.5 ± 0.9		14.9 ± 2.1	20.0 ± 2.7	ns/c	29.0 ± 2.4	b
18:1 (n-9)	27.4 ± 1.8	28.8 ± 1.3		29.3 ± 1.0		26.7 ± 2.7	24.3 ± 3.5		26.2 ± 1.6	
18:2 (n-6)	10.0 ± 4.1	2.8 ± 0.2		5.2 ± 2.0		12.0 ± 2.6	8.6 ± 4.5	ns/a	nd	a
20:4 (n-6)	5.7 ± 1.2	5.1 ± 0.4		4.4 ± 0.3		< 1.0	nd	a/ns	nd	a
22:4 (n-6)	5.8 ± 1.3	4.5 ± 0.7		5.1 ± 0.2		nd	nd		nd	
20:5 (n-3)	nd	1.9 ± 0.1	a/ns	1.7 ± 0.3	a	2.6 ± 0.5	nd	a/ns	nd	a
22:5 (n-3)	1.3 ± 0.1	2.2 ± 0.2	b/ns	2.0 ± 0.2	c	< 1	nd	a/ns	nd	a
22:6 (n-3)	2.0 ± 0.2	3.2 ± 0.2	b/ns	3.6 ± 0.6	c	1.3 ± 0.5	< 1		nd	a
n-6/n-3	5.6 ± 1.3	1.8 ± 0.1	c/ns	2.0 ± 0.3	c	6.2 ± 0.3	3.3 ± 0.7	b/ns	-	

Values are means ± S.E. of observations from 4 pooled ejaculates eachof 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
nd represents not detectable.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.7a. Fatty acid composition of the triacylglycerol of the spermatozoa and seminal plasma at 24 weeks of age.

	Spermatozoa				Seminal plasma			
22:6 (n-3) supplementation	-	+		+/vit E	-	+		+/vit E	
Fatty acid (%w/w of total fatty acid)									
16:0	23.7 ± 1.4	23.0 ± 0.8		26.7 ± 2.8	26.1 ± 1.7	27.0 ± 1.3		24.4 ± 1.9	
18:0	10.3 ± 2.1	17.3 ± 1.5		19.6 ± 0.9	19.5 ± 3.3	22.1 ± 3.8		10.9 ± 4.6	
18:1 (n-9)	31.8 ± 1.0	31.2 ± 0.4		31.6 ± 1.0	32.8 ± 0.6	39.8 ± 6.2		24.4 ± 2.2	
18:2 (n-6)	20.8 ± 1.0	10.6 ± 1.8		17.4 ± 1.4	16.1 ± 1.7	4.1 ± 0.1		10.3 ± 3.6	
20:4 (n-6)	1.3 ± 0.2	2.2 ± 0.1		< 1.0	nd	nd		nd	
22:4 (n-6)	1.3 ± 0.6	2.5 ± 0.1		< 1.0	nd	nd		nd	
18:3 (n-3)	1.4 ± 0.3	nd	a/ns	< 1.0	nd	nd		nd	
22:6 (n-3)	2.1 ± 0.3	4.2 ± 0.5		5.2 ± 1.3	nd	4.2 ± 0.1	a/ns	2.8 ± 0.1	a
n-6/n-3	6.3 ± 1.1	2.9 ± 0.6	c/ns	2.9 ± 2.1	-	1.0 ± 0.2	a/ns	1.2 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [c]P<0.05.
nd represents not detectable.
ns represents not significant.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.7b. Fatty acid composition of the triacylglycerol of the spermatozoa and seminal plasma at 40 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid (% w/w of total fatty acid)										
16:0	22.5 ± 2.8	25.5 ± 1.0		24.1 ± 2.5		32.4 ± 5.4	39.9 ± 4.5		30.1 ± 1.9	
18:0	16.9 ± 3.9	21.4 ± 1.3		17.1 ± 0.7		25.8 ± 6.9	27.2 ± 0.7		28.2 ± 3.5	
18:1 (n-9)	35.0 ± 3.1	31.3 ± 1.2		25.8 ± 1.5	c	28.8 ± 3.7	25.1 ± 0.7	ns/b	33.4 ± 3.0	
18:2 (n-6)	9.3 ± 1.8	4.9 ± 0.6		9.4 ± 2.6		21.6 ± 3.5	nd	a/a	5.7 ± 0.6	b
20:4 (n-6)	3.1 ± 0.4	1.9 ± 0.1	c/ns	2.6 ± 0.9		nd	nd		nd	
22:4 (n-6)	4.2 ± 1.3	4.7 ± 0.7		5.9 ± 2.9		nd	nd		nd	
22:6 (n-3)	2.8 ± 0.4	5.3 ± 1.4		8.0 ± 2.4		nd	10.1 ± 3.3	a/ns	5.1 ± 0.06	a
n-6/n-3	6.1 ± 1.3	3.4 ± 0.9		2.3 ± 0.4	c	-	-		1.1 ± 0.1	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
nd reprsents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.7c. Fatty acid composition of the triacylglycerol of the spermatozoa and seminal plasma at 58 weeks of age.

