ARTIFICIAL BREEDING OF THE DOMESTIC FOWL

A thesis presented in partial fulfilment of the requirements for the degree of MASTER OF AGRICULTURAL SCIENCE in Animal Science at Massey University.

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1967

A C K N O W L E D G E M E N T S

I acknowledge with gratitude the assistance from Mr M.R. Patchell for his supervision of this thesis.

Thanks are also due to the Poultry Research Centre staff; in particular Miss P. Hopkirk for experimental help and Mr J. Hawes for care of the stock.

Presentation of the thesis was made possible with welcome co-operation from the Massey University library and central photographic unit, photographic assistance from Mr D. Laing, and the competent typing of Mrs P. Patchell.

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I N T R O D U C T I O N.

Topic.

Artificial breeding is an interception of natural mating procedure, by the collection of semen and its retention *in vitro* before deposition in the female genital tract.

Semen is usually collected after an induced ejaculation into a suitable receptacle, from the engorged copulatory organ situated in the cloaca of the male fowl.

When removed from their first natural environment in the male genital tract and retained *in vitro*, precautions are needed to preserve spermatozoa function. This is the most artificial phase and the one to which most research has been directed in an attempt to extend semen storage time.

Insemination is the final phase of semen transfer and the techniques for depositing semen in the female genital tract, parallel those used for other species.

A study of artificial breeding in the domestic fowl is presented in this thesis.

Scope of Research.

Three phases of artificial breeding were investigated; semen production and release, semen storage, and artificial insemination.

Semen production was concerned with the ability of cockerels to produce adequate functional spermatozoa. The physiological characteristics of production were measured by age at sexual maturity; increase and persistence of spermatozoa output; semen quality traits such as spermatozoa concentration and the percentage of normal live spermatozoa; and the response to imposed environmental treatments. Semen release was more concerned with the

psychological responses and semen resources of cockerels under varied circumstances.

A few aspects of semen storage were investigated, and evaluated by the potential fertilising ability of semen held *in vitro* for up to twentyfive hours. Some temperature regimens were tested, together with several methods of protecting spermatozoa *in vitro* by varying diluents and dilution rate.

Artificial insemination was studied from two aspects: firstly in the modification of semen to obtain optimum fertility; and secondly, the physical procedures for depositing spermatozoa in the female genital tract.

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CHAPTER 1.

Review of Poultry Reproduction.

Most animal research has been conducted on laboratory and domestic mammals, with scant and often indirect attention to poultry. Until recently poultry farming was not a major livestock industry and the paucity of research into avian anatomy and physiology merely reflected the economic situation. This was accentuated by basic anatomical differences between birds and the large farm mammals. A brief review of the salient features of poultry and bovine reproduction will introduce the thesis topic, and contribute to conclusions expressed in later chapters.

1.1 Male Anatomy and Physiology.

The avian reproductive system like that of higher vertebrates begins with the appearance of primordial germ cells, thought to arise as differentiating buds from the germ wall endoderm. Within about two days of incubation the germ cells have circulated in the embryonic blood system and collected near the future gonad site. They apparently migrate dorso-laterally to either side of the embryo by amoeboid movement and at this stage a permanent assymmetry arises, with a marked majority on the left side. The morphologically indifferent sexual period extends from the third to eighth day of incubation, although physiological or genetic sex differences are already present.

In the avian embryo the reproductive and excretory systems are closely interrelated. Intermediate mesoderm gives rise to the excretory system which develops in three phases, of which the second nephric structure, the mesonephros, contributes to the genital system. Incipient ovarian cortex around the medulla of the left gonad renders this organ potentially bisexual, whereas the right gonad, mainly medullary tissue, is dominantly masculine from the beginning and responsible for the ovotestis in some mature females. Medulla

and cortex of the early gonad arise as successive proliferations of sex cords from the germinal epithelium, and differ in their type and intensity of hormone production. Sexual differentiation occurs with the regression of one component by hormonal action under genetic control. Near hatching time, most of the embryonic testis is composed of proliferated male sex cords made up of seminiferous tubules and germ cells. At hatching all the definitive male sexual organs have appeared.

Spermatogenesis begins at about the seventeenth day of incubation, with a four day phase of intense multiplication and transformation of the primordial germ cells to spermatogonia. As the gonad develops around them the spermatogonia assume their permanent position in the seminiferous tubules, then remain relatively quiescent for several weeks after hatching. The phases of spermatogenesis vary between breeds of fowl; in precocious Leghorn strains, primary spermatocytes appear at five to six weeks of age. Some spermatogonia migrate to the tubule lumen and undergo rapid multiplication to form several layers of smaller cells, which transform to primary spermatocytes. Normally haploid secondary oocytes appear at the lumen centre at about ten weeks in Leghorn chicks. These quickly undergo a second meiotic division to produce small distinctive spermatids, with a spherical nucleus surrounded by clear peripheral cytoplasm. Spermatids are thought to metamorphose directly to the sleek flagellated spermatozoa. Leghorn strains may achieve spermiogenesis by twelve weeks of age, but Parker, McKenzie and Kempster (1942) as cited by Sturkie (1965), claim that fertility is poor until the males are about twenty-five weeks old. Prior to discharge spermatozoa are buried head-first into large non-germinal Sertoli cells which have migrated from the seminiferous tubule walls. Motility is acquired in passage through the epididymal region and vasa deferentia; requiring from one to four days, depending on spermatogenesis and mating activity of the male.

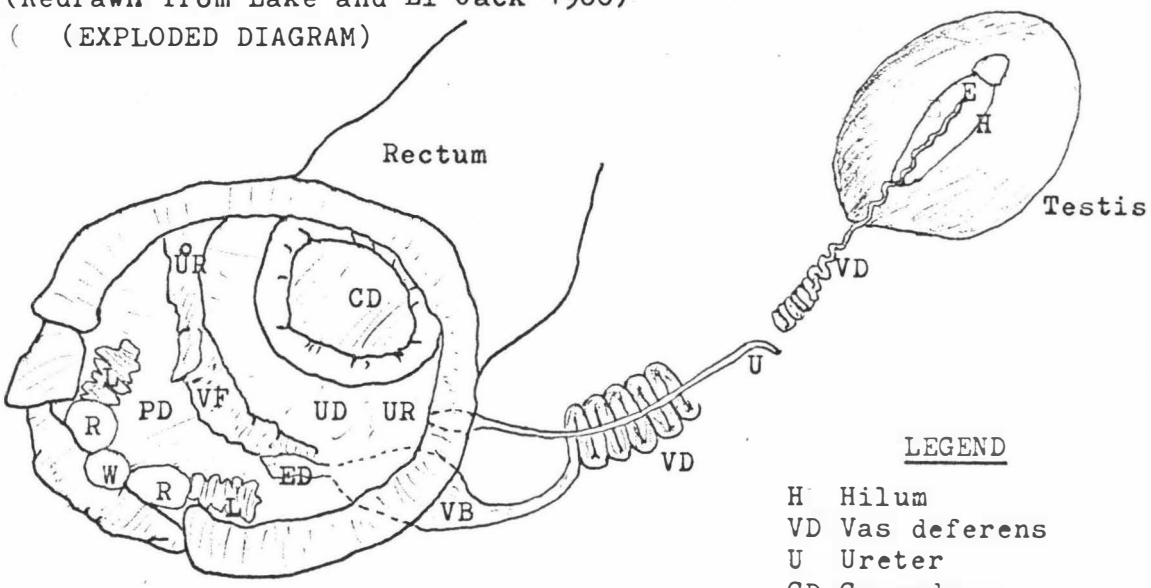
The cockerel reproductive tract is composed of four regions,

FIGURE 1

Reproductive Organs of Cockerel.

(Redrawn from Lake and El Jack 1966)

((EXPLODED DIAGRAM)



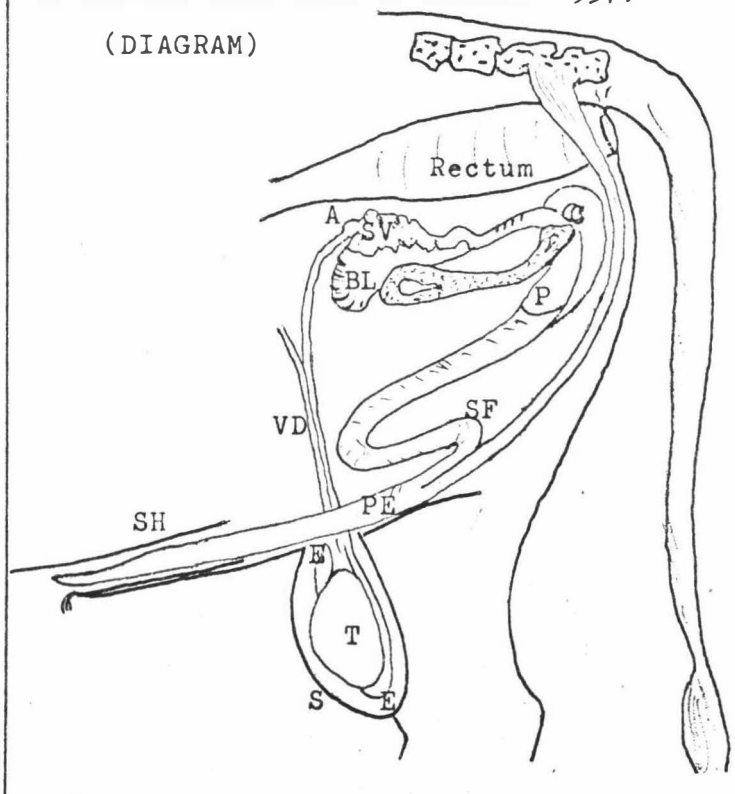
LEGEND

H Hilum
VD Vas deferens
U Ureter
CD Coprodaeum
UD Urodaeum
PD Proctodaeum
UR Ureter opening
ED Ejaculatory duct
VF Vascular fold
L Lymph fold
R Round body
W White body
VB Vascular body
SV Seminal vesicles
BL Bladder
T Testis
E Epididymis
S Scrotum
C Cowper's gland
P Prostate
PE Penis
SF Sigmoid flexure
A Ampulla
SH Sheath

Reproductive Organs of Bull.

(Redrawn from NZDB AB Manual 1957)

(DIAGRAM)



distinguished by their origin or function (Lake 1957 a), (see Figure 1). Paired soft testes are attached by a short mesorchium to the anterior-ventral surface of the kidneys, comprising about one percent of body weight at the peak of reproductive activity. Long convoluted seminiferous tubules are supported by stroma connective tissue ramified with capillaries, arterioles and venules, and a very thin non-septate tunica albuginea encloses each testis. The contents of the seminiferous tubules are discharged into the rete tubules which radiate to the testis hilum. The vasa efferentia and ductus epididymis consist of a small cluster of tubules on the hilum, indicating a relatively minor role in semen maturation in contrast to higher mammals (Mann 1964). The whole epididymal duct system is embedded in connective tissue and firmly anchored to the dorsal abdominal cavity wall. An intense holocrine secretion from parts of the vasa efferentia epithelial lining, has been reported by Lake (1957 a), and other epithelial activity in the sexually fit male, which indicates some functional influence of these tubules. In the same project, the ductus epididymis was concluded to be relatively inert. A long coiled vas deferens serves as a spermatozoa storage organ, and duct from each testis to the copulatory organs in the cloaca. Each vas deferens adheres to the ventral surface of the kidney, becoming thickened by muscle and connective tissue at the distal end to form a conical-shaped ejaculatory duct, which protrudes into the urodaeum. Lake (1957 a) describes the phallus as a central white body plus two adjacent round folds, on the distal lip of the proctodaeum. Two discrete lymph folds in the ventral proctodaeum, lie between the round folds and ejaculatory ducts. A vascular body traverses the ventral boundary of the proctodaeum and urodaeum, between the two ejaculatory ducts. The phallus, lymph folds, vascular body plus ejaculatory ducts, are generally considered to be the avian copulatory organ.

1.2 Female Anatomy and Physiology.

Gonad differentiation in the female occurs from the fifth to seventh day of incubation. The left gonad investment of germinal epithelium becomes several layers thick, and later a second cellular proliferation gives rise to the cortical cords. Small clusters of medullary cord cells may persist in the mature functional ovary, but degeneration of most of the cells proceeds from halfway through incubation. After hatching the right ovary persists in a rudimentary form. If cortical tissue is present, the organ is capable of developing into an ovotestis; but if only medullary cords persist, the organ is potentially a male gonad. By the eighth day of incubation the primordial germ cells are evenly distributed in the germinal epithelium, and begin active division to form oogonia which accumulate in large numbers. Lobules in the deep surface of the germinal epithelium are derived from clusters of three or more oogonia, which swell out as cortical cords on the ninth day of incubation. Before hatching, the ovarian cortex is thickened by connective tissue stroma which surrounds and separates the cords and medullary core.

Oviduct development is closely synchronised to that of the ovary (Van Tienhoven, 1959), which produces stimulative hormones, but the response also depends on vitamin B_{12} and folic acid availability. Normally only the left Mullerian duct in the female gives rise to an oviduct and the right duct degenerates about halfway through incubation, and usually a complete regression occurs in the male embryo.

Oogenesis begins at the eighth to eleventh day of incubation in the female embryo, with a phase of rapid germ cell multiplication. Two or three days later the oogonia transform to primary oocytes, and remain in this state for the remainder of incubation and at least another four to five months, until the first meiotic division just prior to ovulation. An avian egg is characterised by massive proportions of non-germinal, nutritional and protective substances about the tiny germinal disc. The ovarian contributions

can be considered at two levels; nuclear and cytoplasmic, which proceed independently toward a synchronised maturation. The cytoplasmic or yolk accumulation continues very slowly until a rapid acceleration in growth occurs in the fourteen days before the oocyte is due to ovulate. Meiosis in the germinal portion, reduces the primary oocyte to a haploid secondary oocyte about five to two hours before ovulation. The extruded first polar body remains near the germinal disc beneath the oocyte vitelline membrane. If the fowl is mated, the second meiotic division and extrusion of the second polar body occur as a spermatozoon penetrates the germinal area. However, in an unfertilised secondary oocyte, meiosis is thought to be arrested at the second metaphase, and the egg layed as such. Olsen (1967) suggests that diploidy may be restored by the re-entry or nondisjunction of the second polar body, leading to abortive, although occasionally successful, parthenogenetic development.

