FACTORS DETERMINING THE FERTILISING ABILITY OF AVIAN GERM PLASM

DEBABRATA CHAUDHURI

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DEDICATION

This thesis is dedicated to my parents as a token of their blessing, love and affection.

Declaration/Certificate

I hereby certify that the work embodied in this thesis is the result of original research and has not been submitted for any other degree or professional qualification to Edinburgh or any other University.

Dated 12/8/1988. (D. CHAUDHURI)

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NOMENCLATURE

A_{520}	Absorbance of the solution at 520 nanometre
AI	Artificial insemination
ANOVA	Analysis of variance table
ATP	Adenosine triphosphate
AR	Analytical grade reagents
b	regression coefficient
BES	N,N-bis[2-Hydroxyethyl]-2-aminoethane sulphonic acid
cm	centimetre
c.mm	Cubic milimeter
Δ	Delta, finite difference (gain)
g	gram
g	centrifugal force [calculated in relation to speed of rotation, revolution per minute (RPM) and diameter of the spinning rotor of the centrifuge]
hr	hour
i.d.	internal diameter
INT	(2-p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride, or, iodo-nitro-tetrazolium
l	litre
M	molar
min	minute
mg	milligram
ml	millilitre
mm	millimeter
mM	milliMolar
N	Normal
NBT	Nitro-blue-tetrazolium salt
nm	nanometre
nM	nanomolar
μ	micron
μl	microlitre
μM	micromolar
OD	Optical Density
$P \leq 0.05$	Probability less than or equal to 5% level
$P \leq 0.01$	Probability less than or equal to 1% level
$P \leq 0.001$	Probability less than or equal to 0.1% level
pH	A measure of hydrogen ion concentration
PMS	Phenazine methosulphate
r	Correlation coefficient
R^2	Square of correlation coefficient (coefficient of determination)
RPM	Revolution per minute
spin	spinning or centrifuging
t	time
TES	N-tris (Hydroxymethyl)methyl,2-aminoethane sulphonic acid

ABSTRACT

This thesis reports the results of an investigation into the factors affecting the fertilising ability of avian germ plasm. The study was limited to males of a Rhode Island Red control population and females of a commercial white egg-laying hybrid. The stock was replaced annually from the same source.

The investigation was broadly organised according to three major objectives:

1) Development of an objective *in vitro* test to predict fertilising ability.
2) A study of some management and biological factors affecting fertilising ability of semen.
3) Development of a diluent and conditions for extended storage of fowl semen at high temperatures of 20 and 40°C.

A simple, objective, controlled colourimetric technique was developed for estimating the capacity of fowl spermatozoa to reduce a tetrazolium dye to a coloured compound, formazan, which could be measured spectrophotometrically. The reduction of this dye is dependent on number of spermatozoa, incubation temperature and time of incubation. The test is shown to give a quantitative measurement of the metabolic activity of spermatozoa, as judged by their rate of oxygen utilisation, and thus of the quality of semen. The rate of tetrazolium dye reduction was shown to be highly correlated with sperm motility, morphology, ATP content and fertilising ability. The method has many practical advantages over existing tests for the prediction of fertilising ability.

A study of frequency of semen collection, over a period of 4 weeks, showed that the fertilising ability was not significantly different when semen was collected twice a day, once a day or thrice a week. Exchange of seminal plasma between samples of semen from males designated as highly or poorly fertile, or as young or old, did not influence the *de novo* fertilising ability of the original spermatozoal samples.

The investigation of the age of male and female revealed that age of male influenced fertilising ability but not the age of females. However, interaction in fertilising ability between age of male and age of female was significant.

A simple diluent and storage condition was developed for holding fowl semen at high temperatures of 20° and 40°C for 17 hr. A simple method for storing semen for 6 hr in a still condition on a bench top around 20°C, which should be suitable for field situations, is described.

This thesis examines some features of fowl semen physiology concerned with identifying quality of spermatozoa, and some beneficial procedures for the better exploitation of artificial insemination of probable benefit to breeders of commercial poultry.

1. GENERAL INTRODUCTION

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The application of scientific knowledge of genetic selection of farm animals has resulted in practical animal breeding improvement programmes which in turn have made significant improvements in the production of food and fibre from animals. These valuable genetic stocks need to be maintained and even improved further according to needs of regional agroclimatic conditions and human demands. Furthermore of more recent concern is the preservation of genomes for future genetic engineering of farm animals.

Artificial insemination (AI), originally developed as a research technique, is today one of the approved and widely-adopted methods worldwide producing over 70% of all conceptions in cattle. Artificial insemination has become the obligatory method for the commercial production of turkey poults because of the exaggerated sexual dimorphism in body size due to the phenomenal increase in growth rate and breast muscle from genetic selection over the last 25 years. That situation may not be far away in the broiler fowl industry for similar reasons. In the layer-fowl industry, AI is used mainly by primary breeders as an aid to increase the efficiency of the collection of accurate pedigree data in selection programmes involving characters of low heritable traits like egg production or rate-of-lay. Considerable progress has been made over the last 10 to 15 years in obtaining knowledge on poultry semen physiology which can enable a more purposeful exploitation of AI, for stock improvement and more flexible breeding management. For example, more effective use of semen from outstanding sires, saving on the cost of breeding (reduction in floor space and labour; total elimination of breeding pens, trap nests and floor eggs), controlling preferential or incompatible matings due to body size differential or heterologous matings among breeds or species, and also ease of maintaining control populations and the efficient conduct of diallel matings.

Domestic birds are in a continuous reproductive state for many months following sexual maturity, and the success of commercial breeding by AI is determined by succeeding in fertilising each ovum ovulated daily in succession, which commonly involves inseminating several thousand females once per week (an arduous task). The criterion of success of AI in the fowl is to achieve a level of fertility of over 90% fertile eggs from a single insemination lasting over a period of 10 to 14 days. This is unlike mammals where fertilisation of one egg per insemination, or several eggs at one instant directly after insemination, is the criterion of success, and a measure of the quality of the spermatozoa used. Another important factor to bear in mind as determining the success of AI in poultry relates to the fact that a proportion of spermatozoa start losing integrity as soon as collected and the process is progressive. Thus, efficient handling of semen is a necessity to slow down this loss which affects fertility levels. With this in mind there are several factors which have to be recognised as ultimately controlling fertilising ability of fowl semen in the context of successful artificial breeding. They may be broadly classified into two categories:

1. Factors which affect initial quality of spermatozoa.
2. The maintenance of viability of spermatozoa in vitro until they are inseminated into the female reproductive tract.

1. The initial quality of spermatozoa is governed by biological factors including the genetic make-up of the population and age of the bird, management factors, such as diet, and components of the environment under which the birds were reared and maintained in adulthood (including housing, lighting), and the regimen of frequency of collection of semen from the cocks.

To exploit AI to advantage as a tool for making genetic advancements, it is important to be able to assess the inherent quality of spermatozoa and to predict the breeding potential of the male (the fertilising ability of its gametes). However, there are few objective tests available and most of them are very sophisticated and expensive. Many subjective tests have been developed over the years, but most of them are not very sensitive and vulnerable to human error of judgement causing wide variation in predicting fertilising ability.

2. The second category of factors governing the success of the application of AI is concerned with the maintenance of viability of the spermatozoa in vitro; e.g. the effect of the

time between collection of semen and insemination, the dilution rate (if diluted semen needed), constituents and properties of the diluents (pH and osmotic pressure), the temperature and conditions under which semen is stored (if storage is needed), the influence of seminal plasma during storage and insemination, the physical conditions of storage (e.g. size, shape, dimension and composition of the holding vessels in which semen is collected and stored, degree of aeration in semen sample during storage.

Because of the anatomy of the male bird, with no penis as a separate outflow for semen, care must be taken to avoid contamination of semen with faeces, urine and/or a transparent secretory fluid emanating from cloacal tissue, all of which can affect the ultimate fertility obtained with inseminations under particular circumstances. Also, because females are continuously in reproductive condition for many months, eggs are constantly flowing down the oviduct and, therefore, timing of insemination in relation to oviposition, depth of insemination in the vagina, volume of inseminate and numbers of spermatozoa are vital to avoiding regurgitation and loss of spermatozoa, all of which affect the achievement of optimum fertilisation rates.

In the present study, investigations were made into a few of the above factors where more information is needed on semen physiology and the application of techniques for optimising the fertilising ability of fowl semen in artificial breeding under certain circumstances. There were the following objectives:

1) To investigate and develop an objective method of evaluation of the quality of spermatozoa, and to compare the suitability of the proposed method with existing methods for predicting fertilising ability.

2) To test the influence of the age of male, which may be confounded by the age of the female, on the fertilising ability of semen.

3) To examine the influence of the frequency of collection of semen on fertilising ability.

4) Since pooling semen is a common practice in the application of AI in poultry breeding, to examine for possible reciprocal effects of seminal plasma from males of different age, or with different inherent integrity of spermatozoa, on the fertility of a whole semen sample.

5) To develop a diluent and conditions for the handling and storage of fowl semen at high temperature (20° and 40°C) which has great advantages for using AI in selective breeding programmes especially in tropical countries, but also universally.

Thus, the experimental work of the thesis is divided into four different sections: the first two (Sections 4.1 and 4.2) deal with the development and application of an objective method of assessing semen quality for predicting fertilising ability of males and monitoring semen quality with time. It is based upon measuring, spectrophotometrically, the integrity of the metabolic activity (specifically the oxido-reductase enzyme pathway) of the spermatozoa as judged by the reduction of a colourless dye, by electron transfer, to produce coloured formazan. The third section (Section 4.3) deals with factors (natural phenomena and some connected with handling of semen in vitro), like age of donor male, frequency of semen collection and influence of seminal plasma, which may play a part in determining fertilising ability in an artificial breeding situation. The last section (Section 4.4) deals with the development of a simple diluent and conditions for storing fowl semen at high temperatures for up to 17 hr, and a simplified version of the method for handling and storing semen on the bench top at a common room temperature around 20° for up to 6 hr.

2. REVIEW OF LITERATURE

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2.1 INTRODUCTION

The scientific basis for the genetic selection of animals was worked out in Europe and the United States of America in the late 1930s and 1940s. The application of these findings to practical animal breeding improvement programmes has assisted in achieving notable increases in the production of food and fibre per animal. A few high performance breeds have emerged in temperate regions which are gradually displacing the local breeds as well as those in developing countries in the tropics.

Each breed in harsh climatic regions, through natural or man-made selection, has developed characteristics which make them well adapted to the environmental conditions under which the animals have to live and produce. This valuable genetic material needs to be maintained and indeed improved according to the regional agro-climatic conditions. It is necessary to maintain a reasonable balance of exotic and indigenous stocks by utilising modern scientific breeding techniques.

Artificial insemination (AI), originally introduced as a research tool in the study of reproductive biology and controlled breeding, has found wide application in animal improvement programmes. Extensive studies on semen biology and spermatozoal morphology, and the development of appropriate techniques for semen evaluation, dilution and preservation have added further dimensions to the use of AI. As seen today, AI is one of the approved and widely adopted methods throughout the world for genetic improvement particularly in cattle and pigs.

Today all commercial turkey poults are produced by means of AI. This obligatory procedure of breeding arose not to consciously increase the rate of genetic improvement but rather as a consequence of it. Commercial turkey breeders were making such rapid improvements in growth rate and breast conformation in their birds during the 1960s that they were developing anatomical characteristics impairing natural mating ability. Consequently AI had to be used to maintain high fertility levels in turkeys. However, the application of the technique has made possible subsequent genetic improvement in the performance of commercial turkeys.

With fowl, the adoption of AI is becoming more popular in large scale breeding farms with elite and Grand Parent nucleus stocks because of the accuracy of pedigree data available from the cage system of management.

Detailed below are some of the factors which have been responsible for popularising the increasing use of AI in poultry breeding:

a) More effective use of semen

b) Effective use of outstanding sires

c) Transport of semen

d) Use of single sire's semen simultaneously over various locations enabling the covering of a large number of females

e) Saving the cost of breeding pens, space, trapnesting, labour and loss of accuracy of data due to floor eggs

f) Overcoming infertility due to physical incompatability in intra- and inter-species mating. Preferential mating may be avoided by use of AI.

g) Ease of maintaining control populations or conducting diallel matings.

Ideally, for the adoption of AI the reproductive performance of the males and their fertilising ability needs to be monitored constantly. Semen evaluation by *in vitro* tests before actually using the males for breeding should be encouraged. If a poor quality semen producer is selected for breeding, the result can be disastrous and particular families may be eliminated or reduced in size resulting in fewer sires contributing to the next generation.

To achieve maximum desirable fertility in poultry by AI, therefore, evaluation of semen quality could play as major a role as semen collection and insemination techniques, but it is still not widely appreciated and practised.

The objectives of semen quality evaluation are as follows:

1) To assess the fertilising ability of breeder males

2) To evaluate potential breeders before the start of the hatching season

3) To ensure good quality semen suitable for dilution to extend the coverage by each male

4) As an aid in determining which samples of semen should be retained for storage and preservation for long-term use

5) As a general procedure in the selection of males to improve semen quality

6) As a measure of spermatozoal viability during storage experiments or in testing the efficacy of diluents

Fowl semen is a physiological secretion consisting of spermatozoa and seminal plasma (seminal fluid). Unlike mammalian semen, there is no addition to the semen at ejaculation of secretions from accessory reproductive organs, such as the prostate gland, seminal vesicles and Cowper's glands. The fertilising capacity of semen depends upon the morphology, motility, viability and metabolic activity of the spermatozoa. Various tests of semen quality or evaluation can be applied to assess these characters. They are:

1) Semen appearance and colour

2) Volume of ejaculate

3) Initial motility of spermatozoa

4) Density of spermatozoa by

   a) Haemocytometer

   b) Optical density of a suspension of spermatozoa in a given volume

   c) Electronic particle counter

   d) Packed cell volume (spermatocrit)

5) Hydrogen ion concentration

6) Proportion of live and dead spermatozoa

7) Proportions of various other abnormal spermatozoa

8) Dye-reduction time (methylene blue, resazurin) as a measure of metabolic activity and spermatozoal density

9) Speed of movement of spermatozoa

10) Fluorometric determination of the viability of avian spermatozoa (assessing membrane damage by fluorescing DNA)

11) Determination of egg fertilisation rate and hatchability

2.2 DEVELOPMENT OF ARTIFICIAL INSEMINATION

Artificial insemination (AI) is one of the most important techniques devised for the genetic improvement of farm animals. The earliest documented use of AI was in 1780 when Spallanzani (Mann and Mann, 1981), an Italian physiologist, obtained pups by manually introducing semen into the vagina of the bitch. It was not until about 1900 that extensive AI studies with farm animals began in the USSR and from 1900 to 1930 Ivanov developed AI techniques for horses and sheep. The same for cattle was developed in Denmark in the early 1930s (Mann and Mann, 1981). Ivanov first inseminated birds artificially as early as 1902 by killing the male and then drawing the semen from the terminal bulbs of the seminal ducts (Griffin, 1938). Others allowed roosters to mate and either intercepted the semen just prior to mating or removed semen from the hen's cloaca (Amantea, 1922; Payne, 1914, cited by Sexton, 1984).

In fowls, AI has been used for research purposes since Quinn and Burrows (1936) reported that high fertility could be obtained by inseminating 0.1 ml of undiluted semen intravaginally. Economically, the fertility of the male fowl in a breeder flock is of greater importance than that of females. Hays and Sanborn (1939) showed that in most cases in flock matings the males were responsible for infertile matings, since a change of the infertile males resulted in 93% of the infertile matings becoming fertile. The commercial adoption of AI in domestic fowl was very limited until around 1960's though previously it had commercial applications in Israel (Thumin, 1951; Van Weerden and Van der Wal, 1962; Bornstein et al., 1965) and in Japan, where there is a very favourable report on results obtained over a 4-year period comparing natural mating with artificial insemination in the production of commercial chicks (Saeki and Nogami, 1964). During the 1960s, AI in fowls gained momentum in the U.S.A. (McDaniel, 1974) and in Great Britain (Perek, 1966; Cooper, 1969; Lake, 1971). As previously mentioned, turkey breeders use AI extensively to propagate their stock because of their mating difficulty due to body size and broad breast conformation. Large-scale use of AI in duck and goose breeding programmes is widely adopted in the Far East (Huang and Chow, 1974; Tan, 1980a, b; Lake, 1983).

2.2.1 The influence on fertility of basic factors associated with the male in artificial insemination

Lake (1983), in an extensive review, identified two major aspects of the impact of the male on fertility when employing AI:

a) Genetics and physiology of the spermatozoa:

The inherent variation in age at sexual maturity and semen production between species of poultry, breed, strain, lines and between individuals within a strain, line or breed is well known. Shilin (1978) observed that the semen quality of cocks was highest between 6 and 24 months of age in two strains of fowl which matured at 104 and 107 days. Benoff et al. (1981), Fiser and Chambers (1981) and Petitjean et al. (1978) observed similar findings and suggested that there is a possibility of improving semen quality by selection which could improve the efficiency of breeding by AI.

Studies on waterfowl revealed that the efficiency of gamete function takes a long time to become established; the quality of gander semen improved after the first breeding year (Borys et al., 1978; Bielinska et al., 1976; Kurbatov et al., 1976).

A homozygous condition (rose comb) in some breeds of fowl has been identified as producing defective spermatozoa and very poor fertility (Buckland et al., 1969; Petitjean, 1970; Crawford, 1971). Etches et al. (1974) suggested that the spermatozoa of these types of male failed to ascend the oviduct properly due to possible defective carbohydrate metabolism. Froman and Bernier (1987) found that subfertility in a strain of fowl was due to the early death of a proportion of spermatozoa in the ductus deferens.

The number of good quality spermatozoa in a semen sample inseminated into a hen is one important factor determining the fertility level obtained by AI. In this respect it is important to recognise that the proportion of naturally degenerating spermatozoa in the ductus deferens increases with long rests between semen collection (El Jack and Lake, 1966). Also, several studies have been made on the effect of the frequency of semen collection on optimum breeding efficiency (Tan, 1980a; Fuquay and Renden, 1980; Sexton, 1977a). Twice or thrice weekly semen collection is probably optimum in fowl. Brown (1971) found that collecting semen from turkey males at 3 and 4-day intervals resulted in a greater output of spermatozoa than collecting at 2-day intervals. Wishart (1984) investigated lipid peroxide formation in 5 hr-stored fowl semen and its effect on the fertilising ability of spermatozoa. He concluded that the degree of lipid peroxide formation (detected by rate of malonaldehyde formation) differed between males within a genetic strain. The males which produced the least rate of malonaldehyde formation during storage yielded highest fertility and vice versa.

Environmental factors such as photoperiod, light quality, temperature and nutrition (Lake, 1967, 1969, 1971; Menge and Forbish, 1976) play a vital role in influencing semen production. Interaction between light intensity and semen output was confirmed by Jones et al. (1977).

Age and seasonal effects on semen production in ducks, geese, turkey and broiler fowls are more marked than in layer-type fowls (Lake, 1983).

b) The collection and preparation of semen for insemination

Variable amounts of transparent fluid from the cloaca, in addition to faeces and urine, often contaminate semen samples obtained artificially from birds. Lake and Stewart (1978) described a method of collecting semen from fowls without contamination. The presence of transparent fluid often reduces the fertility if the semen sample is held for a prolonged period *in vitro* before insemination (Nishiyama, 1955; Fujihara and Nishiyama, 1976; Lake, 1981). Apart from direct effects on spermatozoa, contamination decreases the density of spermatozoa in semen samples with a danger of causing lower fertility unless the dosage volume of semen is compensated.

An important point to remember is that from the time semen is ejected from the male, a proportion of the spermatozoa begin naturally to lose their integrity and the process continues during storage *in vitro* with a resultant progressive decline in fertility after insemination. The precautions which should be taken when handling and diluting semen to reduce the rate of loss of spermatozoa have been reviewed by Lake and Stewart (1978).

2.2.2 Factors associated with the female influencing fertility: Factors associated with the insemination procedure

Spermatozoa are able to live in the oviduct for at least 5 to 11 days in ducks, geese and fowl and 4 to 6 weeks in turkeys, and during these periods a succession of eggs laid by a hen is fertilised after a single insemination. The criterion of the success of AI in birds is the production of this pattern of prolonged fertility in each female between inseminations (Lake, 1975; Lake and Stewart, 1978). The optimum fertility in a flock throughout a breeding season is maintained by repetitive inseminations at intervals determined by certain factors given below. The maximum hatchability rates may also be partly influenced by aspects of the interaction of female reproductive physiology with the activity of spermatozoa in the oviduct.

a) Number of spermatozoa inseminated into the oviduct

To maintain a constant high level of fertility throughout a breeding season, a minimal number of good quality spermatozoa must be inseminated at regular intervals. The interval depends upon the type of bird, but is generally about one week under farm conditions. It is generally recognised that about 80 to 100 million fresh fowl spermatozoa per insemination are necessary to maintain high fertility under farm conditions, and no advantage is gained by inseminating more than 100 million fresh, good quality spermatozoa into a female at any one time. Compton and Van Krey (1979) found in the fowl that the proportion of utero-vaginal sperm storage tubules filled with spermatozoa was not increased by inserting more than a critical number of spermatozoa. Recently, Wishart (1985) observed that under controlled laboratory conditions as few as 6 to 10 million good quality spermatozoa per insemination produced the desired peak fertility during 2 to 8 days following insemination. This would be well worth exploiting in maximising the use of sires in the future application of AI in poultry breeding.

Although the number of spermatozoa inseminated is the basis for sustaining good fertility, a breeder under farm conditions, where it is not uncommon to have to inseminate several hundred females in a session, generally fixes a dosage of semen to administer to the hens hoping that it always contains the correct number of spermatozoa. This may be one cause of fluctuating fertility levels because, due to several factors, the density of spermatozoa in a semen sample as already mentioned is likely to vary during the course of a breeding period. Individual males may have different inherent quantities of spermatozoa in their male tract from time to time due to physiological changes.

Other factors to bear in mind in influencing the number of viable spermatozoa inseminated in field conditions are: the holding time for semen between collection and insemination, the 'dilution effect' on spermatozoa, if diluted, and the tendency of spermatozoa in diluents to sediment with time.

The frequency of insemination and/or the absolute number of spermatozoa inseminated may need to be increased from the middle to the end of the breeding period to partially overcome a tendency for a progressive drop in the fertilisation potential of semen. It must also be recognised that a change in reproductive ability often occurs in the female simultaneously with a change in the male as reported in broiler fowl by Ansah et al. (1980) which influences the overall fertility observed. In stored semen an increasing proportion of the spermatozoa degenerates depending upon duration and conditions of storage. Thus a greater absolute number of spermatozoa may need to be inseminated to compensate for a progressive loss of spermatozoa if diluted and stored semen is used in a breeding programme. Van Wambeke (1984) used about 200×10^6 spermatozoa with semen stored for 24 hr at 5°C.

b) The deposition of semen and timing of insemination

The distal portion of the vagina needs to be well everted before semen is inserted into the oviduct to deposit spermatozoa as near as possible to the sperm storage tubules in the proximal vagina. The procedure of insemination is well described by Lake and Stewart (1978).

The timing of insemination is important to achieve high fertility and it is generally recommended to take place when no hard-shelled egg is likely to be present in the uterus, or at least within three hours of oviposition (Bornstein et al., 1960; Tanaka and Okano, 1971; Parker and Arscott, 1971; Lee, 1970; Giesen et al., 1980; Christensen and Johnston, 1975, 1977, 1978). The proper insertion of the semen may be impeded when a hard-shelled egg is present in the uterus and some spermatozoa may be washed out of the vagina if an egg is oviposited soon after insemination before reaching the storage tubules.

Semen may be regurgitated from the vagina if hens are treated roughly during capture or release after insemination. Macpherson et al. (1977) showed a lowered fertility in hens which were thrown down on to a litter floor after insemination. Meyer et al. (1980) suggested that it is possible that stress of any kind may interfere with the transport of spermatozoa in the oviduct with a consequent effect on fertilisation rate. However, the mechanism has not been explained.

2.2.3 Aspects of the reproductive physiology of the female governing the activity of spermatozoa in the oviduct

Fertility levels after insemination may be influenced by the effect of oviduct environment on the transport of spermatozoa and the retention of their fertilising ability. In this respect, Lake (1969) reviewed work which suggested that hens laying eggs most intensively laid fewer infertile eggs and had a consistently greater duration of fertility than poor layers. This phenomenon may be associated with hormone status in particular females.

Extracts from the fowl oviduct influence the activity of the spermatozoa (Howarth and Huston, 1974; Vo et al., 1980; Bakst and Pierson, 1981; Lake, 1971) and various studies indicate that bird age, season, management conditions and type of bird in turn condition the oviduct effect.

Ogasawara and Fuqua (1972), Christensen (1981) and Sexton (1977a) observed in the turkey that fewer uterovaginal tubules were filled with spermatozoa late in the breeding season. Using a fixed dosage of semen and failing to pay attention to the possible deterioration of the quality of semen as the breeding period advances is an additional cause of decline in fertility as mentioned in the previous section. Cooper (1955) and Nishiyama and Fujishima (1967) did not find a decline in fertility over an entire year in laying hens when inseminating a correct number of spermatozoa frequently and effectively. Yu and Burke (1979) could improve the fertility in naturally sub-fertile turkeys by intramagnal insemination. This could indicate that a function of the lower region of the oviduct was responsible for poor fertility in this case. Aged spermatozoa in the oviduct is probably a cause of early embryonic death (Fiser and Chambers, 1981). This may be a function of spermatozoal change per se or an interaction with oviduct cells with time.

Controversial opinion has been expressed that the fertility level in the fowl and turkey might be affected in certain circumstances by hens generating antibodies against spermatozoa (Choi, 1976; Burke and Yu, 1979; Yu and Burke, 1979). The work of Cooper (1955) and Nishiyama and Fujishima (1967) showing a constant high fertility level throughout a season when maximal usage of AI was practised would not support such a hypothesis. Lake (1967, 1969) commented on the phenomenon at length and suggested further work was needed on the significance of this important topic.

2.3 EVALUATION OF SEMEN

The form of apparatus for fowl semen collection varies considerably but basically it falls into the funnel (or cup) type and the aspirator type. To evaluate spermatozoa and achieve good results in fertility, the collecting apparatus must be dry, clean and free from detergents. The glassware needs to be thoroughly washed several times in distilled/deionised water to make it free from detergent. The following semen characteristics have been used for evaluation of semen and the limitations and usefulness of each are described.

a) Assessment of semen appearance and colour

The semen of poultry is commonly evaluated on the farm by the spot visual examination of specimens. Thick, creamy-white semen is considered to have good fertilising ability whereas watery or clear semen, or yellowish semen, is considered as poor. MacDaniel and Craig (1959) have examined the relationships between appearance scores and fertilising ability. The method has been used for a long time but it is a subjective method and it has limitations. It has been observed on many occasions that white thick creamy semen can have poor fertilising ability. However, appearance can help in avoiding use of contaminated semen with faeces, urate and blood.

b) Estimation of the volume of ejaculate

Semen volume varies between breeds, between males, and with age and season (Clark and Sarakoon, 1967). The method of collection will markedly influence volume as described previously. In turn this can influence spermatozoal density if transparent fluid contributes to the volume. Lake (1957) developed a technique to collect semen without transparent fluid. Often semen is ejaculated in jets from the papillae of the ducti deferentia which may miss being collected totally, thereby creating the chance of an error in measuring the available volume of semen. In general, it is observed that the higher the volume the lower is the spermatozoal concentration and vice versa. Several reports of the range in semen volume and spermatozoal concentration have appeared in the literature. The volume of semen in the fowl can vary between 0.15 to 1 ml. It is desirable to have a male producing a high volume of semen with a high density of spermatozoa to cover the greatest number of females effectively. However, for the reasons given above, semen volume per se cannot be considered an objective indication of the fertilising ability of the male.

c) Measurement of hydrogen ion concentration (pH) of semen

Initial pH of semen was found to give a high correlation with fertilising capacity of bull semen (Erb et al., 1952). Cooper and Rowell (1957) found a significant correlation of pH with fertility using pooled fowl semen and samples from males of low and high fertilising capacity. However, the result was not confirmed when using individual male samples (Cooper and Rowell, 1958). It was reported that there is a significant correlation between change in pH (over 5 minutes in a poultry house at an ambient temperature of 20 to 28°C) and motility of spermatozoa in semen from White Rock males (Snapir and Perek, 1964). Wilcox (1958) observed a natural marked fall in pH in semen during the first hr of storage, but there was no further change from 4 to 24 hr of storage. This was most marked in semen samples held at 40°C and least in samples held at 10°C. This result provided a possible explanation for the better fertility obtained when semen was held at 10°C rather than at a higher temperature.

Various workers have reported a wide range of pH values of fresh fowl semen samples ranging from 6.25 to 9.00 (Bogdonoff and Shaffner, 1954; Cooper and Rowell, 1958; Dube et al., 1977; Lambert and McKenzie, 1940; Lardy and Phillips, 1943; Parker et al., 1942; Wales and White, 1958; Wheeler and Andrews, 1943; Wilcox and Shaffner, 1957). The very variable and wide ranging pH values reported in the literature leads to controversy over the correlation of pH with fertilising ability on examination of individual semen samples. Perhaps it is not surprising that pH measurement has not become a popular method of evaluating fertilising ability. In practice, a measure of true pH is difficult to obtain because it can be influenced by many uncontrollable factors, e.g. ambient temperature of the testing environment and time after collection (Wilcox and Shaffner, 1957; Wilcox, 1958; Snapir and Perek, 1964).

d) Estimation of the motility of the spermatozoa

Motility is a questionable characteristic to predict fertilising ability of a semen sample under all circumstances. Initial motility of spermatozoa in a fresh semen sample has been found to give a reasonably high correlation with fertility (Shaffner and Andrews, 1948; Cooper and Rowell, 1958; McDaniel and Craig, 1962; Soller et al., 1965a). It has been observed (personal observation) that in diluted semen after standing at room temperature (15°C) for 20 minutes the motility improved considerably if it had been stored previously at 38°C, whereas the motility was slow if observed at 38–40°C.