	Spermatozoa					Seminal plasma		
22:6 (n-3) supplementation	-	+		+/vit E		-	+	+/vit E
Fatty acid (% w/w of total fatty acid)								
16:0	25.7 ± 4.0	22.0 ± 1.6		30.7 ± 4.1		34.2 ± 3.2	25.9 ± 2.0	37.1 ± 4.7
18:0	23.7 ± 4.3	26.7 ± 3.8		22.9 ± 2.9		14.8 ± 1.3	24.7 ± 5.4	20.2 ± 1.7
18:1 (n-9)	26.7 ± 1.3	31.9 ± 3.1		27.6 ± 3.7		30.2 ± 2.3	33.6 ± 1.7	31.8 ± 2.1
18:2 (n-6)	12.3 ±4.4	6.3 ± 1.2		6.1 ± 0.8		8.9 ± 0.8	8.5 ± 1.7	8.6 ± 0.8
20:4 (n-6)	< 1	2.9 ± 0.2	a/a	nd	a	nd	nd	nd
22:4 (n-6)	4.0 ± 0.5	4.6 ± 0.9		5.5 ± 0.5		nd	nd	nd
22:6 (n-3)	nd	6.0 ± 0.6	a/ns	4.6 ± 0.3	a	nd	nd	nd
n-6/n-3	-	2.2 ± 0.3	a/ns	2.3 ± 0.4	a	-	-	-

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.8a. Fatty acid composition of the cholesterol ester of the spermatozoa and seminal plasma at 24 weeks of age.