Considering the complex procedure that daily egg production entails; from the sequential maturation of the oocyte nucleii and massive yolk formation in the ovary, to the ovulation, fertilisation, and passage in the oviduct with rapid deposition of more nutritive and protective substances transported by the blood system, then oviposition; it is assumed that a neuro-hormonal system is responsible for the synchronisation.

Hormones apparently influence the reproductive cycles in fowls much as they do for mammals. The anterior pituitary produces follicle-stimulating hormone (F.S.H.) which stimulates seminiferous tubule growth and spermatogenesis in the male, and ovarian follicle growth in the female. A luteinising hormone (L.H.) similar to mammalian LH, is thought to stimulate development in the testis interstitial cells and cause ovulation in the female. However, investigators disagree over the composition and function of this hormone, or group, which has been named : ovulation-inducing hormone (O.I.H.), or interstitial-cell-stimulating hormone (I.C.S.H.), or avian LH. The hormone is

responsible for stimulation of interstitial Leydig cells in the male and the induction of ovulation in the female. Prolactin hormone, also produced in the anterior pituitary, induces broodiness in susceptible breeds and suppresses FSH output.

At least three gonadal hormones appear in both sexes, but their site of production and exact modes of influence are not clear. Androgens are probably produced in the Leydig or interstitial cells, of the testis and ovarian medulla. The principal androgen, testosterone, is responsible for the striking comb growth in the juvenile male, and together with other genetic and physiological factors, influences the changes in cockerel voice, temperament, libido and plumage. Circulating testosterone has a suppressive feed-back effect on pituitary LH output. Oestrogens have equally dramatic effects on the oviduct, near the time of sexual maturity in the female. They are also responsible for some sexual dimorphism, and influence the accelerated metabolic processes for egg production demands. Oestrogens are probably produced in the theca cells of maturing follicles.

Progesterone is thought to be produced in both sexes. Fraps (1955) has suggested that progesterone could be the 'excitation hormone', secreted from the maturing follicle. He postulated that the hormone accumulated in the blood until there was sufficient to stimulate a diurnally varying neural mechanism, responsible for the release of OIH from the pituitary. The pituitary is responsive from about 11 p.m. to 8 a.m., thus the ovulation which occurs about six hours after OIH release, is confined to 5 a.m. to 2 p.m., with oviposition about twentyfour hours later.

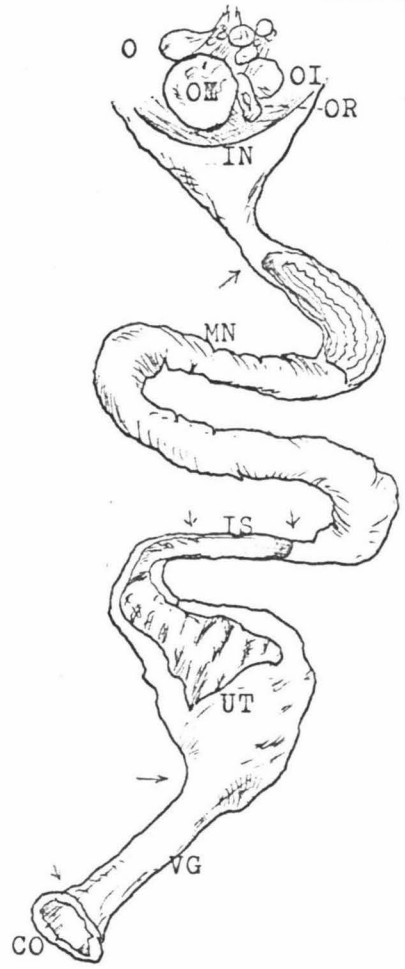
During a laying phase, the avian reproductive tract is geared to daily ovulation, egg formation and oviposition. Normally the right ovary is rudimentary and the right oviduct regresses completely in the adult. The left ovary is suspended by mesorchium near the anterior-ventral surface of the kidneys. It consists of an irregular medullary stroma, histologically similar

FIGURE 2

Reproductive Organs of Pullet.

(Redrawn from Sturkie 1965)

(DIAGRAM)

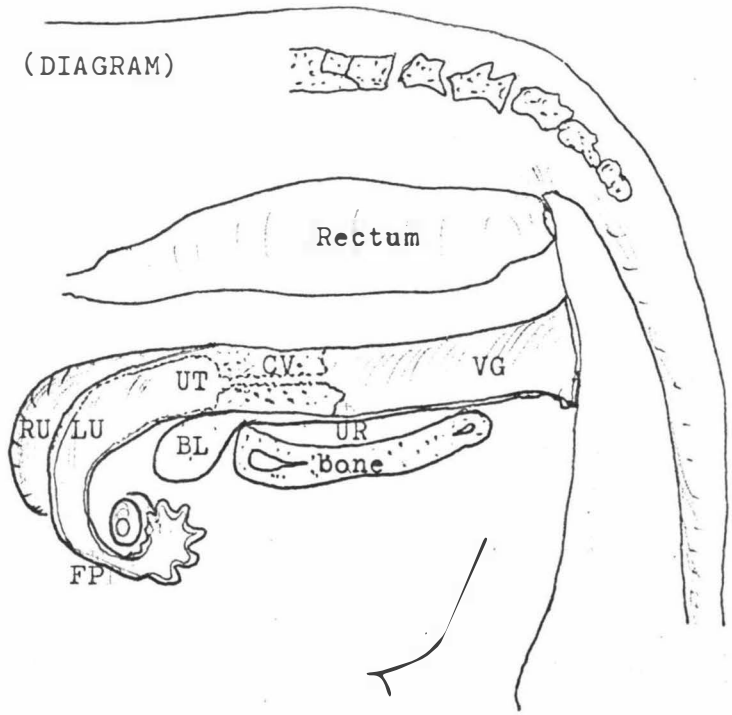


LEGEND

OM Mature ovum
OI Immature ovum
OR Ruptured follicle
IN Infundibulum
MN Magnum
IS Isthmus
UT Uterus
VG Vagina
CO Cloacal opening
O Ovary
UR Urethra
LU Uterus (left horn)
RU Uterus (right horn)
FP Fallopian tube (oviduct)
BL Bladder
CV Cervix

Reproductive Organs of Cow.

(DIAGRAM)



to the testis interstitial cells, surrounded by the cortical tissue. Several thousand vasculated follicles all containing an oocyte, arise from the ovarian cortex, and mature sequentially under hormonal control. Only a small fraction of the follicles are ovulated.

The oviduct consists of five distinct functional parts. A funnel-shaped infundibulum lies partially free-moving in the abdominal cavity below the ovary, and is stimulated to engulf the ovum immediately after ovulation. The magnum is a long and highly glandular portion of the oviduct responsible for egg protein formation. After about three hours the egg passes into the isthmus where it receives the two egg shell membranes. About one hour later the egg moves into the glandular and muscular uterus, where a watery uterine fluid and salt mixture dilate the soft contents as the egg shell components are deposited, over another eighteen to twenty hours. A short junction separates the uterus from the vagina which opens into the cloaca.

1.3 Resemblance of Avian and Bovine Reproduction.

Artificial breeding has been applied to dairy cattle on a large scale; initially to provide a cheap breeding service for small farms, but in recent years some schemes have been extended as part of a genetic programme to improve production traits in the national dairy herd. The research into bovine reproduction and artificial breeding has some application for poultry.

Although the basic reproductive processes are common to all vertebrates, the physiological and behavioural sequences vary. The most important difference is the oviparous reproduction of the fowl and the viviparous gestation in the cow, and these functional differences are reflected in the anatomy of the reproductive tracts (see Figure 2).

Avian and bovine males exhibit more similarity than females, in their reproductive characteristics. Some investigators regard certain structures in the cock as analagous to those in the bull, on the basis of semen biochemistry, but discrepancies arise from different semen collection techniques. The

main anatomical differences are the well-developed accessory organs, and position of the testes, in the bull (see Figure 1). The scrotal sac containing the testes and epididymi is suspended from the abdominal cavity, and thermo-regulatory control is maintained by muscular elevation to the optimum temperature for fertility. Separate vasa deferentia extend from each testis through the inguinal canal, forming dilated ampullae at the opening into the urethra. Paired seminal vesicles and a small diffuse prostate gland, around the ampullae, and paired bulbo-urethral glands, all drain into the urethra. There is some controversy whether the glandular copulatory organs in the cock corresponds to some mammalian accessory organs (Nishiyama 1954), or not (Lake 1957a, Lake and McIndoe 1959), although Lake (1957a) notes some important similarities of the avian ejaculatory ducts and mammalian penis. These anatomical differences are the main cause of variations in semen collection and insemination technique.

Semen biochemistry.

Bovine semen is composed of spermatozoa, plus secretions from the testes and excurrent ducts, and specific secretions from several accessory organs. This represents a higher evolutionary development than the testes plus modified duct system of the fowl, of which one consequence is the superior spermatozoa storage in the bull reproductive tract. Unfortunately fowl semen cannot easily be collected during natural mating, so most analyses are made on semen collected after artificial stimulation, or extracted from the vasa deferentia. Neither method would necessarily obtain the natural ejaculated semen. Mammalian semen composition varies greatly between species, partly due to the relative contributions of their accessory glands.

Examples of mammalian and avian semen volumes and spermatozoa concentrations, of ejaculated semen (from Mann 1964), are shown on the following page:

TABLE A.

Species	Vol. Ejaculate (ml.)	Sp. Density (per µl.)
Bat	0.05	6,000,000
Boar	250	100,000
Bull	4	1,000,000
Cock	0.8	3,500,000
Drake	0.3	3,500,000
Man	3.5	100,000
Turkey	0.3	7,000,000
Ram	1	3,000,000

Seminal plasma in the bull is composed of secretions from the epididymi, vasa deferentia, ampullae, seminal vesicles, prostate, bulbo-urethral and urethral glands; and serves as a medium and vehicle for spermatozoa. Accessory organ secretions are initiated and controlled by androgens produced in the testes. The secretions are characterised by the presence of such substances as citric acid, fructose and glycerylphosphoryl-choline, which rarely occur in such high concentrations elsewhere in the body. Granules and globules, apparently of cellular origin, in the secretions indicate continual changes in the epithelial structure of the glands.

Some of these features apply to the fluid arising from the cloacal tissues of the fowl, during semen collection. Nishiyama (1954) suggested that the copulatory organs correspond to the accessory glands of mammals with respect to physiological function, from his experiments recording the effect of androgen on the cloacal tissue. Lake and McIndoe (1959) considered that natural seminal plasma was derived from the testes, epididymi, vasa efferentia, and vasa deferentia, with small and incidental additions from the cloaca during mating.

A natural ejaculation in the bull is instantaneous, but with electric stimulation it is possible to collect several split-ejaculate fractions, which can be attributed to one or more regions of the genital tract. Citric acid and fructose originating mainly from the seminal vesicles, and glycerylphosphory-choline from the epididymi, are at particularly high levels in bull seminal

plasma, yet negligible or absent in cock plasma. Chemical analyses of semen are presented in Table B, from tables compiled by Mann (1964), Lake, Butler, McCallum and McIntyre (1958a), and Lake and El Jack (1964). The wide range of most values indicates the large variation incurred by artificial methods of semen collection.

TABLE B.

(Bull values are semen concentrations, cock values are seminal plasma concentrations).

	Cock mg/100 ml.	Range	Bull mg/100 ml.	Range
Calcium	8	6.9 - 9.3	34	24 - 45
Chloride	205	197 - 212	371	309 - 433
Citric Acid	negligible		720	340 -1,150
Creatine	92	72 - 112	3	
Fructose	none from testes or ducts		540	280 - 770
Glutamic acid	1,067	890 -1,340	0.35	
Glycerylphosphoryl-choline			350	110 - 496
Lactic Acid	34		35	20 - 50
Non-protein nitrogen	177	140 - 275	48	
Potassium	43	39 - 49	288	150 - 415
Pyruvic Acid	2.9		4.7	
Sodium	393	379 - 428	109	57.- 201

Mating

In domestic dairy cattle spermatogenesis and breeding proceed throughout the year in most environments, with the peak of sexual activity during the summer in temperate climates. The cow displays relatively uniform twenty one day oestrous cycles, throughout the year. Only during a short oestrous which lasts for about eight to twentyfour hours, depending on environmental conditions and the stock, will she accept the bull for mating.

Ovulation occurs from two to twentysix hours after the end of oestrous and the ovum is capable of fertilisation for up to six hours, in the fallopian tubes. Hence functional spermatozoa must be present in the tubes in sufficient numbers at this time, or a successful conception cannot occur for at least another three weeks. Spermatozoa are thought to require at least 6 hours in

the female tract for capacitation, and retain their fertilising capacity only twentyfive to thirty hours after ejaculation. Conception from natural mating therefore is very dependent on the animals recognising the oestrous, having access to each other, and at least one satisfactory mating before or soon after, ovulation. Under natural or farm conditions the bull is required to mate far less than a cock, but it is imperative that functional spermatozoa are available when an oestrous occurs. If conception does not occur the cow will continue oestrous cycles with ovulations every three weeks, but if fertilisation and implantation are successful, the cycles will not normally resume until at least three weeks after calving.

Important differences in avian and bovine reproduction can be noted here: the domestic hen ovulates daily and has the capacity to retain functional spermatozoa in-vivo for about fourteen days and sometimes up to thirty days, while the cow ovulates every three weeks and can not store sperm in the oviduct over twentyeight hours. Whereas the cow will only mate near the time of ovulation, the hen will mate at any time virtually irrespective of egg production or the number of spermatozoa already in the oviduct.

Fewer differences distinguish male mating behaviour. Mating is initiated by the cock, and subject to time of day and environmental stimuli, rather than any condition of the hen. Spermatogenesis proceeds at an extremely high level in the cock and there is relatively low storage potential in the male reproductive tract, so it usually mates with many females throughout the year. In contrast, the bull can not mate an anoestrous cow, spermatogenesis proceeds at a relatively lower rate, and the bovine epididymi are equipped to store sperm.

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CHAPTER 2.

Semen Production and Release.

A. Review.

An insemination programme is dependent on the ability of the male fowl to release functional semen when it is artificially stimulated. Two sets of factors are responsible: intrinsic effects on semen production, and interacting mental and physical influences on the release of semen. Where poultry management is satisfactory, semen release is usually the limiting factor during a collection.

2.1 Spermatozoa Production.

Physiological aspects.