McDaniel and Craig (1962) believed motility to be the best single criterion to predict fertilising ability. However, in experiments on spermatozoal storage it has been observed that motility does not always predict the precise ability of the spermatozoa to fertilise eggs. Stored frozen spermatozoa regain their motility on thawing but generally the semen has substantially reduced capacity to fertilise eggs (Polge et al., 1949). Morgan (1969) showed that irradiated spermatozoa under certain circumstances did not lose their motility but they lacked the capacity to fertilise eggs. McCartney (1956) and Wall and Boone (1973) did not find a significant correlation of spermatozoal motility with fertility but Cooper and Rowell (1957) found that a bivariate analysis using motility and dye-reduction time (a measure of spermatozoal metabolism) significantly explained much of the variation in fertility. A positive correlation between motility and fertility has been reported by many investigators (Parker et al., 1942; Allen and Champion, 1955; Kamar, 1960; McDaniel and Craig, 1962; Soller et al., 1965b, Boone, 1968; Kammerer et al., 1972; Monsi et al., 1975). Motility has been shown to be negatively and significantly correlated with dye-reduction time (McDaniel and Craig, 1962; Marini and Goodman, 1969) and percent dead and abnormal spermatozoa (Marini and Goodman, 1969). Kammerer et al. (1972) found that the number of progressively motile spermatozoa per ejaculate was the most consistent and significant single semen trait correlated with fertility. They concluded that it could be used to identify potentially low fertile males for removal from the breeding flock as well as to indicate males with high fertilising potential.

Evaluation of initial motility is done using a score system, using a scale of numbers. This method has limitations, in that evaluation must be carried out by experienced persons, otherwise scoring can be very subjective and misleading. Bilgili et al. (1985b) made a similar comment with regard to evaluating spermatozoal morphology. Moreover, this test has to be done very quickly after semen collection, otherwise often it dries under the cover slip and a zero motility can then be given even to a potentially good sample.

Wishart and Ross (1985) developed for the first time an objective method of estimating the motility of fowl and turkey spermatozoa by passing them through a flow cell in a spectrophotometer and utilising their rheotactic and light-scattering properties as a measure of viability. The method is highly correlated with the production of fertile eggs (Wishart and Palmer, 1986b). From a recorded trace of the optical density of diluted semen before and after stopping the passage of spermatozoa through a flow cuvette measures of the density of spermatozoa, the percentage of motile spermatozoa and a reflection of the vigour of the motility of spermatozoa are determined simultaneously.

e) Measurement of the density of spermatozoa

The concentration of spermatozoa can be estimated with a haemocytometer (Sampson and Warren, 1939), but this is time-consuming and tedious. The optical density created by a mass of cells has been used as a measure (Carson et al., 1955; Kosin and Wheeler, 1956) but when using this method it is essential that the semen sample should be free of accessory fluids, urates and other contaminations as this may lead to an error in the estimate of the concentration of spermatozoa (Kosin and Wheeler, 1956; McCartney and Brown, 1959; Quicke and De Meulenaere, 1958). Taneja and Gowe (1961) found a highly significant correlation of +0.96 and a repeatability estimate of 0.97 between optical density and haemocytometer counts, and reported a spermatozoal concentration of $6.1 \times 10^6/\mu l$ for good quality fowl semen which is similar to estimates by Lake (1957) on samples free of contaminants. An electronic counter has been used to determine the density of bull semen (Glover and Phipps, 1962), and Jones and Wilson (1967) reported how efficient it was for estimating the density of spermatozoa in fowl semen.

Recently Wishart and Ross (1985) developed a technique to measure the spermatozoal concentration whilst simultaneously measuring the motility activity of the spermatozoa. The optical density (OD) of a sample of semen when flowing through a light path is measured. A good correlation (0.82) was found between OD and spermatozoal number actually checked and calculated with a haemocytometer.

f) <u>Measurement of packed cell volume (spermatocrit)</u>

Shaffner and Andrews (1943) and Arscott and Kuhns (1969) estimated spermatozoal density using a micro-haematocrit centrifuge spinning at 10,000 RPM for 15 minutes. A correlation of packed cell volume with optical density of semen of 0.92 was obtained and a repeatability of 0.92 by Taneja and Gowe (1961).

Brillard and McDaniel (1985) studied four methods to estimate the concentration of spermatozoa, e.g. haemocytometer, Coulter Counter, optical density and spermatocrit and concluded that the optical density and Coulter Counter methods were the most reliable and most efficient (less time-consuming) in estimating the concentration of spermatozoa in fowls. The reliability expressed as coefficient of variation for haemocytometer, Coulter Counter, optical density and spermatocrit were 17.9%, 1.57%, 2.24% and 9.95%, respectively.

g) Determination of the proportion of live and dead spermatozoa in semen

The percentage of live spermatozoa revealed by staining a smear of semen and observing with a microscope is an indication of the viability of spermatozoa and their fertilising ability. They are assessed and quantified microscopically. The so-called live spermatozoa are those which remain unstained following the mixing of semen with a stain for a given period of time. A number of stains have been suggested for use, nigrosin-eosin (Cooper and Rowell, 1957, 1958; El Jack and Lake, 1966) and bromphenol blue-nigrosin (Kamar, 1959). Chatterjee et al. (1967) compared nigrosin-eosin, bromphenol blue-nigrosin and congo red-nigrosin and found nigrosin-eosin was the most satisfactory with turkey semen. A difficulty met in selecting stains is in obtaining sufficient contrast between the stained and unstained spermatozoa and the intensity of the background stain. A further difficulty with live-dead staining is that the stain solvents can influence results as observed by Cooper (1969).

Haye (1984) suggested a stain of eosin scarlet and aniline blue and the result is quite satisfactory to the eye and comparable to nigrosin-eosin.

A reduction in fertility has been reported with an increase in the percentage of dead spermatozoa but 'dead sperm' alone is not always the cause of the reduced fertility. An increasing number of abnormally-shaped spermatozoa also contributes to reduced fertility, so the proportion of dead and abnormal spermatozoa in a semen sample will be a better estimate of semen viability than taking live (unstained) spermatozoa counts alone.

h) Measurement of the proportion of morphologically-abnormal spermatozoa in semen

Various morphological abnormalities have been observed in fowl spermatozoa. Lake and Stewart (1978) described several abnormal forms. Kamar and Badreldin (1959) reported that tail abnormalities accounted for 25% of 30% total abnormal spermatozoa. Saeki (1960) reported crooked neck (CN) forms. Bent, coiled heads and bulbs on the head are other abnormal types. These increase on storage of spermatozoa. The proportion of normal, after deducting the dead and various classified abnormal, are a guideline for a good semen quality.

In certain experimental circumstances, samples of semen with a high percentage of morphologically-normal spermatozoa fail to produce acceptable fertility, e.g. irradiated spermatozoa (Wishart and Dick, 1985) and spermatozoa treated with some specific enzymes like neuraminidase (Lake and Ravie, 1988). However, in most field conditions a sample of semen showing 90% (and above) normal-shaped spermatozoa is generally expected to be a good sample having high fertilising ability.

i) Estimate of the mean speed of travel of spermatozoa in vitro in a semen sample

Birrenkott et al. (1977) modified a technique used in human fertility studies and presented it as a simple, inexpensive and quantitative method for fowls. Capillary tubes of 75 mm length and 1.2 mm diameter are used. The tube is filled 90% of the length with thin albumen at room temperature. The semen sample, diluted 10-fold with thin albumen, is introduced and the other end of the tube was sealed with clay. The tubes were incubated for 45 minutes in a horizontal position. The distance which the foremost spermatozoa moved was recorded under a microscope and an estimate of the movement of spermatozoa in relation to time, μ/minute, was made. It was concluded that a group of males whose spermatozoa travelled at 37 to 48 μ/minute gave fertility percentages of 82% and above. This method does not take into account the number of spermatozoa in the original semen sample and so it is not an ideal objective method.

j) Fluorometric determination of avian sperm viability and concentration

Fluorometric analysis of avian semen quality involves the quantification of spermatozoal membrane permeability to ethidium bromide (Bilgili and Renden, 1984). In this assay, sperm concentration and viability were simultaneously recorded by measuring the fluorescence of ethidium bromide which is bound to nucleic acid. Bilgili et al. (1985a) obtained a high, positive correlation between the eosin and nigrosin staining technique (morphology test) and the fluorometric assay for estimating the percentage dead spermatozoa which was artificially created by mixing several concentrations of frozen-thawed dead and freshly collected spermatozoa. A similar high, positive correlation was also achieved between dead spermatozoa and glutamic oxaloacetic transaminase (GOT) activity in seminal plasma. But, the correlation between flurometry and fertility was not reported. However, Bilgili et al. (1987) commented, "To date, no subjective or objective laboratory assay for semen quality, including fluorometry has been proven to accurately predict fertilising potential of spermatozoa".

k) Measurement of the metabolic activity of spermatozoa

The time taken for a mixture of semen with methylene blue or resazurin to change colour (dye-reduction time) is generally interpreted as a measure of dehydrogenase activity in the spermatozoal population which is part of the oxidative metabolism system of the cell (Beck and Salisbury, 1943). Shaffner and Andrews (1948) were the first workers to compare the reduction-time (using methylene blue) and fertility in turkeys. Cooper and Rowell (1958) using resazurin, by a modification of the method of Erb et al. (1952), with first and second year Rhode Island Red (RIR) males and De Silva (1963), with inbred White Leghorn males, obtained highly significant correlations between reduction-time and fertility. McDaniel and Craig (1962) were not able to get a high correlation using a White Leghorn strain though they did succeed with RIRs.

Bogdonoff and Shaffner (1954) showed that reduction-time was significantly affected by pH. It is common in performing dye-reduction tests to add liquid paraffin on the top of the solution to eliminate the entry of atmospheric oxygen as the reaction continues. But using an oxygen electrode it has been shown that liquid paraffin itself contains enough oxygen to aid the dye-reduction process for some time. This may explain why although the dye reduction test has been developed for more than 40 years, it has not been widely adopted in practice because it can contribute to inconsistent results reported from time to time.

However, basically the dye reduction test should be a very effective and potentially simple method to evaluate the metabolic activity of the spermatozoa which should be correlated with their fertilising ability. It is well worth perfecting as an objective, simply-performed test of the quality of a unit of spermatozoa in a semen sample.

To summarise, all the above mentioned semen evaluation tests are intended to identify sires giving semen with spermatozoa of good morphology, high motility and optimum metabolic activity as an indicator of good viability liable to produce optimum fertility. However, all of the tests have certain limitations because most are subjective and qualitative. There is still a need to develop an objective simple test which can take into account the number as well as quality of normal viable spermatozoa and predict absolutely the fertilising ability. The test must be quick, efficient and have high precision. At the same time it should be economical and have wide applicability under field conditions so that males can be ranked on a quantitative scale.

The most final and reliable test for measuring the fertilising capacity of the actual spermatozoa and the potential fertility of the male is the magnitude and duration of fertility following a single insemination of his semen (Shaffner and Andrews, 1948; Gowe and Hutt, 1949; Williams and McGibbon, 1956). This is the so-called "Fertility Trial". In so doing, one has to accept a possible female factor in the overall fertility and it is suggested that to test a single male's likely contribution his semen should be used to inseminate a specified number of females thereby ensuring a reliable estimate is obtained. It is often unrealistic to consider testing every male on a farm with a large number of females. It would be highly desirable, therefore, to be able to screen the males using in vitro tests. Initially, this means conducting a fertility trial with a limited number of males on a desired number of females to confirm correlation of fertilising ability using a fixed number of spermatozoa.

2.4 SEMEN DILUTION AND PRESERVATION

Undiluted semen will deteriorate within 30 to 45 minutes of collection, or sooner depending upon the initial quality. Semen must be diluted and kept under particular conditions if the fertilising ability of the spermatozoa is to be preserved for longer periods *in vitro* before insemination. The objectives of semen preservation are varied. Most are concerned with aiding in genetic improvement plans where the semen of the same male can be used over several birds distributed between several locations. In these circumstances there is a need for methods of short-term storage up to about 48 hr. Interests in the long-term storage of avian semen have been gaining momentum; and the application of cryopreservation is now available to breeders and scientists. The following are a few cases where cryopreservation might be applied: progeny testing, measuring genetic progress at intervals of 3 to 4 years, maintaining control populations, test-crossing and backcrossing, conserving desirable genes (reference strains and inbred stock) by primary breeders, preserve genes to be used in the future (sex-linked dwarf or sex-linked feathering), insurance against losing elite sires due to disease outbreak, wild life conservation projects for loss of old breeds (Lake, 1986).

Short-term storage of semen at 5°C

In general, several common physiological saline solutions suffice for diluting semen that is to be inseminated within about 15 minutes. Good fertility can be obtained with uncontaminated fowl semen stored above 0°C for up to 24 hr (Lake and Ravie, 1979; Van Wambeke, 1967, 1972). Fertility was depressed when semen was stored for 48 hr (Lake and Ravie, 1981) using the same absolute number of spermatozoa as for the fresh semen. The same authors (Lake and Ravie, 1983) reported the successful storage of fowl semen for 72 hr at 5°C and obtained 82.7% fertility which is highly promising for a short-term storage period. Under the particular conditions used the semen was found to store best in diluents containing glucose rather than without it.

Storage of semen at 37°/40°C.

Wishart (1984) held fowl semen at 37°/40°C *in vitro* for up to 6 hr for physiological and biochemical studies of spermatozoal activity. With selected males with spermatozoa producing lipid peroxides in small amounts, he obtained fertility similar to fresh semen after keeping diluted fowl semen in an agitated Erlenmeyer flask in a water bath at 37°C and inseminating a large number of spermatozoa. Previously, it was found that rooster spermatozoa lost their fertilising ability within 7 hr when preserved in static tubes *in vitro* at temperatures above 30°C (Garren and Shaffner, 1952; Schindler *et al*., 1955). Fujihara and Howarth (1980) reported that rooster spermatozoa, when incubated at 41°C with fluid removed from tissue-cultured oviducal cells, maintained their motility for 4 to 5 days when assessed at room temperature. Fertilising ability was retained to some extent for one week after culture for two days at 41°C. This result was thought to indicate that the fluid removed from the tissue culture cells contains a product(s) capable of prolonging *in vitro* the motility and fertilising ability of spermatozoa. Ashizawa and Nishiyama (1977, 1983) were able to culture spermatozoa with shell gland epithelia in a diffusion chamber for 72 hr at 41°C in the presence of 95% O_2:5% CO_2 and after insemination obtained fertility of 76% from days 2 to days 8 after insemination.

Long-term storage of semen (cryopreservation)

An unsuccessful attempt to freeze semen in liquid air was made in 1939 by N.M. Nelson of Ohio State University (quoted by Shaffner et al., 1941). Shaffner et al. (1941) were the first to freeze fowl spermatozoa in the presence of fructose using solid carbon dioxide-alcohol mixture but the spermatozoa regained only limited powers of motility on thawing. Shaffner (1942) was able to obtain a few fertile eggs with frozen-thawed semen but the embryos did not survive beyond 15 hr of development. In the late 1950s and early 1960s, Polge et al. (1949) and Polge (1951) made extended investigations and obtained several live fowl chicks for the first time with frozen-thawed semen by using glycerol as a cryoprotective agent. But, the presence of glycerol in the inseminate had a contraceptive effect. Polge (1951) dialysed thawed fowl semen to reduce the concentration of glycerol and improved the fertilising ability of the spermatozoa. Shaffner (1964) devised a system of dilution and centrifugation for reducing the glycerol concentration gradually after thawing semen in order to minimise cell membrane damage. Lake (1967) commented that AI with frozen semen could be a commercial proposition.

Compared with cattle, the revival of a much higher proportion of spermatozoa is needed in fowl semen after freezing to give acceptable fertility for at least 10 days after a single insemination intravaginally. In cattle, one offspring per insemination into the uterus is considered wholly satisfactory. Lake et al. (1981) developed a method of cryopreservation that showed that the use of frozen semen under a particular regime is a feasible commercial proposition under practical farm conditions. In this case, the semen of individual males was frozen and stored, thawed and inseminated and achieved over 90% fertile eggs with a 90% hatchability. More than an adequate number of female chicks required per family was obtained to satisfy a particular practical breeding demand.

Simultaneously, Lake et al. (1981) and Sexton (1981) indicated that there is still need to improve the method because the absolute number of spermatozoa that needs to be inseminated after thawing intravaginally to produce a desired fertility is much higher than when fresh diluted semen is used (500 to 700 x 10^6 vs. 50 to 100 x 10^6). Also a single insemination of cryopreserved semen gives an overall lowered fertility (Lake and Stewart, 1978; Lada-Gorzowska et al., 1978; Sexton, 1978; Ravie and Lake, 1984) than with multiple inseminations (Lake et al., 1981).

Effect of cryopreservation on the fertilising capacity of spermatozoa

The average motility of fowl spermatozoa in a semen sample after freezing and thawing is reduced by 30-60% compared with a fresh sample when measured soon after thawing (Watanabe, 1967; Westfall and Harris, 1975; Bakst and Sexton, 1979; Scott et al., 1980). Other light microscope tests revealed 32 to 50% reduction in spermatozoa retaining normal gross morphology (Lake, 1968; Wishart and Palmer, 1986a). An electron microscope study estimated that 60% of fowl spermatozoa showed irreversible damage after thawing (Bakst and Sexton, 1979). It would be expected on the basis of these observations that sufficient good spermatozoa should be available in a thawed sample to produce fertility of over 90% on insemination with a single insemination of about 700 x 10^6 spermatozoa. However, Wishart (1985) observed recently that the fertility of cryopreserved semen is reduced to 1.6% of that of fresh semen with the same given fixed dose of spermatozoa. The result agreed with other observations (Wishart and Palmer, 1986a) which showed very low metabolic activity in samples of thawed spermatozoa and only a small number of intact cells by in vitro tests.

Lake (1986) in a detailed review suggested there was a need and much scope for future work on cryopreservation of avian semen. Lake and Ravie (1986) used a new cryoprotective agent, dimethylacetamide, and produced similar levels of fertility as those of deglycerolised semen. The advantage of the new agent is that insemination can be done in the presence of dimethylacetamide. However, further work with this new agent is needed before it becomes a commercial proposition.

The work reviewed above shows the available general knowledge relating to poultry semen studies and fertility relevant to exploiting AI as a means of breeding. In sections of this thesis, further work will be discussed in relation to the chosen specific topics of study, i.e. developing a new method of evaluating the semen of sires, investigating certain factors determining the fertilising ability of spermatozoa manipulated in vitro and storing semen at high ambient temperature.

3. MATERIALS AND GENERAL PROCEDURES

3. MATERIALS AND GENERAL PROCEDURES

3.1 BIRDS

3.1.1 Males

The males used were of a pure Rhode Island Red strain randomly bred since 1977 when they were obtained from Ross Breeders Ltd, Newbridge, Midlothian, Scotland. Prior to that they were selected as the male line for increased egg number, weight and size. For all three generations of males in the present study, 60 were used each time from the same hatch. Those used for the years 1985-86, 1986-87 and 1987-88 were hatched on 10th December 1984, 5th December 1985 and 17th December 1986, respectively.

3.1.2 Females

The females used were of a commercial white egg laying strain obtained from Ross Breeders Ltd, Newbridge, Midlothian, Scotland. The hatch dates corresponded to those of the males in each generation. On each occasion a population of about 200 birds were obtained from a single hatch.

3.1.3 Housing and management

For each year, separate sex brooding and rearing were organised in an environment-controlled house in floor pens with *ad libitum* feeding of a recognised diet. At 16 weeks of age the birds were transferred to individual cages with 14 hr light/24 hr with *ad libitum* feeding until 20 months of age. As adults, each hen had

1350 sq cm floor space in a 3-tier battery cage system, each cage had dimensions of 45 cm length, 30 cm breadth and 45/53 cm height. The males were in a 2-tier cage system, individually caged with 2025 cm^2 floor space with dimension of 45 cm length, 45 cm breadth and 54 cm height.

3.2 MONITORING PRODUCTION AND QUALITY OF SPERMATOZOA

For all of the experiments it was essential to be acquainted with the quality of semen of each male, to guard against false treatment effects due to variation in semen quality between males. At puberty around 20 weeks of age, the males of each generation were trained for semen collection and individual samples were collected (in batches of 10) and evaluated for their spermatozoal quality. Periodically, throughout each year the males were re-tested to confirm their consistency of semen quality.

Motility test in hanging drop of semen

The motility of fresh spermatozoa was observed in a drop of semen on an inverted coverslip on a cavity slide under the low power microscope (objectives X 10 and X 40). A reasonable estimate of the liveability of spermatozoa was noted in the hanging drop by observing a combination of the vigour of the swirl of the total spermatozoa and the depth of penetration of the swirl into the centre of the drop, the proportion of spermatozoa moving and involved in the swirl (in this assessment a note was taken of the degree of stillness in the centre of the drop) and the general picture of the morphology of the mass of spermatozoa in the drop (bent spermatozoa and obvious degenerative types are observed at the edge of the drop). A sliding scale of marks was devised (0-4) to record the overall impression of the sample. The above described motility test is useful for screening out very good and very poor samples of semen within a short time at reasonable ambient temperature. A score is given which is correlated with fertilising ability provided the observer is experienced and recognises the factors which can influence spermatozoal motility in the circumstances of the hanging drop. Very high density of spermatozoa, the composition of some diluents, temperature (including local heat produced by some microscope lighting systems), and dilution effects can all inhibit general vigour of motility which might give a bad impression to an inexperienced observer.

(b) Objective motility test

Wishart and Ross (1985) devised an objective method of estimating motility and the observations were well correlated with fertilising ability of individual semen samples (Wishart and Palmer, 1986b). A 200-fold diluted semen sample is passed through a 1 cm light path flow cell stabilised at 30°C in a spectrophotometer set to read absorbance at 550 nm. The flow is allowed for 30 seconds at the rate of 6 ml per minute when the pump was stopped. From a 4 minute recorded trace of the changes in optical density of the diluted semen before and after stopping the passage of spermatozoa (Fig. 3.1), three independent constants are determined: Maximum

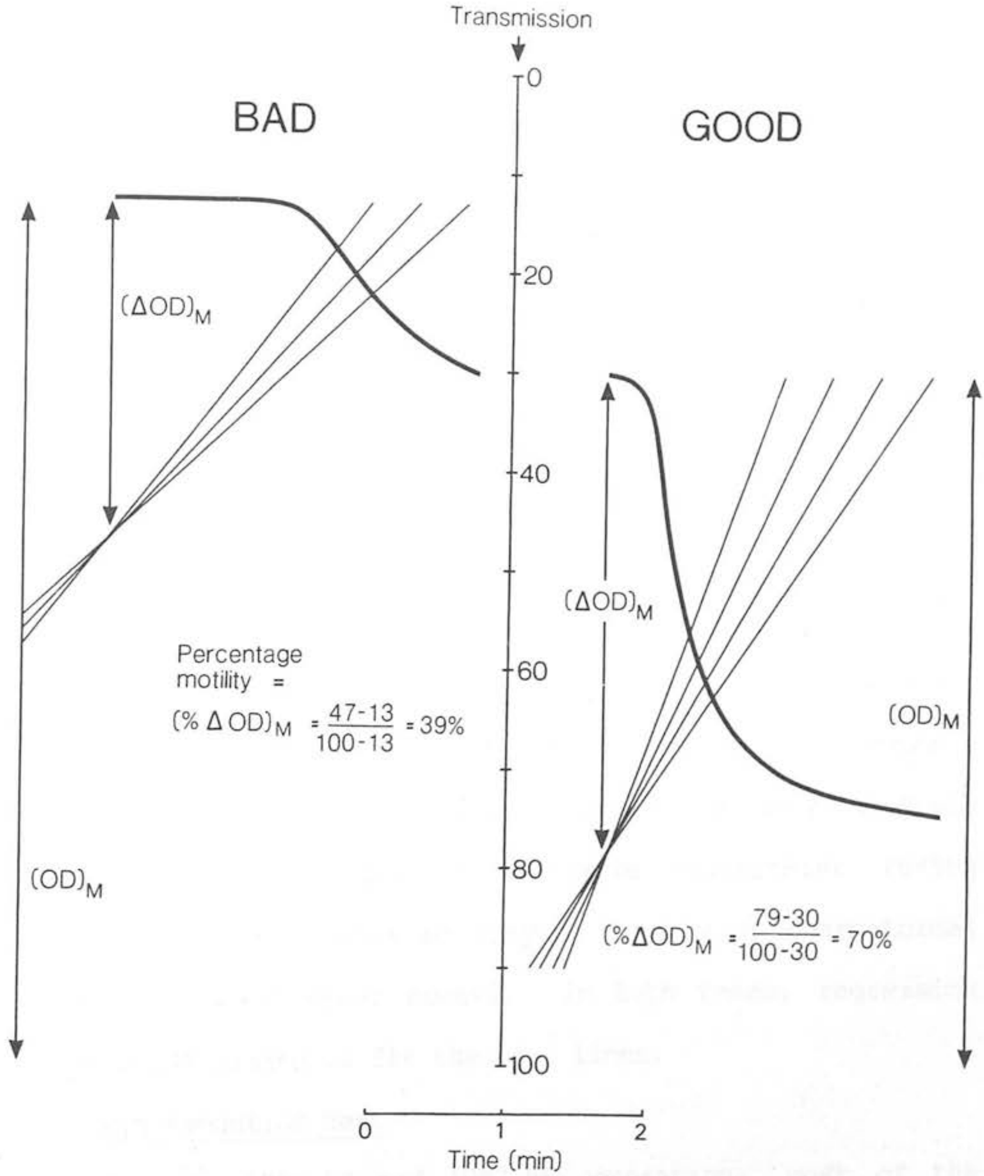

Fig.3.1 A sample spectrophotometer trace of motility from good and bad semen samples.

optical density of the semen flowing through the cell, $(OD)_M$, which is a measure of the initial density of spermatozoa in the semen sample; the change of optical density in relation to time ($t^{\frac{1}{2}}$), which is a measure of the rate of movement of the spermatozoa; and $(\Delta OD)_M$ which is a measure of the proportion of spermatozoa actually motile.

The constant, $\%(\Delta OD)_M$, which is derived from the ratio $(\Delta OD)_M/OD_M$ gives a useful measure of percentage motile sperm independent of the concentration of sperm in the sample.

(c) Measurement of spermatozoal concentration

Spermatozoal concentration was determined either during the previously described objective motility test after correlating the optical density readings with actual spermatozoal number, determined previously with the haemocytometer, or from a graph recording absorbance readings on a simple colorimeter (using 100-fold diluted semen samples of varying density of spermatozoa) against actual haemocytometer counts. In both cases, regression equations were calculated to fit the best lines.

(d) Dye-Reduction Test

For the 1986-87 and 1987-88 generations, most of the birds were further evaluated using the objective colorimetric Dye-reduction Test, which reflects the integrity of the metabolic activity of spermatozoal function, and which was developed in the early part of the thesis work (page 57-91 & 211).

(e) Morphology test

The morphological integrity of the population of spermatozoa in semen samples was judged from time to time using a stained semen-smear technique using nigrosin-eosin (Lake and Stewart, 1978) or aniline blue-eosin dyes (Haye, 1984). Normal-structured spermatozoa and those with defects were counted differentially under an oil immersion objective and quantified (Lake and Stewart, 1978).

3.3 CHARACTERISATION OF MALES FOR QUALITY OF SPERMATOZOA AND CONSISTENCY OF SEMEN PRODUCTION PERFORMANCE

In each year, the semen of all the males were tested and categorised into three distinct groups according to the quality of their semen production: i) 'Good', ii) 'Medium', and iii) 'Poor'. During 1985, they were tested by the objective motility method, ATP content (page 54) and the morphology method. In 1986 and 1987 the Dye-reduction Test, which was developed as part of the early thesis work, was employed together with the morphology method. Sample data for Good, Medium and Poor categories of males are presented in Table 3.1.

The males of each category were monitored in 1986 and 1987 once every three months to ensure that their inherent spermatozoal quality had not changed. It is important to mention that when the males aged, some of them had lowered spermatozoal concentrations, but no drastic change in morphology or metabolic quality was observed, indicating that the spermatozoa were themselves unchanged.

3.4 MONITORING FEMALE FERTILITY

Individual, daily, egg-laying records were maintained for all the birds. Before using the hens in fertility trials it was ensured that the birds were not inseminated for at least 3-4 weeks beforehand to guard against residual fertility. Only hens laying more than 5 good-shelled eggs per week were used from the population at any time and no hens laying soft-shelled eggs within 2 weeks of a trial were used. The hens were allotted randomly to the treatments. Eggs were collected for up to 2-3 weeks from the second day of the insemination and stored in a cold room at 10°C. The eggs were set in an automatic incubator every week ensuring that no egg was held in the cold room for more than 7 days. Evidence of egg fertilisation was ascertained by candling between days 4 and 7 after the start of incubation when developing early embryos were visible. The white eggs made inspection easy. Any cracked eggs or eggs showing doubtful embryonic development on incubation were confirmed by break-out and inspection of the blastoderm for evidence of fertilisation (Kosin, 1944).

The incubation was always terminated within a week. In general, no hatchability data were required because the main objectives of the present study were to investigate the fertilising ability of the spermatozoa. Moreover, to carry out meaningful

Table 3.1. The quality of semen samples of males used for the 1985 generation

Category of male		Sperm Conc. ($x10^6$/c.mm.)	ATP (nmol/10^9 sperm)	% Motility by objective test	Morphology (% normal sperm)	Formazan* produced (nmol/min/ 10^9 sperm)
Good	1	3.7	74.67	78.3	92	17.54
	2	4.5	53.32	66.5	91	16.90
	3	3.4	77.48	66.9	88	16.36
	4	5.2	27.23	49.4	87	14.88
	5	4.9	49.92	42.8	81	14.50
	6	4.6	87.03	72.5	95	15.28
	7	3.3	90.52	74.6	94	15.46
	8	3.8	77.12	59.6	89	16.48
	9	2.6	77.97	61.2	90	15.43
	10	2.4	69.62	73.0	87	16.95
	11	3.0	63.91	70.4	85	17.48
	Mean±SE	3.8±0.3	68.07±5.6	65.0±3.3	89±1	16.11±0.32
Bad	1	2.5	11.68	1.0	17	8.39
	2	3.0	9.78	1.0	21	6.10
	3	2.6	10.26	20.8	38	7.70
	4	3.0	18.97	18.5	58	9.75
	5	3.3	28.06	19.5	52	9.62
	6	2.9	23.17	19.4	54	10.51
	Mean±SE	2.8±0.1	16.99±3.1	13.4±3.9	40±7	8.68±0.66

Table 3.1. (continued)

Category of male		Sperm Conc. ($x10^6$/c.mm.)	ATP (nmol/10^9 sperm)	% Motility by objective test	Morphology (% normal sperm)	Formazan* produced (nmol/min 10^9 sperm)
Medium	1	3.2	46.56	42.6	58	12.7
	2	4.3	46.55	33.7	73	11.8
	3	3.2	55.35	58.6	70	15.2
	4	2.9	53.14	41.3	66	13.5
	5	3.5	32.99	32.2	65	14.6
	6	2.7	40.83	44.0	66	13.9
	Mean±SE	3.30±0.2	45.9±3.3	42.1±3.9	66±2	13.62±0.51

* Dye test was conducted at 40°C for 20 minutes.