	Spermatozoa			Seminal plasma				
22:6 (n-3) supplementation	-	+	**+/vit E**	-	+		**+/vit E**	
Fatty acid (% w/w of total fatty acid)								
16:0	20.5 ± 1.9	20.3 ± 1.5	16.0 ± 1.3	18.0 ± 1.3	24.4 ± 2.8		24.4 ± 1.9	
18:0	13.8 ± 2.1	14.9 ± 1.4	13.6 ± 2.9	6.0 ± 0.8	7.0 ± 0.8		10.9 ± 4.6	
18:1 (n-9)	41.6 ± 3.2	45.9 ± 3.0	30.9 ± 3.2	19.8 ± 0.4	23.6 ± 2.0		24.4 ± 2.2	
18:2 (n-6)	12.2 ± 1.5	9.8 ± 0.8	28.2 ± 9.7	53.0 ± 4.1	31.5 ± 1.0	**b/ns**	26.7 ± 2.6	b
20:5 (n-3)	nd	nd	nd	nd	5.7 ± 0.6	**a/ns**	4.9 ± 0.4	a
22:6 (n-3)	4.5 ± 0.8	6.5 ± 0.8	7.2 ± 1.4	nd	13.1 ± 1.0	**a/ns**	8.8 ± 1.5	a
n-6/n-3	2.9 ± 0.8	2.0 ± 0.1	3.9 ± 1.6	-	2.0 ± 0.4	**a/ns**	2.3 ± 0.4	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.8b. Fatty acid composition of the cholesterol ester of the spermatozoa and seminal plasma at 40 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		+/vit E		-	+		+/vit E	
Fatty acid (% w/w of total fatty acid)										
16:0	17.8 ± 1.1	26.8 ± 4.4		25.5 ± 2.9	c	23.8 ± 2.1	42.2 ± 5.9	c/ns	28.4 ± 3.9	
18:0	13.3 ± 1.6	18.9 ± 3.1		13.0 ± 1.0		11.2 ± 1.7	19.8 ± 3.4		15.7 ± 2.4	
18:1 (n-9)	18.8 ± 4.2	29.8 ± 3.3		28.9 ± 8.1		25.3 ± 5.2	23.2 ± 11.5		25.7 ± 3.1	
18:2 (n-6)	9.3 ± 1.7	9.2 ± 0.2		9.1 ± 2.4		30.7 ± 1.7	12.6 ± 1.4	a/ns	14.8 ± 0.5	a
20:4 (n-6)	4.0 ± 1.4	2.5 ± 0.5	ns/a	nd	a	nd	nd		nd	
22:4 (n-6)	6.0 ± 2.9	6.2 ± 4.0	ns/a	nd	a	nd	nd		nd	
22:6 (n-3)	3.7 ± 1.1	9.2 ± 0.9	b/ns	13.0 ± 1.9	b	9.6 ± 0.2	8.0 ± 5.7		13.4 ± 2.1	
n-6/n-3	6.4 ± 1.0	1.3 ± 0.2	b/ns	1.5 ± 0.2	b	3.5 ± 0.2	3.0 ± 2.0		1.2 ± 0.3	a

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

Table 7.8c. Fatty acid composition of the cholesterol ester of the spermatozoa and seminal plasma at 58 weeks of age.

	Spermatozoa					Seminal plasma				
22:6 (n-3) supplementation	-	+		**+/vit E**		-	+		**+/vit E**	
Fatty acid (% w/w of total fatty acid)										
16:0	29.5 ± 6.1	31.1 ± 6.5		31.8 ± 4.3		35.7 ± 3.3	29.4 ± 0.9	**ns/c**	36.2 ± 2.1	
18:0	12.9 ± 2.0	33.7 ± 2.2	**a/b**	18.9 ± 1.2	**c**	23.1 ± 3.5	31.1 ± 5.2		26.3 ± 0.5	
18:1 (n-9)	32.1 ± 7.0	35.2 ± 4.3		32.0 ± 6.4		28.7 ± 1.8	29.9 ± 4.0		32.2 ± 4.4	
18:2 (n-6)	33.1 ± 0.9	nd	**a/a**	13.2 ± 4.1	**a**	15.0 ± 4.6	nd	**a/ns**	nd	a
20:4 (n-6)	nd	nd		nd		nd	nd		nd	
22:4 (n-6)	nd	nd		nd		nd	nd		nd	
22:6 (n-3)	nd	nd		nd		nd	nd		nd	
n-6/n-3	-	-		-		-	-		-	

Values are means ± S.E. of observations from 4 pooled ejaculates each of 3 birds.
Significance of difference between groups : [a]P<0.001 ; [b]P<0.01 ; [c]P<0.05.
ns represents not significant.
nd represents not detectable.
Only major fatty acids and n-6 and n-3 fatty acids have been included.

PUBLICATIONS ARISING FROM THIS WORK

Full papers in refereed Journals

Lipid and antioxidant changes in semen of broiler fowl from 25 to 60 weeks of age. ***Journal of Reproduction and Fertility***. (1995). 106 : 201 - 206.

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