It is difficult to assess the precise effects of nutrients on sexual activity, from the literature available.

Restricting protein to growing males has been shown to reduce live weight gain and delay sexual maturity, but full restoration of spermatogenesis occurs when adequate protein is available (Wilson, Waldroup, Jones, Duerre and Harms 1965). Jones, Wilson, Harms, Simpson and Waldroup (1966), demonstrated that sexual maturity was delayed by an average eight weeks, when protein was restricted below 9% in growing cockerels. No significant differences in semen characteristics persisted when 17% protein was fed from twentyone weeks of age. Arscott and Parker (1963), recorded small differences in the fertilising capacity of semen from mature males fed from 6.9% to 16.9% protein for thirtythree weeks, but concluded that the range was insufficient to cause important changes as all amino acids were above minimum maintenance levels in the low-protein ration.

A restriction in energy intake causes a more severe decline in spermatogenesis. Parker and Arscott (1964), reported significant declines

in semen volume, semen fertilising capacity, body weight and testes weight, where daily energy intake was decreased from 230 Calories to 143 Calories. They suggested that an energy restriction responsible for an 11 to 16% loss in liveweight would also decrease semen volume, and a 30% loss in weight would preclude infertility.

Vitamin deficiencies produce quite spectacular effects in the sexual activity of male fowl. Paredes and Garcia (1959), reported severe effects from hypovitaminosis A on reduced spermatozoa motility and density, increased % abnormal cells, and a tendency to temporary sterility. Apparently spermatozoa quality was particularly sensitive to vitamin A levels, as semen volume and general health were not significantly changed. Vitamin E is thought to protect the integrity of spermatozoa as an anti-oxidant. Arscott and Parker (1966), demonstrated that vitamin E was effective in countering the effects on reduced fertilising capacity and spermatozoa concentration, of high levels of unsaturated linoleic acid. Semen volume, hatchability of fertile eggs, body weight and testes weight were not significantly affected by hypovitaminosis E (Arscott, Parker and Dickinson 1965). Recently vitamin C has been implicated in semen decline during high temperatures. Perek and Snapir (1963), found supplementary ascorbic acid during the summer months in Israel, stimulated faster growth rate and earlier sexual maturity, and maintained higher semen production in mature males. Perek (1965, 1966), suggested that under certain conditions fowls are incapable of synthesising their ascorbic acid requirements, and depend upon exogenous supplies to maintain sexual activity.

Many diseases adversely affect the sexual activity of males, but specific influences on spermatozoa formation and quality, have not been investigated.

Genetic variations in semen production have been well documented. Differences between strains in the onset of spermatogenesis and sexual activity,

sexual maturity (Williams and McGibbon 1956, McDaniel and Craig 1959, Saeki 1963b, Tindell and Arze 1965, Fomin 1966), and the persistence of semen production (Perek and Snapir 1963), indicate inherited traits. Some factors of semen quality are considered to be genetically determined; Cooper and Rowell (1958), reported that most of the variation between cocks' infertility was attributable to spermatozoa motility, % dead or metabolic activity, which are themselves related. Soller, Snapir and Schindler (1965), derived the heritability and repeatability of semen volume, 0.41 and 0.78; spermatozoa concentration, 0.46 and 0.79; and spermatozoa motility, 0.87 and 0.83; respectively. Saeki (1962 b), reported highly significant differences between turkey males in the percentage of crooked-neck spermatozoa in fresh semen.

The physical attributes of male fowl seem to reflect rather than determine sexual activity. Fransen, Andrews and Carrick (1955), reported highly significant correlations between testes weight and comb weight, comb weight with body weight, and testes with body weight. Grosse and Craig (1960), recorded that sexual maturity and natural mating behaviour, were retarded in cockerels which were dominated during rearing, but without impairment of potential fertilising capacity. Goodwin, Cole, Hutt and Rasmussen (1955), considered inherited endocrinal abnormalities of cockerels, could have been partly responsible for low fertility in a strain of Leghorn fowls.

There is some doubt whether semen quality is affected by the frequency of ejaculation and release of semen. Boone (1964), compared two-per-month and three-per-week semen collection frequencies and found no significant differences in semen volume, spermatozoa density, spermatozoa per ejaculate, motility or vigour, but a tendency to declining body weight in the latter group. However, El Jack and Lake (1966), reported an increasing percentage of abnormal spermatozoa from males rested more than two

weeks, and a return to normality after frequent ejaculation.

Lake (1962 a), concluded that spermatozoa production was maintained in the adult fowl, and fluctuated less than the seasonal changes of mating behaviour.

Environmental Aspects.

Temperature effects on semen production are closely related to other environmental factors, particularly the light and season. Apparently spermatogenesis is not curtailed by a wide range of ambient temperatures; Lamoreux (1942), observed no relationship between temperature and infertility over five years, when the weekly mean ranged from 23° to 50°F. Long and Godfrey (1952), found no evidence for reduced fertility or natural mating of males, in temperatures ranging from 12° to 88°F. providing there was no freezing injury, but observed that mating declined in periods of high minimum temperature. Probably some temperature influences on semen production are indirectly effective through feed consumption, humidity, light and ag ing. In Israel, Perek and Snapir (1963), and Perek (1966) found no correlation between temperature and spermatozoa concentration or semen volume. Kamar and Badreldin (1959), suggested that environmental factors influenced semen through endocrinological paths; temperature via the thyroid gland, and light via the pituitary. They found no restricted breeding season with Fayomi cocks at Giza, Egypt, but thought that seasonal effects on qualitative traits could differ from those on quantitative aspects such as semen volume or total spermatozoa, because of different anatomical origins of semen. Higher temperatures were demonstrated to accelerate sexual maturation in cockerels, but after maturity caused a refractoriness in spermatogenesis which was accentuated by excess light (Ingkasuwan and Ogasawara 1966). In a controlled and specific experiment Boone and Huston (1963), increased ambient temperature by 10°F. per hour, to 104°F. and 65% relative humidity, but failed to get any constant significant

effects on mature White Rock males. Semen volume and spermatozoa concentration declined in some trials, but without any correlated fertility effects. Apparently there was no relationship between spermatozoa quality and temperature, as motility, vigour and fertilising capacity were not affected by the changes in body temperature.

The duration of light is considered to influence semen production by stimulating increased gonodotrophin secretion from the pituitary (Benoit and Assemnacher 1959), and by providing the bird with long feeding time. Growing cockerels respond to light stimulation by accelerated sexual maturation (Parker and McCluskey 1965, Ingkasuwan and Ogasawara 1966), and once fully mature, semen production is strongly influenced by light-temperature interactions. Lamoreux (1943), demonstrated the variation in semen volumes from males subjected to artificial light regimes. He concluded that the threshold for semen production was between nine and twelve hours daily, and that intermittent light was more stimulatory than a single exposure per day. This was confirmed by Wilson, Siopes and Mather (1965) with Japanese quail. Hays (1954), reported that temporary supplementary lighting increased the fertilising capacity of aged cocks in natural matings, but had no significant effect on young stock receiving adequate light.

Most investigators agree that excessive light stimulation produces sexual refractoriness and attribute this to increasing concentrations of gonadal hormones suppressing the sexual activity of the pituitary gland. The experiments of Parker and McCluskey (1964), suggest that the fertilising potential of adult male fowls is not directly significantly affected by photoperiodism, and that reduced light regimes may favour extended semen production. Bajpai (1963), reported significant improvement in the semen quality and quantity of mature cocks when their daily light period was increased from twelve to sixteen hours, and a corresponding decrease in semen quality from a comparable decline to eight hours per day. However

significant changes in the percent live or normal and density of spermatozoa do not necessarily preclude variations in fertilising capacity of semen.

Light cycles, probably with associated influences of feeding and mating activity, are considered to produce a diurnal rhythm in semen production indicated by mitotic activity in the testes (Lake and Woodcush 1956). MacCartney (1942), detected a significantly higher number of dividing cells in the period between 1330 and 0400 hours, than between 0600 and 1130 hours, with maximum activity at midnight.

Seasonal influences are considered to arise from age, light, temperature, and other non-specific factors which vary with stock, management and location. Investigations in the temperate latitudes suggest that semen fertilising potential is highest in the spring, but it is difficult to generalize further (Parker and McSpadden 1943 a, Schindler, Volcani and Weinstein 1957, Kamar and Badreldin 1959, Perek 1966).

Housing, management and sexual activity, have been implicated in environmental effects on semen production. When individually caged, males have no natural opportunity to release semen, and El Jack and Lake (1966), reported a decline in spermatozoa quality which they attributed to senescence in the reproductive tract. Alternatively (Fomin 1966), postulated that excess semen release accelerated spermatogenesis, resulting in the production of weaker spermatozoa with reduced fertilising capacity. Both investigations suggest that spermatozoa quality is dependent on an optimum release rate of semen.

The effects of housing males away from the females, in separate cages or flocks, have not been clearly resolved. Siegel and Beane (1963), and Woodward and Abplanalp (1966), recorded significantly larger semen volumes from caged cockerels and turkey males, respectively, although Parker and McCluskey (1959), considered semen volume was not affected. All

the reports confirmed that fertility was not impaired.

2.2 Semen Release and Collection.

Collection of semen satisfactory for insemination is relatively easy in the fowl. If functional spermatozoa are present in the reproductive tract, the bird can be sexually stimulated and induced to ejaculate them. The success in obtaining adequate and pure semen, depends on a physical and psychological interaction between bird and man.

Release.

The psychology of ejaculation in fowls is not well documented. Most authors assessed the mental state of the male during artificial semen collection or natural matings, by the display of libido, courtship, social aggressiveness, and other non-sexual emotions such as fear. Lake (1957 b), classified male temperament and reaction to collection, as quiet, excitable and frightened, and related the composition of the semen ejaculated to the temperament, handling method and stage of breeding cycle. McDaniel and Craig (1959), estimated the phenotypic associations of social aggressiveness, and sexual effectiveness or libido in cockerels and crouches elicited in pullets, as being significantly correlated with one another in natural matings. For caged males, the genetic determination of libido may be less effective in modifying semen release than the response to handling or training habits (Wood-Gush 1960).

Ejaculation is extremely rapid during mating or artificial collection from the cockerel. Semen in the bulbous distal vasa deferentia, is expelled through the ejaculatory ducts onto the erectile tissue of the proctodaeum probably by muscular contraction of the anal sphincter. After relaxation of the muscles, abdominal pressure forces semen from the proximal vasa deferentia into the bulbous distal end (Burrows and Quinn 1937).

Physical responses during artificial stimulation vary; massage is not necessarily followed by spontaneous ejaculation and some pressure near the vent is usually required to express the semen. Further responses to massage are progressively harder to elicit and the quality of the ejaculate usually deteriorates (Lake 1962a, 1967).

Artificial collection.

Several different methods of extracting or collecting functional semen from cockerels, have been reviewed by Burrows and Quinn (1937), and Kamar (1958). Apart from killing the males and squeezing semen from the vasa deferentia, semen maybe extracted from the cloaca after mating, or intercepted by artificial cloacas or contraceptives during a natural mating. Most of the recent methods are one or two-man adaptations of the massage and stimulation technique described by Burrows and Quinn (1937). Gabriel (1957) outlined a method of restraining males in an inverted cone during collections, and some investigators have strapped the birds to small trestles to reduce resistance and obtain good quality semen, sometimes without touching the copulatory organ (Kamar 1958).

The frequency of semen collection from individually caged males, influences both the semen quality and the learned response to handling. There is general agreement that good and frequent collections predispose faster and more natural cockerel responses, but the relationship between semen turnover and quality is not resolved (McCartney, Chamberlin, Carter and Wyne 1958, Boone 1964, El Jack and Lake 1966).

2.3 Semen composition and evaluation.

Physical and chemical characteristics.

Semen consists of solids and fluid arising from several anatomical structures, mixed at varying concentrations in an ejaculate.

Lake (1957b), considered that some components of semen were artifacts, or contaminants which arose from unnatural movements during collection. He isolated ten components in semen, which were attributed to sources associated with the reproductive tract. Spermatozoa from the testes plus an opaque fluid secreted in the epididymi and vasa deferentia, constituted true semen. A clear mucinous continuous secretion from the epithelial lining of the erectile tissue in the cloaca, was thought to be a passive supplement to true semen. A white solid precipitate of uric acid, semi-transparent buff coloured fluid containing urates, and clear fluid containing uric acid, were all thought to originate from the ureters. Yellowish fatty material observed in the semen from some young cockerels was considered to be debris from the maturing duct system. Blood transudates and red blood cells which were squeezed from superficial sinuses in the erectile tissue, and faeces, were attributed to poor collection technique.

Bacterial contamination occurs as the semen passes through the cloaca and during storage in-vitro. Gale and Brown (1961), demonstrated that turkey vasa deferentia semen was free of bacteria, but obtained concentration estimates of from nil to 2,700 million bacteria per sample of ejaculated semen.

Chemical analyses of semen reflect the variations between ejaculates, particularly in the proportions of cloacal fluids. A more comprehensive record of analyses is presented in Table C, which augments the introduction in Section 1.3. Table C is shown on the following page.

Spermatozoon structure.

Fowl spermatozoa are cylindrical motile cells, about 100 microns in length. The head region is composed of a large dense nucleus which is capped anteriorly by acrosome material (see Figure 3), including a

TABLE C. Chemical Analyses of Fowl Semen.