Table 3.1. (continued) The performance of males used for the 1986 generation tested at 3 different periods (Aug. '86, Dec. '86, April '87)

Category of males		Sperm Concentration ($x10^6$/c.mm. of semen)			Formazan produced (nmol/min/10^9 sperm)		
Medium to good	1	4.5	5.7	4.5	14.35*	5.06**	4.16**
	2	5.1	5.1	4.4	15.56	3.85	4.44
	3	4.4	5.1	5.3	15.78	10.08	5.10
	4	4.2	4.1	5.7	15.41	11.07	5.39
	5	3.8	3.8	5.1	12.89	9.98	4.66
	6	4.8	3.8	3.3	14.76	10.33	8.59
	7	4.4	4.6	2.1	13.09	11.02	6.51
	8	5.3	6.3	3.0	14.42	8.92	9.33
	9	4.4	4.1	4.8	12.07	10.02	7.02
	10	5.1	6.3	4.3	11.97	8.55	8.62
	11	4.8	5.1	3.8	12.07	11.14	11.33
	12	4.4	4.8	5.1	12.05	9.39	5.67
	13	3.8	3.8	5.4	13.5	12.48	10.22
	14	5.4	5.1	5.7	11.88	10.08	8.99
	Mean±SE	4.6±0.14	4.84±0.23	4.46±0.29	13.56±0.39	9.43±0.62	7.15±0.63

Note: No bad males were available in this generation
* Dye test was conducted at 40°C for 20 minutes.
** Dye test was conducted at 20°C for 20 minutes.
By morphology test, on all the three occasions, 80% and above normal spermatozoa were produced by the males.

Table 3.1. (continued) The quality of semen samples of males used for 1987 generation tested over 3 different periods (Aug. '87, Dec. '87 & March '88)

Category of males		Sperm Concentration ($x10^6$/c. mm of semen)			Formazan produced (nmol/min/10^9 sperm)			Morphology (% normal)		
Good:	1	4.4	4.6	3.8	10.17	8.5	7.02	80	91	85
	2	4.2	4.6	3.8	6.82	7.3	7.54	82	93	91
	3	2.4	3.8	3.5	8.70	6.51	6.15	82	78	89
	4	4.6	5.75	5.5	7.49	6.90	6.50	81	86	94
	5	4.4	4.6	3.4	6.80	7.62	8.23	83	94	87
	6	4.6	4.2	4.05	7.62	9.21	6.75	92	96	84
	7	4.2	5.5	4.4	7.43	6.61	6.65	81	84	82
	8	5.5	5.25	5.25	7.20	6.92	6.44	84	80	85
	9	3.4	5.5	4.60	7.85	7.66	8.61	82	80	90
	10	4.2	3.40	3.15	6.82	6.52	8.06	78	74	75
	11	4.2	3.95	4.8	7.89	6.92	6.10	80	90	91
	12	4.2	5.0	5.0	7.89	5.89	5.60	82	82	87
	13	5.0	4.4	4.2	6.24	6.51	7.58	80	81	91
	Mean±SE	4.25±0.2	4.66±0.2	4.27±0.2	7.61±0.2	7.16±0.25	7.02±0.25	83±1	85±2	87±1

Table 3.1 (continued)

Category of males		Sperm Concentration ($x10^6$/c. mm of semen)			Formazan produced (nmol/min/10^9 sperm)			Morphology (% normal)		
Bad:	1	1.5	1.1	0.9	4.4	*	*	9	7	0
	2	1.0	1.0	0.7	*	*	*	0.3	1	4
	3	3.8	3.9	2.0	3.8	4.86	3.2	2.7	45	30
	4	1.0	3.4	3.9	0.8	4.62	4.53	3.0	32	36
	5	1.75	4.6	4.6	1.99	*	*	3.0	7	8
	6	0.02	0.02	0.06	*	*	*	1.0	5	2
	Mean±SE	1.51±0.5	2.34±0.7	2.12±0.7	1.85±0.08	1.58±1.0	1.29±0.08	3.17±1.3	16.2±7.3	13.3±6.3

* The formazan content is so low the colour is beyond the limit of the spectrophotometer
Note: During this year the formazan was developed by incubation of the samples at 20°C for 20 minutes.

hatchability studies, the maximum number of fertile eggs per treatment was too small to ensure against variation due to incubator conditions and other problems affecting proper incubation of eggs. It has been estimated that for standard hatchability trials each treatment needs to have at least 12000 hatching eggs per treatment (Laughlin and Lundy, 1974) which was not possible in conducting the present study.

All inseminations were conducted between 10.00 and 15.00 hr (the lighting switched on 04.00 hr and off 18.15 hr) so that no hard-shelled egg was present in the lower reproductive tract during insemination. This prevents improper spermatozoal establishment in the storage tubules of the oviduct (Christensen and Johnston, 1975, 1977, 1978). The doses of inseminates (volume and number of spermatozoa) were calculated and delivered with a positive-displacement digital pipette with disposable cannulae. They were deposited with care to a depth estimated to be near the utero-vaginal sperm-storage tubules which is known to greatly minimise the loss of spermatozoa by regurgitation.

3.5 DATA MANAGEMENT

Statistical procedure and design of experiment

The fertility data, from individual birds, calculated as the proportion of fertile eggs to total eggs laid, was based on eggs laid from days 2-8, 9-15 and 2-15 following single inseminations. Treatment groups in which not a single fertile egg was laid were excluded from further statistical analysis. Exclusion was necessary and legitimate because such data would affect the error mean square of the analysis of variance by greatly reducing the precision of the estimates. The arc sin $\sqrt{\%}$ transformation was applied to fertility percentage data to convert them to normal data. Analysis of variance, regression, correlation, mean and standard error were calculated according to a Minitab programme using a Prime P550 computer. Where a significant difference among treatment means was indicated by the F ratio in the analysis of variance the individual means were tested by 't test' for identifying the significance between two particular treatment means (it is recognised that this method has inherent dangers). The specific designs of the individual experiments adopted are explained later in each case.

3.6 CHEMICALS AND GLASSWARE

All chemical compounds used in this study were of AR reagent grade. Pyrex glassware was used throughout and any disposable pipette tips or other accessories were made of inert materials relevant to maintaining and not harming spermatozoal activities. All solutions (semen diluents) were prepared using glass-distilled water and stored at 4°C for not more than 2 weeks. Care was taken to discard stock solutions and prepare fresh supplies on signs of deterioration. Routinely, diluents were warmed to room temperature (about 20°C) before mixing with semen at the same temperature. Care was taken to rinse glassware with distilled water several times after initial cleaning processes and before drying and storage for subsequent use, as traces of detergents are harmful to spermatozoa.

3.7 STANDARD TESTS AND PROCEDURES USED DURING STUDY

3.7.1 pH Measurement

The hydrogen ion concentration of solutions as well as semen samples were recorded with a pH meter (Ionalyser/Model 399A, manufactured by "Orion Research"), standardised for temperature and reading in certain pH ranges. The probe was a combined glass and reference electrode small enough to take measurements in a 0.3 ml volume of unknown solution with accuracy to two decimal places.

3.7.2 Measurement of osmotic pressure

The osmotic pressures of solutions were measured as freezing point depression, with an "Advanced Digimatic Osmometer", model 3DII, (Advanced Instruments Inc., Massachusetts) capable of taking measurements in 0.2 ml volume of unknown solution.

3.7.3 Measurement of ATP content or synthesis in spermatozoa

ATP content was measured by comparing chart traces from individual semen samples and standard ATP solutions. The chart recorder was attached to the LKB 1250 Luminometer. A Flow Diagram for the procedure of measuring ATP is presented in Fig 3.2. The ATP content of the initial semen sample was measured as nmol of ATP/ml of neat semen and was corrected for individual spermatozoal

EXTRACTION OF LUCIFERASE

50 mg (vacuum dried) Firefly Lantern tails

+

50 mg Mg $So_4.7H_2O$

|

Grind in pestle and mortar

↓

Dissolve in 5ml (ice cold) 0.1M $Na_3 Aso_4.12H_2O$ adjusted to pH 7.4 with 1N HCl

↓

Spin (700g, 10 min, room temp)

↓

Collect supernatant

↓

Dilute 5 fold in distilled water (ice cold)

↓

Inject 800µl into Luminometer sample chamber simultaneously with 200µl of either semen extract or ATP standard

EXTRACTION OF SEMEN EXTRACT FOR ATP MEASUREMENT

Collect individual semen sample, record spermatozoal concentration from Aliquot. with other Aliquot. make 4-fold dilution in 150 mM NaCl and 20 mM TES buffer, pH 7.4 with 1N NAOH.

↓

Keep in shaking water bath at 37°C for 20 min.

↓

Inject 100µl into 300µl distilled water in glass tube in boiling water bath for 1-2 min.

↓

Cool tube to room temperature

↓

Spin (700g, 5 min, room temperature)

↓

Collect supernatant → Store in freezer (-20°C) → Thaw

↓

* Take 100 µl ← Thaw

add 100µl distilled water and inject whole into Luminometer

N.B. ATP STANDARDS :

Serial dilutions of a standard ATP stock solution (1mM) were made with distilled water in final volumes of 200µl. These were injected into the Luminometer simultaneously with the firefly extract (800µl) and different traces obtained according to the concentration of ATP. These were used to calculate the concentration of ATP in unknown sample of semen. For accurate determination of the unknown it was desirable to use for comparison ATP standard and unknown trace readings towards the middle of the chart

* If the reading on the chart recorder is off the scale the volume taken is reduced and diluted to 100µl and the ratio noted for final correction of calculations based on unit sperm number.

Fig.3.2 Flow diagram for measurement of ATP content of semen sample.

concentrations for making valid comparisons. The procedure of ATP measurement was that of Strehler (1974) adapted for use with avian spermatozoa by Wishart (1982).

3.7.4 Measurement of oxygen consumption of spermatozoa

The oxygen consumption of semen samples was measured using a Clark type electrode which was placed in 1 ml 4-fold diluted samples in a jacketed well warmed at 37°C as described by Robinson and Cooper (1970) as adapted for fowl semen samples by Wishart (1982).

4. EXPERIMENTAL

4.1 Development of a dye-reduction test to assess and predict the quality of spermatozoa

4. EXPERIMENTAL WORK

4.1 DEVELOPMENT OF A DYE-REDUCTION TEST TO ASSESS AND PREDICT THE QUALITY OF SPERMATOZOA

The development of an objective colourimetric in vitro method for assessing the metabolic activity of fowl spermatozoa

4.1.1 INTRODUCTION

Artificial insemination (AI) is the only method for the propagation of the modern turkey, and many breeders in the broiler industry today are seriously considering a more directed use of AI for stock improvement, particularly in France, Canada and the USA. The 'Grand Parent' farms in the breeding of commercial egg-laying hybrids also adopt AI for implementing their breeding programme efficiently.

It is a great advantage to be able to monitor constantly the reproductive performance of males and their fertilising ability in the practice of AI. In vitro tests of semen evaluation can help to monitor breeding performance as well as identify the potential individual breeder male in the beginning of the breeding season. However, this is not widely practised because most of the available tests are subjective and are not consistent in their performance in terms of repeatability and precision.

The objectives and advantages of semen evaluation tests are: 1) To identify the best breeding males in terms of quality and spermatozoal concentration and volume of semen before the hatching season, 2) To assess periodically the fertilising ability of the breeder males once in use to avoid fluctuations in fertility in the flock, 3) To identify the best quality of semen suitable for dilution to extend its coverage per unit of male, and 4) To aid in the determination of the suitability of samples of semen for storage and long-term preservation, if needed. Dilution and storage of semen can contribute greatly to the flexibility of the use of AI in the breeding programme for making genetic improvements as well as propagating stock.

The ideal tests for measuring the quality of poultry semen should be objective, quantitative, simple and efficient to perform and interpret, inexpensive and, of course, able to predict fertilising ability. It is very difficult to get all these criteria in a single test.

An effort was made to develop an in vitro test to fulfill some or all of these criteria. This section describes the developmental procedure and final assay technique. It is concerned with exploring the integrity of the well-known oxido-reductase enzyme pathway vital to the normal metabolism of cells (Giese, 1973; Fig. 4.3, page 59) as it might reflect the fertilising ability of fowl spermatozoon.

4.1.2 REVIEW OF LITERATURE

Leeuwenhoek (1677) postulated that there might be an association of the existence of spermatozoa in human semen and male

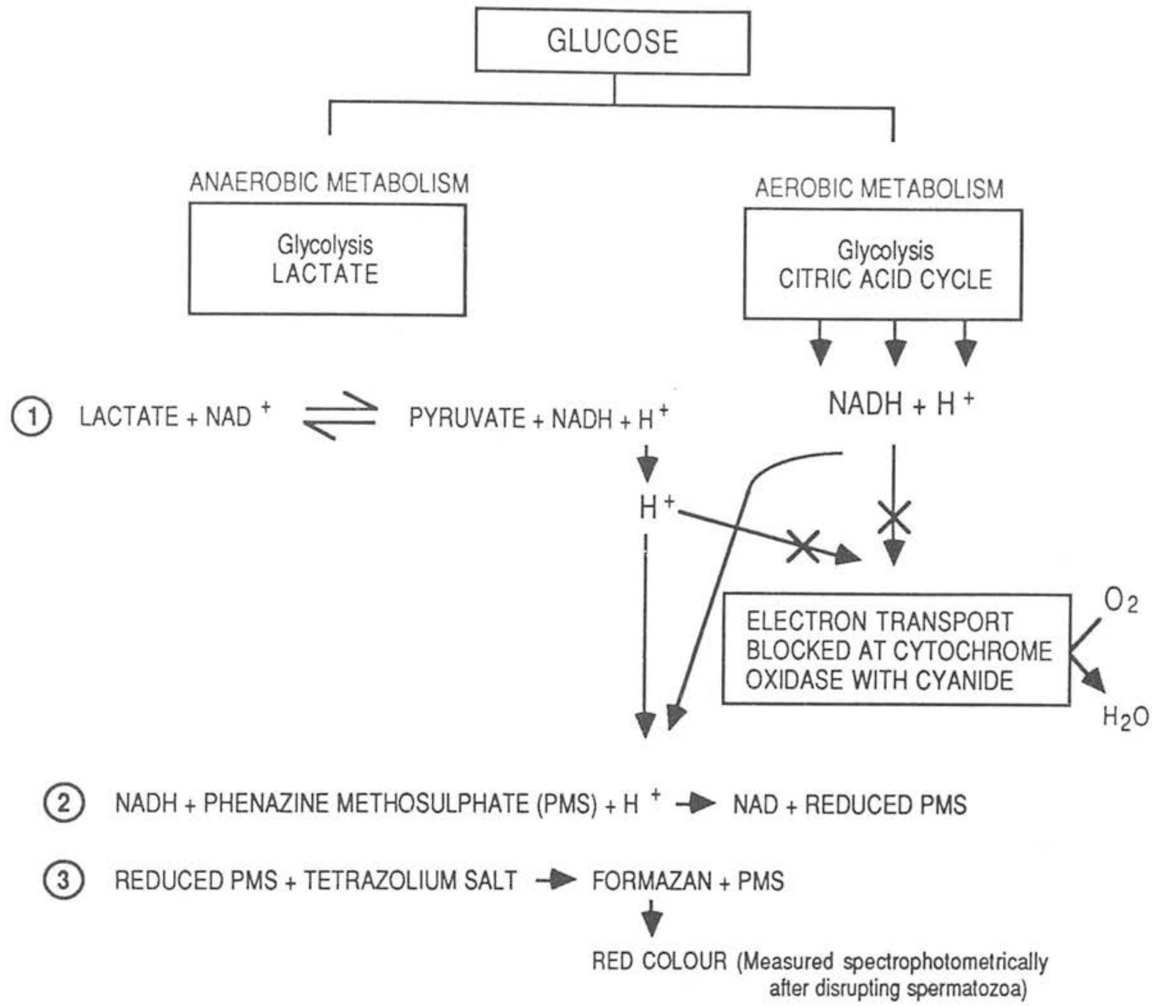

Fig.4.3 Schematic representation of the exploitation of the oxido-reductase system in spermatozoa to develop a dye-reduction test of their viability. The object was to divert the hydrogen, from the final step in the production of water from H^+ and O_2, to reduce a tetrazolium salt to a coloured formazan.

fertilising ability. He also anticipated another basic concept that spermatozoa, as well as being motile, must also be sufficiently energetic to survive in the female tract for a certain period to produce fertility.

Successful fertilisation of eggs is conditional upon the attainment by spermatozoa of certain qualitative and quantitative standards. For the proper application of AI to breeding it is essential to identify criteria applicable to ejaculated spermatozoa, which can form the basis of assessing their ability to fertilise eggs. In the avian situation, there is a fundamental difference compared with mammals. Usually with the latter, the fertilisation of one egg after an insemination is considered successful. With the bird, the fertilisation of a succession of eggs after a single insemination over a period of 1 to 3 weeks is the criterion of success. Thus, the final test for measuring the fertilising capacity of the semen of the bird and the potential fertilising capacity of a male is the magnitude and duration of fertility following a single insemination of his semen (Shaffner and Andrews, 1948; Gowe and Hutt, 1949; Williams and McGibbon, 1956). There is the possibility of females partly influencing overall fertility. In poultry breeding where large numbers of males can be used in a farm's breeding programme, to select a male on fertility potential he should ideally be used to inseminate several females for a reliable estimate to be obtained. It is unrealistic to consider testing every male on a farm in such a way. It is sensible to be able to screen each male initially using in vitro tests.

Many laboratory tests of avian spermatozoal function have been designed with the purpose of establishing a characteristic, measurable in vitro, which can absolutely reflect the fertilising ability of poultry semen (see Wilson et al., 1979; Bilgili and Renden, 1984; Stove-Schimmelpfennig and Flock, 1985; Wishart and Palmer, 1986b). Such tests have included semen appearance and colour (MacDaniel and Craig, 1959); volume of ejaculate (Clark and Sarakoon, 1967); hydrogen ion concentration (pH) of the semen (Erb et al., 1952; Cooper and Rowell, 1957, 1958); motility of the spermatozoa (Shaffner and Andrews, 1948; Cooper and Rowell, 1958; McDaniel and Craig, 1962; Soller et al., 1965b); density of spermatozoa by haemocytometer (Sampson and Warren, 1939), optical density of a diluted semen sample (Carson et al., 1955; Kosin and Wheeler, 1956), electronic particle counter (Glover and Phipps, 1962; Jones and Wilson, 1967) or in a flow cell in a spectrophotometer (Wishart and Ross, 1985); measurement of Packed Cell Volume (Shaffner and Andrews, 1943); determination of the proportion of morphologically-abnormal spermatozoa by staining a smear of semen (Cooper and Rowell, 1957, 1958; Kamar and Badreldin, 1959; Saeki, 1960; El Jack and Lake, 1966; Lake and Stewart, 1978); an estimate of the mean speed of travel of spermatozoa in thin albumen (Birrenkott et al., 1977) which is similar to the cervical mucus penetration test used for human semen (Kremer, 1965); fluorometric determination of avian sperm viability and concentration (Bilgili and Renden, 1984; Bilgili et al., 1985a,b; 1987); measurements of the metabolic activity of spermatozoa (Beck and Salisbury, 1943; Shaffner and Andrews, 1948; Erb et al., 1952; Cooper and Rowell, 1958).

Most of the abovementioned tests have been subjective, relying on the experience of the operator to judge, for example, the scoring of sperm motility in semen viewed under the microscope (see Wishart and Palmer, 1986b) or noting the time for a colour change to occur in a dye-reduction test (Shaffner and Andrews, 1948; Cooper and Rowell, 1958). It may be suggested that this subjectivity, with resultant lack of standardisation is a major reason that such tests, although available for over 30 years, have not been widely used.

Dye-reduction tests, in principle, have been shown to satisfy some of the criteria to predict fertilising ability. For example, Cooper and Rowell (1958) showed good correlation of the rate of dye-reduction, using resazurin, with fertilising ability. However, the main disadvantage is that the tests required the operator to judge, visually, when a particular colour disappears. As mentioned above, this is quite subjective and will vary between operators. Moreover, the tests have also been performed under conditions in which the spermatozoa are able to utilise oxygen and hence did not reduce the dye efficiently.

Tetrazolium salts, such as Nitro Blue Tetrazolium (NBT) and Iodonitro Tetrazolium (INT) have been used widely in both cytochemical (see Altman, 1972) and biochemical (e.g. Bergmeyer and Bernt, 1974) studies of dehydrogenase enzymes functioning in the oxido-reductase system in cells. Upon reduction they produce highly coloured formazans which can be measured by instrumentation. These salts have also been used to estimate the reducing capacity, and thus metabolic activity of whole cells, e.g. soil microorganisms (Casida *et al*., 1964) and granulocytes (Baeher and Nathan, 1968), the amount of formazan formed within the cells being measured after organic extraction. It was considered worthwhile to find out whether fowl spermatozoa showed similar behaviour in reducing the tetrazolium salt, INT, and if so whether the property of reducing INT to formazan could be utilised to develop an effective objective *in vitro* test of spermatozoal function among males reflecting their fertilising ability. The system explored is given in Fig. 4.3, page 59.

4.1.3 MATERIALS AND METHODS

Birds

The male fowl were of a Rhode Island Red control strain (for details of their management, see Materials and General Procedures, Birds, Page 40).

Semen collection and dilution

Semen was collected free of transparent fluid as described by Lake (1957). Pooled samples from 3 to 12 birds were either diluted 4-fold in 0.15 M NaCl with 20 mM TES (adjusted to pH 7.4

with 1 N NaOH) and kept at 40°C for not more than one hour in 25 ml conical flasks in a horizontal shaking water bath (200 times to and fro per minute) or used fresh within 20 minutes of collection whilst aspects of the development of the method were explored.

Procedures in the development of the assay

Spermatozoal ATP concentration was measured by the luciferase method (Strehler, 1974) in extracts of boiled semen (for details see Materials and General Procedures, Measurement of ATP content, Page 54). Oxygen utilisation by spermatozoa was measured with a Clark type electrode as described by Robinson and Cooper (1970) (for details see Materials and General Procedures, Oxygen Consumption of Spermatozoa, Page 56).

All solutions for exploring the development of the dye-reduction test were prepared from AR Grade reagents in glass distilled water (Table 4.1). They were kept at 4°C for not more than 3 weeks. INT and PMS solutions were kept in dark glass bottles for protection from direct light.

To develop the proper conditions for performing the assay, various experiments were conducted to finalise the optimum incubation time, the temperature of incubation, the volume of semen and the concentration of spermatozoa.

The optimum INT and PMS concentrations were tested. To stop the INT reduction after a fixed incubation time, various detergents were explored which would disintegrate the spermatozoa and liberate the formazan. Five biological non-ionic detergents

Table 4.1. Constituents of assay mixture and the preparation of solutions for the dye-reduction test

	Stock solution	Ingredients	Concentration
I	Chemical Solutions		
	1) Salt/TES pH 7.4	8.70 g NaCl 4.58 g TES/L Adjust to pH 7.4 with 1 N NaOH	150 mM NaCl 20 mM TES
	2) Glucose	180 mg/10 ml	100 mM
	3) Calcium chloride. $6H_2O$	0.219 g/10 ml	100 mM
	4) Potassium cyanide	0.065 g/10 ml	100 mM
	5) PMS	10 mg/100 ml	0.33 mM
	6) INT	10 mg/5 ml	4.0 mM
II	Semen		
	4-fold diluted semen	50 µl neat semen + 150 µl salt/ TES solution (1)	
	or undiluted semen	50 µl	
III	Acid Triton		
	5% Triton X-100 in 0.1 M HCl	5 ml triton X 100 in 100 ml of 0.1 M HCl	5% Solution of Acid Triton

were explored, Lubrol, Numidiol, Nonidet, Sorbitan and Triton X-100, and the latter was found most suitable and effective overcoming the viscosity, cost and handling problems. With the addition of 200 μl of 5% Triton X-100 in 0.1 M HCl to the incubation mixture, spermatozoa were seen microscopically to be completely disrupted for the release of the formazan for subsequent measurement spectrophotometrically.

A calibration curve with authentic formazan was constructed to determine the absorbance limits which could be measured accurately with the spectrophotometer. The relationship between oxygen consumption and INT reduction was investigated as oxygen consumption is considered to be a good indicator of metabolic activity of spermatozoa as well as an indicator of spermatozoal viability. In the aliquots of the same sample of semen, ATP synthesis, oxygen consumption and INT reduction in the presence of various levels of cyanide were examined to determine the optimum concentration of cyanide which could be safely used to allow maximum reduction of INT without affecting ATP synthesis (See Fig. 4.6, page 76).

It would sometimes be useful to refer formazan production to a fixed density of spermatozoa for comparison between males. In the field it would be useful to measure density of spermatozoa in an aliquot of the same sample of semen from a male. This can be a very small volume (0.05 ml) and so a calibration curve of spermatozoal concentration was explored utilising a very low semen volume (50 μl) before the addition of PMS and INT in the assay procedure (See Appendix, page 211) and using a 1 mm path-cell in the spectrophotometer.

4.1.4 RESULTS

4.1.4.1. Calibration of formazan concentration and absorbance at 520 nm (A_{520})

The colourimetric assay explored in the present study was based on that described by Bergmeyer and Bernt (1974). Oxidoreductase enzyme activity reduces tetrazolium salt, INT, to a strongly coloured formazan, which is measured spectrophotometrically at between 500 and 550 nm.

In the present work, 520 nm was adopted for exploring the best conditions of applying the assay to evaluate the functional integrity of the spermatozoal oxidoreductase activities. To determine the range of concentrations of formazan that could be measured satisfactorily by absorbance at 520 nm, 3 different samples of authentic formazan were prepared in 5% Triton X-100 in 0.1 M HCl by initially dissolving the formazan in ether, adding the triton/HCl and then removing the ether. Each sample was diluted to give 10 concentrations of between 10 and 100 nmol of formazan/ml and the absorbances measured. A linear regression between A_{520} and formazan concentration was calculated which produced a regression equation of A_{520}= 0.006 + 0.0155 nmol formazan/ml (R^2=99.9%). The molar extinction coefficient of formazan was 15.5×10^3.

4.1.4.2. Estimation of the concentration of spermatozoa in the assay

The light-scattering of 20 incubation mixtures (before the addition of PMS and INT - see Table 4.1 and Appendix) each from different semen samples with spermatozoal concentrations ranging from 1.00 to 5.50 X 10^9/ml was measured at 520 nm using a 1 mm light-path cuvette. A linear regression of the optical densities against the numbers of spermatozoa was calculated giving a regression equation of: OD=0.033 + 0.153 sperm X 10^9/ml with R^2=95.2%. An estimation of spermatozoal concentration (from a very low volume semen - 50 μl) was obtained, with high accuracy, using the equation:

$$\text{Concentration } (\times 10^9 \text{ spermatozoa/ml semen}) = \left[\frac{(OD - 0.033)}{0.153} \right]$$

4.1.4.3. The effect of varying INT and PMS concentration

During the initial stages of the development of the dye-reduction test with fowl spermatozoa, it was observed that INT was reduced very slowly, prolonging the incubation time when used alone. The method described by Bergmeyer and Bernt (1974) suggested the use of INT along with PMS which acted to accelerate the reaction in the electron transport system similar to a catalytic agent. Nachlas *et al*. (1960) suggested a colourimetric method for the measurement of lactic dehydrogenase activity in serum and tissue homogenates utilising phenazine methosulfate (PMS) as an intermediate agent for electron transfer from NADH to a tetrazolium salt (INT) (Fig. 4.3). They studied several compounds for their ability to act as an intermediate electron transfer reagent, but concluded that none was superior to PMS. It was necessary to determine the optimum combination of concentrations of INT and PMS for the present work.

The mean absorbance at 520 nm of 3 aliquots of pooled samples incubated for 20 minutes with 7.7 μM PMS and 40, 80, 160 and 320 μM INT were 0.24, 0.38, 0.49 and 0.48, respectively. The mean absorbance of similar replicates incubated with 160 μM INT and 1.9, 3.8, 7.7 and 15.4 μM PMS were 0.15, 0.29, 0.42 and 0.43, respectively. The optimal combinations of INT and PMS concentration subsequently chosen was 160 and 7.7 μM, respectively.

4.1.4.4. The effect of temperature on the rate of INT reduction

For standardisation of the conditions of the proposed test it was necessary to explore the optimum temperature under which the dye-reduction test could be conducted. Six separate semen samples were collected, each of which was a pool contributed by 4 different males. From all the samples, the dye-reduction test was conducted in triplicate. Incubation was carried out for 10 minutes at 3 different water bath temperatures (20°C, 30°C and 40°C). The mean absorbance and standard error at 520 nm for 20°C, 30°C and 40°C were 0.23 $\pm$ 0.05, 0.50 $\pm$ 0.03 and 0.72 $\pm$ 0.06, respectively.

It was clear that at 40°C, within 10 minutes of incubation, a very high amount of formazan was formed. To magnify the differences in the function of spermatozoa between males, it is suggested that the reaction might be better carried out at 20°C rather that at the higher temperatures. Also, it was easier to get the absorbance reading in the middle of the scale after 20 minutes incubation, thus ensuring more accurate readings.

For application in the field, it is an advantage to be able to conduct tests at 20°C. It avoids an expensive water bath as incubation could be done at room temperature provided it is equilibrated around 20°C.

4.1.4.5. The effect of time of incubation and concentration of spermatozoa on INT reduction

In the course of the development of the dye-reduction method with fowl spermatozoa, it was observed that beyond a certain time the colour development (INT reduction) frequently reached its saturation point and absorbance readings were off the scale. In part, this appeared to be due to samples of semen with high densities of spermatozoa. Thus, to optimise the range of volume of semen and incubation time suitable for the test, and also to investigate whether the relationship of colour development was linear with time of incubation and with number of spermatozoa, the following experiments were conducted:

Five different pooled samples of semen, each contributed from 4 cocks, were utilised for the test. Incubation was started in 5 tubes simultaneously for each sample. The incubation was stopped

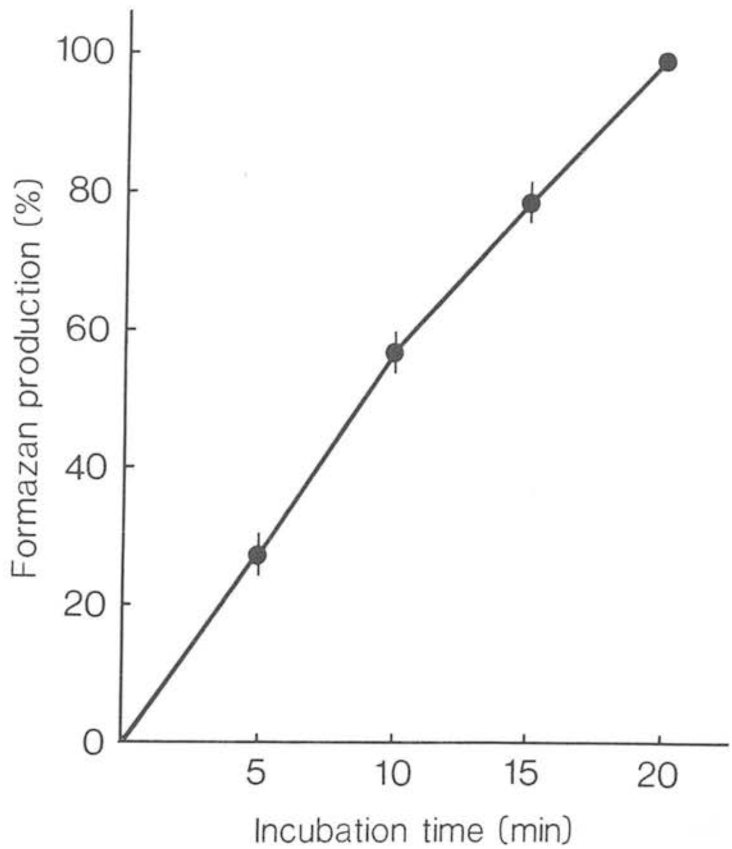

Fig.4.4 Relationship between incubation time and the rate of INT reduction. Formazan produced by five different pooled samples of semen was measured after 5, 10, 15 and 20 min of incubation. Results were expressed as a percentage of the 20 minute value for each of the different samples, the means of which are shown (vertical bar; SEM). A linear regression of formazan formation against time gave a regression equation: % formazan formation = 6.02 ± 4.78 min (R^2=93%).

by the usual procedure of adding acid triton (200 μl of 5% Triton X 100 in 0.1 M HCl) at 0, 5, 10, 15 and 20 minutes. The absorbance recorded for each sample at 20 minutes was considered as 100% and the respective percentage of colour development at other times (15, 10, 5, 0 minute) was recorded. The mean and SE (for 5 samples) of percentage development of colour/INT Reduction at 5, 10, 15 and 20 minutes were 27.4 ± 2.3, 56.8 ± 4.5, 79.0 ± 5.3 and 100.00, respectively (Fig. 4.4).

Similarly with 5 different pooled semen samples (each contributed by 4 different males), the percentage of colour development was recorded after 20 minutes incubation using 50, 100, 150 and 200 μl of 4-fold diluted semen in salt/TES solution (150 mM NaCl/20 mM TES adjusted to pH 7.4 with 1 M.NaOH) considering 100% development of colour in the tube with 200 μl of semen. The means and SE of the percentage of colour development/INT reduction calculated from 5 samples each with 50, 100, 150 and 200 μl of 4-fold diluted semen were 20 ± 3.4, 52 ± 3.7, 77 ± 1.1 and 100.00, respectively (Fig. 4.5).

It can be seen that colour development at a fixed concentration of spermatozoa is linear with time of incubation (Fig. 4.4) whilst that for a fixed incubation time is linear with the concentration of spermatozoa (Fig. 4.5).

To verify the fact that INT reduction/colour development was due only to the spermatozoa and not the seminal plasma, the test was conducted on spermatozoa free from seminal plasma and on the

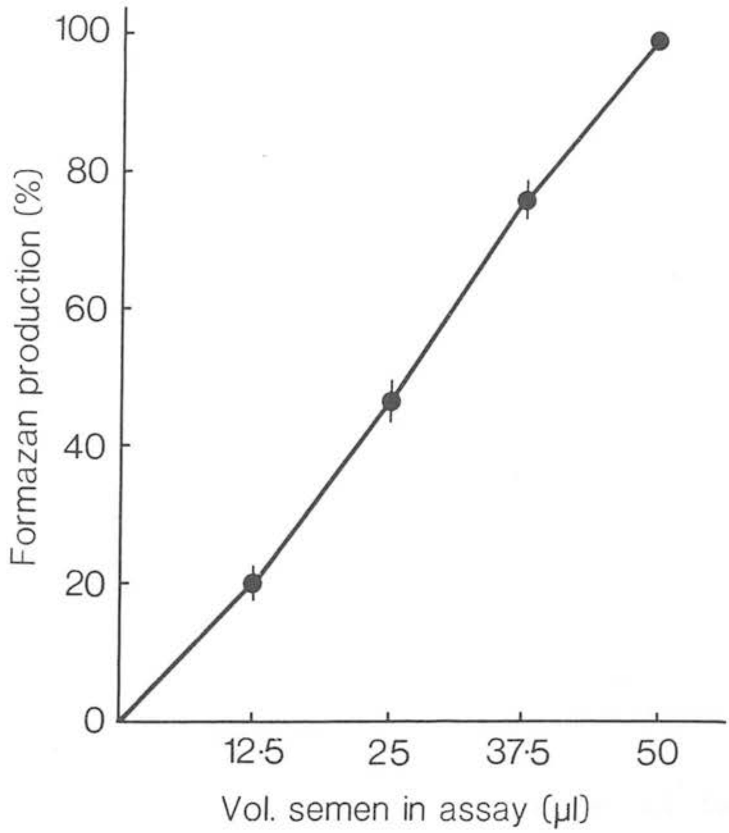

Fig.4.5 Relationship between concentration of spermatozoa and rate of INT reduction. The formazan produced in 20 min by 12.5, 25, 37.5 and 50µl of 5 different samples of pooled semen were measured and expressed as a percentage of the 50µl value. The mean ± SEM are shown for the respective volumes. A linear regression for formazan development against semen volume gave a regression equation: % formazan formation = -4.49 ± 0.532µl (R^2=96.5%).

seminal plasma alone. The absorbance of the former after 20 minutes incubation read 0.85 whilst that of seminal plasma was less than 0.01 indicating virtually no reduction due to seminal plasma.

4.1.4.6. Relationship between the rates of oxygen consumption by spermatozoa and INT reduction

It was necessary to investigate the relationship between the rate of oxygen consumption and rate of INT reduction, simultaneously, from the same sample of semen so that the data on INT reduction/colour formation could be related to 0_2 consumption of the spermatozoa. For a living cell, normal metabolic activity can be reflected in its ability to utilise oxygen under appropriate conditions. The standard oxygen content of 1.2 ml salt/TES buffer in a 10 mm internal diameter tube equilibrated at 40°C was 220 ± 4 nmol/ml (N=5).

On seven different occasions, batches of four separate pooled semen samples were collected and diluted 4-fold instantly with salt/TES solution. The rate of oxygen consumption was measured and the dye-reduction test carried out simultaneously. A linear regression of nmol formazan produced per minute = 24.4 ± 0.125 nmol of oxygen utilised/minute per ml of semen. The regression was highly significant ($P \leq 0.001$). The correlation coefficient (r) between these characteristics was 0.805, which was also highly significant ($P \leq 0.001$). The coefficient of determination (R^2) was 64.7%. The evidence of a very high association between INT reduction and oxygen utilisation suggested that this test is a predictor of the metabolic function of the spermatozoa.

4.1.4.7. The effect of cyanide (CN^-) on spermatozoal oxygen consumption, ATP concentration and rate of INT reduction

The concern in developing the present test was that a colourless dye should be reduced to a coloured compound efficiently in a controlled manner by virtue of the functioning of the oxido-reductase system in the spermatozoa. In the normal process of electron transport, the electron is passed through a series of reactions involving NADH. At the end of the chain, the electron is passed normally, via cytochrome oxidase, when a hydrogen ion binds with oxygen to form water (Fig. 4.3). This chain reaction can be stopped by inclusion of cyanide in an incubation mixture so that in the electron transport chain, cyanide binds the haem (or iron) group in cytochrome oxidase thereby preventing the transfer of electrons to oxygen. In such a situation INT receives the electron through PMS and NADH resulting in the rapid formation of formazan (Fig. 4.3). However, for the application of the dye-reduction test for testing spermatozoal viability, they should not be destroyed by CN^- and it is essential not to prevent synthesis of ATP necessary to maintain motility and other metabolic activity for the duration of the test. So it was essential to work out the optimum concentration of cyanide which would allow cellular ATP synthesis whilst at the same time enhancing electron transport to reduce the INT to formazan

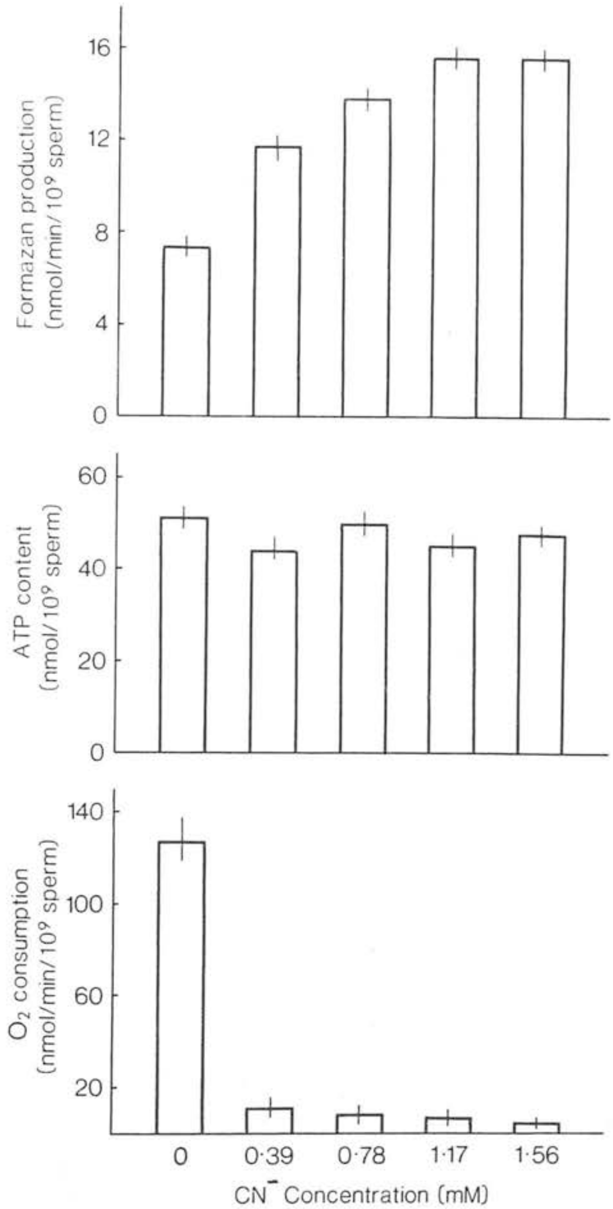

Fig. 4.6 The effect of cyanide on sperm oxygen consumption, ATP concentration and rate of INT reduction (which were measured in aliquots of each of 4 samples of semen in the pres ence of 0 to 1.56 mM cyanide). Each result shows the mean ± SEM for each cyanide concentration.

and not permitting the combination of hydrogen and oxygen to form water.

For this purpose, on four separate occasions pooled semen samples were collected and oxygen consumption, ATP content and formazan production by spermatozoa were recorded simultaneously in the presence of 0, 0.39, 0.78, 1.17 and 1.56 mM cyanide. It was observed that at a concentration of 1.56 mM, cyanide enhanced the rate of INT reduction and greatly inhibited oxygen consumption and did not reduce sperm ATP content in the presence of glucose (Fig. 4.6). In the presence of 0, 0.39, 0.78, 1.17 and 1.56 mM cyanide the means and standard error of nmol of formazan formed per minute per 10^9 sperm were 7.29 $\pm$ 1.1, 11.65 $\pm$ 1.3, 13.81 $\pm$ 1.5, 15.59 $\pm$ 2.1, 15.53 $\pm$ 1.6, respectively. The 0_2 consumptions (nmol/minute/10^9 sperm) with these concentrations of cyanide were 127 $\pm$ 36.6, 11 $\pm$ 4.96, 8.4 $\pm$ 4.2, 6.4 $\pm$ 3.2, 2.46 $\pm$ 0.9 and ATP synthesis (nmol/10^9 sperm) were 50.6 $\pm$ 3.7, 43.8 $\pm$ 4.9, 49.4 $\pm$ 11.4, 45.3 $\pm$ 6.3, 47.3 $\pm$ 3.4, respectively.

4.1.5 DISCUSSION

In cytochemical as well as in biochemical studies, the tetrazolium salts (INT & NBT) have been used widely (Altman, 1972; Bergmeyer and Bernt, 1974). They produce highly coloured formazan dyes on reduction by dehydrogenase enzymes such as lactate dehydrogenase and succinic dehydrogenase. Reducing capacity reflecting the level of metabolic activity of whole cells, e.g. soil microorganisms (Casida et al., 1964) and granulocytes (Baeher and Nathan, 1968) was estimated using tetrazolium salts, the amount of formazan formed within the cells being measured after organic extraction. In the method described here, the latter step was accomplished by the simple solubilisation of the formazan with a biological non-ionic detergent, Triton X 100, in acid media.

In the past, the metabolic activity of poultry spermatozoa was estimated by following the reduction of both methylene blue (Shaffner and Andrews, 1948; Cherms, 1968) and resazurin (Cooper and Rowell, 1958; McDaniel and Craig, 1962). In at least one case (Cooper and Rowell, 1958) this type of test has been shown to be successful for predicting the fertilising ability of fowl semen. However, for reasons stated previously, mostly due to lack of objectivity, these tests have not been widely accepted.

The present work shows that the reduction of INT may be used to measure the metabolic activity of fowl spermatozoa in a controlled, quantitative objective test and that the rate of dye reduction is correlated with the rate of oxygen utilisation by the spermatozoa, a good measure of viability in vivo.

The method has several advantages over previous dye-reduction tests used with fowl spermatozoa (e.g. Shaffner and Andrews, 1948; Cooper and Rowell, 1958): primarily it is objective, measuring the colour change spectrophotometrically rather than by eye; it also measures the appearance, rather than disappearance, of colour allowing higher substrate levels of dye to be used and thus a faster reaction rate to be achieved.

The inclusion of cyanide clearly enhanced the rate of INT reduction presumably by inhibition of cytochrome oxidase and thus preventing oxygen utilisation. This occurred under conditions where substrate-level ATP synthesis, and thus spermatozoal viability, was unaffected. In previous dye-reduction tests applied to spermatozoa (using methylene blue and resazurin), the reaction mixture was covered with a layer of liquid paraffin. This was assumed to prevent oxygen utilisation by invoking anaerobic conditions, but in our experience this method was quite inefficient and often liquid paraffin was found to contain oxygen.

Calcium was included in the reaction medium since it has been shown to increase the motility of fowl spermatozoa (Wishart and Ashizawa, 1987) and thus, presumably, their metabolic rate and it accelerated the reaction time for the in vitro test. The production of formazan was accelerated by increasing the incubation temperature. However, from an examination of many different semen samples containing varied qualities of spermatozoa, it is recommended that 20°C be adopted routinely as it allows optimal differentiation of colour development between particular semen samples. This indicates that the test may be carried out at room temperatures, provided that these are standardised and around 20°C. In general, 20 minutes incubation time is recommended because in this time, with 50 μl of moderately good semen samples containing 150 million spermatozoa incubated at 20°C, absorbances A_{520} are around 0.80 which is within a suitable part of the scale. Moreover, 20 minutes gives enough time in between to handle the starting and stopping of the reaction of a few samples simultaneously with the chance of reducing error in estimating the rate of formazan development per minute per 10^9 sperm in all samples. However, for extremely active or inactive samples the sensitivity of the test may be adjusted by varying the concentration of spermatozoa or the incubation time. It was observed under the microscope that the spermatozoa in the incubation mixture, inclusive of cyanide, survived for up to an hour.

Preliminary work has shown that the method may be used to determine the reducing ability of semen from turkeys and indeed mammalian species (tested on a few ram semen samples). It has already been shown (Wishart, 1982; Wishart and Palmer, 1986b) that _in vitro_ tests of ATP synthesis and oxygen utilisation by fowl and turkey spermatozoa have enabled the prediction of their fertilising ability. The next section will describe how this dye-reduction test can be used for predicting the fertilising ability of fowl spermatozoa.

The final procedure worked out for using the dye-reduction test developed in this section is given as an Appendix to the main thesis (Page 211).

4.2 Use of the dye-reduction test to predict the fertilising ability of avian semen

4.2 USE OF THE DYE-REDUCTION TEST TO PREDICT THE FERTILISING ABILITY OF AVIAN SEMEN

A comparison of a simple colourimetric test with other methods for predicting the fertilising ability of fowl semen

4.2.1 INTRODUCTION

The fertility of the male fowl in a breeder flock is of greater economic importance than that of the females because the male is responsible for fertilising the eggs from a number of females. Hays and Sanborn (1939) showed that in most cases the males in flocks were responsible for infertile matings since changing them resulted in 93% of the infertile matings becoming fertile. In commercial breeding flocks, males of low or questionable fertility may go undetected because, in many instances, they are sexually aggressive and otherwise appear normal and healthy. Elimination of sterile males and those producing low quality semen can improve the fertility of a flock dramatically (Perek, 1966). Although the majority of fertile eggs in both the layer and broiler industry in the world are still produced from natural matings, there has been increasing interest in artificial insemination (Perek, 1966; McDaniel, 1974; Lake, 1971). The use of stored semen (Lake and Ravie, 1979; Van Wambeke, 1967, 1972; Lake and Ravie, 1981, 1983) has opened up new dimensions in the use of AI allowing transportation of semen between satellite breeding farms. The cryo-preservation of avian semen was first achieved by Polge et al. (1949) but the post-thaw fertility was not good. Over 3 decades many improvements and developments have been made in this area and today it is feasible as a commercial proposition (Lake et al., 1981). Another landmark in avian semen management is the development of a method for the storage of semen at high temperature (40°C) for up to 17 hr (part of the work of this thesis, See Pages 138-174) previously only possible for up to 5 hr (Wishart, 1984). Until this discovery it was generally acknowledged that rooster spermatozoa lost their fertilising ability within 7 hr of collection when preserved in vitro at temperatures above 30°C (Garren and Shaffner, 1952; Schindler et al., 1955). All the above findings on storage of semen could revolutionise the fowl industry by the greater adoption of AI, thus producing economies on space requirements, the use of fewer males, the higher accuracy of breeding data and feed savings. Many more females could be covered by a single male which would permit its use for more purposeful stock improvement.

It would be beneficial if the commercial breeder could evaluate the semen of roosters before using them in his flock, whether the flock be naturally mated or artificially inseminated. The selection of such males might be facilitated if some substantial correlation could be found between an in vitro measure of semen quality and fertility. Wilson et al. (1979) suggested benefits of evaluating the quality of semen from poultry, either for selecting breeding males or for routinely monitoring their performance throughout a season.

In the previous section, the various semen evaluation tests available and their merits and demerits were discussed. The development of an objective colourimetric technique for measuring the metabolic activity of fowl spermatozoa was described and discussed. It was suggested that this technique has several advantages over previous tests of semen quality, which have been mainly subjective relying on the experience and judgement of the operator.

In this section the ability of the new objective dye-reduction test to predict the fertilising ability of fowl semen is examined. Results of the new test are compared with those of other tests of spermatozoal function - motility, ATP content (Wishart and Palmer, 1986b) and morphology (see Lake and Stewart, 1978) vital for fertilising ability.

4.2.2 MATERIALS AND METHODS

Birds

The 19 males used in this trial were of a Rhode Island Red type control strain and females were of a commercial laying strain (for details of management, see Materials and General Procedures, Page 40).

Semen collection and treatment

Semen, free of transparent fluid (Lake, 1957), was collected on a thrice weekly routine from 19 males selected randomly from a population of 48 males. In batches, semen samples from 7 individuals were collected separately and diluted 4-fold (0.15 ml semen + 0.45 ml diluent) with a buffer containing 0.15 M NaCl and 20 mM TES adjusted to pH 7.4 with 1 N.NaOH in 18 mm diameter poly-carbonate vials. These were held in a shaking water bath at 40°C and all tests were accomplished within one hr.

4.2.2.1 Assays conducted on spermatozoa

1) Dye-reduction test

The ability of the spermatozoa to reduce the dye INT to formazan was measured as described in the previous section with 20 minutes incubation at 40°C with 200 μl 4-fold diluted semen. The final value was recorded as nMol of formazan produced/per minute/per 10^9 spermatozoa as mentioned in the previous section.

2) Morphology of spermatozoa

40 μl of 4-fold diluted semen was dropped and mixed into 9 drops of ice-cold stain mixture (Nigrosin and Eosin) (Lake and Stewart, 1978) for 2 minutes. Smears were made in duplicate on glass (grease-free) microscopical slides by putting a drop of stain semen mixture on to them and drawing up another slide to the drop at an angle of 30° and then pushing it away. The smears were instantly dried with a hair dryer blowing cold air. Three hundred spermatozoa were individually counted on each slide and classified as having normal or abnormal structure.

The percentage normal was recorded as described by Lake and Stewart (1978).

3) Motility of spermatozoa

Spermatozoal motility was estimated in semen samples diluted a further 50-fold (100 μl 4-fold + 4.9 ml of buffer containing 0.15 M NaCl, 20 mM TES and 8 mM $CaCl_2$) at 40°C. The 200-fold diluted semen sample was passed through a flow cell at 40°C in a spectrophotometer as described by Wishart and Ross (1985) - see Material and General Procedures, page 42).

4) ATP content

The ATP content of spermatozoa was measured in boiled extracts of semen according to the method outlined in Material and General Procedures, page 54.

5) Fertility trial

The concentration of spermatozoa in a semen sample from each male was estimated and the volume of a 20-fold dilution of semen containing 10 x 10^6 spermatozoa was calculated (between 48 μl and 117 μl). For each sample, the correct volume was inseminated into 8 hens. Fertility testing was based on 19 individual males and 152 hens laying a total of 2660 eggs. The average rate of lay between the group of 8 hens was 83.33% during the fertility test over days 2-21 after insemination. The minimum and maximum number of eggs laid by a group of 8 hens between days 2-21 after a single insemination were 116 and 157. Eggs were stored for up to 4 days at room temperature

(18–20°C) before being incubated. The fertility of eggs was estimated by candling (See Material and General Procedures, page 46).

All percentage data on motility measured by spectrophotometer and on normal spermatozoa for each male were transformed to arc sin$\sqrt{\%}$ values for further calculation.

The correlation and regression between the fertilising ability and measures of semen evaluation tests, including the dye-reduction test, were calculated by standard statistical methods.

4.2.3 RESULTS

The mean fertility data for 3 periods and semen evaluation data from all the *in vitro* tests are given in Table 4.2.

The correlation coefficients among the fertility data and semen quality data by various tests including the transformed data are presented in Table 4.3.

The rate of INT reduction per minute/10^9 spermatozoa is significantly correlated ($P<0.001$) with the fertilising ability (2–15 days after insemination) and their ATP content, motility and morphology as seen in Table 4.3 and Fig. 4.7. The magnitude of correlation of fertilising ability with ATP synthesis as well as with INT reduction appears to be of equivalent value as evidenced in Table 4.3. A very high significant correlation ($P<0.001$) was observed between ATP synthesis and the dye-reduction test (0.93)

Table 4.2. The fertility data and results of the *in vitro* tests, including the dye-reduction test

Male	C-1	C-2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
1	53.33	29.78	20.00	8.87	18.80	29.55	43.2	0.41	26.44	88.06	2.98	0.55
2	12.24	6.45	4.65	6.70	0.00	12.71	17.7	0.31	19.99	37.88	2.98	0.55
3	10.41	5.20	3.44	5.35	0.00	11.76	16.3	0.27	17.41	38.25	3.25	0.60
4	56.60	39.04	26.75	8.89	26.60	41.90	47.0	0.56	36.12	170.12	4.06	0.73
5	22.91	12.63	8.39	7.93	0.03	21.60	39.0	0.24	15.48	42.12	1.95	0.39
6	53.19	30.43	20.28	10.92	29.50	56.26	63.2	0.53	34.18	176.12	3.13	0.58
7	80.43	52.08	35.91	9.53	36.36	51.01	59.2	0.38	24.51	131.10	2.57	0.49
8	83.33	53.68	35.86	10.83	47.60	68.55	73.0	0.64	41.28	261.20	3.81	0.69
9	79.59	60.67	40.60	12.66	75.47	81.83	77.0	0.76	49.02	316.71	3.87	0.70
10	92.68	69.51	50.00	13.58	85.20	122.02	92.6	0.75	48.37	434.41	3.56	0.65
11	84.44	61.70	42.44	14.06	71.21	130.15	88.0	0.65	41.92	387.85	2.98	0.55
12	76.59	45.74	30.06	10.30	45.83	75.74	91.6	0.54	34.83	256.01	3.38	0.62
13	83.67	58.00	38.15	10.49	46.98	58.95	87.3	0.68	43.86	246.45	4.18	0.75
14	85.10	53.19	34.72	13.39	50.98	99.15	95.6	0.43	27.73	205.25	2.07	0.41
15	82.50	50.61	33.60	15.57	94.28	136.09	70.3	0.50	32.25	281.72	2.07	0.41
16	83.67	53.53	36.24	13.65	73.52	114.35	89.3	0.36	23.22	194.40	1.70	0.35
17	82.60	58.51	40.00	9.77	77.58	91.80	90.0	0.55	35.47	333.26	3.63	0.66
18	89.36	56.38	37.06	11.22	53.85	96.10	94.0	0.35	22.57	193.13	2.01	0.40
19	46.80	23.91	15.71	7.37	26.47	44.09	73.0	0.23	14.83	88.64	2.01	0.40

C1, C2, C3 - Fertility percentage 2-8, 2-15, 2-21 days after insemination; C-4-Formazan development per min/10^9 sperm; C-5-Percentage (ΔOD_M); C-6-ATP synthesis in nmol/10^9 sperm; C-7-Percentage normal spermatozoa; C-8-A520 of formazan; C-9-Formazan per minute/per ml of neat semen; C-10-nmol of ATP/per ml of neat semen; C-11-Sperm concentration x 10^9 per ml of neat semen; C-12-(OD_M).