	Seminal Plasma		Spermatozoa		Reference
	Average	S.D or Range	Average	S.D	
Calcium (ckl)	3.3 mEq/L	± 0.45			1
" (cock)	3.8 "	± 0.85			1
"	8 mg/100ml	± 0.9			1
"	4.2 "	± 0.2			5
"	7.0 "	± 0.4			4
"	2.0 mEq/L				8
Sodium (ckl)	171 "	± 8			1
" (cock)	187 "	±16			1
"	393 mg/100ml	±17			1
"	383.1 "	± 7.2			5
"	337.7 "	±17.9			4
"	161 mEq/L				8
Potassium (ckl)	12.6 "	± 1.1			1
" (cock)	17.0 "	± 1.7			1
"	43 mg/100ml	± 4			1
"	50.3 "	± 1.3			5
"	44.8 "	± 2.76			4
"	13.0 mEq/L				8
Phosphorus	7.5 mg/100ml	± .71	902 mg/100ml	±48	3
Chlorine	205 mg/100ml	± 7			1
"	131.2 "	± 4.1			5
"	497.3 "	±27.7			4
"	37.0 mEq/L				8
Magnesium	6.1 mg/100ml	± 0.2			5
"	5.0 mEq/L				8
HPO_4^{--}	2.0 "				8
HCO_3^-	20.0 "				8
Non protein nitrogen	176.8 mg/100ml	±37.5			2
Pyruvic acid	2.9 "	± .05			5
Lactic acid	34 "	± 1.2			5
2-oxo-glutarate	21.1 "	± 2.05			5
glutamic acid	1067.5 "	± 136			2
"	1033 "	±91			1
"	1178 "		192.5 mg/100ml		7
creatine	92.4 "	±31.7			2
arginine	18.5 "		10.7 "		7
lysine	2.4 "		12.8 "		7
ammonia	3.2 "		15.8 "		7
urea	29.4 "		0		7
nitrogen	180 "				4
inositol	20.4 "		8.8 "		6
glucose	3.1 "		11.2 "		6
glycerol	2.8 "				6
erythritol			5.0 "		6

References to Table C: (1) Lake, Butler, McCallum and MacIntyre (1958); (2) Lake and McIndoe (1959); (3) Lake (1962b); (4) Hammond, Boone and Barnett (1964); (5) Lake and El Jack (1964); (6) Ahluwalia and Graham (1966 a); (7) Ahluwalia and Graham (1966 b); (8) Lake and El Jack (1966).

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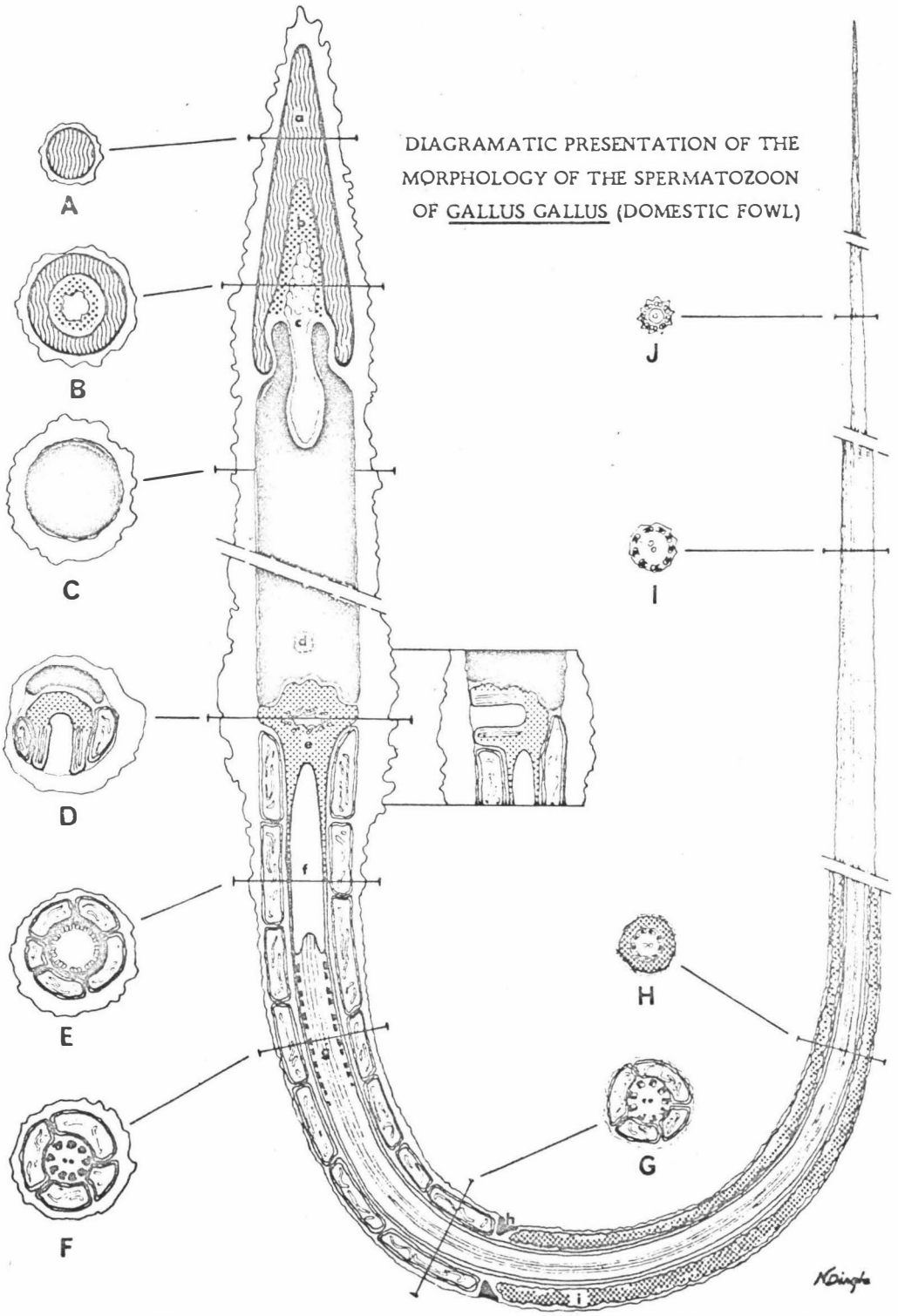
From DINGLE & McCOSKER (1967).

FIGURE 3



A-J: Cross-sections of the spermatozoon at the regions indicated. Opposite D is a longitudinal section taken at right angles to the section of the whole spermatozoon.

A, Acrosome cap; b, Amorphous layer; c, Acrosome spine; d, Nucleus; e, Implantation plate surrounding the proximal centriole; f, Distal centriole; g, Axial cylinder; h, Annulus; i, Tail sheath.

spine which extends into the nuclear material and is characteristic of poultry spermatozoa.

The midpiece region contains two centrioles and axial cylinder enveloped by a mitochondrial helix of seven gyrae, (Dingle and McCosker 1967). An implantation plate at the junction of the head and midpiece surrounds the proximal centriole which is orientated at right angles to the longitudinal axis of the cell. The distal centriole, also a tubular structure, lies along the anterior midpiece, and posteriorly is transformed into the axial filament. As in mammalian spermatozoa, the axial filament is composed of two central fibres surrounded by a ring of nine doublet fibres.

An annulus marks the beginning of the principle piece of the tail, where the axial filament is covered by an amorphous tail sheath which gradually diminishes in thickness until it exposes the axial material at the endpiece.

A plasma membrane surrounding the whole cell appears loosely attached at the head and midpiece, and probably causes the confusion over cytoplasmic drops and sheaths in the investigations of fowl spermatozoa.

Semen evaluation.

In spite of extensive research over many years, investigations have not been able to predict the fertilising capacity of spermatozoa from laboratory examination of normal ejaculates. Extreme features of aspermic, grossly contaminated or degenerated semen, can usually be detected at the time of collection.

The main criteria of semen quality currently used are: semen volume; spermatozoa concentration; spermatozoa motility (Cooper and Rowell 1957, 1958, Perek 1966); resazurin reduction time, or other methods of indicating spermatozoa metabolism by chemical changes; percent live spermatazoa (Bonadonna, Pazzi and Olgiati 1954); percent abnormal spermatozoa (Saeki and Brown 1962 b); resistance to temperature shock (Wales and White

1959); and changes in initial hydrogen ion concentration (Snapir and Perek 1964).

B. Research.

2.4 Materials.

1. Stock.

Males from two strains of single-comb White Leghorns, referred to as White Control (WC) and M Line (ML), together with one Black Australorp strain (AO), were retained from March 1966 to August 1967 solely for thesis experiments.

The WC males were a heterogeneous group derived from reciprocal crosses of a large-bodied unproductive strain which had been developed at Massey University, and the M Line. The cockerels averaged 5.8 lb. live weight on one year of age.

ML stock had been imported from an Australian C.S.I.R.O. line selected for high egg production, in 1960. The cockerels were phenotypically uniform, tight feathered, alert, and slightly smaller than the W.C.

The AO males were a large-bodied, loose feathered and heterogeneous group derived from three New Zealand strains. Average live weight at one year of age was 8.5 lb., but the range extended from 6.7 to 10.7 lb.

All the males were intensively reared on deep litter, in one controlled environment shed. From hatching, the stock received ten hours light per day provided by dull red bulbs. All cockerels were debeaked at one or two days of age.

At nineteen weeks of age, during March 1966, all the stock were moved to another controlled environment shed, where they remained for their productive lives. The cockerels occupied individual cages adjacent to six three-tier batteries containing three thousand laying hens.

PLATE 1.

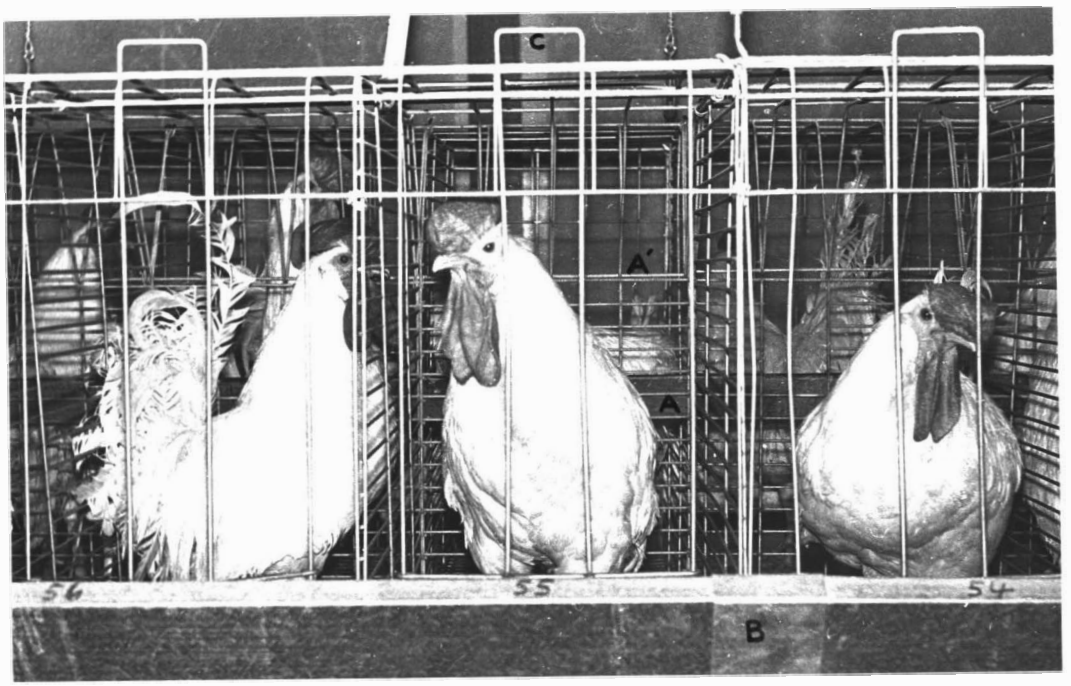


PLATE 1. Cockerel cages.

A : water trough

A' : position of original water trough

B : feed trough

C : top of sliding door to cage

In December 1966, twenty of the cocks were retained for natural matings over the summer, During this time, two cocks and approximately fifteen hens per pen, were housed on deep litter in small open front laying pens. Twelve of the original sixty males, were returned to individual cages in March 1966 for the remaining experiments.

2. Environment.

The cockerels were reared in mixed strain and sex of approximately 300 chickens. Natural matings occurred frequently during the juvenile stage, however it was impracticable to assess sex or strain social relationships.

Cockerel cages were situated five yards from the nearest, parallel laying battery. Individual cages were one foot wide and two feet deep and high, with vertical wire slide doors six inches above the cage floor (see Plate 1). The sixty cages were suspended two and one half feet from the floor, in two rows of thirty fused back to back. Running water was provided in a V-shaped trough which traversed the rear partitions of the cages. Long feed troughs suspended below the cage doors were subdivided during nutrition experiments.

Some temperature control was maintained by the manipulation of low wall vents and reversible fans at the apex of the shed. The mean diurnal range varied less in the winter months, as under local conditions the building was better equipped to conserve than repel heat. Daily maximum and minimum temperatures near the cockerel cages were recorded over the 1966 experimental period; the fortnightly average values are presented in Table D and Figure 4. Table D is shown on the following page.

Feed was distributed thrice weekly and available ad lib to the cockerels. The ration, a layer's mash, was estimated to contain

TABLE D.

	May 1-15-31		Jun. 1-15-30		Jul. 1-15-31		Aug. 1-15-31		Sep. 1-15-30		Oct. 1-15-30		Nov. 1-15-30		Dec. 1-15
Max.	63	60	61	58	58	57	58	59	61	64	64	66	68	67	69
Mean	57	55	56	53	54	52	53	53	56	57	57	60	57	60	63
Min.	51	48	50	48	49	47	47	47	50	51	50	53	54	53	57

15% crude protein, 2.7% fat, 2.7% ash, and 1331 calories metabolisable energy per lb. Wheatmeal, at 55% was the main grain constituent. The premix contained santoquin, 2 oz. Trelcovite (containing 8 million I.U. vitamin A per lb.; 2 million AOAC units vitamin D_3 per lb.; and 4 g. vitamin B_2 per lb), plus 0.6 oz. selenium premix plus 1 g. vitamin E plus 2 g. manganese sulphate, per 100 lb. of feed; and stone grit was always available.

Routine activities in the vicinity of the cockerels included daily automated feeding of the pullets, daily egg collections, and twice daily removal of manure trays. Special activities, which tended to excite the cockerels, included semen collection and insemination handling; spraying deodorant; cleaning fan cases; moving feed, and visitors.

Lighting was provided by three pairs of white 40 watt bulbs suspended three feet above each row of the cockerel cages, spaced about ten feet apart. The daily light period was increased to twelve hours during March when the stock entered the laying shed at nineteen weeks of age. When aggregate egg production reached 70%, in early May, the light period was increased by thirty minutes per week to sixteen hours daily and held constant thereafter.

3. Equipment.

Pyrex centrifuge tubes were used to collect and store semen. All the tubes were hand calibrated with a diamond pencil to metered water volumes from a burette. A reading accuracy of 0.05 ml. under 0.5 ml., and 0.1 ml. from 0.5 ml. to 1.5 ml., could be obtained, and normally only one ejaculate was collected in a tube. The tube capacity of 15 ml. and stem length of over four inches, allowed for diluting and mixing semen.