Table 4.3. The correlation coefficients among fertilising ability and semen evaluation tests

	C-1	C-2	C-3	C-4	C-5	C6	C7	C8	C9	C10	C11	C12	C13	C14	C15	C16	Abbreviation
C2	0.98																C-1 % fertility 2-8 days
C3	0.97	0.99															C-2 % fertility 2-15 days
C4	0.81	0.80	0.79														C-3 % fertility 2-21 days
C5	0.84	0.87	0.87	0.89													C-4 formazan per min/ 10^9 sperm
C6	0.83	0.82	0.82	0.93	0.94												C5 % (ΔOD_M)
C7	0.90	0.87	0.85	0.73	0.79	0.81											C6 ATP in nmol/ 10^9/sperm
C8	0.63	0.73	0.74	0.57	0.61	0.51	0.51										C7 % normal spermatozoa
C9	0.63	0.73	0.74	0.57	0.61	0.51	0.51	1.00									C8 Dye test OD
C10	0.81	0.88	0.89	0.77	0.87	0.84	0.76	0.85	0.85								C9 Formazan per min/per ml of neat semen
C11	0.08	0.20	0.21	-0.14	-0.02	-0.16	-0.04	0.71	0.72	0.36							C10 ATP in nmol/ per ml of neat semen
C12	0.09	0.21	0.22	-0.14	-0.01	-0.16	-0.03	0.71	0.72	0.37	1.00						C11 concentration of spermatozoa
C13	0.99	0.98	0.97	0.81	0.84	0.84	0.91	0.63	0.63	0.81	0.07	0.08					C12 - OD_M
C14	0.98	0.99	0.99	0.80	0.86	0.82	0.88	0.72	0.72	0.86	0.19	0.19	0.99				C13 Arc sin transformed fertility 2-8 days
C15	0.98	0.99	0.99	0.80	0.86	0.82	0.87	0.73	0.73	0.87	0.19	0.19	0.98	0.99			C14 Arc sin transformed fertility 2-15 days
C16	0.87	0.88	0.87	0.89	0.98	0.92	0.82	0.60	0.60	0.85	-0.04	-0.03	0.88	0.88	0.88		C15 Arc sin transformed fertility 2-21 days
C17	0.98	0.86	0.84	0.72	0.77	0.81	0.99	0.48	0.48	0.75	-0.06	-0.05	0.90	0.87	0.86	0.80	C-16 Arc sin transformed % (ΔOD_M)
																	C17 Arc sin transformed percentage normal spermatozoa

Table Value of r at (n-2) = 17 d.f. $P<0.05 = 0.455$, $P<0.01 = 0.575$, $P<0.001 = 0.69$

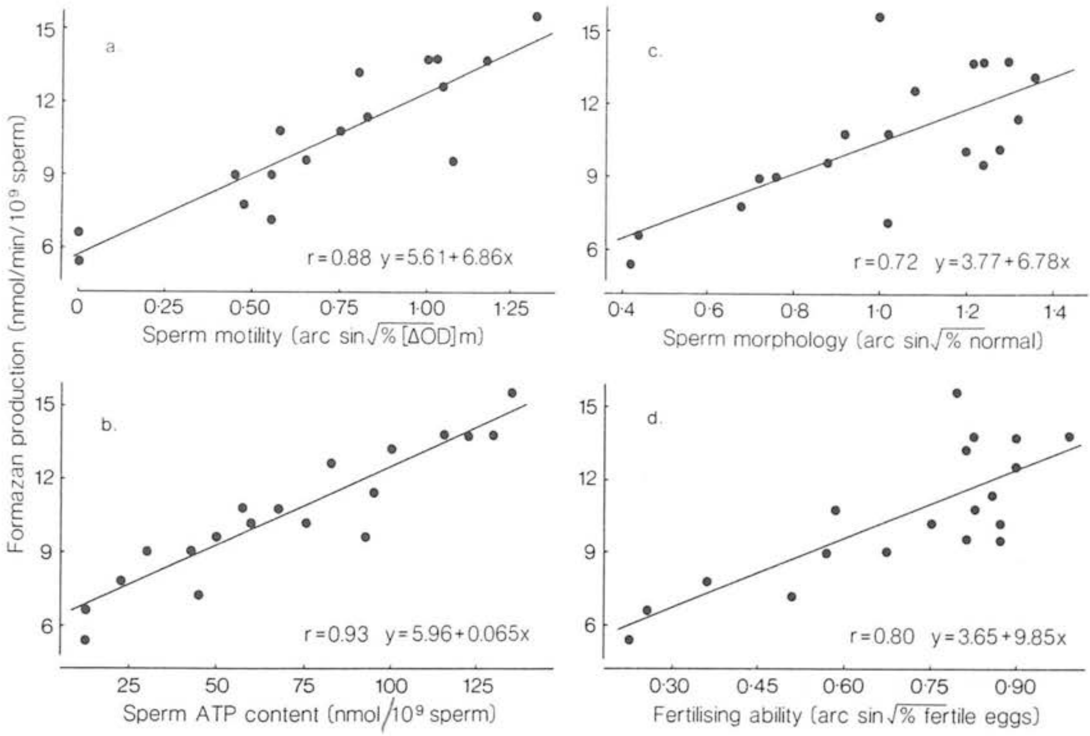

Fig.4.7 Relationship between the rate of INT reduction and motility (a), ATP content (b), morphology (c), and fertilising ability (d) of spermatozoa. Each point represents measurement from a sample of semen from an individual male. Fertilising ability is shown as the mean transformed Arcsin $\sqrt{\%}$ fertility of eggs laid by groups of hens between days 2-15 post insemination.

which shows that this test is a real indicator of metabolic activity of the spermatozoa.

4.2.4 DISCUSSION

Significantly high degrees of correlation, as evidenced by the results of the dye-reduction test, with fertilising ability and the other semen evaluation tests suggests that the new dye-reduction test developed and described in the previous section can serve a useful purpose for semen and/or sire evaluation. In a previous study (Wishart and Palmer, 1986b), it was observed that morphology was less significantly correlated with fertilising ability than ATP content of spermatozoa, whilst motility showed the most significant correlation (0.82). However, differences in the magnitude of these correlations may be of little importance: the use of a non-saturating insemination dose such as 10×10^6 spermatozoa is essential for discerning fine differences between the fertilising ability of samples of semen (Wishart, 1985), but also exaggerates the individual variation in the hen's response, i.e. the production of fertile eggs. This has been shown to account for about 90% of the variation between the observed fertilising ability and that predicted from an in vitro test of spermatozoal function and fertilising ability (Wishart and Palmer, 1986b).

Other considerations can be taken into account to demonstrate the value of the present semen evaluation test. The assessment of spermatozoal morphology requires some experience and even then is extremely tedious and time consuming; measurement of sperm ATP content is quantitative and objective but involves pre-incubation, preparation of extracts of spermatozoa and the use of expensive equipment, although several samples can be processed consecutively. The motility assay (Wishart and Ross, 1985) is quantitative and objective, but requires expensive equipment and samples must be treated individually, each taking at least 3 to 5 minutes.

Measurement of the reduction of INT by spermatozoa is the simplest of the four tests described here. For example, the semen samples can be incubated at room temperature so that water baths are not required; the results can be read on an inexpensive colourimeter (green filter, wavelength range 500-540 nm) and it is a simple matter to process 20-25 samples consecutively - the whole process taking less than 45 minutes. Primarily of course, the main advantage of the test is its objectivity, allowing controllable, consistent and operator-independent assessment of the quality of fowl spermatozoa thereby enabling selection and monitoring quality of breeding males.

4.3 Factors determining the fertilisation rate in artificial breeding

4.3 FACTORS DETERMINING THE FERTILISATION RATE IN ARTIFICIAL BREEDING

4.3.1 EFFECT OF AGE OF MALE AND FEMALE ON FERTILITY

4.3.1.1 INTRODUCTION

Reproductive ageing in adult breeders is the cumulative results of functional transformations in the male and female reproductive organs, and the gametes might be expected to be affected. That fertility, defined as production of fertile eggs, declines with ageing is an observation that has been made by several workers for many years. The possible causes of this decline in fertility can be attributed to male and/or female factors. In the male, there is a seasonal decline in spermatozoal output and quality, due to reduced testicular function, accompanying physiological changes influenced by environmental conditions, especially marked in wild birds. Individuals will vary in rate of decline of reproductive ability due to their genetic make-up. Similarly, the reproductive decline in the female, modulated by individual genetic make-up, is associated with a seasonal decline in egg production as a result of a decreased rate of production of the large yellow follicles in the ovary, follicular atresia, failure of ovulated ova to enter the oviduct or the laying of soft-shelled eggs. The retention of spermatozoa within the uterovaginal sperm-storage sites in the females is also affected. At any given time in individual females there is a positive correlation between rates of egg production and fertility. Therefore, it is important to investigate the possible influences of sex and age on total fertility. Herein, spermatozoal quality and interaction of gametes is the main concern.

4.3.1.2 REVIEW OF LITERATURE

Work on effect of age and sex on avian reproduction is limited. Most of the work has been carried out in turkeys and a little in broiler-type fowls. In layer-type fowl no work appears to be recorded. Observations over a 3-year period of fertility following AI using split semen samples from young and old turkey males on females in the first five months compared to the next five months of prduction showed data to be significant only for female effects (Harper and Arscott, 1969). Harris (1984) also commented that reproductive efficiency normally declined as the turkey aged. Fertility declined at a slower rate during the last 10-week period of lay than during the first 10 weeks. However, the decreased rate of decline in the latter period could have been due to the fact that the frequency of insemination was increased from once fortnightly to once weekly.

Kirk et al. (1980) reported that fertility and hatchability of fertile eggs reached an early peak and then decreased by about 9% by 60 weeks of age in broiler breeder females naturally mated. Lake (1967) reported that effects of ageing in the male on semen production are probably confounded with seasonal factors in broilers. In this respect, Ansah et al. (1980) conducted a study under constant climate conditions, which reduced the confounding effects between age and season in broiler breeder males and showed that semen production peaked and then declined significantly with advancing age.

Sexton (1977a) showed that the number of weeks that a turkey hen had been in egg production changed the minimum number of spermatozoa that were required to maintain maximal fertility with unstored semen. The fertility of hens inseminated with a fixed number of spermatozoa showed a steady decline with age (Sexton, 1977a, 1986). Lorenz et al. (1959) suggested that inherited differences and age were factors accounting for some of the variations in the maintenance of spermatozoal viability in individual turkey hens and stated that, "sperm have a shorter life in the old hen's oviduct than in the young hen". Van Krey et al. (1967) also showed that late season decline in fertility is possibly due to non-retention of spermatozoa within the uterovaginal sperm storage sites. Sexton and Giesen (1983) suggested that the quality of spermatozoa declined as male turkeys aged, so that fewer viable spermatozoa were inseminated late in the egg production period, if dose of semen was not altered, which leads to lower fertility. Krueger (1984) suggested that as the production of viable spermatozoa in the male decreased with age, the female's requirement for a minimum number of spermatozoa increased.

In spite of the above-mentioned general knowledge, there is still a need to verify the involvement of a male and female factor and a possible interaction in relation to lowered fertility with age. Much of the evidence is anecdotal. Often the phenomenon has been described in a population of males and females growing old together, or sometimes workers have used semen from different males at different ages on a batch of females at a given time, or semen from a male at particular age on batches of females of different ages and genetic make-up. It would be preferable to examine for the effect of individual sexes by simultaneously inseminating batches of semen collected from the same males at different ages into the same females at different ages with a fixed insemination dose, which is physically impossible with freshly collected semen. Recent advances in frozen semen technology has made it possible to store fowl semen indefinitely without change in semen quality (Lake et al., 1981). Thus, advantage was taken of this finding here to pursue further the possibility of male and female factors affecting the total fertility picture.

4.3.1.3 MATERIALS AND EXPERIMENTAL PROCEDURES

The males used were a control Rhode Island Red population of the 1986 generation and females used were of a commercial white egg-layer strain of the 1987 generation (for details see Materials and General Procedures, Birds, Page 40). At sexual maturity, the population of males was screened by in vitro tests for semen quality and 14 males were chosen out of a population of 60 and categorised as good quality semen producers (for details see Materials and General Procedures, Categorisation of Males, Page 45).

At 28 weeks of age (designated as young age of the male), pooled semen was collected from 14 males and frozen in glass ampoules in a glycerolised medium, each ampoule containined 0.15 ml semen and 0.45 ml medium and frozen at a cooling rate of 3°C per minute from +5°C to -35°C before submerging in liquid nitrogen at -196°C (see method described by Lake et al., 1981). Sufficient semen was collected during the week to build up a stock of 60 ampoules. This was accomplished by 3 collections from the males on alternate days, each time preparing 20 ampoules. The procedure was repeated when the same males were 46 weeks (middle age) and 67 weeks (old age) old.

With the 1987 generation females at 24 weeks of age (young age), frozen semen portions from each age period of the males were thawed and deglycerolised at 5°C by the procedures described by Lake et al. (1981). On average 24 birds were inseminated each with 75 μl semen (150 million spermatozoa, approximately) and another 24 birds with 50 μl semen (100 million spermatozoa, approximately) with semen samples from each of the age groups. The inseminations were accomplished over three consecutive days. Two sessions per day, one in the morning and one in the afternoon, were necessary for the task. The different doses of semen from each age class were randomly inseminated. However, the time of insemination was constant each day either between 12.00-12.30 hr or 14.00-14.30 hr. The whole procedure was repeated when the females were 41 weeks (middle age) and 54 weeks (old age) of age. That is to say, frozen semen collected from the same males when young, middle and old age were deglycerolised and inseminated over 3 consecutive days, using two different doses of spermatozoa at each female age. In general about 8 to 12 glass ampoules of frozen semen were handled for deglycerolisation and insemination in each session, which allowed enough precautions to be taken to thaw and handle semen properly to prevent any risk of losing samples of semen due to processing difficulties in such a large scale operation. Every possible effort was made for each hen to receive, in turn, semen of males taken at each age. All the females were in good laying condition at the time they were used for test inseminations, they had laid 5 or more eggs in the previous week. At the young age, 142 females were used from a population of 178 and, at middle age and old age, 147 and 131 hens, respectively, were inseminated from the same population.

For each treatment combination, data from a minimum of 23 hens were available except for the higher insemination dose in old age females where with young males only 21 hens and with old males only 14 hens could be inseminated because a few ampoules exploded during thawing. Following inseminations, all the eggs were collected from days 2 to 15 and tested for fertility by incubation (for details see Materials and General Procedures, Fertility trials, Page 46).

<u>Statistical analysis of data</u>

The factorial design analysis was conducted on transformed data as degrees (y) when (x) is the fertility percentage, so that, $x=100\ (\sin(y))^2$ or, $y=\sin^{-1}\ (\sqrt{x/100})$. The "genstat" software was used for the analyses.

The statistical model for the factorial experiment was:

$$Y_{ijkl} = \mu + a_i + b_j + c_k + ab_{ij} + ac_{ik} + bc_{jk} + abc_{ijk} + e_{ijkl}$$

where, μ = population mean

a_i = male age effect i=1 to 3 (young, middle and old)

b_j = female age effect, j=1 to 3 (young, middle and old)

c_k = dose of inseminations, k=1 to 2 (100 million and 150 million spermatozoa)

ab_{ij} = interaction between age of male and female

ac_{ik} = interaction between age of male and dose

bc_{jk} = interaction between age of female and dose

abc_{ijk} = Interaction among age of male, age of female and dose

e_{ijkl} = random error with mean 0, and variance σ^2, which is normally and independently distributed

Y_{ijkl} = lth individual transformed fertility data with i-th male, j-th female and k-th dose

4.3.1.4 RESULTS

The fertility obtained by insemination of the semen collected from males when they were young, middle and old age into young females are presented in Table 4.4. The percentage fertility ranged from 63.8 to 66.6 for the lower (100 million spermatozoa) and 63.7 to 70.6 for the higher insemination dose (150 million spermatozoa) for the period 2-8 days after insemination. During the period 9-15 days the respective fertility percentages ranged from 11.3 to 17.4 and 12.5 to 17.4 for the two doses, whereas during 2-15 days the corresponding ranges were 37.8 to 42.9 and 39.5 to 41.3, respectively. For all the three periods, the fertility with the higher dose of spermatozoa was superior to the lower, although, the magnitude was marginal. The pooled fertility for the semen of all the three male ages on the young females was similar.

The fertility data for the semen collected from different aged males used on middle age females and on old age females are presented in Tables 4.5 and 4.6, respectively. The fertility produced by the males when young was considerably lower than when they were middle and old aged. The higher insemination dose produced higher fertility than the lower dose and it was evident with females of all ages.

In general, the overall fertility obtained in this experiment with frozen semen was not as high as would be expected with fresh semen. However, a few treatment combinations resulted in

Table 4.4. The fertility data obtained by inseminating young hens (24 wk) with two different insemination doses (100 and 150 million spermatozoa) of semen collected and stored frozen from the same males at 3 different ages; young (28 wk), middle (48 wk) and old (67 wk) age

plan of Insemination	PERCENTAGE FERTILE EGGS LAID DURING PERIODS AFTER INSEMINATION								
	2–8 days Insemination Dose		Average	9–15 days Insemination Dose		Average	2–15 days Insemination Dose		Average
	100 mil	150 mil		100 mil	150 mil		100 mil	150 mil	
Young male X	63.88 (92/144)*	70.67 (94/133)	67.15 (186/277)	11.34 (16/141)	12.50 (17/136)	11.91 (33/277)	37.89 (108/285)	41.11 (111/269)	39.45 (219/554)
Young females (N)	24	24	48						
Middle male X	66.66 (90/135)	63.75 (95/149)	65.14 (185/284)	17.46 (22/126)	13.14 (18/137)	15.21 (40/263)	42.91 (112/261)	39.51 (113/286)	41.13 (225/547)
Young females (N)	23	24	47						
Old male X	63.82 (90/141)	65.21 (90/138)	64.52 (180/279)	13.14 (18/137)	17.39 (24/138)	15.27 (42/275)	38.84 (108/278)	41.30 (114/276)	40.07 (222/554)
Young females (N)	24	23	47						
Pooled mean	64.76 (272/420)	66.42 (279/420)	65.59 (551/840)	13.86 (56/404)	14.35 (59/411)	14.11 (115/815)	39.80 (328/824)	40.67 (338/831)	40.24 (666/1655)
(N)	71	71	142						

* No. eggs fertile/total eggs laid during period.

Table 4.5. The fertility data obtained by inseminating middle aged (41 wk) hens with two different insemination doses (100 and 150 million spermatozoa) of semen collected and stored frozen from the same males at 3 different ages; young (28 wk) middle (48 wk) and old (67 wk) age.

plan of Insemination	PERCENTAGE FERTILE EGGS LAID DURING PERIODS AFTER INSEMINATION								
	2-8 days Insemination Dose		Average	9-15 days Insemination Dose		Average	2-15 days Insemination Dose		Average
	100 mil	150 mil		100 mil	150 mil		100 mil	150 mil	
Young male X	28.67 (41/143)*	58.45 (83/142)	43.35 (124/285)	2.72 (4/147)	7.33 (11/150)	5.05 (15/297)	15.51 (45/290)	32.19 (94/292)	23.88 (139/582)
Middle female (N)	24	26	50						
Middle male X	58.20 (78/134)	72.91 (105/144)	65.82 (183/278)	15.82 (22/139)	23.61 (34/144)	19.78 (56/283)	36.63 (100/273)	48.26 (139/288)	42.60 (239/561)
Middle female (N)	24	25	49						
Old male X	78.94 (105/133)	79.28 (111/140)	79.12 (216/273)	19.56 (27/138)	23.77 (34/143)	21.70 (61/281)	48.70 (132/271)	51.23 (145/283)	50.00 (277/554)
Middle female (N)	24	24	48						
Pooled	54.63 (224/410)	70.18 (299/426)	62.56 (523/836)	12.50 (53/424)	18.07 (79/437)	15.33 (132/861)	33.21 (277/834)	43.80 (378/863)	38.59 (655/1697)
mean	72	75	147						

* No. eggs fertile/total eggs laid during period.

Table 4.6. The fertility data obtained by inseminating old age (54 wk) hens with two different insemination doses (100 and 150 million spermatozoa) of semen collected and stored frozen from the same males at 3 different ages; young (28 wk), middle (48 wk) and old (67 wk) age.

plan of Insemination	PERCENTAGE FERTILE EGGS LAID DURING PERIODS AFTER INSEMINATION								
	2-8 days Insemination Dose		Average	9-15 days Insemination Dose		Average	2-15 days Insemination Dose		Average
	100 mil	150 mil		100 mil	150 mil		100 mil	150 mil	
Young male									
X	42.42 (56/132)*	52.67 (59/112)	47.13 (115/244)	7.14 (10/140)	13.00 (13/100)	9.58 (23/240)	24.26 (66/272)	33.96 (72/212)	28.51 (138/484)
Old female (N)	24	21	45						
Middle male									
X	54.10 (79/146)	72.53 (103/142)	63.19 (182/288)	15.38 (22/143)	24.84 (39/157)	20.33 (61/300)	34.94 (101/289)	47.49 (142/299)	41.32 (243/588)
Old female (N)	24	24	48						
Old male									
X	52.00 (78/150)	71.42 (50/70)	58.18 (128/220)	15.73 (23/152)	15.79 (12/76)	15.35 (35/228)	33.44 (101/302)	42.46 (62/146)	36.38 (163/448)
Old female (N)	24	14	38						
Pooled mean	49.77 (213/428) 72	65.43 (212/324) 59	56.51 (425/752) 131	12.64 (55/435)	19.21 (64/333)	15.49 (119/768)	31.05 (268/863)	42.01 (276/657)	35.79 (544/1520)
Overall pooled over young, middle and old age females (N)	56.35 (709/1258) 215	67.52 (790/1170) 205	61.73 (1499/2428) 420	12.98 (164/1263)	17.10 (202/1181)	14.97 (366/2444)	34.63 (873/2521)	42.19 (992/2351)	38.27 (1865/4872)

* No. eggs fertile/total eggs laid during period

79% fertility which is considered very satisfactory with the number of spermatozoa inseminated.

The analysis of variance on transformed fertility data of the factorial design with 3 factors (age of male-3 levels, age of females-3 levels and dose-2 levels) are presented in Table 4.7. It was evident that for all the three periods of fertility measurement, age of the male was highly significant ($P \leq 0.001$); whereas, age of female was not significant in any of the periods. The effect of dose was highly significant ($P \leq 0.01$) for the period 2-8 days and 2-15 days following insemination, whereas, for the period 9-15 days this was less significant ($P \leq 0.05$). The male and female age interaction was significant for all the three periods of fertility measurement. However, the level of significance varied for different periods, $P \leq 0.05$ for the period 2-8 days; $P \leq 0.10$ for the period 9-15 days and $P \leq 0.01$ for the period 2-15 days. All other interactions (2 factor interaction; male age x dose and female age x dose and 3 factor interaction; male age x female age x dose) were not significant.

The adjusted mean and standard error for unequal numbers of the transformed fertility data are presented in Fig. 4.8. It is observed that the fertility produced by higher insemination doses was significantly higher than lower insemination doses. The fertility obtained with old age females and middle age males at the higher insemination dose was superior for all the three periods of fertility measurement, but this was not evident at the lower

Table 4.7. The factorial analysis of the effect of male age, female age and dose effect on transformed fertility data

Analysis of Variance

Source of variation	d.f.	Mean Squares			Variance ratio		
		2–8 days	9–15 days	2–15 days	2–8 days	9–15 days	2–15 days
M age	2	6604.8	4334.5	3280.7	9.13***	14.18***	10.65***
F age	2	462.0	140.5	244.7	0.64	0.46	0.79
Dose	1	5472.6	1698.1	2177.4	7.57**	5.56*	7.07**
M age x F age	4	2242.1	693.0	1049.1	3.10*	2.27+	3.41**
M age x Dose	2	701.3	3.7	275.4	0.97	0.01	0.89
F age x Dose	2	897.3	639.7	276.0	1.24	2.09	0.90
M age x F age x dose	4	925.3	80.8	151.8	1.28	0.26	0.49
Error	402	723.4	305.6	308.0			
Total	419	778.3	329.3	331.4			

+ $P \leqslant 0.10$; * $P \leqslant 0.05$; ** $P \leqslant 0.01$; *** $P \leqslant 0.001$

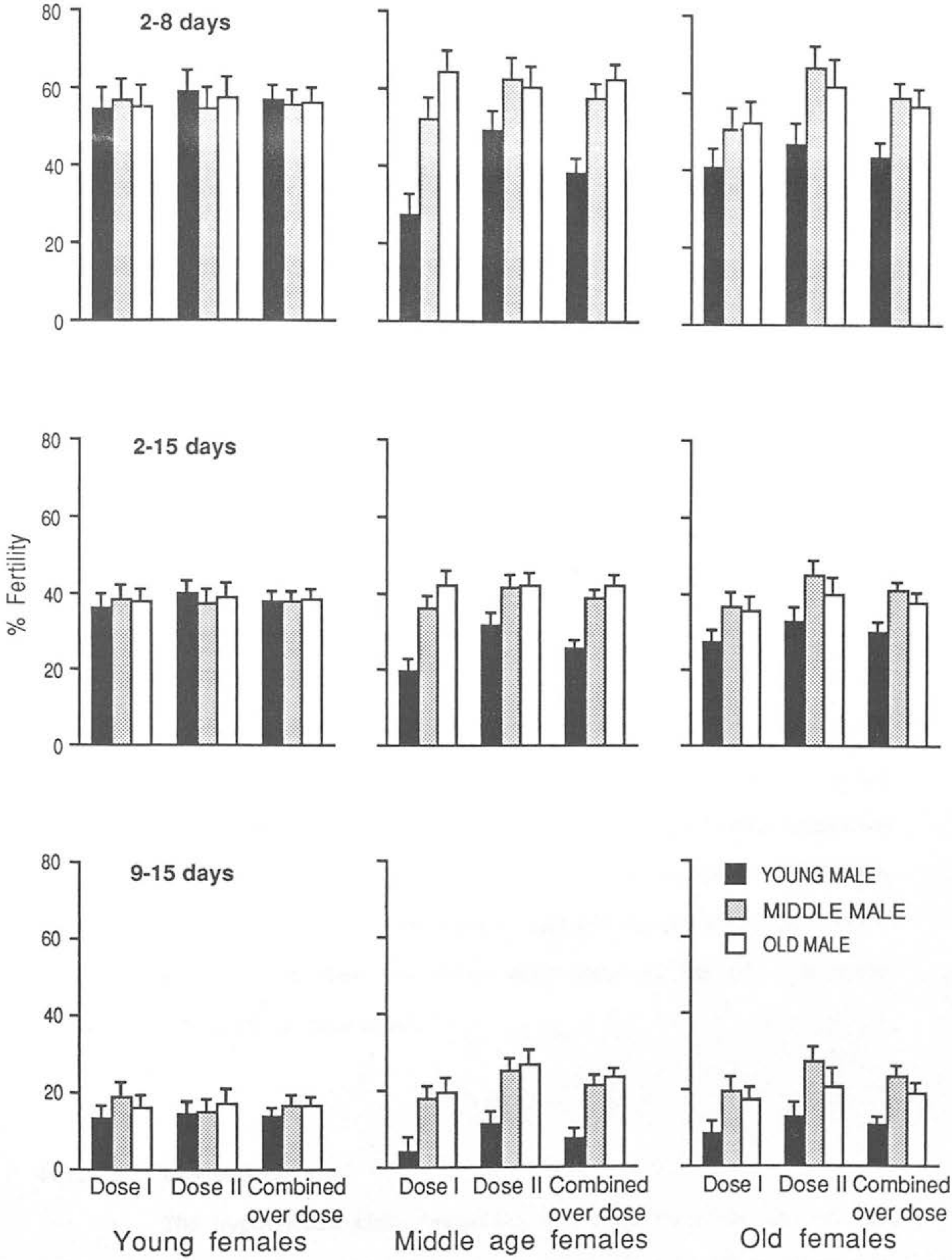

Fig.4.8 Transformed fertility Mean and SE at three ages of the male and female and at two different insemination doses and also on combined data over doses.

insemination dose. However, when data for the doses were combined the superiority of middle aged males was evident which was more prominent for the 2-15 day and 9-15 day periods than for the 2-8 day period. The fertility produced with old age males and middle age females was superior to that with young and old age females inseminated with semen from old age males, and was consistent for all the fertility periods and doses of spermatozoa except during the 2-8 day period with the higher insemination dose.

The fertility produced with young age males with middle and old age females was significantly lower than that obtained with young age males and young age females, and was evident in all the three periods of measurement of fertility with both doses of spermatozoa. The fertility produced with young age males with young age females was no different from middle age or old age males with young age females, but the fertility produced by middle age males with young age females was much inferior to the fertility obtained with middle age males with middle age or old age females. Also this trend was evident with old aged males, and prominent with the higher insemination dose and when the doses were combined for all the three periods of fertility measured.

4.3.1.5 DISCUSSION

The hypothesis that fertility declines with the age of the male was not proved in this experiment with frozen semen which was collected from males at 67 weeks of age and inseminated into females at 54 weeks of age. However, semen collected and frozen from males when they were young (28 weeks of age) resulted in *

This may be due to the fact that at the young age with very high testicular function, many immature spermatozoa were produced. With fresh insemination this is not detected but with frozen semen, when a great proportion of spermatozoa are lost due to freezing and thawing, this is evidently revealed especially with the low insemination doses chosen for the experiment. This effect was not detected when using young females which may indicate that they have a higher fertility potential than when middle or old aged.

Evidence has been obtained from the analysis of variance that the age of the male and the dose of spermatozoa are highly significant in affecting fertility. The age of the female is not significant but there is a male age x female age interaction. Harper and Arscott (1969) concluded in turkeys that only the age of the female was likely to contribute to a seasonal fall in fertility. However, in making a comparison, it has to be kept in mind that the reproductive physiology in turkey is different from the layer-type fowl. Also, in this experiment the old age of the female was chosen as 54 weeks when the birds were quite capable of laying for another 20 weeks. Harper and Arscott (1969) reared their turkeys in a range house when they found marked seasonal decline in fertility. In the present study the birds were maintained throughout in an

*a reduced fertility compared with semen from older males when inseminated into middle (41 weeks) and old (54 weeks) age females.

environment-controlled house. It is also possible that effects of season may be greater on turkeys than on egg-type fowl.

Lorenz et al. (1959) working with turkeys stated that "spermatozoa have a shorter life in old hens". In the present experiment with fowl, semen from middle and old aged males produced good fertility with old and middle aged females. In the present study both males and females were chosen as good quality producers of semen and eggs which was not the case in the studies of earlier workers. It enabled the use of more uniform gamete quality in the present work to test effect of age.

To summarise and conclude, it is found that semen from too young males tends to be inferior. Above that age the semen quality stabilised and maintained good quality up to old age (in this case 67 weeks). A female effect on fertility could not be observed up to 54 weeks of age. In the present study the males and females were not growing old together, therefore preventing confounding effects of ages and sexes.

Thus, for preserving semen in semen banks it is desirable to exclude the semen from very young males to be certain of good fertilising ability.

4.3.2 EFFECT OF FREQUENCY OF SEMEN COLLECTION ON FERTILISING ABILITY

4.3.2.1 INTRODUCTION

Artificial insemination (AI) is in much greater use in the fowl industry today than a decade ago. The turkey industry depends 100% upon AI because of the anatomical configuration and large size of the male. With the ever increasing body size of the broiler breeder nowadays, we may not be far away from having to adopt compulsory AI in their breeding. In the layer industry, so far, AI is limited to selecting and breeding the stock of elite populations, and to 'Grand Parent' operations involved in basic breeding with an objective of achieving higher accuracy in maintaining pedigree data as well as gaining on economic returns by reducing necessary floor space and numbers of breeding males.

With the development of modern semen management technology, new targets can now be set up to maximise the required number of progeny from a fewer number of quality cocks with the desired genetic make up. This will be achieved partly by using the minimum semen dose containing the minimum number of spermatozoa per insemination to derive optimum desired fertility. This aim will be helped by introducing the use of diluted and stored semen.

Recent research has shown that good fertility can be obtained from a single insemination with low numbers of good quality spermatozoa (about 10 million) (Wishart, 1985; Lake and Ravie, 1987). With the use of diluents this would enable an elite sire with good semen quality to cover many more hens than is usually thought possible. Another advantage for enabling the spreading of the influence of an elite sire would be to be able to collect semen very frequently without affecting the quality of semen otherwise obtained.

The libido of the domestic male fowl has been observed by a number of workers; Hauser (1916), Penquite et al. (1930) and Guhl (1951) reported that mating frequency ranged from 0 to 41 per male per day. It is questionable whether all these matings were complete with production and ejaculation of viable spermatozoa capable of producing optimum fertility. At the other extreme, it has been observed that resting cocks for too long (more than 2 weeks) affects their semen quality. Increasing rest between ejaculations causes an increase in the proportion of morphologically-abnormal spermatozoa in semen samples (El Jack and Lake, 1966). Parker et al. (1940) stated that in natural matings, as observed for 15 min periods following 36 hr rest, repeated continuously monthly for seven consecutive months, the volume, density and numbers of spermatozoa decreased with each successive ejaculation and very few were observed after three or four ejaculations. In artificial ejaculations, Parker et al. (1942) suggested that collecting semen daily from a male fowl would probably yield the greatest number of total spermatozoa over a short unspecified period. Lorenz et al. (1955) found that for turkeys the number of spermatozoa decreased gradually during the first week of daily collection. McCartney et al. (1958) reported that daily semen collection from turkeys had no adverse effect on fertility, hatchability or spermatozoal concentrations, but there appeared to be a gradual decrease in semen volume as frequency of collection was increased from 1 to 5 successive days. McDaniel and Sexton (1977) studied broiler males over a 13-week period, and males from a layer Leghorn strain over 20 weeks, for effects of frequent semen collection. It was collected daily, twice daily and three times weekly from broilers, and weekly, three times weekly and 5 times weekly from layer type males. It was concluded that the frequency of semen collection had a definite effect on semen volume and density of spermatozoa but not on fertility. This conclusion was drawn after fresh insemination of 175 million or more spermatozoa (0.05 ml of undiluted semen) per female. Tan (1980a) conducted a trial on the semen production of Muscovy ducks over 24 consecutive days. With different frequency of collection, e.g. twice daily, once daily, once every two days and once every 3 days, it was observed that though twice daily collection gave the highest total semen volume, there was little difference between once daily and twice daily collection in the total yield of spermatozoa. The recommendation was that daily collection was the most efficient for production of spermatozoa for artificial insemination for a 24-day period.

At present most of the commercial farms breeding fowl practise thrice weekly collection of semen when using artificial insemination. The objective of the present experiment was to see how such a practice compared with more, or less, frequent collections of spermatozoa as judged by their sensitivity to being stored *in vitro* regarding their quality either in the frozen or liquid state.

4.3.2.2 MATERIALS AND EXPERIMENTAL PROCEDURES

Males were of a Rhode Island Red strain and hens were of a commercial egg-laying strain (for details see Materials and General Procedures; Birds, Page 40) housed in individual cages at 18 weeks of age and given a constant light 14 hr/24 hr and fed a proprietary breeder ration *ad libitum.*

From a population of 60 males, 20 medium to good category males were selected on the basis of laboratory *in vitro* tests of semen quality (for details of categories of males and evaluation tests see Materials and General procedures, Page 45).

Four males were allotted randomly to each of the 5 different treatments (frequencies of semen collection). The treatment was continued for 28 days, which involved collecting semen:

1. Twice daily (09.00 and 16.00 h),
2. Once daily (09.00 h),
3. Thrice a week (09.00 h),
4. Once a week (09.00 h),
5. Once in 2 weeks (09.00 h)

On the 28th day, pooled semen was collected from each group of males. Pooled semen was used for each treatment, to obtain sufficient semen from each group to measure concentration of spermatozoa, to inseminate a group of hens with fresh semen and to have at least 1 ml to store for 24 hr. This would not be otherwise possible using individual males considering the normal average volume of an ejaculate.

In rotation, a portion of semen (1 ml) from each group was diluted 2-fold in a glutamate based diluent (pH 7.1) and placed in storage at 5°C for 24 hr in a refrigerated bath. The diluted semen was contained in long pyrex tubes, 12 mm i.d. The diluent is recognised as suitable for long-term storage of semen at low ambient temperature, 5°C (Lake and Ravie, 1979). Next, a portion of each of the semen samples was diluted immediately 4-fold in a diluent containing 150 mM NaCl and 20 mM TES adjusted to pH 7.4 with 1N.NaOH. With this the concentration of spermatozoa was measured (for method see Page 44) for each group and was found to be 4.9, 5.2, 5.6, 3.9 and 3.4 million/c.mm, respectively. These samples were further diluted 5-fold (final dilution, X 20) and volumes of the diluted semen were inseminated, adjusted to give 10×10^6 spermatozoa to each hen allotted to the different treatment groups. Wishart (1985) demonstrated that this number of spermatozoa was optimum for examining treatment differences as it avoided masking the presence of defective spermatozoa due to "swamping" with excessive spermatozoa. The hens were allotted randomly to treatment (for details of procedure see page 46).

After 24 hr, 50 x 10^6 stored spermatozoa were inseminated at the same time of the day as for previous fresh insemination.

Because it was not possible to freeze specimens of semen on the same day that the semen for the above sets of treatments was handled, the schedule of different frequencies of collection was continued for another 14 days with the same groups of males. Samples of semen were then collected and frozen in glass ampoules after 4-fold dilution in a freezing medium according to the method of Lake et al. (1981). The cooling rate was 3°C per minute from +5°C to -35°C and then the ampoules were plunged into liquid nitrogen for storage. After nine months, the frozen semen was thawed in alcohol at 5°C for 5 minutes, the concentration of glycerol in the diluted semen was reduced to below 1% by serial dilution with a glutamate-based buffer described by Lake et al. (1981). The samples were then spun at 5°C (700 g, 15 min), the supernatant removed and the pellet of spermatozoa reconstituted in non-glycerolised diluent (Lake et al., 1981).

The whole experimental procedure was repeated once.

Approximately 160 million frozen and thawed spermatozoa were inseminated into each hen. In normal practice for frozen semen, 300 to 350 million or more are inseminated. In the present experiment, a lower dose was chosen hoping to magnify any differential treatment effect on fertility due to frequencies of collection of semen.

The data for analysis of fertility differences was based on 746 fertile eggs out of 1217 eggs laid by 116 hens for fresh insemination, 766 fertile out of 1338 eggs laid by 122 hens for insemination of semen stored at low ambient temperature and 614 fertile out of 1096 eggs laid by 117 hens for insemination of frozen semen. Thus, the statistical analysis involved comparing 5 different frequencies of collection and 3 storage conditions and was based on 2126 fertile eggs out of 3651 eggs laid by 355 hens.

The fertility data for individual hens was transformed by arc sin $\sqrt{\%}$ transformation on which the statistical analysis was carried out (see General Procedures, Page 53).

The statistical model used was an analysis of variance - two way analysis with interaction:

$$Y_{ijk} = \mu + x_i + y_j + (xy)_{ij} + e_{ijk}$$

where μ = population mean

x_i = ith frequency of collection (i=1-5; twice daily, daily, thrice weekly, weekly and fortnightly)

y_j = jth storage condition (j=1-3; control - fresh, 5°C or frozen)

$(xy)_{ij}$ = Interaction between ith frequency and jth storage condition

e_{ijk} = random error with mean 0, and variance σ^2, which is normally and independently distributed

Y_{ijk} = transformed data of fertility of kth individual hen of the ith frequency of collection and jth storage condition.

4.3.2.3 RESULTS AND DISCUSSION

The mean and standard error of the mean (SEM) of percentage fertility are presented in Table 4.8. The fertility was classified as fertile eggs laid by the hens during days 2-8, 9-15 and 2-15 after insemination. The mean fertility of eggs laid over 2-15 days between different "frequency groups" ranged from 54.09 to 71.70 for fresh, 51.46 to 64.86 for 24 hr stored and 42.43 to 71.20 for frozen stored semen. During 2-8 days the results were 81.28 to 94.93 for fresh, 77.87 to 89.53 for stored and 67.39 to 92.57 for frozen semen, respectively. A knowledge of the fertility expected during this latter period is important in practice where semen is normally inseminated once a week to maintain optimum fertility over an extended breeding period of many months.

The mean squares of transformed fertility data obtained by analysis of variance (two way classification) for different periods are presented in Table 4.9. The data indicate that there were no significant differences due to storage of semen for all the three periods of fertility. Significant differences between different frequencies of semen collection was evident for the 2-8 days period ($P \leq 0.05$) and the periods 9-15 and 2-15 days ($P \leq 0.01$; both periods) following insemination. Overall, the 2-15 day fertility data is the best indicator of treatment effects; the 2-8 day period covers only the peak fertility and the 9-15 day period covers the rate of reduction of fertility. However, the interaction component, storage x frequencies, was significant for the 2-8 day period ($P \leq 0.05$) and the 2-15 day period ($P \leq 0.01$) but not for day 9-15. This indicates that there was an association between storage conditions and frequencies of collection.

The transformed fertility mean and SEM for individual treatment groups and overall mean for each period (pooling the data for 3 storage conditions) are presented in Table 4.10, for comparison between pair of treatments. Table 4.11 shows the levels of significance of the differences after application of the "t test" for the pairs of treatments which were significantly different. It was observed that the semen collected least frequently (once in one or 2 weeks) differed significantly from those collected most frequently (twice daily, daily and thrice weekly; see Tables 4.10 and 4.11); ($P \leq 0.05$) in fresh and 24 hr stored sample; and ($P \leq 0.01$) in frozen and overall (pooling the data of all 3 storage situations). The significant differences of the pooled means of transformed fertility between least frequent (once in one and 2 weeks) and most frequent (twice daily, daily and thrice weekly) were 0.0975, 0.1261 and 0.1384, respectively, for fresh insemination

Table 4.8. The mean and SEM of fertility data obtained with fresh, stored and frozen semen collected from males subjected to different frequencies of collection

Frequency of collection		% Fertile eggs + SEM during the periods after insemination		
		2-8 days	9-15 days	2-15 days
Twice-a-day	Fresh (23)*	83.55+5.14	28.18+6.18	54.09+4.88
	Stored (25)	77.87+5.30	28.41+4.63	51.46+4.43
	Frozen (14)	88.33+4.51	43.57+8.15	71.20+5.07
Once-a-day	Fresh (23)	94.52+2.56	37.80+5.11	66.23+3.28
	Stored (25)	83.67+5.21	32.96+4.68	57.89+4.25
	Frozen (23)	67.39+6.61	16.85+4.63	42.43+4.91
Thrice-a-week	Fresh (23)	82.50+4.45	27.22+4.79	56.10+4.45
	Stored (25)	78.13+5.56	27.69+5.05	51.58+4.75
	Frozen (26)	75.64+6.23	24.94+5.03	51.31+4.70
Once-a-week	Fresh (24)	81.28+5.07	37.14+6.97	59.05+5.57
	Stored (22)	89.53+2.60	38.78+5.77	64.86+3.72
	Frozen (24)	92.57+2.87	32.98+5.29	64.71+3.48
Once-in-2-weeks	Fresh (23)	94.93+3.78	50.09+6.08	71.70+4.23
	Stored (25)	86.63+3.82	39.17+4.92	63.76+3.69
	Frozen (30)	88.71+3.89	34.33+4.94	61.35+3.15

( )* Number of hens

Table 4.9. Analysis of variance of transformed fertility data due to different frequencies of semen collection and different storage conditions

Source	df	Mean squares at periods after insemination		
		2-8 days	9-15 days	2-15 days
Between storage	2	0.2874^{NS}	0.3144^{NS}	0.1418^{NS}
Between frequencies of collection	4	0.3905*	0.5484**	0.3343**
Interaction (storage x frequency)	8	0.3426*	0.2321^{NS}	0.2133**
Error	340	0.1349	0.1421	0.0658

* Significant at $P \leq 0.05$, ** significant at $P \leq 0.01$

Table 4.10. Transformed fertility means and SEM for different periods after insemination compiled for various semen storage situations with semen collected at different frequencies

Frequencies of collection	Treatment No.	PERIOD FOLLOWING SINGLE INSEMINATION: 2-8 days				9-15 days				2-15 days			
–		Fresh	Stored	Frozen	Overall	Fresh	Stored	Frozen	Overall	Fresh	Stored	Frozen	Overall
Twice-a-day	1	1.267 ±0.40 (23)*	1.175 ±0.41 (25)	1.352 ±0.31 (14)	1.252 ±0.38 (62)	0.438 ±0.43	0.484 ±0.35	0.662 ±0.41	0.508 ±0.39	0.821 ±0.28	0.790 ±0.26	1.014 ±0.22	0.849 ±0.27
Once-a-day	2	1.458 ±0.22 (23)	1.286 ±0.42 (25)	1.025 ±0.47 (23)	1.257 ±0.42 (71)	0.643 ±0.33	0.553 ±0.33	0.313 ±0.35	0.507 ±0.36	0.968 ±0.20	0.871 ±0.23	0.682 ±0.30	0.841 ±0.27
Thrice-a-week	3	1.249 ±0.33 (23)	1.196 ±0.43 (25)	1.157 ±0.48 (26)	1.199 ±0.42 (74)	0.465 ±0.35	0.458 ±0.35	0.422 ±0.38	0.457 ±0.36	0.849 ±0.23	0.795 ±0.29	0.779 ±0.31	0.806 ±0.28
Once-a-week	4	1.246 ±0.38 (24)	1.336 ±0.25 (22)	1.421 ±0.26 (24)	1.337 ±0.30 (70)	0.614 ±0.51	0.631 ±0.39	0.541 ±0.37	0.595 ±0.42	0.915 ±0.35	0.955 ±0.22	0.944 ±0.19	0.938 ±0.26
Once-every-2 weeks	5	1.484 ±0.27 (23)	1.317 ±0.31 (25)	1.347 ±0.36 (30)	1.378 ±0.32 (78)	0.795 ±0.38	0.639 ±0.32	0.592 ±0.39	0.667 ±0.37	1.041 ±0.27	0.935 ±0.20	0.920 ±0.23	0.960 ±0.24
Overall		1.342 ±0.34 (116)	1.260 ±0.37 (122)	1.257 ±0.41 (117)	1.286 ±0.38	0.592 ±0.42	0.556 ±0.35	0.498 ±0.39	0.549 ±0.38	0.919 ±0.27	0.867 ±0.25	0.857 ±0.27	0.881 ±0.27

* ( ) number of hens.

Table 4.11. Comparisons of the transformed fertility means due to different frequencies of collection with their levels of significance

Storage Situation during insemination	Period following single insemination 2-8 days	9-15 days	2-15 days
Fresh insemination	1-5* 3-5* 4-5*	1-5** 3-5*	1-5** 3-5* [1,2,3]-[4,5]*
24 hr stored insemination	NS	NS	1-4* 1-5* 3-4* [1,2,3]-[4,5]*
Frozen	1-2** 2-4*** 2-5** 3-4*	1-2** 2-4* 2-5**	1-2** 1-3** 2-4*** 2-5*** 3-5* [1,2,3]-[4-5]**
Overall	1-5* 2-5* 3-4* 3-5**	2-5*** 3-4* 3-5***	1-4* 1-5* 2-5** 3-4** 3-5*** [1,2,3]-[4-5]**

Legend 1 = Twice-a-day 4 = once-a-week
2 = Once-a-day 5 = once in 2 weeks
3 = Thrice-a-week

*$P \leq 0.05$ **$P \leq 0.01$ ***$P \leq 0.001$

($P \leq 0.05$), 24 hr stored ($P \leq 0.05$) and frozen ($P \leq 0.01$) for the 2-15 day period (Table 4.11). Although the fertility produced by the "twice-a-day" group was superior to other treatments, it was considered due to a random chance and probably might be due to sample sizes; number of hens (n=14) inseminated in comparison to other treatments (n=25). A higher number of females could not be inseminated for "twice-a-day" group due to low semen volume. Comparison of twice-a-day frequency with daily and thrice-a-week frequency of collection revealed that there is no significant difference either between fresh or 24 hr stored, as well as on overall (Table 4.11) for all the three periods of fertility measured. However, with frozen semen there was evidence of significant differences ($P<0.01$). But comparison of means (Tables 4.8, 4.10 and 4.11) showed the fertility produced by twice-a-day groups was superior to once a day for all the three periods of fertility but for thrice-a-week groups only for the 2-15 day period. This may also be attributed as random sampling error due to low number of hens in twice-a-day group.

In conclusion, it is evident that the fertility from semen collected least frequently (once in one or 2 weeks) is significantly different from those collected most frequently (twice daily, daily or thrice weekly). Also twice daily collection produced superior fertility indicating that during a breeding season, breeders may adopt this frequency when demand for semen is more, and can achieve good fertility at least for a short spell of 4 weeks duration, and is suitable not only in fresh insemination but also in stored (liquid or frozen) conditions.

Considering the possible economic and biological benefit of using males on a schedule of high frequency semen collection, albeit for short breeding periods, the objective of the present experiment was to determine whether there would be a significant adverse effect of high frequencies of collection on the fertilising ability of semen in fresh or stored conditions. McDaniel and Sexton (1977) concluded that frequency of semen collection had a definite effect on semen volume and spermatozoal output but not on fertility per se. In the present study a similar result was obtained with fresh and stored semen samples when it was shown that, except in the case of once-a-week and once-a-fortnight, most of the other frequencies did not produce semen of different fertilising quality. Thus, for a short breeding period at least twice-a-day collection could be adopted in lieu of the common thrice-a-week regime of frequencies of collection when in an emergency when there is an intense demand for chicks and the number of good quality sires is limited. However, if it is desired to collect and freeze-bank elite semen from suitable males available only for a short period, it can be done with very frequent collection of semen provided the dose of spermatozoa is increased over 160×10^6 as there were signs of some difference in the quality of a proportion of spermatozoa rendering them unable to withstand freezing.

The general findings about the fowl spermatozoal quality being satisfactory with very frequent collection from the male, at least if inseminated in the short term, supports the observations that the fowl reproductive organs are capable of generating mature spermatozoa at a much faster rate than mammals (De Reviers, 1968, 1975; Lake, 1984). Changing the frequency of semen collection from once weekly to once daily was found to be associated with greater motility of the ejaculated spermatozoa when tested after storage for 5 hr at 4°C (Reviers, 1975). Lake (1984) suggested that it would have been interesting for De Reviers to have examined the fertilising ability of the more-frequently collected spermatozoa, because "younger" cells, post-testicular, may remain viable longer in vitro. The findings of the present work support this contention. At the other extreme, it is interesting to recall that an increased number of degenerating spermatozoa were found in semen samples when collected very infrequently (El Jack and Lake, 1966).

4.3.3 EFFECT OF STORING SPERMATOZOA IN THE PRESENCE OF RECIPROCAL SEMINAL PLASMA FROM MALES OF DIFFERING REPRODUCTIVE QUALITY AND AGE

4.3.3.1 INTRODUCTION

It has been reported that fowl seminal plasma contains factor(s) that have some stimulatory effects on spermatozoa (Fewlass et al., 1975; Terada and Watanabe, 1978; Terada et al., 1981; Ashizawa and Okauchi, 1984; Lake et al., 1985; Wishart and Ashizawa, 1987). Weakley and Shaffner (1952) showed that fowl semen used freshly could be diluted up to 10-fold with homologous seminal plasma before the fertility dropped significantly below that using whole semen. Gavora and Hodgson (1976) showed that when fresh fowl semen was diluted excessively (up to 100-fold) with whole turkey semen and a small number of spermatozoa (1.4 to 2.7×10^6) were inseminated, there was an apparent enhancement of the rate of fertilisation compared with using an aqueous diluent. They suggested that perhaps unidentified factors in heterologous semen supplied some necessary physiological conditions to support the viability of spermatozoa in vitro, and/or that foreign spermatozoa could aid in the act of fertilisation by the homologous spermatozoa. However, Lake and Ravie (1987) diluted fowl semen 10-fold and 46-fold with either aqueous diluent, fowl seminal plasma, turkey seminal plasma or whole turkey semen and concluded that with freshly diluted semen the best fertility was obtained in the group where fowl semen was diluted with fowl seminal plasma. Dilution with turkey semen components produced fertility no different from that with aqueous diluent when the dose of spermatozoa inseminated was low. Also, no difference was found between using turkey seminal plasma and whole turkey semen. They confirmed that 5.5 to 10 x 10^6 good quality spermatozoa are sufficient to produce acceptable fertility in weekly inseminations of fresh semen. This would enable good quality semen from chosen sires to be highly diluted with advantage in selective breeding programmes requiring many dams per sire. However, at high dilution rates they suggested that there is a need to reconsider the ideal composition of semen diluents, with respect to considering the need for inclusion of as yet unknown factor(s) provided by homologous seminal plasma. Munro (1938) suggested long ago that a pure aqueous diluent may be a less favourable medium for highly diluting fresh fowl semen than a natural fluid per se in order for the spermatozoa to establish themselves in the oviduct for the usual long sojourn.

In commercial breeding, as distinct from selective primary breeding, it is routine practice to use pooled semen samples for insemination. It was considered possible that some of the males in the population may be of poor quality in terms of the semen produced by them, even although they produce a high volume of semen. It is thus of interest for breeding to find out whether these types of males can thereby influence the total fertilising ability of a pooled sample of semen.

It was decided to investigate whether the reciprocal exchange of seminal plasma between semen samples from males designated as good and poor semen producers (see page 45) had any reciprocal effect on the fertilising ability of the suspended spermatozoa. Also, it was decided to investigate whether ageing of males changed the quality of their seminal plasma measured by its influence either directly or during storage for 48 hr on the spermatozoa of young males. Ageing has been suspected to contribute to a lowering of fertility often noticed towards the end of a breeding season (Harris, 1984; Sexton, 1977a). So seminal plasma of aged males was also investigated for possible effects contributing to this deterioration process.

4.3.3.2 MATERIALS AND METHODS

The male fowl used were of a R.I.R. control strain and females were of a commercial white egg-laying strain (age 9 months) (see page 40). The semen quality of individual males was monitored frequently by in vitro tests (see page 41) to ensure that semen was uniform and always of high inherent quality.

Experimental Procedures

Processing of semen to extract seminal plasma

From old males (related males of the previous generation), aged 20 months, of the good and medium category of semen producers (see page 45), seminal plasma was collected separately over several days and stored at -20°C until sufficient was obtained for the experiments. Seminal plasma from the young males (aged 9 months) was collected from designated good and bad males. Some of it was prepared just before use and some was collected 24 hr previously and stored at 4°C. Seminal plasma was obtained by centrifuging semen at 5°C (700 g, 20 min). A flow diagram for the separation of seminal plasma from the different categories of males and the experimental procedure are given in Figs 4.9 and 4.10.

Details of the insemination

Inseminations of the control semen preparations (Groups E, F, Z, Table 4.12; Fig. 4.10) were completed within 1 hr from the collection of semen. Inseminations of semen stored for 48 hr in the presence of the different seminal plasmas were completed within 15 min after removal from the 5°C water bath.

4.3.3.3 RESULTS

The fertility data obtained with spermatozoa stored in the presence of reciprocal seminal plasma, and data about the controls are presented in Table 4.12. The spermatozoa from the inherently poor males gave either no or very few fertile eggs (Groups X, Y and Z) whether inseminated after storage or in fresh condition. Spermatozoa from good males in the presence of seminal plasma from good males gave very good fertility in fresh insemination (Group F) with a very low number of spermatozoa (14 million; 6 μl semen dose). After 48 hr storage, 9 to 17% fertility was obtained with 100 million spermatozoa from good males in the presence of various

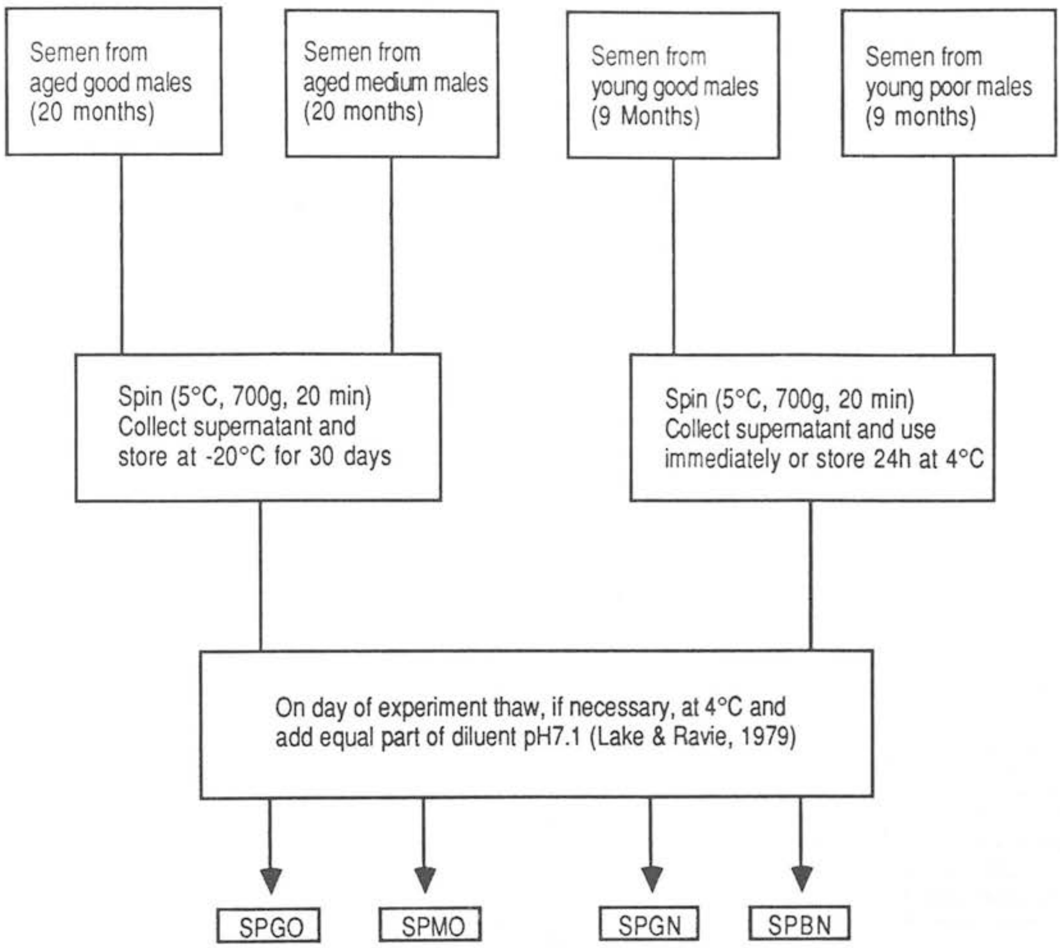

Fig 4.9 Flow diagram for the separation of seminal plasma from different types of males.

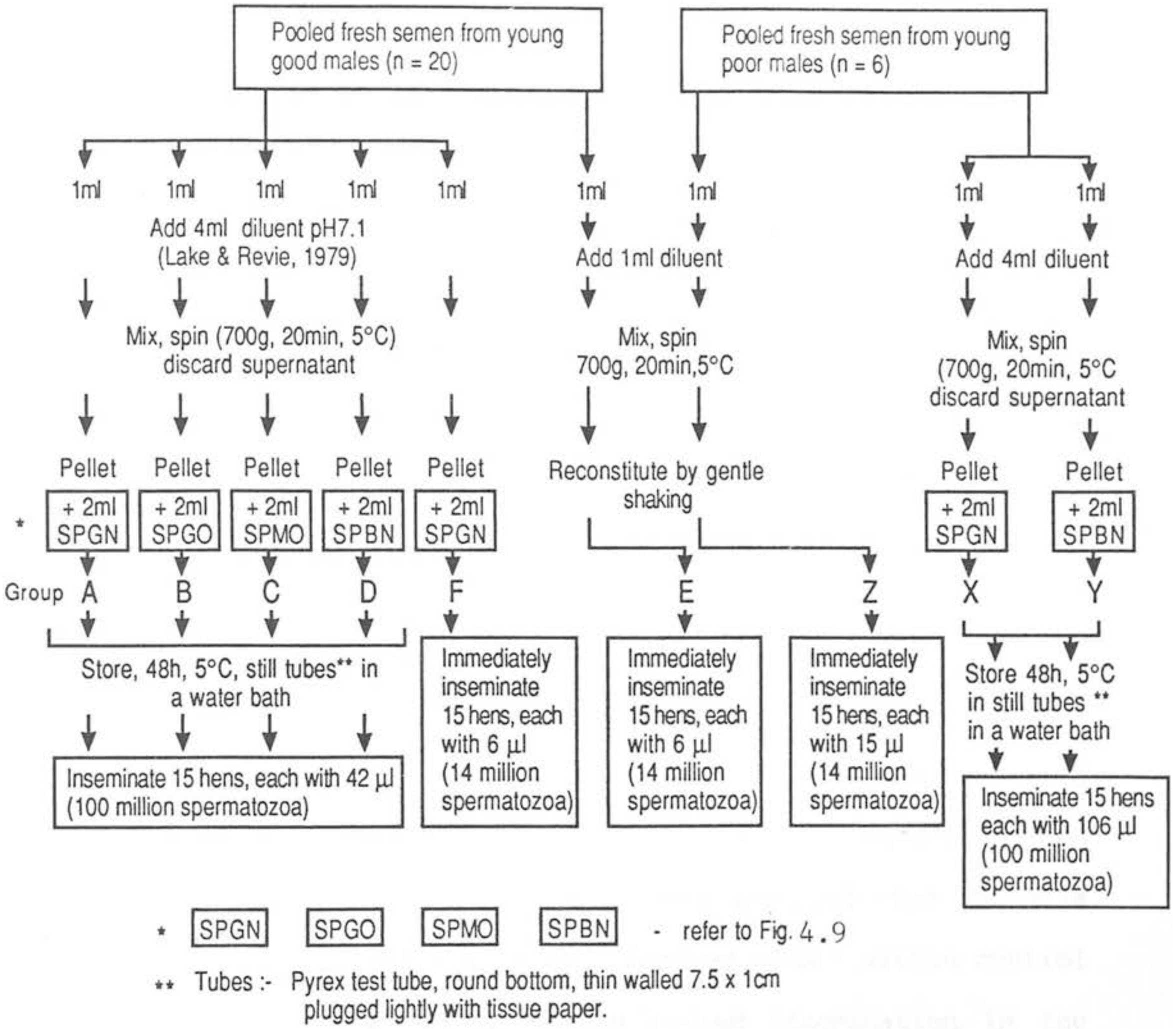

Fig. 4.10 Diagram of the experimental procedure for examining the effects of the reciprocal exchange of seminal plasma.

categories of seminal plasma (Groups A to D). The general level of fertility was low, but this was expected because of the prolonged storage period (48 hr) and the low number of spermatozoa inseminated (100 million) for stored semen. In practice, to achieve high fertility with stored semen about 250 million spermatozoa would be inseminated. The spermatozoa had also been centrifuged which leads to the destruction of a proportion of spermatozoa. However, the level of spermatozoa inseminated was judged to be more likely to differentiate between treatment effects. Surplus spermatozoa can mask the integrity of the treatment effects (Wishart, 1985).