PLATE 2.

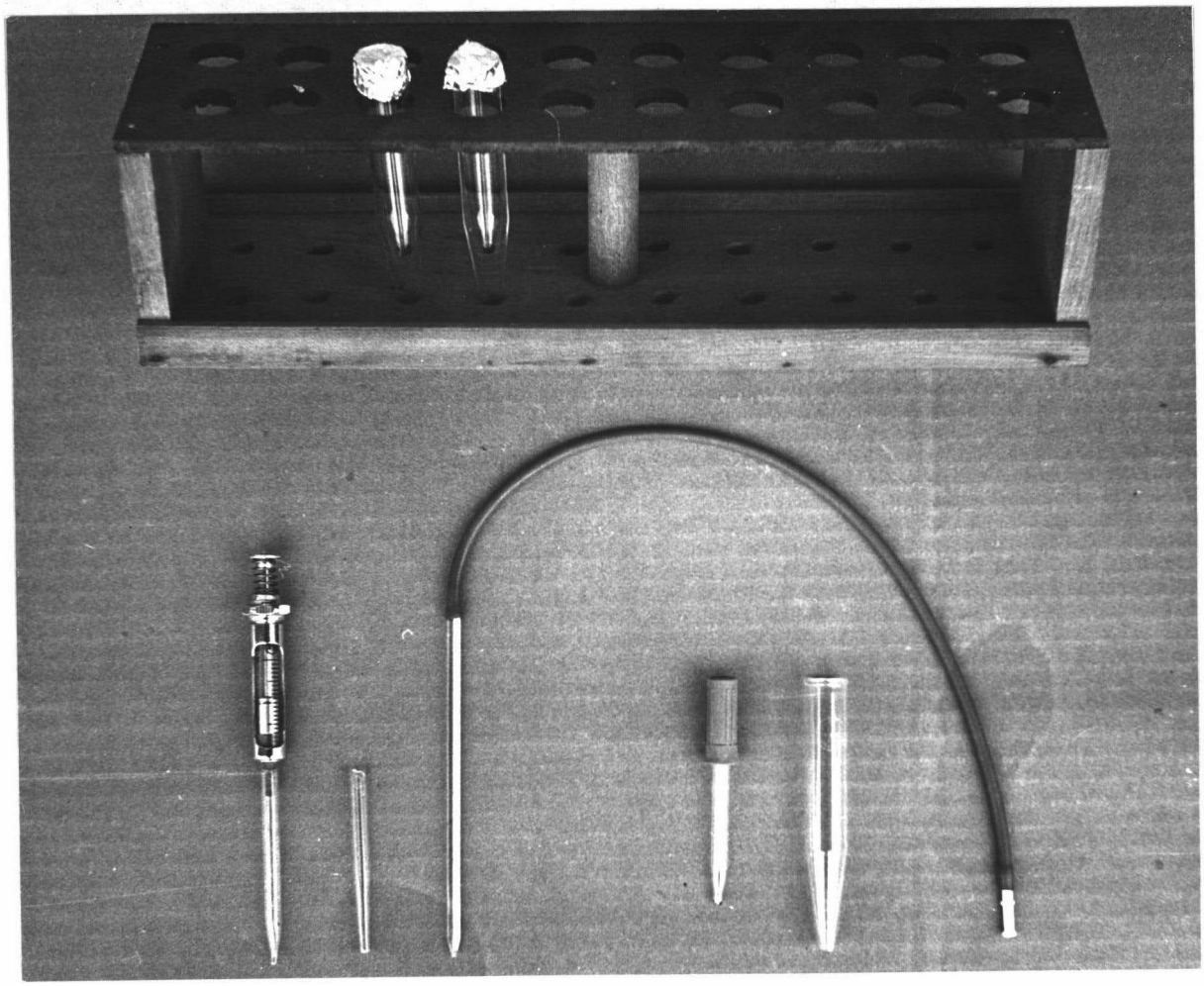


PLATE 2. Equipment used for semen collection and artificial insemination

Top : tinfoil capped centrifuge tubes in rack.

Left : insemination syringe with affixed cannula.

Centre : rubber blow tube and micropipette of 0.025 ml. capacity.

Right : alternative glass cannula and 15 ml. centrifuge tubes used for the collection and storage of semen.

When the samples were exposed to dust, tinfoil milk bottle tops were pressed over the tube tops (see Plate 2). Syringes and small pipettes were used for inseminations. Insulin syringes with a 2 ml. capacity were adapted for rapid use by inserting a return spring on the plunger and a brass tube around the bore, which limited the capacity to a metered fraction of a millilitre. Glass cannulae, about three inches long and a quarter inch bore, or eye droppers with tapered bulbous tops, were fitted onto the syringes to hold and convey semen into the vagina. Two pipettes with capacities of 0.025 ml. and 0.10 ml., were attached to a rubber blow tube for use in experimental inseminations.

Several diluents were investigated. Evaluations of pasteurised whole milk, Tyrode solution, Locke's solution and a sodium-phosphate buffer, are recorded in later chapters. The chemical composition of all the diluents are presented in Appendix B.

A nigrosin-eosin dye, composed of 30 g. nigrosin plus 5 g. eosin in 300 ml. distilled water, was used throughout the study.

A "Olympus", Model ECBI light microscope with WF 10X eyepiece and 10X, 40X, and 100X objectives, was used for all microscopic assay. The 100X objective was used with Cargille grade A oil, for immersion studies of stained smears.

A "Assistent" bright line improved, double Naubauer ruling, haemacytometer, was used for spermatozoa concentration counts. The chamber consisted of one square millimetre divided into twentyfive squares, all of which contained sixteen squares with an area of 0.0025 square mm. A gap of 0.1 mm. separated the chamber and coverslip.

Glass capillary tubes of one eight inch internal bore and four inches long, were used to contain semen in packed cell volume estimates.

An incubator run at 100°F. was used for fertility tests.

PLATE 3.

PLATE 3. Semen collection.

Pressure around the vent being applied during the second phase of stimulation during collection. After ejaculation the left hand is moved from massaging the cockerel's back, over the tail, to direct the semen release.

2.5 Methods.

1. Collection.

Ejaculates were collected by a one-man adaptation of the method proposed by Burrows and Quinn (1937). Cockerels were removed feet first from their cages and placed breast down on the operator's lap. The bird's legs were held above the hocks by the right hand, leaving the left hand free to massage the sacral-lumber regions and direct the ejaculation which followed (see Plate 3). Semen flow was guided into a tube by gentle pressure on the extruded copulatory organ (see Plate 4). The tube base was firmly gripped by the thumb and forefinger of the right hand. This allowed for large variations in the size and anatomy of cockerels, and conveniently warmed the part of the tube where the semen quickly settled. Tubes were then placed upright in a rack, and incompletely sealed with tinfoil caps.

The physical response and psychological state of every male during semen collection, were assessed by the number of stimulations required to elicit semen release, and the display of excitement after handling, respectively. A stimulation consisted of one or more massages and slight pressure around the copulatory organ if the cockerel ejaculated. Sexual desire of cockerels was subjectively scored from 0 to 5, as libido. The assessment was made on the intensity and duration of courtship behaviour, such as strutting and clucking, immediately after semen release when the cockerels were returned to their cages. Where possible, males were handled in the same manner and sequence.

2. Semen evaluation.

1. Semen volume was recorded immediately after ejaculation, when the fluid had settled in the centrifuge tube. Faecal and urinary contaminations and debris, and the proportion of cloacal fluid, were noted during the collection.

PLATE 4.

PLATE 4. Semen collection.

Semen is being expressed from the tip of the copulatory organ by gentle pressure and streams into the collecting tube. Characteristically, ejaculation was induced quickly and easily from the Australorp cockerel and the copulatory tissue subsided and reverted unless forcibly restrained. The semen illustrated, has been deposited exteriorly and appears relatively concentrated and uncontaminated by excess cloacal fluid or excreta.

2. (a) Subjective estimates of spermatozoa concentration were made by determining the opacity of a fresh semen sample swirled in natural light. Opacity was scored from 0 to 11, and concentration derived from a regression line presented in Figure 9.

(b) Packed cell volume estimates of spermatozoa concentration, were made by centrifuging two or three inches of whole semen in a capillary tube, at 3000 RPM (1277 RCF), for fortyfive minutes. A semen sample was sucked into the tube, the lower end was flame sealed, and two samples per ejaculate were centrifuged. Solid matter and supernatant plasma were clearly demarcated, and were measured in the tube, to an accuracy of 0.03 inches, or by the shadow cast on graph paper.

(c) For haemacytometer counts semen was drawn into a red blood cell pipette to the 0.5 mark, or 1.0 mark if very dilute, and mixed with diluent drawn up to the 101 mark. One drop of the mixture was released onto the counting chamber and immediately contained by a coverslip. When the fluid was stationary, entire spermatozoa in forty small squares were counted. Five large squares of each row and column were randomly chosen, and eight of the sixteen small squares within these were observed for spermatozoa.

TABLE E. Haemacytometer squares observed for spermatozoa.

			4	
6				
		13		
				20
	22			

Large squares

1		3	
	6		8
9		11	
	14		16

Small squares.

In a few instances two separate samples were drawn and counted for heterogeneous ejaculates. Spermatozoa were considered to lie in a square if the head and midpiece lay wholly within or extended over the right or lower margins.

Spermatozoa concentration was calculated by:

$$\frac{\text{No. sperm x dilution rate}}{\text{No. squares counted}} \times \frac{1}{\text{area square (mm).}} \times \frac{1}{\text{depth of charge (mm).}}$$

per cubic millimetre. Counts were made with 400 x magnification.

3. Dead and abnormal spermatozoa were detected in samples stained in nigrosin-eosin, at 1000 x magnification. About 0.1 ml. of semen was diluted $\frac{1}{2}$ or $\frac{1}{3}$, depending on viscosity, with diluent, and mixed in the bulb of a red blood cell pipette with an equal volume of dye. Two drops of the mixture were released onto a glass slide, smeared and air dried, and studied under oil immersion.

A total of five to seven hundred spermatozoa were counted in several fields of observation, to estimate the percent live. If the smear was irregular or inadequate, another sample was drawn from the ejaculate. Abnormal spermatozoa were recorded at the same time.

4. Motility was subjectively assessed from 100X and 400X magnifications of one drop of whole semen under a coverslip. Two 0 to 5 scales were used; the first for general swarming motion, and the second for the proportion of spermatozoa displaying movement.

3. Artificial insemination.

A method of insemination without wholly removing the pullets from their cages, was adopted after trial techniques of everting the cloaca (see Plate 7). The pullet, pulled feet first beneath the feed trough so that the abdominal region could be manipulated, remained in a familiar environment. One operator held both legs above the hocks, in the left hand, and exerted constant pressure anteriorly from the vent with the right hand. Extra pressure

was obtained by manipulating the bird's hocks. A few recalcitrant pullets could not be made to evert until they were lifted from their cages and strong constant pressure was applied to the lower abdominal region (see Plate 8). A second operator carefully penetrated the everted vaginal opening for four to five centimetres with a glass cannula attached to the syringe. When pressure was released, semen was expelled as the cloaca reverted and the abdomen relaxed.

4. Fertility tests.

For most experiments fertility was computed on an individual pullet basis, as the percentage of eggs which were fertile. Treatment fertility was computed as the average fertility of the pullet replications.

Eggs for fertility tests were collected from the second day after insemination, and stored stationary in a cool room at 10°C. for not more than eight days. After twentyfour hours incubation, the eggs were broken out onto an illuminated glass platform and the germinal discs were examined macroscopically. Those displaying normal embryonic development were classified fertile. Doubtful discs were examined under magnification and compared with diagrams of fertile and unfertilised, unincubated germinal discs.

2.6 Experiments.

Investigations of several aspects of semen production and release, were extended over twelve months for convenience of labour and assessing age and seasonal influences.

All the tables of results and analyses are presented in Appendix A.

Tables are composed of original data, although an arcsin transformation of all percentage results was considered necessary for analyses and comparison between means. Where comparisons were significant, the transformed treatment means and significant differences between them, are included in the text. With few exceptions, only the 1% and 5% probability levels of significance were applied to differences between means, correlations or t tests.

Experimental methods and results are reported here, and a discussion of the results follows in section 2.7

Semen production:

Experiment 1 Nutrition : protein and energy level effects on semen volume.

" 2 Light : pre-collection light stimulation.

" 3 Age and season influences on semen quality.

" 4 Genetic : semen characteristics of three strains.

Semen release:

Experiment 5 Hour of semen collection.

" 6 Collection techniques.

" 7 Semen exhaustion with frequent collections.

" 8 Evaluation of spermatozoa concentration estimates.

EXPERIMENT 1.

Methods.

Four combinations of dietary protein and energy levels were tested on cockerel semen production. Two crude protein levels, referred to as high protein (HP) 16.30%, and low protein (LP) 10.35%, were achieved by varying the casein level from 1% to 8% of the diet. Untanalised pine sawdust was used to dilute the energy levels and up to 27% sugar to increase them. The high energy (HE) level was estimated at 1298 Calories metabolisable energy per pound, and low energy (LE) at 946 Calories ME/lb. The contrived differences between the rations were as follows:

Ration A	10.35% cr.protein;	946 Cal./lb;	Cal/prot. = 90
Ration B	10.35% " ;	1298 " ;	" = 125
Ration C	16.39% " ;	946 " ;	" = 58
Ration D	16.30% " ;	1298 " ;	" = 80

Complete ration analyses are presented in Appendix C.

The experiment extended for seven weeks from October 1966, and was divided into six, eight day periods for time trend analysis.

Twenty males of each breed; Australorp and WC White Leghorns (WL), in contiguous cages, were tested with five randomly selected replications per ration. Initial and final group live weights are presented in Table 1B.

All the rations were fed ad lib; they were weighed-in thrice weekly and cleared every three weeks. Feed consumption and estimated protein and caloric intake, averaged over five cockerels per treatment group, are presented in Table 1B.

Semen collections were taken every fourth day at 1430 to 1600 hours, using the same apparel and sequence of collection every time.

Individual semen volumes were recorded for every collection and are presented in Table 1A as the average of two consecutive collections per eight day period. Spermatozoa concentration estimates were made on the first and last collections. Spermatozoa per ejaculation, were computed from volume and concentration, and are presented in Table 1C.

Results.