Individual bird fertility data were converted to arc sin $\sqrt{\%}$ for Groups A to F (Table 4.12). The data for Groups X, Y and Z were not included for statistical analysis as the results were unequivocal. Therefore, one-way analysis of variance was conducted separately within controls (Groups E and F) and within stored (Groups A, B, C, D) samples and the results are presented in Table 4.13. No significant difference was observed either within control insemination (Groups E, F) or within stored insemination in the presence of reciprocal seminal plasma (Groups A, B, C, D). Thus, centrifugation per se or exchange of heterologous seminal plasma from good or aged males did not cause any degradation of spermatozoa.

To summarise, it was observed that with fresh sperm from good quality males either in the presence of its own seminal plasma or replaced by seminal plasma from good males which had been stored

Table 4.12. Comparative fertility obtained with spermatozoa stored for 48 hr at 5°C in the presence of reciprocal seminal plasma between males judged previously to be of good and poor fertilising potential, and which were young or old

Group	Spermatozoa	+	Seminal plasma (S.P.)	Insemination Dose	Percentage fertility during periods after a single insemination: 2-8 days	9-15 days	2-15 days
Stored Spermatozoa:							
A	Young "good" Spermatozoa	+	S.P. young good males (SPGN)	42 μl ($100 x 10^6$ sperm)	17.72 (14/79)*	1.13 (1/88)	8.98 (15/167)
B		+	S.P. Aged good males (SPGO)	42 μl ($100 x 10^6$ sperm)	9.57 (9/94)	0.00 (0/83)	5.08 (9/177)
C		+	S.P. Aged medium males (SPMO)	42 μl ($100 x 10^6$ sperm)	12.08 (11/91)	2.22 (2/90)	7.18 (13/181)
D		+	S.P. Young poor males (SPBN)	42 μl ($100 x 10^6$ sperm)	12.35 (11/89)	1.11 (1/90)	6.70 (12/179)
X	Young "poor" Spermatozoa	+	S.P. young good males (SPGN)	106 μl ($100 x 10^6$ sperm)	0.00 (0/94)	0.00 (0/83)	0.00 (0/177)
Y		+	S.P. young poor males (SPBN)	106 μl ($100 x 10^6$ sperm)	1.06 (1/94)	0.00 (0/82)	0.56 (1/176)

Table 4.12. (continued)

Group	Spermatozoa + Seminal plasma (S.P.)		Insemination Dose	Percentage fertility during periods after a single insemination		
				2-8 days	9-15 days	2-15 days
Controls, fresh spermatozoa:						
E	Young "good" semen	Spin and Reconstituted	6 μl ($14x10^6$ sperm)	89.65 (78/87)	51.06 (48/94)	69.61 (126/181)
Z	Young "poor" semen		15 μl ($14x10^6$ sperm)	0.00 (0/95)	0.00 (0/83)	0.00 (0/178)
F	Young "good" spermatozoa	+S.P. Young good males (SPGN)	6 μl ($14x10^6$ sperm)	94.50 (86/91)	47.05 (40/87)	71.59 (126/176)

* ( ) Number of fertile eggs/total eggs laid during the period

Table 4.13. Analysis of variance of transformed fertility data obtained with spermatozoa treated with reciprocal seminal plasma between young, aged, poor and good insemination.

Source	d.f.	Mean Squares 2-8 days	9-15 days	2-15 days
Stored spermatozoa (groups A to D, Table 4.12)				
Between seminal plasma	3	0.0239^{NS}	0.0079^{NS}	0.059^{NS}
Error	56	0.0528	0.0109	0.103
Control, freshly diluted spermatozoa (groups E, F, Table 4.12)				
Between seminal plasma	1	0.052^{NS}	0.0134^{NS}	0.0014^{NS}
Error	28	0.115	0.0784	0.0659

at 4°C for 24 hr, there was no difference in the fertilising ability, thus demonstrating that storage of seminal plasma alone does not affect fertility. The exchange of seminal plasma between good young, good old, medium old or young poor males and subsequent storage of the spermatozoa for 48 hr at 5°C did not influence the fertilising ability.

4.3.3.4 DISCUSSION

The physiological significance of seminal plasma in the natural mating process of the bird is open to question. It acts as a vehicle to help transfer spermatozoa to the storage tubules in the uterovaginal junction of the oviduct. All contact with seminal plasma is likely to be lost soon after ejaculation. From here proportions of spermatozoa travel daily to the upper reaches of the oviduct accounting for the phenomenon of the fertilisation of eggs daily in succession in the fowl for up to 10 to 12 days after a single insemination in exceptional breeding strains (Lake, 1975). All contact by the spermatozoa with seminal plasma is likely to be lost soon after ejaculation, and therefore, it is likely that, if anything, seminal plasma, by virtue of its composition, may serve only to stimulate the active motility of spermatozoa to give them a good chance of reaching the storage tubules. Further movements of spermatozoa thereafter are dependent upon muscular peristaltic movements and possibly constituents of oviduct secretion for assistance. _In vitro_ storage of spermatozoa imposes an additional stress on spermatozoa depending upon the diluent and conditions under which it is kept before insemination. It is likely that if stimulants are necessary to ensure the establishment of spermatozoa in the oviduct, then it is important to establish the natural factors so that proper treatments in vitro can be devised to provide the stimulatory properties to ensure good fertility upon eventual insemination into the female.

Several in vitro studies have been made of stimulants to the motility of spermatozoa and conditions of insemination procedure vital to achieving optimal fertility upon insemination. However, it should be borne in mind that the extrapolation from such studies to explaining natural function, assumes that spermatozoal membrane structure and metabolism are not altered in vitro and that extra types of stimulation are not needed in the natural circumstances.

In commercial poultry breeding practices, commonly semen pooled from several males is used for artificial breeding. Often the individual males are not pre-examined for semen quality and so it was of interest to find out whether intermixing of semen of different quality was likely to be one factor influencing the fertilising ability of a combined semen sample. Also whether age leads to changes in seminal plasma deleterious to spermatozoal activity. The conclusions to be drawn from the results are that the semen of males containing spermatozoa of poor fertilising ability is not reflected in the production of a factor in the seminal plasma harmful to spermatozoa. The spermatozoa from poor males always gave low fertility and the addition of seminal plasma from good males in this case did not have a beneficial (stimulatory) effect. Ageing did not appear to affect seminal plasma in that seminal plasma from aged males was not different from that of young males with respect to influencing fertility.

The fact that spermatozoa from good males were not influenced in any way by seminal plasma either from good, medium, poor or aged males, and the fact that the spermatozoa from poor males always produced low or no fertility, suggests that the fertilising ability of spermatozoa is genetically determined.

4.4 Development of a simple diluent and uncomplicated conditions suitable for storing semen at high temperature (20 and 40°C)

4.4 DEVELOPMENT OF A SIMPLE DILUENT AND UNCOMPLICATED CONDITIONS SUITABLE FOR STORING SEMEN AT HIGH TEMPERATURE (20 and 40°C)

4.4.1 INTRODUCTION

There is well established technology available for indefinite storage of fowl semen by cryopreservation; the technique is costly and of use for establishing semen banks for selective breeding, genetic conservation and long distance transportation of semen (see review Lake, 1986). There are also methods available for the storage of fowl semen at low ambient temperature (5°C) for up to 72 hr (Lake and Ravie, 1979, 1981, 1983; Van Wambeke, 1967, 1972; Sexton, 1977b). For such procedures, a refrigerated water bath is necessary, which is expensive and difficult to use under field conditions, especially in tropical countries. Ashizawa et al. (1976) and Fujihara and Howarth (1978), investigating the motility of fowl spermatozoa at 41°C showed that it could be prolonged for up to 7 days when the spermatozoa were incubated in culture in the presence of oviducal cells/fluid in an atmosphere of 95% oxygen: 5% carbon dioxide. Ashizawa et al. (1976) after culturing spermatozoa for 4 days under such conditions achieved 88% fertile eggs over 7 days after a single insemination of 150×10^6 spermatozoa with the extracted spermatozoa from culture medium. However, culturing of spermatozoa needs sophisticated laboratory facilities and is unsuitable for using stored semen in breeding under field conditions.

Various workers have investigated the possibility of storing fowl semen in conventional tubes at high temperature. Garren and Shaffner (1952) stored fowl semen at 10, 20, 30 and 40°C for 4 hr and inseminated 100 μl neat semen and obtained 40% fertility (eggs laid 2-8 days after insemination) for the sample which was stored at 10°C, the other samples recorded negligible fertility (less than 20% for up to 3 hr storage period and almost no fertility at 4th hr). Schindler et al. (1955) achieved 96% fertility with a large dose of 0.1 ml semen after holding semen for 4 hr at 10°C either undiluted or diluted in Ringer or Locke's solution. However, when semen was held at 41°C for the same time only 3% fertility was achieved. Wishart (1982) showed that under aerobic conditions in the presence of glucose, fertility of 90% or more (eggs laid 2-8 days after a single insemination of 160 million spermatozoa) could be obtained with semen stored for up to 3 hr at 40°C. Under anaerobic conditions, within one hr the fertility dropped to 20% in the absence of glucose, whereas 80% fertility was maintained for up to 3 hr in the presence of glucose. The medium used by him contained chloride, glutamate and phosphate and was buffered to pH 7.4. The ionic constituents of the medium were similar to those found in the fowl seminal plasma. Wishart (1984) investigated semen samples from groups of males varying in tendency to form the products of lipid peroxidation during 5 hr aerobic incubation at 40°C. The formation of malonaldehyde, as an indicator of lipid peroxide formation in the different semen samples, was assayed.

With one particular group of males in which the least malonaldehyde was recorded, 100% fertility was obtained with the semen after 5 hr of incubation in a medium containing glutamate and antibiotics and buffered to pH 7.4. Only 40 to 50% fertility was obtained with other groups in which malonaldehyde formation in semen was of a medium concentration. Almost no fertility was obtained with semen from males where very high concentrations of malonaldehyde were formed.

It would be useful to have a simple methodology to store fowl semen from random males for short periods up to 6-7 hr and longer at ambient room temperature between 20° to 40°C. Flexibility could be introduced into breeding practices on farms. For example, semen could be collected in the morning and utilised in the evening. In the short time, semen could also be sustained for transportation from one farm to another. Also, it would be an advantage for practising AI in tropical countries.

With this objective in view, a first experiment was undertaken to develop a suitable simple diluent and uncomplicated storage conditions to store fowl semen for up to 7 hr at temperatures of 20° and 40°C. It was then of interest to attempt to extend storage to about 16-17 hr so that semen could be collected in the afternoon and utilised in the forenoon of the following day under the same temperature conditions. Apart from the above objectives, the following criteria were set for the development of a method for high temperature storage. The diluent should be simple in composition, economical and easy to produce and keep, and be easy to use under field conditions in temperate and tropical countries. It should not cause a great loss of spermatozoal viability during the storage period so that the minimum number of spermatozoa need be inseminated per insemination.

To achieve the final goal different experiments were carried out sequentially, each investigating factors that might be expected to influence the survival of spermatozoa in vitro until an acceptable method was accomplished. The initial experiments involved only in vitro screening of the activity of spermatozoa as affected by different conditions. The later experiments involved full fertility trials.

4.4.2 MATERIALS, METHODS AND EXPERIMENTAL PROCEDURES

Initially, experiments were set up to compare different salt components, buffer and pH on spermatozoal activity. All the reagents were of analytical grade and glass distilled water was used. The pH and osmotic pressure was measured as described previously in Materials and General Procedures (Page 54).

The male birds used were from a layer-type control strain of Rhode Island Reds and hens were from a commercial white egg laying hybrid. They were housed in cages and fed a commercial breeders ration ad libitum and were subjected to 14 hr light/24 hr. (For details see Materials and General Procedures, Management of Birds (Page 40). Only pooled semen, judged to be of good and medium quality from males, was used for the semen storage study after certain tests (Page 45).

During the course of investigation, diluted semen samples were kept in glass tubes, glass conical flasks (10 or 25 ml capacity), petri dishes (of different diameter) and either placed on the bench top or in a controlled water bath pre-set to either $5^{\circ}C$, $20^{\circ}C$ or $40^{\circ}C$, with facilities for shaking if required. The neck of the flasks were covered with a layer of parafilm, tubes with loosely packed tissue paper and petri dishes with their respective cover to prevent loss of volume due to evaporation. In early sets of experiments the viability of the spermatozoa after storage was assessed by the in vitro tests as previously described (motility by hanging drop, dye-reduction test, morphology test). Later, fertility trials were done as described on Page 46. The statistical analysis of the fertility data was only conducted on arc Sin $\sqrt{\%}$ transformed data of individual hen fertility records. Analysis of variance (either one-way or two-way) was conducted with interaction model, depending on the experiment, to test certain hypotheses.

4.4.3 RESULTS AND DISCUSSION

4.4.3.1 Investigation of salts and conditions used in high temperature storage

Sodium glutamate is considered to be a suitable salt component for storing fowl semen at $5^{\circ}C$ (Lake and Ravie, 1979, 1981, 1983). Isotonic sodium chloride solution alone has been found suitable for diluting and inseminating fowl semen at ambient temperature provided the semen was inseminated quickly after dilution (personal observation). At alkaline pH 7.4 after anaerobic storage for 24 hr at 5°C at a 2-fold dilution rate in still tubes (round-bottomed, thin walled, 7.5 X 1 cm), it was found that there was a tendency for over-stimulation of spermatozoa and a reduction of fertility compared with storage in diluents buffered at pH 7.1 or 6.8 (Lake and Ravie, 1979). Aeration of fowl and turkey spermatozoa during storage is a factor which controls the retention of the fertilising ability of semen (Lake and Wishart, 1984; Wishart, 1982).

Thus, firstly, it was of interest to see whether a solution of pH 6.8 would be of benefit in conserving spermatozoal energy at high temperature during storage for a short duration of 5 hr. Four simple solutions were prepared without glucose (A, B, C, D; Table 4.14). Pooled semen was collected from males and aliquots were diluted 4-fold (1 ml semen + 3 ml diluents in duplicate in 25 ml flasks) with each of the diluents, A, B, C and D. Other aliquots of the same pooled sample were diluted 2-fold (1.0 ml semen + 1.0 ml diluent in 1 cm X 7.5 cm round bottom tubes) in each of the diluents A, B, C and D. One set of diluted semen in flasks and a set in tubes were kept at 40°C for 5 hr in a thermostatically controlled water bath. Other sets of the samples in flasks were kept in a horizontal shaker (140 times to and fro/min) maintained at 40°C for the same period. Anaerobic conditions could be expected to prevail in diluted semen stored in tubes under this condition as detected by tests with an oxygen electrode (Wishart, 1981).

After 5 hr of storage, motility of the spermatozoa was judged by the hanging-drop method. Also, the change in pH as well as the morphological status of the samples were recorded by a pH meter and under a microscope (300 spermatozoa were observed on a stained slide under oil immersion), respectively. The metabolic integrity of the spermatozoa were also assessed by the 'dye-reduction test' developed as part of the thesis (Page 211). This was done only on the few samples which showed promising results by the motility test (hanging-drop method).

The results are presented in Table 4.15. With buffers of this strength all the samples except those in still tubes became greatly alkaline in the 5 hr period. From previous experience (Lake and Ravie, 1979) alkalinity would be expected to be associated with causing a reduction in fertility at low temperature storage. However, according to motility, morphology and the metabolic integrity (shown by the dye-reduction tests), solution 'D', Table 4.15, could be considered the most suitable for storing spermatozoa under the conditions of this experiment at 40°C. Under still conditions, the semen stored poorer than under shaking conditions and was also poorer in tubes than in flasks at this temperature.

Next an attempt was made to prevent the rise of pH to find out whether it would improve the survival of spermatozoa. The solutions were modified to include glucose to encourage anaerobic

Table 4.14. Composition of the diluents used for storing fowl semen at high temperature

	Diluents									
Compound	A	B	C	D	E	F	G	H	I	J
Sodium glutamate H_2O	2.84	-	2.84	-	2.84	-	2.84	-	-	2.31
Sodium chloride	-	0.872	-	0.872	-	0.872	-	0.872	0.70	-
TES*	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46	-	-
Glucose	-	-	-	-	0.60	0.60	0.60	0.60	0.55	0.55
1N NaOH (ml)	0.24	0.32	0.84	0.94	0.28	0.36	0.86	0.96	3.30	2.95
pH	6.80	6.80	7.40	7.40	6.8	6.8	7.4	7.4	6.8	6.8
BES**	-	-	-	-	-	-	-	-	2.132	2.132
Osmotic pressure (mOsmol/Kg H_2O)	312	309	321	312	340	340	363	344	398	401

Values in g, unless otherwise stated, dissolved and made up to 100 ml with distilled water.
* N-Tris (Hydroxymethyl)methyl-2-amino ethane sulphonic acid.
** N-Bis (2 Hydroxymethyl)-2-amino ethane sulphonic acid.

Table 4.14 (contd). Composition of the diluents used for storing fowl semen at high temperature

Compound	Diluents								
	K	L	M	N	O	P	Q	R	S
Sodium glutamate (H_2O)	-	2.31	-	-	-	-	-	2.56	1.52
Sodium chloride	0.70	-	0.80	0.80	0.80	0.80	0.80	-	-
TES*	2.293	2.293	1.374	1.374	1.374	1.374	1.374	1.374	-
Glucose	0.55	0.55	0.60	-	-	0.60	1.20	0.60	0.60
1N Sodium hydroxide (ml)	4.65	4.20	2.75	2.75	2.75	2.75	2.75	2.75	5.60
BES**	-	-	-	-	-	-	-	-	3.05
Antibiotic*** (ml)	-	-	-	-	0.1	0.1	0.1	0.1	0.1
pH	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.4	7.1
Magnesium acetate ($4H_2O$)	-	-	-	-	-	-	-	-	0.08
Tri Potassium citrate (H_2O)	-	-	-	-	-	-	-	-	0.128
Osmotic pressure (mOsmol/Kg H_2O)	405	405	380	345	346	382	421	379	407

Values in g, unless otherwise stated, dissolved and made up to 100 ml with distilled water.
* N-Tris (Hydroxymethyl)methyl-2-amino ethane sulphonic acid.
** N-Bis (2 Hydroxymethyl)-2-amino ethane sulphonic acid.
*** 5 ml stock prepared with 0.25 g Streptomycin sulphate and 0.30 g of penicillin sodium in distilled water.

Table 4.15. The change in pH of semen and the motility, metabolic activity and morphology of spermatozoa after storage for 5 hours at 40°C in 4 different diluents, A, B, C and D, and in different storage vessels and storage conditions

In Vitro Tests		Diluents											
		A			B			C			D		
		St.T	St.F	Sh.F	St.T	St.F	Sh.F	St.T	St.F	Sh.F	St.T	St.F	Sh.F
1. pH	0 hr	6.8	6.8	6.8	6.8	6.8	6.8	7.4	7.4	7.4	7.4	7.4	7.4
	5 hr	6.96	7.46	7.73	7.05	7.56	7.66	7.45	7.90	7.88	7.56	7.93	8.03
2. Motility	5 hr	0	0-0.5	0.5-1.0 (poor)	0	0-0.5	1.00-1.50 (moderate)	0	0-0.5	1.00-1.50 (moderate)	0	0-0.5	1.5-2.00 (moderate)
3. Morphology (% normal sperm)	5 hr	54	48	50	35	43	64	46	56	61	44	62	87
4. Dye-reduction test	5 hr												
i) optical density (A_{520})		-	-	0.44	-	-	0.67	-	-	0.68	-	-	0.75
ii) nMol of formazan produced/min		-	-	28.38	-	-	43.22	-	-	43.87	-	-	48.38
iii) nMol of formazan* produced/min/ 10^9 sperm		-	-	7.27	-	-	11.08	-	-	11.24	-	-	12.40

St.T: still tubes, St.F: still flask, Sh.F: shaking flask
* Incubated at 40°C for 20 minutes.

glycolysis and lactic acid production by the spermatozoa. Four different solutions were prepared with the addition of glucose and buffered at 2 different pHs, 6.8 and 7.4 (Table 4.14, Solutions E, F, G. H).

Pooled semen samples were collected and 4-fold dilutions made in duplicate and stored in 25 ml conical flasks at 40°C under still or agitated conditions as described previously. The pH of semen, and the motility (by hanging-drop method), morphology and the metabolic integrity of spermatozoa, judged by the dye-reduction test, were examined after 6 hr and the results are presented in Table 4.16.

It was observed that all the samples became very acidic after storage under still conditions. Semen samples stored under agitated conditions appeared to be superior to others as judged by the motility and metabolic integrity of spermatozoa. The morphology test revealed a similar result in diluent 'H' for the still and shaking, whereas in the other 3 diluents, E, F and G, still samples were poorer than shaking.

These results show that shaking helped in aerobic metabolism and the better preservation of spermatozoa. Also pH 7.4 is superior to 6.8 at high temperature and the presence of chloride is superior to glutamate.

As a result of the above observations it was decided that agitation was of benefit for high temperature storage and that the buffering of the solutions might be improved with benefit. Thus, an

Table 4.16. The change in pH of semen and motility, metabolic activity and morphology of spermatozoa after storage for 6 hr at 40°C in 4 different diluents, E, F, G and H, and in different storage vessels and storage conditions.

In *vitro* tests		Diluents							
		E		F		G		H	
		St. F	Sh. F	St. F	Sh. F	St. F	Sh. F	St. F	Sh. F
1 pH	0 hr	6.8	6.8	6.8	6.8	7.4	7.4	7.4	7.4
	5 hr	5.45	6.52	5.49	6.61	5.51	6.64	5.38	6.62
2 Motility	5 hr	0	2.0	0	2–2.5	0	2–2.5	0	2.5
3. Morphology	5 hr	42**	62	27	64	47	65	74	76
4. Dye-reduction test	5 hr								
i) Optical density (A_{520})		0.33	0.39	0.11	0.46	0.13	0.50	0.39	0.54
ii) nmol of formazan produced/min		20.90	24.77	6.70	29.29	8.0	31.87	24.77	34.45
iii) nmol of formazan produced/min/10^9* sperm		5.22	6.19	1.67	7.32	2.00	7.96	6.19	8.61

St. F: still flask, Sh. F: shaking flask,
* Incubated at 20°C for 20 minutes.
** % age normal spermatozoa.

experiment was performed with higher concentrations of the buffer components. Four solutions were prepared (Table 4.14, I, J, K, L) and 4-fold diluted semen samples were stored in 25 ml-capacity conical flasks for up to 6 hr at 40°C, some were kept well aerated by shaking and others were kept still for comparison. The buffering of the solution was changed to reduce the drop in pH that occurred during storage which adversely affected the spermatozoa.

After storage for 6 hr, the motility of the spermatozoa, their metabolic integrity and the change in hydrogen ion concentration (pH) in the diluted semen were observed and the results are presented in Table 4.17. On the evidence of the in vitro tests, it was considered that a diluent at pH 7.4, with sodium chloride instead of sodium glutamate, was best (diluent 'K', Table 4.14) for the retention of spermatozoal viability. Also, aeration during storage was better. Also changing the buffer concentration resulted in keeping the pH towards neutrality.

4.4.3.2 Storage of fowl semen for up to 5 hr at 40°c

As a result of the foregoing in vitro tests, it was considered that the NaCl/TES/glucose solution buffered at pH 7.4 [Solution 'K', Table 4.14] was the most suitable for storage at high temperature (40°C), and the agitation created the most favourable conditions to sustain the spermatozoa. However, before attempting to extend the storage of semen beyond a few hr it was decided to modify the formula to reduce the osmotic pressure within the range of 370-380 mOsmol/kg water and adjust the sodium content similar to that in natural seminal plasma. Two solutions, one with and one without glucose, were prepared [solutions 'M' and 'N', Table 4.14] having a sodium content of 0.3719 mg/100 ml fluid. Aliquots of pooled semen were diluted 4-fold (1 ml semen + 3 ml diluent) with each of the diluents [solutions 'M' and 'N', Table 4.14] and placed in 25 ml conical flasks. Some samples were kept still and others shaken. Storage was for 5 hr at 40°C. After storage, 50 μl volume diluted semen (containing 40 X 10^6 spermatozoa) was inseminated into groups of 6 hens as a preliminary investigation. The pH of each diluted sample of semen was measured after storage. Table 4.18 shows the fertility data after storage of semen in diluents at 40°C for 5 hr. Fertility with semen, using the diluent containing NaCl and TES with glucose, buffered at pH 7.4 (diluent 'M', Table 4.14) and agitated, was the best (Table 4.18). Individual fertility data were transformed (see Page 53) and an analysis of variance (two-way, with interaction) revealed that both agitation ($P<0.001$) and the presence of glucose ($P<0.01$) gave significantly better fertility for 2-15 days following insemination. However, the interaction component was not significant as tested for all the three periods (2-8, 9-15 and 2-15 days) of fertility (Table 4.19) indicating that the presence of glucose and agitation of the sample influence independently the resulting fertility and there is no relationship between them.

Table 4.17. The change in pH in diluted semen, the metabolic activity of the spermatozoa and their motility observed after 6 hr storage in 25 ml-capacity flasks at 40°C

Test results		Diluents I	J	K	L
pH, 0 hr storage		6.8	6.8	7.4	7.4
pH, 6 hr storage	still	5.5	5.5	6.2	6.1
	shaking	6.7	6.7	7.0	6.9
Motility	still	NIL	NIL	poor	moderate
	shaking	poor	poor	moderate to good	moderate
Dye-reduction test (A_{520})	still	0.21	0.10	0.15	0.53
	shaking	0.55	0.57	0.85	0.68
nmol of formazan produced/min	still	13.16	6.06	9.29	33.80
	shaking	35.09	36.38	54.45	43.48
nmol of formazan* produced/min/10^9 sperm	still	3.13	1.44	2.21	8.04
	shaking	8.35	8.66	12.96	10.35

* Incubated at 20° for 20 minutes.

Table 4.18. Fertility data using semen that had been stored for 5 hr at 40°C with diluents containing sodium chloride and TES, with and without glucose, buffered to pH 7.4. Samples kept still or agitated in 25 ml flasks.

Diluent	Storage conditions	pH after storage	Percentage fertility during the periods following a single insemination		
			2-8 days	9-15 days	2-15 days
'M', Salt/TES with glucose, pH 7.4	Shaking	7.0	100.00 (30/30)	56.00 (14/25)	80.00 (44/55)
	Still	6.7	27.58 (7/29)	17.39 (4/23)	21.15 (11/52)
'N', Salt/TES without glucose, pH 7.4	Shaking	7.6	89.28 (25/28)	26.08 (6/23)	60.78 (31/51)
	Still	7.5	0.00 (0/27)	0.00 (0/27)	0.00 (0/54)

(   ) Number of fertile eggs/total eggs laid during the period
For composition of diluents 'M' and 'N' refer to Table 4.14.

Table 4.19. Analysis of variance of data in Table 4.18 to test the effect of agitation and the presence of glucose for storage (5 hr) of fowl semen at high temperature (40°C).

ANOVA

Sources of variation	Degree of freedom	Mean squares of transformed fertility data		
		Periods		
		2-8 days	9-15 days	2-15 days
Bet glucose	1	0.4685*	0.6851**	0.6255**
Bet shaking	1	10.0183***	2.0983***	4.0952***
Glucose X shaking (interaction)	1	0.0781	0.0554	0.0197
Error	20	0.0636	0.0669	0.0433

* $P<0.05$, ** $P<0.01$ and *** $P<0.001$ level of significance

4.4.3.3 Storage of fowl semen up to 7 hr at 20° and at 40°C

Having established a basic procedure for high temperature storage of semen, it was next decided to test the effect of adding antibiotics to diluents when attempting to extend semen storage at high temperature beyond 5 hr when it would be expected that micro-organisms may reach a toxic level in the diluted semen. The fertility during the second week (9-15 days) after insemination might thereby be expected to be improved. From previous experience a reduction in fertility at this time often reflects a high death rate of spermatozoa during storage. Additionally, it was thought worth- while investigating conditions for storing semen at an ambient room temperature of 20°C, as distinct from higher temperatures common in some tropical countries.

For this purpose solutions [M, N, O, P; Table 4.14] were prepared with and without glucose and antibiotics, keeping other properties of the diluents constant. Fertility examinations were set up to study the efficacy of the diluents and storage conditions. Chosen treatments were all the 4 diluents [M, N, O, P; Table 4.14] at 40°C and 2 diluents [M, P; Table 4.14] at 20°C. Storage period was 7 hr and the dilution ratio was 4-fold (1 ml semen + 3 ml diluent). The samples were kept in 25 ml flasks and agitated as described previously.

At the end of storage, 36×10^6 spermatozoa (40 μl diluted semen) were inseminated per hen, with 15 hens per treatment. The results (Table 4.20) show that for up to 7 hr, each of the diluents

M, N, O, P; Table 4.14, under the storage conditions yielded reasonably good fertility. Transformation of the fertility data on individual hens and conducting an analysis of variance (two-way) with interaction model, to test the effect of the presence of glucose and antibiotics, on the data for the 40°C storage, revealed that there was no significant differences with and without glucose or antibiotics. The interaction, glucose X antibiotics, was also not significant for any of the fertility periods tested (Table 4.21). Similarly on examination of the transformed fertility data for the 20° storage it was revealed that there was no significant effect of antibiotics between treatments (Table 4.22) which is probably due to the fact that insufficient microbes were produced in 7 hr to damage the fertilising ability of the spermatozoa at the high temperature. Also, during 7 hr there was no benefit in having glucose in the diluent as a substrate. Presumably, sufficient energy was obtained through the operation of oxidative metabolic pathways utilising internal substrates in the spermatozoa.