Semen volumes were analysed as a three-factor experiment with randomised blocks in Table 1A. Figures are the average volume in millilitres, of two collections per eight day period from individual cockerels.

Average semen volumes in millilitres

Period	Mean	Ration	Mean	Breed	Mean
3	.7223	D (HP.HE)	.7988	AO	.7903
2	.6893	A (LP.LE)	.5958	WL	.4941
4	.6545	B (LP.HE)	.5880	$D_{.01}$ =	.0928
1	.6100	C (HP.LE)	.5262		
5	.5913	$D_{.05}$ =	.1307		
6	.4960	$D_{.01}$ =	.1584		
$D_{.05}$ =	.1773				
$D_{.01}$ =	.2094				

1. There were no significant interactions between the factors tested.

2. Semen volumes were significantly higher in period 2 ($P<0.05$), and period 3 ($P<0.01$), than period 6.

3. Semen volumes were higher ($P<0.01$) from cockerels fed ration D, than rations A, B and C, which themselves were not significantly different.

4. Australorps volumes were significantly higher than Leghorn.

Average number of spermatozoa per ejaculation in millions.

Collection	Mean	Breed	Mean
First	2224.8	AO	2334.3
Last	1525.2	WL	1415.4

5. There were no significant ration effects or interactions between the factors tested, for spermatozoa per ejaculation.

6. Average spermatozoa release was higher ($P<0.01$), from the first collection.

7. Average Australorp spermatozoa release was higher ($P<0.01$), than Leghorn release.

8. From Table 1B, positive correlations were obtained between semen volume with initial live weight (+ 0.871**), and percent increase in live weight with caloric intake (+ 0.785**); for rations within strains.

9. A moderate but non-significant correlation (+0.561) between semen volume and daily caloric intake, was much higher than that with daily crude protein intake (+0.213); for rations within strains.

EXPERIMENT 2.

Methods.

The effects on yearling cocks of bright illumination for two hours prior to collection, were assessed by the subsequent daily semen release.

After three days' preliminary collections, light treatment was applied for six consecutive days to group I males, and after cross-over, for six days to group II males.

Five WC yearling cocks in contiguous cages were used in each group.

The extra illumination for Treatment A, was provided by two white 150 watt bulbs suspended two feet above the test males, which were switched on at 1400 hours each day. Control males on Treatment B, shielded from these lights, received a constant sixteen hours light per day from the shed 40 watt bulb circuit. From the seventh to twelth day of the experiment the 150 watt bulbs were transferred to the group II control males, and group I males reverted to Treatment B.

Semen volumes were individually recorded from collections taken at about 1600 hours daily.

Results.

Data treated by crossover analysis of light treatments A and B, on male groups I and II, are presented in Table 2. Figures represent the summed semen volume in millilitres, of five males per group on each of the twelve days under test.

Average daily semen volume per group, in mls.

Treatment	Mean
A	1.329
B	1.174

1. Moderately higher semen volumes ($P<0.10$), were obtained from cocks under light Treatment A.

2. There were no significant semen volume effects attributable to group or day of collection.

3. No significant changes in libido or stimulation grades were observed during the fifteen days of daily semen collection.

EXPERIMENT 3.

Age and seasonal influences on semen production and release were mainly assessed from accumulated data of other experiments. From these

general observations it was impossible to distinguish individual light, temperature or aging effects. In two specific tests, comparisons were made between the semen characteristics of a group of males at twelve, and at nineteen months of age.

Method.

The first collection was taken when the cocks were twelve months old, during late spring in November 1966, and the second under similar conditions during late autumn, in May 1967.

Five WC, five ML and two AO yearling cocks were used for the comparison; one ML cock also underwent another semen exhaustion test similar to that reported in Experiment 7.

After preliminary settling periods the males were handled and massaged under similar circumstances and one ejaculation from each was collected for analysis.

Results.

Original data are presented in Tables 29A and 29B. The two ages of twelve and nineteen months, are referred to as 1966 and 1967 respectively. The strain groupings are as follows: males 1 to 5 White Control; males 6 to 10 M Line; and males 11 and 12 Australorp.

Average ejaculation volume, in mls.

Year	Mean
1966	0.4750
1967	0.2875

1. Moderately higher semen volumes. ($P<0.10$), were released by the cocks at twelve months age.

Average spermatozoa concentration, at 10^9/ml.

Year	Mean
1966	3.950
1967	3.383

2. There were no significant changes in spermatozoa concentration.

3. The average spermatozoa release per ejaculation, was higher ($P<0.05$) at twelve months than nineteen months of age.

4. Positive correlations were obtained between individual semen volumes (+ 0.646*), and spermatozoa concentrations (+ 0.684*), of the two collections.

A comparison of semen characteristics from exhaustion tests is presented in Tables 30A and 30B. Both tests were made during July; the first at nine months, and the second at twenty-one months of age. Only one ML male; referred to as male 9 in Expt. 3 and male B in Expt. 7, was used in the test. Four semen collections were made within three hours, at 1400, 1500, 1555 and 1650 hours.

Average semen volume, in mls.

Year	Mean	Hour	Mean
1967	0.4625	1400	1.15
1966	0.3250	1555	0.80
		1500	0.70
		1650	0.50

5. No significant age or hour differences in semen volume were obtained.

6. A large but non-significant correlation (+ 0.824) between the volumes of equivalent collections was derived.

Average spermatozoa concentration, at 10^9/ml.

Year	Mean	Hour	Mean
1966	0.80	1400	1.33
1967	0.51	1500	0.72
		1555	0.30
		1650	0.27

7. A significant decline in average spermatozoa concentration ($P<0.05$), occurred between the first and fourth collections.

8. Average concentrations were moderately higher ($P<0.10$), at nine months of age in 1966, than at twentyone months of age in 1967.

9. A positive correlation (+ 0.9762*) was obtained between the concentrations of equivalent ejaculations.

General age and seasonal influences are derived from the semen volume data in Table 36, and light and temperature changes graphed in Figure 4.

Monthly average semen volumes in mls., of all experimental cockerels.

Month	Mean		
July	0.587		
October	0.570	$D_{.05}$	= 0.123
June	0.520	$D_{.01}$	= 0.159
November	0.507		
December	0.503		
September	0.470		
August	0.403		

10. Semen volumes obtained in July and October were significantly higher ($P<0.01$), than those in August.

11. The monthly trends in semen volume were very similar for the three strains, as illustrated in Figure 4.

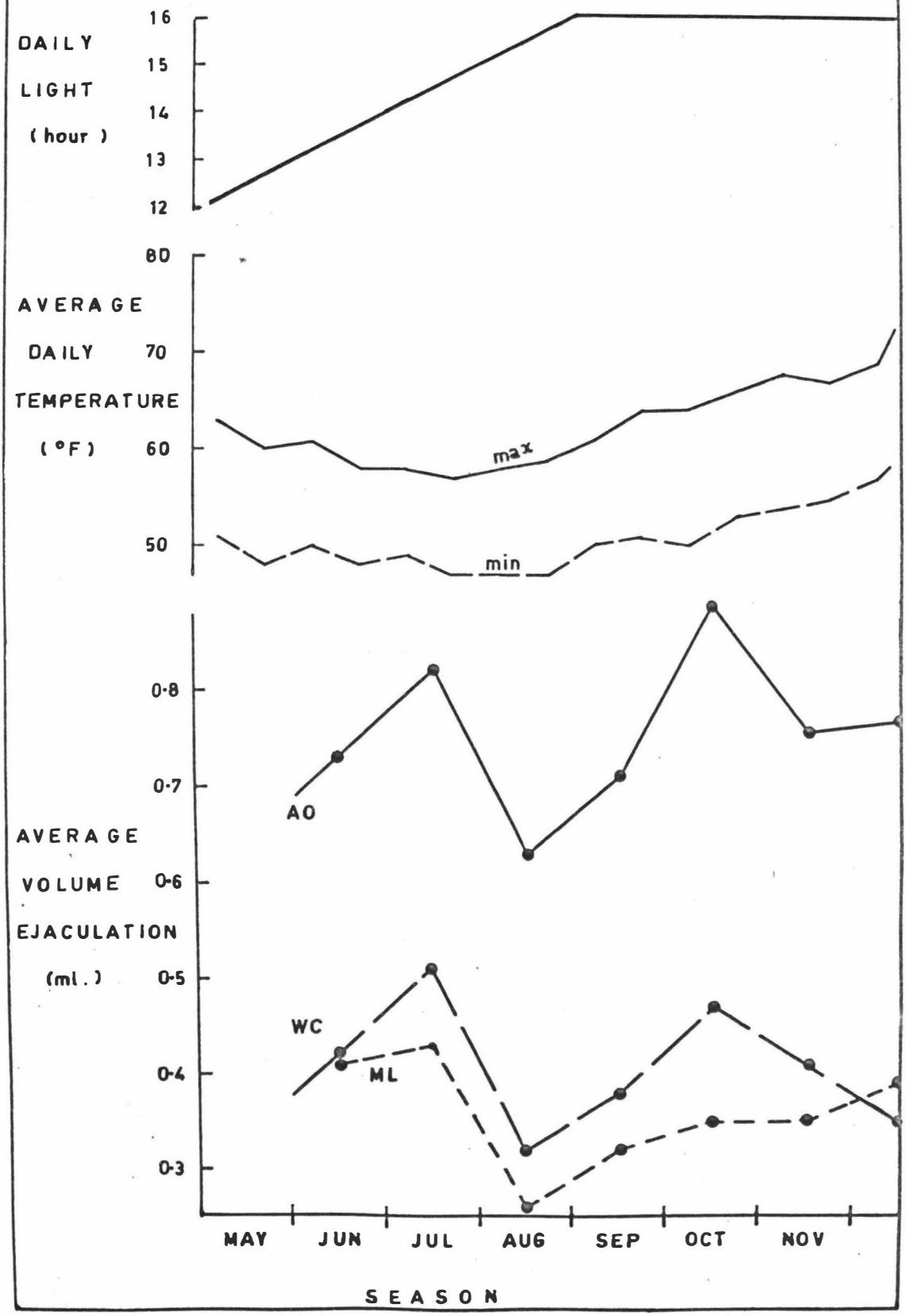
12. In the four months from July to October, the changes in average semen volume paralleled those of ambient temperature, and to some extent the changes in diurnal temperature range. The May-June semen values could be attributed to training, but the November-December values could not be statistically related to temperature or light changes.

EXPERIMENT 4.

Strain or genetic variations in semen production and release, were statistically significant in many of the experiments intended to test other

FIGURE 4

SEASONAL SEMEN PRODUCTION



factors.

A complete table of individual semen collection statistics from the thirty WC, ten ML and twenty AO cockerels, was compiled from May to December 1966. The monthly average semen volumes are presented in Table 36; strain averages are illustrated in Figure 4.

Results.

Significant comparisons obtained from Table 36 are fully reported in this experiment, but those extracted from other experiments are explained in their respective contexts.

Average semen volume, in mls.

Strain	Mean	
AO	0.7133	$D_{.05}$ = 0.1700
WC	0.4115	$D_{.01}$ = 0.2140
ML	0.3440	

1. The average semen volume released per collection by Australorp cockerels from the age of six to thirteen months, was higher ($P<0.01$) than that from Leghorn cockerels.

2. Within the Leghorn strains; the WC averaged 16% higher volumes than the ML, but were not significantly different ($P<0.05$).

3. Seasonal trends in semen release were similar for all strains, as illustrated in Figure 4. The ML exhibited relatively less fluctuation than the other two strains and maintained increasing semen volumes until December, when the collection ceased.

4. From Expt. 1, Tables 1A and 1C, significantly higher semen volumes ($P<0.01$), were obtained from AO than WC cockerels. There were no significant interactions between strain and nutritional factors tested.

Average semen volume, in mls., summed over three cockerels per group. (Table 25A).

Strain	Mean	
AO	1.580	$D_{.05}$ = 0.383
WC	0.933	$D_{.01}$ = 0.502
ML	0.787	

5. From Expt. 5, Table 25A; significantly higher semen volumes ($P<0.01$), were obtained from AO, than WC or ML males, as illustrated in Figure 8. In the same experiment, strain spermatozoa concentrations, were not significantly different. There were no significant interactions between strain and the time of semen collection, although Leghorn spermatozoa concentrations increased relatively more in the afternoon collections, than Australorp.

From Expt. 6, Tables 26A and 26B; several semen volume and concentration differences were obtained between the AO and ML strains.

Average semen volume, in mls. (Table 26A)

Strain	Mean	
AO	0.569	
ML	0.389	$D_{.01}$ = 0.106

6. Average AO semen volume was higher ($P<0.01$) than ML.

Average semen volume, in ml.: (Table 26A)

Strain-Method	Mean	
AO - B	0.617	
ML - B	0.556	$D_{.05}$ = 0.150
AO - A	0.522	$D_{.01}$ = 0.184
ML - A	0.223	

7. The ML response to collection Method A, was significantly lower ($P<0.01$) than that of AO cockerels. No other significant differences in strain response to collection method occurred.

Average semen volume, in mls. (Table 26A).

Strain	Method	Phase	Mean
ML	B	Revd.	0.750
AO	B	"	0.744
AO	A	Norm.	0.604
AO	B	"	0.489
AO	A	Revd.	0.439
ML	A	Norm.	0.400
ML	B	"	0.362
ML	A	Revd.	0.045
	$D_{.05}$ = 0.251		

8. In this three factor interaction, two points emerge : firstly that an increase in semen volume was elicited from both strains by a change to Method B, which was significant ($P<0.05$) for the ML cockerels. Secondly, a decline in semen volume arising from a change to Method A, was significant for the ML, but negligible for the AO cockerels. The normal phase of collection was that to which the cockerels were accustomed, while the reversed phase was an entirely new collection technique to them.

Average spermatozoa concentration, at 10^9/ml. (Table 26B)

Strain	Mean	
AO	4.663	$D_{.05}$ = 0.494
ML	3.963	

9. Australorp spermatozoa concentrations estimated with semen pooled from ten cockerels, were higher ($P<0.05$) than ML concentrations but were influenced by the treatments imposed.

Average semen volume, in mls. (Table 23).

Strain	Male	Mean
AO	D	0.675
AO	C	0.650

Continued....

Average semen volume, in mls. (Table 23). Continued

Strain - Male		Mean
ML	A	0.350
ML	B	0.325

10. From Expt. 7, Table 23; AO ejaculate volumes were significantly ($P<0.01$) higher than ML.

Average spermatozoa concentration, at 10^9/ml.

Strain - Male		Mean	
AO	C	3.68	
ML	A	3.51	$D_{.05} = 1.99$
AO	D	2.49	$D_{.01} = 2.69$
ML	B	0.80	

11. From Expt. 7, illustrated in Figure 5, significant differences in spermatozoa concentration between cockerels were obtained. Significant variations within strains usually obscured genetic differences in spermatozoa concentration between them.

EXPERIMENT 5.

Method.

Semen collections were made at three hours of the day, to determine variations in cockerel semen release.

The experiment was conducted in August 1966, when the cockerels were nine months of age and had been trained to afternoon collection for at least three months.

Nine cockerels in contiguous cages from each strain, were divided into three groups (I, II and III), which were tested over the three treatments.

The treatment consisted of semen collections at 0855, 1335 and 1630 hours. The three groups, each comprising three Ml, WC and AO cockerels, were rotated over the treatment hours on the first, third and fifth days of the

experiment. In this way the sequence of collections was maintained, all the cockerels were tested at each hour, and one day intervals reduced carryover effects. Other environmental influences such as feeding and human activity, were normalised as far as possible.

Individual semen volumes were recorded and spermatozoa concentration estimations made on the semen pooled from each strain group of three cockerels.

Results.

A factorial analysis of variance is presented in Table 25A. Figures are derived from the pooled semen volumes of each strain group. Spermatozoa concentrations in Table 25B are treated the same way, and all the results are illustrated in Figure 8.

Average semen volumes per strain group, in mls.

Strain	Mean	Hour	Mean
A0	1.580	1630	3.34
WC	0.933	1335	3.32
ML	0.787	0855	3.24

$D_{.05}$ = 0.383

$D_{.01}$ = 0.502

1. Significantly higher semen volumes ($P<0.01$), were obtained from the A0 than the Leghorn strains.

2. The hour of collection had no significant effects on the volume of semen released.

Average spermatozoa concentration, at 10^9/ml.

Hour	Mean	Strain	Mean
1630	5.40	A0	5.1
1335	5.30	WC	4.8
0855	3.80	ML	4.6

$D_{.01}$ = 1.192

3. Higher spermatozoa concentrations ($P<0.01$), were obtained from

the afternoon collections.

4. Insignificant spermatozoa concentration differences between strains occurred, but a moderate strain by hour interaction arose from fluctuations in AO concentrations.

EXPERIMENT 6.

Method.

The merits of two semen collection techniques were assessed by semen release and libido.

The experiment was conducted in August 1966, when the cockerels were nine months of age and each group had been trained to its respective collection technique for at least three months.

Ten ML plus ten AO cockerels in contiguous cages, were randomly selected from each groups of males. One group, used solely for experiments, had been trained to the one-man collection technique Method B by one operator. Method B has been described in Section 2.5 and illustrated in Plates 3 and 4. The cockerels trained to Method B, referred to as Group B, were handled more frequently and surrounded by more human activity than Group A. The second group of cockerels had been trained to Method A, a two-man technique similar to that described by Burrows and Quinn (1937), with several operators. In Method A; one operator's right hand restrained a cockerel by the legs, breast down on a plywood sheet which rested on the base of the cage door. Stimulation was applied by massaging the sacral-lumbar region with the left hand, which later moved to the vent region to direct the semen release after ejaculation. The second operator held a tube below the copulatory organ to collect the semen.

In the experimental period four semen collections were made on each cockerel. The first collection on day 1 was normal, with times, sequences and apparel similar for both groups and operators. The next two collections on the third and sixth days were reversed, with Method A applied to Group B cockerels and vice versa. Another normal collection was made on the eighth day to

investigate psychological carryover effect.

Individual semen volumes, pooled group-strain semen concentrations, stimulation grades and libido; were recorded at each collection.

Results.

Original data and analyses of variance are presented in Tables 26A and 26B. Results of the two normal and reversed collections for each cockerel replication, have been averaged and presented as one figure for each phase in Table 26A. Spermatozoa concentrations estimated from pooled semen for each strain-group, are presented separately for each phase as replicate 1 and 2, in Table 26B. A comparison of the two methods is illustrated in Figure 6.

Average semen volume, in mls.

Method	Mean	Strain	Mean
B	0.511	AO	0.569
A	0.395	ML	0.389
$D_{.01}$ =	0.106	$D_{.01}$ =	0.106

1. Higher semen volumes ($P<0.01$) were obtained with Method B.
2. Australorp volumes were higher ($P<0.01$) than M Line.
3. There was no significant phase effect.

Average semen volume in mls., of 2-factor interactions.

Method - Phase		Mean	Method - Strain		Mean
B	Revd.	0.747	B	AO	0.617
A	Norm.	0.502	B	ML	0.556
B	"	0.426	A	AO	0.522
A	Revd.	0.242	A	ML	0.223
		$D_{.05}$ =	0.150		
		$D_{.01}$ =	0.184		

4. Method B induced higher volumes ($P<0.01$) from reversed cockerels than Method A.

5. Method B induced higher volumes ($P<0.01$) from cockerels trained to Method A than the latter method.

6. Method A obtained lower volumes ($P<0.01$) from cockerels trained to Method B than the latter method.

7. Method A applied to ML cockerels obtained lower volumes ($P<0.01$) than when applied to AO cockerels, or compared to Method B with either strain.

Average semen volume in mls., of 3-factor interaction.

Method	Strain	Phase	Mean
B	ML	Revd.	0.750
B	AO	Revd.	0.744
A	AO	Norm.	0.604
B	AO	Norm.	0.489
A	AO	Revd.	0.439
A	ML	Norm.	0.400
B	ML	Norm.	0.362
A	ML	Revd.	0.045
	$D_{.05}$ =	0.251	

8. Method A obtained lower volumes ($P<0.05$) with Group B ML cockerels, than any other combination.

9. Method B induced higher volumes from both strains of Group A cockerels, than the normal Method A; these were significant ($P<0.05$) for the ML.

10. Group B AO cockerels responded similarly to both collection methods.

Average spermatozoa concentration, at 10^9/ml.

Strain	Mean	
AO	4.663	$D_{.05}$ = 0.494
ML	3.963	

11. Average spermatozoa concentrations were higher ($P<0.05$) from

AO cockerels.

12. There were no significant concentration differences between methods or phases.

Average spermatozoa concentrations, at 10^9/ml.

for 2-factor interaction.

Method	Phase	Mean	
B	Revd.	4.65	
A	Norm.	4.60	
B	"	4.38	$D_{.05} = 0.978$
A	Revd.	3.63	

13. Higher concentrations ($P<0.05$) were obtained by Method B than Method A, for reversed collections. From Table 26B it is clear that both strains showed similar responses.

EXPERIMENT 7.

Method.

Some effects of frequent semen collection on cockerels, were assessed by the physical and psychological responses.

The experiments were conducted during July 1966, when the cockerels were eight months old and well trained to handling for semen collection.

Two ML and two AO cockerels were chosen after a preliminary period of selection, for one male capable of concentrated ejaculates and one of dilute ejaculates, in each strain. The ejaculate types were referred to as I and II, respectively. The ML cockerels were labelled A (I) and B (II); and the AO cockerels C (I) and D (II). All four cockerels were normally friendly and responsive to handling.

After the testing period, four semen collections from each cockerel were made within three hours; at approximately 1410, 1500, 1600 and 1656 hours on the first day. A single interim collection was made on the fourth day. On

the seventh day, collections similar to those on the first were made, and each ejaculate was immediately used for four 0.1 ml. inseminations of semen diluted 1/2 with milk. Four AO pullets per ejaculate were inseminated and eggs collected from the second to fifth day were tested for fertility. All the cockerels were treated normally for collection, and semen was obtained after not more than three stimulations and the usual application of pressure to the vent region.

Semen release and quality were evaluated by individual volume, spermatozoa concentration and fertility tests; cockerel response was estimated by stimulation grade and libido.

Results.

Average strain results are illustrated in Figure 5. An analysis of fertility results is presented in Table 22. Figures represent the percentage of fertile eggs from each pullet, but the percentages were transformed for analysis and comparisons between means.

Average percent fertility (transformed).

Strain	Mean	Type	Mean
AO	69.36	I	69.85
ML	55.56	II	55.08

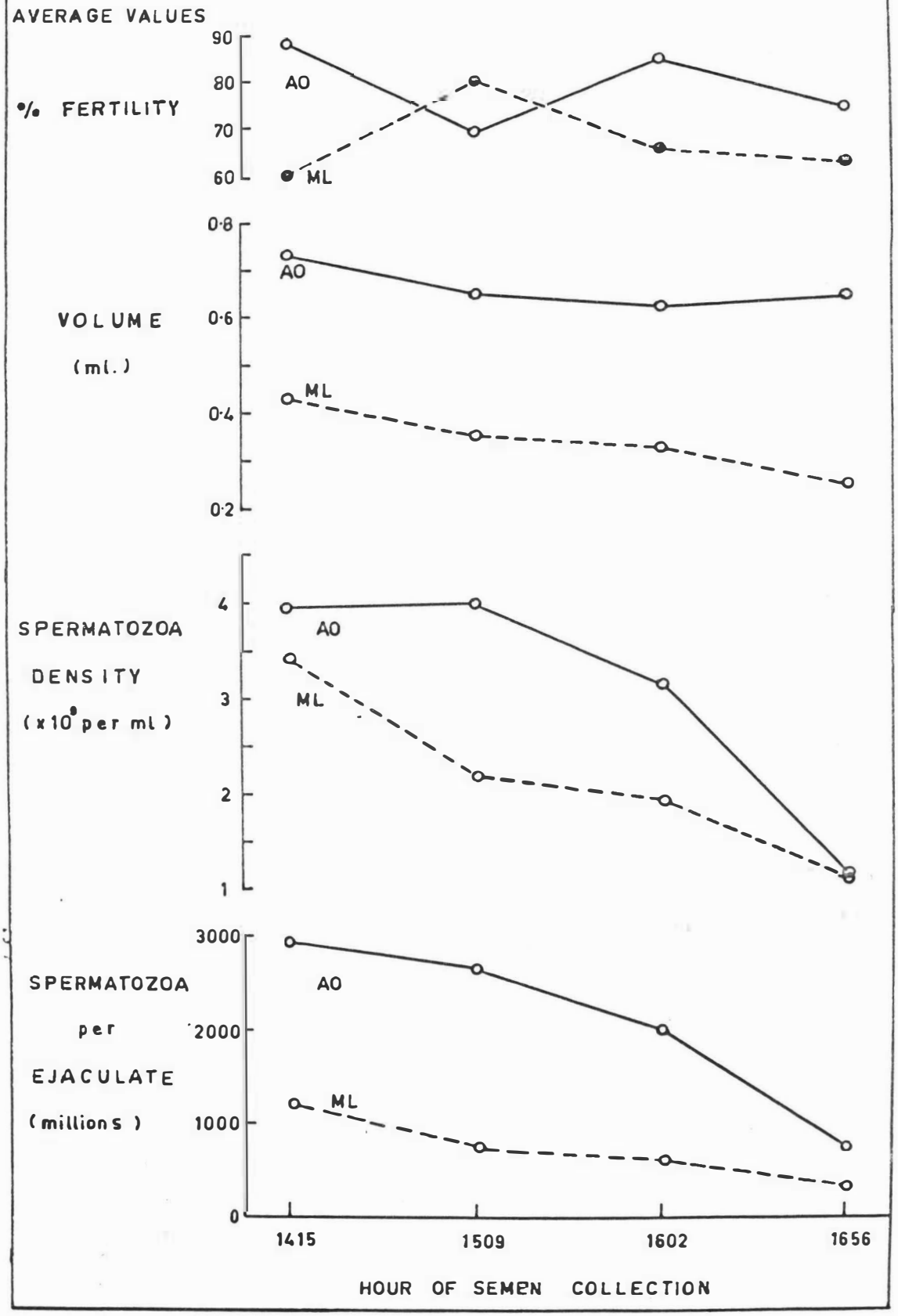
1. Moderately significant genetic differences ($P<0.10$) were obtained; higher fertility was achieved by the AO cockerels, and those two in the Type I or concentrated semen classification. There were no significant differences in the average fertility from successive collections.

Average semen volume, in mls.

Male	Mean
D	0.675
C	0.650
A	0.350
B	0.325

FIGURE 5

COCKEREL SEMEN EXHAUSTION



2. From Table 23 highly significant strain differences in volume ($P<0.01$) were obtained; AO cockerels released higher volumes than ML, but there were no type differences.

3. A gradual non-significant decrease in average volume occurred from the first to fourth collection.

Average spermatozoa per ejaculate, in millions.

Male	Mean	Hour	Mean
C	2554	1407	2189
D	1591	1456	1681
A	1278	1556	1310
B	289	1656	530
	$D_{.05} = 1558$		$D_{.05} = 1558$

4. From Table 24, higher spermatozoa releases ($P<0.05$) were obtained at the first collection than the last.

5. Male C (AO, I) released significantly, ($P<0.05$) more spermatozoa than Male B (ML, II).

Average spermatozoa concentration, at 10^9/ml.

Male	Mean	Hour	Mean
C	3.68	1407	3.67
A	3.51	1456	3.09
D	2.49	1556	2.54
B	0.80	1656	1.16
	$D_{.05} = 1.99$		
	$D_{.01} = 2.69$		

6. From Table 23B significantly higher spermatozoa concentrations ($P<0.01$) were obtained from the two Type I males A and C, than Male B.

7. Higher concentrations were obtained in the first collection ($P<0.05$), than the last.

EXPERIMENT 8.

The accuracy of spermatozoa concentration measurements and the relationship between the three methods of estimation, were assessed by multiple counts and sampling.

Secondly, the relationship of libido and stimulation grade with semen characteristics, was studied.

Methods.

1. Spermatozoa concentration estimations with a haemacytometer as outlined in Section 2.5, were made on ejaculates from four cockerels. The ejaculates were adjusted to equal volumes, coded, and counted in four serial samples; the first twenty minutes after collection and the last series four hours later. All ejaculates were stored aerobically at 10°C. in the original tubes with a tinfoil cap to reduce dessication.

2. Concurrent estimations were made with haemacytometer counts and the two other methods of determining concentration; centrifuged packed cell volume and opacity score. All calculations were made from individual fresh ejaculates, as outlined in Section 2.5.