4.4.3.4 Storage of fowl semen for up to 16 hr at 20° and 40°C

Next, it was decided to see whether the storage time could be further extended to 16 hr. Also, it was thought necessary to see whether glucose concentration needed to be increased for extended storage at the high temperature. For this purpose 3 diluents were prepared containing no glucose, conventional 33.33 mM or double the concentration, 66.66 mM, of glucose [solutions O, P, Q; Table 4.14].

Table 4.20. Fertility data using semen that had been stored in 25 ml flasks (agitating) for 7 hr at 40°C with diluents containing sodium chloride and TES with and without glucose and antibiotics, buffered to pH 7.4. Data also showing effect of storage at 20°C in the presence of glucose, with or without antibiotics.

Diluents	Storage temperature	Percentage fertile eggs laid during the period		
		2-8 days	9-15 days	2-15 days
Salt/TES - Gl (N)	40°C	83.95 (68/81)	36.36 (28/77)	60.75 (96/158)
Salt/TES + Gl (M)		87.14 (61/70)	30.37 (24/79)	57.04 (85/149)
Salt/TES - Gl + AB (O)		88.31 (68/77)	39.51 (32/81)	63.29 (100/158)
Salt/TES + Gl + AB (P)		89.18 (66/74)	40.78 (31/76)	64.66 (97/150)
Salt/TES + Gl (M)	20°C	89.02 (73/82)	21.25 (17/80)	55.55 (90/162)
Salt/TES + Gl + AB (P)		75.64 (59/78)	25.00 (19/76)	50.64 (78/154)

(   ) Number of fertile eggs/total eggs laid during the period
For composition of diluents 'M', 'N', 'O' and P refer to Table 4.14.

Table 4.21. Analysis of variance on data in Table 4.20 to test the effect of the presence of glucose and antibiotic for storage (7 hr) of fowl semen at high temperature 40°C.

ANOVA*

Sources of variation	Mean squares of transformed fertility data			
	d.f	Periods		
		2-8 days	9-15 days	2-15 days
Bet glucose	1	0.026^{NS}	0.009^{NS}	0.0107^{NS}
Bet antibiotic	1	0.052^{NS}	0.208^{NS}	0.0313^{NS}
Glucose X antibiotic (interaction)	1	0.024^{NS}	0.000^{NS}	0.0047^{NS}
Error	56	0.140	0.136	0.0545

* ANOVA was conducted with transformed data.

Table 4.22. Analysis of variance to test the effect of antibiotic for storage (7 hr) of fowl semen at temperature 20° C.

ANOVA*

Sources of variation	Mean squares of transformed fertility data			
	d.f	Periods		
		2-8 days	9-15 days	2-15 days
Bet antibiotics	1	0.309^{NS}	0.070^{NS}	0.0097^{NS}
Error	28	0.160	0.127	0.0746

*ANOVA was conducted with transformed data

Pooled semen was collected and duplicate aliquots diluted 4-fold in 25 ml flasks (1 ml semen + 3 ml diluent) with each of the 3 diluents. Sets were stored with agitation at two different temperatures (20 and 40°C) for 16 hr. For this extended storage time semen was collected in the evening (17.30 hr) and insemination for fertility testing was carried out on the following morning, around 10.30 hr, when very few or no hard-shelled eggs were present in the uterus of the hen. Forty five million spermatozoa (50 μl diluted semen) were inseminated into groups of 15 hens per treatment. The fertility obtained is presented in Table 4.23. Best fertility was recorded with semen stored at both temperatures, i.e. 20 and 40°C, when the diluent contained 33.33 mM glucose. Moderate to low fertility was obtained in the absence of glucose indicating that a proportion of spermatozoa were able to metabolise oxidatively using internal substrates. If the fertility data of this experiment of 16 hr storage is compared with that of the previous experiment, 7 hr storage (Table 4.23 and Table 4.20), it is seen that there is reduction (absolute fertility levels) of fertility with 16 hr storage compared to 7 hr when the diluent was free of glucose, indicating that for the short period spermatozoa will survive by oxidative metabolism with internal substrates. In presence of glucose during storage lactic acid is being produced during metabolism of spermatozoa and the longer the storage time the higher is the lactic acid production. But due to the strength of TES buffer the change of pH to more acid side was controlled. A high concentration of glucose (66.66 mM) caused more acidity in the diluent and it was associated with lowered fertility. The fertility achieved at 20°C incubation was similar to that obtained at 40°C incubation indicating that both temperatures are equally acceptable for storage.

On transformed data on individual hen fertility, an analysis of variance revealed highly significant effects ($P < 0.001$), during all periods of fertility, due to glucose levels, whereas components of variance due to temperatures, or to an interaction between levels of glucose and temperature, were not significant (Table 4.24). This indicates that there is no relationship between temperature and glucose levels for effects on fertility during storage of spermatozoa for 16 hr.

The comparison of individual transformed fertility means, due to glucose levels at each temperature for different periods after insemination, and their levels of significance are presented in Table 4.25. The results confirm the superiority of 33.33 mM glucose for storage at high temperature.

4.4.3.5 Simulation of aerobic conditions, created earlier by agitation in a 25 ml flask, in a vessel in the still state for 6 hr

Now it was considered that a diluent and method for storing fowl semen for 16 hr at high temperature (both at 20 and 40°C) had been developed. For practical application it would be

Table 4.23. Fertility data using semen that had been stored for 16 hr at 20°C and 40°C with diluents containing sodium chloride and TES buffered to pH 7.4, with 33.33 and 66.66 mM glucose and without glucose. Semen stored in 25 ml flasks and placed in a shaking water bath.

Diluent	Storage temperature	Percentage fertile eggs laid during the period 2-8 days	9-15 days	2-15 days
Salt/TES - Gl (O)	40°C	67.12 (49/73)	16.17 (11/68)	42.55 (60/141)
Salt/TES + 33.33 mM (P) glucose		86.11 (62/72)	31.34 (21/67)	59.71 (83/139)
Salt/TES + 66.66 mM (Q) glucose		44.59 (33/74)	9.09 (7/77)	26.49 (40/151)
Salt/TES - Gl (O)	20°C	65.51 (38/58)	18.66 (14/75)	39.09 (52/133)
Salt/TES + 33.33 mM (P) glucose		87.50 (49/56)	40.00 (26/75)	61.98 (75/121)
Salt/TES + 66.66 mM (Q) glucose		40.57 (28/69)	9.58 (7/73)	24.65 (35/142)

For composition of diluents refer to Table 4.14

Table 4.24. Analysis of variance on transformed fertility data of Table 4.23 to test the effect of glucose and temperature of storage (16 hr) on fowl semen.

ANOVA

Sources of variation	d.f	Mean squares of transformed fertility data Periods		
		2-8 days	9-15 days	2-15 days
Between glucose levels	2	3.022***	1.448***	1.311***
Between temperatures	1	0.004^{NS}	0.001^{NS}	0.034^{NS}
Glucose X temperature Interaction	2	0.035^{NS}	0.085^{NS}	0.022^{NS}
Error	84	0.232	0.098	0.094

*** $P \leq 0.001$ level of significance.

Table 4.25. The transformed fertility means of data in Table 4.23 (recorded for the periods 2-8, 9-15 and 2-15 days after insemination), following insemination of semen stored for 16 hr at 20 and 40°C in the presence of different levels of glucose. Also shown is a comparison of their levels of significance.

Diluents	Periods of fertility and temperature of storage						
	2-8 days		9-15 days		2-15 days		
	20°C	40°C	20°C	40°C	20°C	40°C	20° & 40°C combined
1) Without glucose	1.0451	0.9696	0.3772	0.2589	0.6471	0.6733	0.6602
2) With 33.33 mM glucose	1.2432	1.3035	0.5600	0.6438	0.8111	0.9097	0.8604
3) With 66.66 mM glucose	0.6534	0.6291	0.1504	0.1898	0.4468	0.4382	0.4425
Comparisons of means 1-2	NS	**	*	***	*	**	*
and their levels of 1-3	**	**	**	NS	**	**	**
significance 2-3	***	***	***	***	***	***	***

* is significant at $P \leq 0.05$, ** is significicant at $P \leq 0.01$ and *** is significant at $P \leq 0.001$.

more convenient and cheaper if the aeration conditions produced by shaking motion could be simulated in a still vessel. This was achieved by Lake and Wishart (1984) for turkey semen stored at 5°C by increasing the surface area to volume ratio of diluted semen in a container. For the present work, pooled semen was diluted 4-fold [1 ml semen + 3 ml diluent (diluent P, Table 4.14)] and one batch was placed in a 2" diameter petri dish to form a flat film and another batch was placed in a 25 ml conical flask. Precaution was needed so that in the vessel the diluted semen was not in too thin a film to allow evaporative dehydration. The vessels were kept on a bench top for 6 hr at a room temperature around 20°C. After storage, 46 million spermatozoa (50 μl volume) were inseminated into groups of 15 hens.

The fertility data and results of the in vitro tests on spermatozoal integrity are presented in Table 4.26, which showed that there was a greater reduction in normal spermatozoa in the sample stored in a 25 ml flask than in a petri dish, and also there was a reduction in metabolic activity as judged by the dye-reduction test. However, fertility produced under the two storage conditions showed similarity when eggs laid 2-15 days after insemination were considered. The possible explanation for achieving 100% fertility from a sample which showed 63% normal spermatozoa (during the 1st week) may be attributed to the insemination dose of 46 million spermatozoa when enough good quality spermatozoa were still available to obtain such fertility.

Table 4.26. The *in vitro* tests of spermatozoal viability and fertility data after 6 hr storage of 4-fold diluted semen in diluent P, kept on a bench top around 20°C room temperature in still condition in 25 ml flask and in 2" diameter petri dish.

Fertility and In vitro tests		Storage vessel 25 ml flask	2" diameter petri dish
Fertility percentage	2- 8 days	100.00 (66/ 66)	92.20 (71/ 77)
	9-15 days	49.38 (40/ 81)	55.55 (40/ 72)
	2-15 days	72.11 (106/147)	74.49 (111/149)
Motility by hanging drop method (0-4 scale)		2	2
pH at 7 hr		6.95	7.3
Morphology (% normal spermatozoa)		63*	83
nmol of formazan produced per min/10^9 sperm		6.0	9.5
Percentage motility by spectrophotometer method		50.54	58.42

* Many bent sperm were observed.

Table 4.27. Analysis of variance of data in Table 26 to test the storage vessel conditions for holding fowl semen for 6 hr on bench top around 20°C.

Source of variation	d.f	Mean square of transformed data periods		
		2-8 days	9-15 days	2-15 days
Between storage vessels	1	0.2376**	0.297^{NS}	0.0788^{NS}
Error	28	0.0258	0.100	0.0410

** Significant at $P<0.01$.

Using transformed data on individual hen fertility, an analysis of variance revealed (Table 4.27) that there was no significant difference in fertility between storage vessels for the periods 2-15 days and 9-15 days following insemination. However, for the period 2-8 days there was significant difference ($P<0.01$) which could be attributed to random chance because of the lack of variance for the set of data for the semen in the flask where all eggs were fertile. It can be concluded, that both the storage vessels can be recommended for storage of spermatozoa without agitation on the bench top for 6 hr around 20°C.

4.4.3.6 Exploration of the effect of glutamate and chloride on the preservation of fowl spermatozoa at high temperature

Glutamic acid is found in very high concentration in the seminal plasma of the fowl and turkey (Lake and MacIndoe, 1959; Graham et al., 1964). Glutamate is the chief anionic component of diluents that have been successful in storing fowl semen at low ambient temperature at 5°C (Lake and Ravie, 1979; Sexton, 1977b; Van Wambeke, 1967, 1972). Data in Tables 4.15, 4.16 and 4.17 indicated that glutamate was not as suitable as chloride in supporting spermatozoal activity at 40°C. To explore this observation further, three diluents were prepared [diluents P, R, S: Table 4.14]. One was a traditional diluent (diluent S) used for low temperature storage [Lake and Ravie (1979)-"7.1 diluent"], the other was the present diluent (diluent P, Table 4.14) developed for high temperature storage and the 3rd diluent was similar to diluent P in every respect except that sodium chloride was replaced by sodium glutamate (diluent R, Table 4.14). The storage conditions tested were: at 5°C under still conditions [2-fold dilution (1 ml total volume) in tubes], all the 3 diluents P, R and S; at 40°C under agitated condition (4-fold dilution, 4 ml total volume in 25 ml flask), all the 3 diluents P, R and S; and at 20°C under still condition (4-fold dilution, 4 ml total volume in 25 ml flask), the diluents P and S. After 17 hr storage of aliquots of the same pooled sample under the above conditions, 50×10^6 spermatozoa (22 μl volume for 2-fold dilution and 44 μl volume for 4-fold dilution) were inseminated into groups of 15 hens for each treatment. Also, aliquots of samples were examined for metabolic integrity (dye-reduction test) morphology status (% normal spermatozoa) and motility (by hanging-drop method) of spermatozoa.

The results of the in vitro tests are presented in Table 4.28 and the corresponding fertility data are presented in Table 4.29. On examination of the in vitro test results (Table 4.28) it is seen that diluent 'S' [diluent 7.1, Lake and Ravie (1979)] with 2-fold dilution of semen was best under conditions at low temperature (5°C, still) whereas the chloride diluent (diluent 'P') was best at high temperature (40°C, shaking). The simple glutamate diluent (diluent 'R') did not preserve the spermatozoa at high temperature whereas at 5°C (still) there was moderate spermatozoal activity and viability. The fertility data (Table 4.29) show that

Table 4.28. Data on *in vitro* tests of spermatozoal viability, as judged by motility, morphology and dye-reduction, after 17 hr storage in diluents P, R and S at 5°C (still; 2-fold dilution) and at 40°C (agitated; 4-fold dilution) and in diluents P and T at 20°C (still; 4-fold dilution).

In vitro tests	Diluents P (Chloride/TES)			Diluents R (Glutamate/TES)			Diluents S (Traditional 7.1)		
Temperature (°C) Storage condition	40° shaking	20° still	5° still	40° shaking	20° still	5° still	40° shaking	20° still	5° still
Morphology (percentage normal spermatozoa)	71	16	10	0	-	43	0	11	69
Motility in hanging drop, scale of 0-4	2-3	0	2	0	-	3	0	0-1	3-4
nmol of formazan produced per min/ 10^9 spermatozoa in dye-reduction test	9.31	6.08	6.22	0	-	5.94	0	5.10	8.33

For composition of diluents 'P', 'R' and 'S' refer to Table 4.14.

Table 4.29. Fertility data for semen stored for 17 hr with diluents containing chloride/TES (P), glutamate/TES (R) and complex of ions (S) at 40°C (shaking; 4-fold dilution) and at 5°C (still; 2-fold dilution), and also at 20°C (still; 4-fold dilution) with diluents (P) and (S).

Diluents	Percentage fertile eggs laid during the period								
	2-8 days			9-15 days			2-15 days		
	40° shaking (1+3)	20° still (1+3)	5° still (1+1)	40° shaking (1+3)	20° still (1+3)	5° still (1+1)	40° shaking (1+3)	20° still (1+3)	5° still (1+1)
Chloride/TES (P) pH 7.4	71.26 (62/87)	0.00 (0/93)	0.00 (0/97)	17.77 (16/90)	0.00 (0/94)	0.00 (0/ 91)	44.06 (78/187)	0.00 (0/187)	0.00 (0/188)
Glutamate/TES (R) pH 7.4	0.00 (0/93)	-	6.52 (6/92)	0.00 (0/91)	-	0.00 (0/97)	0.00 (0/184)	-	3.17 (6/189)
"7.1" (S)	0.00 (0/92)	0.00 (0.94)	63.44 (59/93)	0.00 (0/99)	0.00 (0/91)	13.18 (12/91)	0.00 (0/191)	0.00 (0/185)	38.58 (71/184)

(   ) Number of fertile eggs/total eggs laid during the period.

Table 4.30. Analysis of variance of data in Table 4.29 to test effects of diluents for 17 hr storage; Diluent "7.1" (S) at 5°C, still, 2-fold dilution and chloride/TES solution (P) at 40°C, shaking, 4-fold dilution.

		ANOVA*		
Sources of variation	d.f	Mean squares of transformed fertility data		
		Periods		
		2-8 days	9-15 days	2-15 days
Between diluents at storage condition	1	0.052^{NS}	0.0184^{NS}	0.014^{NS}
Error	28	0.288	0.0799	0.107

* ANOVA was conducted with transformed data

at 40°C (shaking) reasonable fertility (71.26%) was achieved with the diluent containing chloride (diluent P), compared to 63.44% fertility obtained by diluent 'S' at 5°C (still). No fertility was obtained with diluent 'S' or 'P' in other temperature and storage conditions. Diluent 'R' produced few fertile eggs at 5°C (still condition) and no fertility at 40°C. On comparison of transformed fertility data on individual hens for diluent 'S' at 5°C (still) and diluent 'P' at 40°C (shaking), an analysis of variance revealed that no significant difference existed for all the three different periods of fertility measurement (Table 4.30), confirming that these two diluents are equally efficient in holding quality of spermatozoa for up to 17 hr under particular conditions. The failure to produce good fertility with diluent 'R' at low temperature raises an interesting question as to the precise reasons for the glutamate and chloride effects at low and high temperature, respectively. Little information exists in the literature to explain the precise effects of chloride and glutamate on the function of spermatozoa. El Zayat and Van Tienhoven (1961a, b) gave equivocal results on the effect of chloride and glutamate on spermatozoal respiration and morphology. However, glutamate tended to depress respiration rate after 2 or 3 hr at 5°C. The possibility that, after storage at high temperature, glutamate depresses metabolism below a minimal level compatible with maintenance of the membranes of spermatozoa should be explored.

In summary, a simple diluent containing 138 mM sodium chloride 60 mM TES and 33.33 mM glucose adjusted to pH 7.4 with 1 N NaOH and antibiotics was developed which could hold fowl semen for 17 hr at temperatures between 20-40°C under agitation (140 times to and fro) which resulted in acceptable fertility with insemination of 45-50 million spermatozoa. With the same diluent a simplified method of holding semen at room temperature (around 20°C) was developed either holding in petri dish or in a 25 ml flask. The present diluent produced comparable fertility to a traditional diluent used for low temperature storage. When the sodium chloride component in the new diluent was replaced with sodium glutamate the retention of viability of spermatozoa was lost at high temperature (40°C), but at 5°C a few fertile eggs were obtained.

5. CONCLUDING DISCUSSION AND SUMMARY

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Inherent variation between males exists with regard to fertilising ability of semen samples. This is easily revealed in birds when inseminated artificially with a single insemination of a low dose of spermatozoa, when a hen would be expected to produce fertile eggs daily in succession for a period of 7 to 10 days, or more, with good quality semen. Other males will produce shorter fertile periods to different degrees.

Artificial insemination in poultry could play an important role in the future in directed breeding towards making more rapid genetic gains in desirable performance traits and for conservation of genotypes. This study aimed to examine some aspects of semen evaluation, semen handling, semen harvesting and semen storage which could help in promoting the wider use of AI in temperate and tropical climates.

Prediction of fertility level and control of the production of fertile eggs in an AI programme is only possible with an efficient in vitro test of semen quality. This should now be possible as a result of the present work. Male management factors can influence total fertility success and they are magnified in artificial breeding situations. Present knowledge is limited with regard to some of these factors. In some field situations more flexibility in the use of techniques of extending and storing semen would be an advantage in maximising the use of individual males and pooled semen.

The objectives in the present study were:

1) To develop an efficient, inexpensive and simple *in vitro* test to predict the fertilising ability of semen with high precision;

2) To investigate the influence of seminal plasma from males of different ages and fertilising ability on spermatozoa of known high and low fertilising ability;

3) To study the influence of different frequencies of semen collection on the fertilising ability of fresh, stored and frozen spermatozoa;

4) To investigate the effect of semen collected from males of different ages on fertility with females of different ages;

5) To develop a suitable diluent and simple method for storage of fowl semen at high temperature.

The study was mostly performed with layer-type fowl. The males were a control population of Rhode Island Reds and the females were a commercial white egg-laying strain. All birds were replaced annually from the same source with young genetic stock. At puberty, the males were screened by several *in vitro* tests and sub-groups were designated within the population as 'good', 'medium' and 'poor' quality semen producers. This was an advantage because experiments needing large quantities of pooled, good quality semen for split-sampling could be guaranteed. Repetitive testing revealed that males maintained their ranking with respect to basic spermatozoal quality, even although some individuals varied in the spermatozoal concentration of their semen with age.

With regard to (1) exploration of a colourless dye (INT), [2-(p-iodophenyl)- 3- (p-nitrophenyl)-5-phenyltetrazolium chloride] to exploit a measure of spermatozoal metabolic activity to predict fertilising ability was made. Phenazine methosulphate (PMS), an intermediate electron transfer agent was added to a buffer mixture (containing 116 mM sodium chloride, 15 mM TES, 7.8 mM glucose, 3.1 mM calcium chloride and 1.56 mM potassium cyanide) to facilitate the oxido-reductase electron transfer system in the spermatozoa to cause conversion of the colourless INT by spermatozoa to a coloured formazan compound within 20 minutes at 20°C. Incubation was terminated and formazan released in the assay solution by disintegration of spermatozoa with a biological detergent (5% solution of Acid Triton X100). To measure colour development proportional to the integrity of the spermatozoa, a clear solution was prepared by centrifuging the assay mixture. The colour development was measured at 520 nm with a simple spectrophotometer. Various aspects of the method were investigated to determine the optimum concentration of the dye (INT), PMS, temperature of incubation, time for the incubation, volume of spermatozoa for the asay and cyanide concentration in the assay mixture. Inclusion of cyanide was necessary in the incubation mixture to prevent the normal transfer of reducing equivalents to oxygen to form water, which occurs in normal oxidative metabolism in cell energy production, so that the reducing equivalents were available to INT (through NADH chain via intermediate electron transfer agent, PMS) to be reduced to a non-reversible coloured formazan compound. The concentration of cyanide in the assay mixture was also important because only sufficient cyanide was required which would allow ATP synthesis to continue under the assay conditions without poisoning the spermatozoa.

Extraction of the formazan in the incubation mixture was optimised by investigating suitable biological detergents and their concentration to disintegrate the spermatozoa and liberate the formazan prior to centrifugation. Authentic formazan was used to calibrate a suitable concentration of spermatozoa for accurate measurement in the spectrophotometer at a suitable wavelength. The relationship between the rate of oxygen utilisation by spermatozoa and the rate of INT reduction were established to verify that the in vitro test is a good indicator of the metabolic activity of the spermatozoa. Fertility trials with individual semen samples, and simultaneously a variety of in vitro tests including the dye-reduction test, showed a very highly significant correlation ($r=0.80$; $P<0.001$) between the INT reduction and fertilising ability, and INT reduction, ATP synthesis, motility and proportion of morphologically-normal spermatozoa (highly significant correlation coefficients of $r=0.93$, 0.89 and 0.72, respectively; $P<0.001$).

This confirmed the suitability of the dye-reduction test with other tests for evaluating semen. However, the advantages of the dye-reduction test are many especially as it is most objective, is simple to apply and efficient for handling many samples of semen (30-40) simultaneously in a short time (in one hour).

With regard to (2), the reciprocal exchange of seminal plasma between semen samples of different integrities in terms of fertilising quality (due to age of male or inherent factors) revealed that there was no influence on the fertilising ability of spermatozoa. This was verified with fresh and stored semen. Thus, it is concluded that inherent high fertilising ability of spermatozoa from particular males when part of a pooled semen sample would not be expected to be adversely affected by the inclusion of semen from males known to be less fertile. This is an important finding for the use of pooled semen commonly used in commercial breeding by AI.

With regard to (3), the results of the study on the frequency of semen collection indicated that twice daily collection did not result in significantly lowered fertility. This was true of frozen semen suggesting that at least over a short duration up to 4 weeks during a breeding season, higher frequencies of semen collection than normal may be used when increased demands for semen is necessary to cover higher than normal numbers of females. This knowledge indicates that it is possible to use selected genetic males intensively for breeding instantly, or for banking frozen semen, without concern for loss of spermatozoal quality due to frequent semen collection.

With regard to (4), the investigation, using frozen semen taken from the same males at different ages, to study the influence of the effect of the age of males (28, 46 and 67 weeks) or the age of females (24, 41 and 54 weeks) on fertilising ability, revealed that there was no significant influence of the age of the females per se. However, male age and male and female age interaction appeared to have an influence on fertility. The insemination doses also significantly affected fertility.

Pertinent to making the maximum use of the semen of elite sires was the finding that males initially selected in a breeding period as good quality semen producers persisted for up to 67 weeks of age with a constant supply of good quality semen which was shown by fertility trials to produce excellent fertility. Moreover, this was achieved with a low insemination dose (100 million spermatozoa) of frozen semen. One precaution is necessary, because it was found that semen samples from the very young males (28 weeks of age) tended to produce lower fertility than when they were middle-aged and onwards. This was detected with middle-aged and old-aged females. Thus, for semen banks it is desirable to freeze from well matured males.

With regard to (5), of great benefit to breeding by AI, a simple diluent has been composed and a technique for holding fowl spermatozoa at high temperatures (20 and 40°C) developed. The composition of the diluent is: 136.9 mM sodium chloride, 59.95 mM TES, 33.33 mM glucose, 27.5 mM sodium hydroxide. Antibiotics were added and the final osmotic pressure was 382 mOsmol/kg of water and pH 7.4. With 4-fold dilution of semen (1 ml semen + 3 ml diluent) in a 25 ml conical flask, and keeping it either at 20° or 40°C in a shaking water bath for up to 17 hr, good fertility was obtained with 40-50 million spermatozoa. Under still conditions, a simplified method for 6 hr storage on a bench top at ambient temperatures around 20°C using the same dilution rate and insemination dose, was devised which also resulted in satisfactory fertility. This development could be expected to be very useful under field conditions, especially for application in selective breeding practices using AI in tropical countries. The technique, however, also offers greater flexibility in operating AI in temperate climates and consequently is likely to prove universally beneficial to poultry breeders.

6. REFERENCES

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7. APPENDIX

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The application of the dye-reduction (INT) method for estimating the rate of formazan formation as a measure of fowl semen quality and a predictor of the fertilising ability of fowl spermatozoa.

1) Prepare 40 ml stock premix solution so that a millilitre of the premix contains 0.84 ml of (1), 0.10 ml of (2), 0.04 ml of (3) and 0.02 ml of (4) (See Table 4.1, Page 65) This solution should be stabilised at 20°C.

2) Mark three sets of 20 tubes (4 ml capacity) according to bird number to be tested. Dispense 1 ml premix in each of a 1st set of 20 tubes for dye-reduction test. Dispense 0.5 ml premix in each of a 2nd set of 20 tubes for measuring concentration of spermatozoa. Dispense 0.3 ml salt/TES, pH 7.4 (1) (See Table 4.1, Page 65) in each of a 3rd set of 20 tubes for making 4-fold dilution of semen.

3) In the animal house collect the semen from 20 cocks and immediately dispense 0.1 ml semen from each into the 3rd set of tubes containing 0.3 ml salt/TES solution to make 4-fold dilution.

4) Return the diluted semen to the laboratory and at 20°C dispense 200 ul 4-fold diluted semen (or 50 ul neat semen) into each of the 1st set tubes (containing 1 ml premix) and 100 ul of 4-fold diluted semen into the 2nd set of tubes (containing 0.5 ml premix) according to bird number. Shake each tube for the

proper mixing of the spermatozoal suspension. In using neat semen, in step I instead of 0.84 ml of (1), 0.99 ml of solution (1), to be used in premix).

5) In the 1st set of tubes, add 30 μl PMS solution (5) (See Table 4.1, Page 65) to each tube. Add 50 μl of INT solution (6) (See Table 4.1, Page 65) taking care that this step lasts no longer than 1 min (use a stopwatch from the 1st tube). Mix thoroughly by shaking. The tubes will show a slight colouration due to formazan formation within a minute of the addition of INT. Allow the incubation to continue at 20°C for 20 mins.

6) Add 200 μl Acid Triton (Table 4.1) to all the 20 tubes after 20 min incubation, taking care that this step is completed within 1 minute (use a stopwatch from the 1st tube). Mix while adding acid triton to ensure breaking up of the spermatozoa and the liberation of formazan in the solution. Leave for 5 minutes.

7) Spin at 700 g for 10 min at room temperature to precipitate the sperm debris. Extract the supernatant without disturbing the precipitate and place in 10 mm light-path cell.

8) Read the absorption of formazan suspension at 520 nm and calculate the formazan content of each tube by using the regression equation:

$$A_{520} = 0.006 + 0.0155$$

Using the 2nd set of tubes from step (2) above, record the absorption of the spermatozoal suspensions at 520 nm in a 1 mm light-path cell. This step may be carried out in between the time when incubation was started in the 1st set of tubes, by the addition of INT dye, and before the incubation was terminated by the addition of acid triton. Calculate the spermatozoal concentration by using the regression equation:

A_{520} = 0.033 + 0.153 spermatozoa x10^9/per ml of neat semen

If the number of semen samples to be evaluated at any time is small, or semen produced by a cock is low in volume, then before the addition of PMS and INT to the 1st set of tubes (step 4 above), take out 0.3 ml with a syringe and measure the concentration of spermatozoa as above and then quantitatively return all to the respective tubes for the continuation of the dye-reduction test.

Finally calculate the nmol of formazan produced per minute/10^9 sperm for each semen sample under test. This value will be the criterion of comparison of sperm quality between males to predict the fertilising ability.

8. PAPERS ACCEPTED FOR PUBLICATION

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1. CHAUDHURI, D. and WISHART, G.J. (1988) Predicting the fertilising ability of avian semen. I. The development of an objective colourimetric method for assessing the metabolic activity of fowl spermatozoa. Brit. Poult. Sci. (to be published in December 1988).

2. CHAUDHURI, D., WISHART, G.J., LAKE, P.E. and RAVIE, O. (1988) Predicting the fertilising ability of avian semen. II. A comparison of a simple colourimetric test with other methods for predicting the fertilising ability of fowl semen. Brit. Poult. Sci. (to be published in December 1988).

3. CHAUDHURI, D. and LAKE, P.E. (1988) A new diluent and methods of holding fowl semen for up to 17 hr at high temperature. (to be published in Proceedings of the 18th World Poultry Congress and Exhibition, Nagoya, Japan, 4-9 September 1988).