3. Libido, stimulation grade, opacity score and semen volume, were measured on individual ejaculations from ten WC yearling cocks undergoing daily semen collections.

Results.

Data for the accuracy of haemacytometer estimations are presented in Table 33. Figures represent the number of spermatozoa counted in two determinations from five randomly selected large squares. Each determination was made on four of the sixteen small squares within each of five large squares.

1. No significant differences between samples, within males, were obtained for the two ML ejaculates.

Mean no. spermatozoa per determination.

Male 83		Male 84	
Sample	Mean	Sample	Mean
3	28.1	4	24.7
1	23.0	3	16.4
4	19.3	2	16.3
2	10.7	1	14.8

$D_{.05} = 5.46$

$D_{.01} = 6.66$

2. From Male 83; significantly higher counts were obtained from Sample 3 ($P<0.01$) than Samples 4 or 2, and from Sample 1 ($P<0.05$) than Sample 2.

3. From Male 84; Sample 4 count was significantly higher ($P<0.01$) than all others.

Data on the three methods of estimating spermatozoa concentration are presented in Table 37 and illustrated in Figure 9.

4. A significant regression ($P<0.01$) was obtained of percent packed cell volume on haemacytometer count:

b = 1.989 % P.C.V. per 10^9 spermatozoa/ml. with 1% confidence limits: $1.5132 \leq \beta \leq 2.4646$ % P.C.V.

5. A significant regression ($P<0.01$) was obtained of opacity score on haemacytometer count.

b = 1.514 units score per 10^9 spermatozoa/ml. with 1% confidence limits: $1.0589 \leq \beta \leq 1.9695$ units score

Data on the associations between libido, stimulation grade, opacity score and semen volume, are presented in Table 38.

6. A negative correlation (-0.3065**) was obtained between volume and stimulation, and volume with libido (-0.2631*).

7. A positive correlation (+0.2140*) was obtained between stimulation and libido.

8. A negative correlation (-0.3714**) was obtained between volume and opacity score.

2.7 Discussion.

Significant results obtained in the experiments generally confirmed some of the hypotheses discussed in the literature review. Some results suggested a different emphasis, particularly aspects of cockerel response to semen collection, but there was no conflict with established theories.

With respect to practical artificial breeding, the main conclusions drawn are: the high spermatogenic capacity of cockerels; the seasonal influence on spermatogenesis; the importance of adequate dietary energy levels for spermatogenesis; the disparities between natural mating and artificial semen collection; and the psychological domination of semen release.

Unfortunately the experiments on semen production suffered from the same problems as many of those reported in the literature. They were neither precise nor large enough to produce the anticipated effects, and most of the conclusions, as in the literature, are theories based on reports and some statistically significant results. In this thesis the research into semen production lacked sufficient environmental control or background data on the cockerels during rearing, puberty, and summer semen production.

****

The criteria of semen quality, though few, were useful in the circumstances of an essentially practical approach to artificial breeding. A more sophisticated study of semen was never envisaged, partly because of the limitations to thesis research and also because the literature showed almost unanimously insignificant associations between fertilising capacity and

laboratory evaluations of 'normal' semen.

Methods used for measuring ejaculate characteristics, appeared satisfactory when all the data was recorded by one operator.

Semen volumes, measured immediately after ejaculation in the collection tubes, were quickly and easily recorded. This method avoided the inaccuracies arising from surface wetting and dessication with funnels, transfer pipettes or weighing apparatus. A possible source of error from reading volumes off the faint calibrations in dull light, was unavoidable but probably systematic.

Spermatozoa concentrations were measured by three ways described in Section 2.5, but only regularly by haemacytometer count. This method provided opportunity to see the spermatozoa, their motility and peculiarities, and debris in the ejaculate. The main problems with haemacytometer counts were: sampling from the ejaculate; cellular disintegration due to ethanol added to kill the spermatozoa - a procedure that was later discarded; tight clumps of spermatozoa, which greatly affected the concentration if lying in the counting chamber, yet were considered by some workers to be functionally useless; differences in motility between samples, with highly motile spermatozoa causing an over-estimation; concentration differences, where the dilute samples were easily counted compared to the dense samples which tended to be underestimated; and the high proportion of cloacal transudate and spermatozoa blobs in some ejaculates which were difficult to disperse for sampling. The accuracy tests of haemacytometer counts, showed insignificant variation in repeated samples from the two ML ejaculates. However, the highly significant variations for the two AO ejaculates are attributed to sampling error and the presence of spermatozoa clumps. A concentration effect which could have arisen with evaporation of seminal fluid as the ejaculates were stored, did not eventuate. No significant increase in estimation accuracy was obtained from counting twice the number of large squares per sample.

Estimating concentrations by the percentage packed cell volume, was

quicker than haemacytometer counts with a large number of ejaculates, and avoided some errors by the use of larger and replicated samples. Centrifugation for fortyfive minutes at 1277 RCF was found to be adequate and easy to supervise. However the method entailed some important disadvantages, particularly because the semen was not examined microscopically and the nature of spermatozoa motility, or contaminants, were unknown. Preparing the capillary glass and sucking in the semen and sealing one end of the tube was laborious for a few samples, and accurate measurements of the centrifuged fractions were frequently baffled by a distortion of the tube end caused by the sealing process. Nevertheless, a highly significant correlation between count and packed cell volume, and the regression line illustrated in Figure 9, made this method a useful substitute to the former.

Surprisingly, the third method of estimating concentrations, the opacity score, was also highly significantly correlated to haemacytometer counts. Under the conditions of frequent and personal recording of data this subjective estimation was extremely useful for rough assessments and quick dilution calculations. The simple swirling of individual ejaculates was quick and enabled the operator to detect gross contamination or excessive transudate. In routine semen collections the score allowed the operator to judge the volume in better perspective, and avoided apparent fluctuations in spermatozoa release arising from collection technique. The disadvantages are equally imposing: the scoring was purely subjective and liable to large variation between operators; the natural light conditions for swirling the samples were frequently dissimilar on different days; inaccuracies arose from inadequate mixing and near-white opaque contaminants; and a volume effect meant that very small or aspermic ejaculates appeared more concentrated after swirling, and that dense samples tended to be under-estimated. In spite of the inherent errors, opacity scoring proved to be a useful approximation to spermatozoa concentration.

PLATE 5.

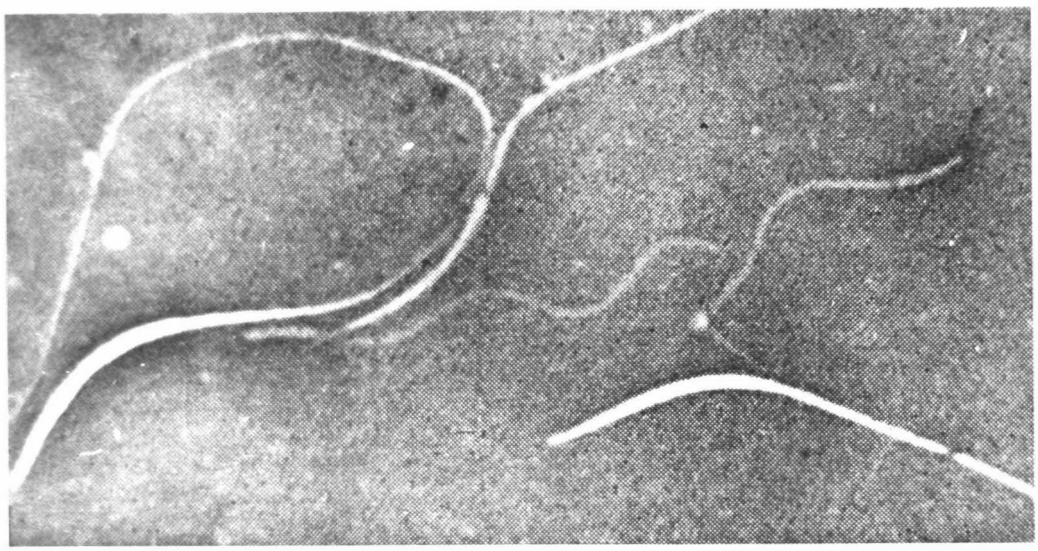


PLATE 5. Spermatozoa stained with Nigrosin and Eosin.

The two lower spermatozoa, alive at the time of staining, have remained impervious to the nigrosin but the centre spermatozoa has stained chromosome material. Two granulated tails and midpieces have also taken up the dye. (From: Lake, P.E., (1954), Proc. Tenth Worlds' Poultry Congress pp 79-85). Scale x 952

Methods for estimating the percentage dead or abnormal spermatozoa with a light microscope, were neither accurate nor particularly useful for semen evaluation. The smears taken were liable to the same sampling errors as haemacytometer counts and since all the spermatozoa were eventually killed, the error could not be discovered by reference to the opacity score. Other problems arose from the irregularities of counting fields associated with the density of spermatozoa and clumps; variations in the degree of staining arising from semen composition and the mixing procedure which rendered some spermatozoa faintly pink and difficult to classify; and distorted spermatozoa which were possibly artifacts of the staining and smearing technique. Excepting some analyses with stored semen, few significant variations in the percentage of dead spermatozoa were obtained. Only two clear abnormalities of spermatozoa were noted. The first was a swollen head and midpiece, distinguishing up to one quarter of the spermatozoa of one AO cockerel without significant changes in fertilising potential. The second abnormality was found in numerous ejaculates, where spermatozoa were bent at the midpiece and occasionally the head was nearly parallel to the tail. There is reasonable evidence from the literature to suggest that either of the abnormalities could be caused by an incompatible ion balance in the diluent, or the staining and smearing techniques. Further doubt is expressed in the report by Saeki (1960), suggesting that a light microscope with 1000 X magnification is inadequate for detecting some abnormalities which can be found with an electron microscope. Staining spermatozoa did not contribute to predictions of semen fertilising capacity in this study, but possibly has a role in the analysis of semen from infertile males in an artificial breeding scheme.

Motility tests were confined to stored semen. The scores were not sufficiently precise for predictions with fresh semen and sometimes irrelevant with stored, as spermatozoa motility was usually retained long after the functional capacity had been lost. Assessments were purely subjective and

although very easy to conduct, observations had to be rapid to avoid a thermal-activating effect from the warm microscope platform.

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Semen Production.

One important conclusion drawn from the nutrition experiment is that dietary energy has influential and quite rapid effects on the semen production of mature cockerels. The results indicate that only ration D, with moderate and balanced energy level, was capable of at least maintaining similar spermatozoa releases throughout the experiment. In contrast, ration C with low and unbalanced energy levels produced a notable decline in spermatozoa release in the last third of the experiment. Parker and Arscott (1964) reported similar results when energy intake was reduced from 230 to 143 Calories per day, and that a loss in body weight was associated with a decline in semen volumes. The comparitively muted variations in Expt. 1 are not surprising considering that a 'low' energy intake in this experiment averaged 320 Calories ME per day, and a 'high' energy intake 407 Calories ME per day. Although the two sets of energy levels may not be exactly equivalent, clearly 320 Calories ME per day is not a low energy intake. Unlike the restricted feed consumption imposed in Parker's experiments, the thesis cockerels were fed ad lib and probably tried to eat to their energy requirements. Under these circumstances differences in semen characteristics could be attributed to the energy-protein balance. Rations A and D, with supposedly well-balanced C/P ratios produced the highest spermatozoa releases in the final weeks, in spite of the 'low' energy and protein levels in the latter. Rations B and C, with equally extreme imbalances, although not significantly lower tha n Ration A, appeared to be inadequate diets. The response to Ration B with its high energy content is particularly puzzling.

All the treatments show a similar trend in semen volume, as illustrated

FIGURE 6

SEMEN COLLECTION TECHNIQUES

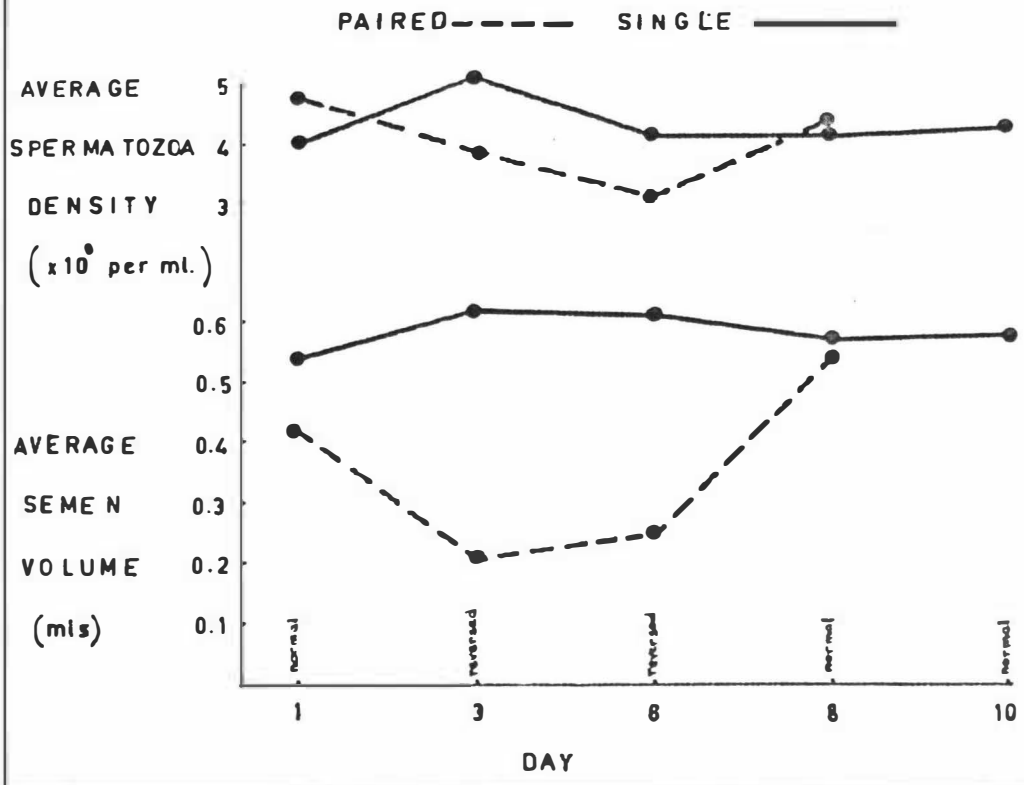
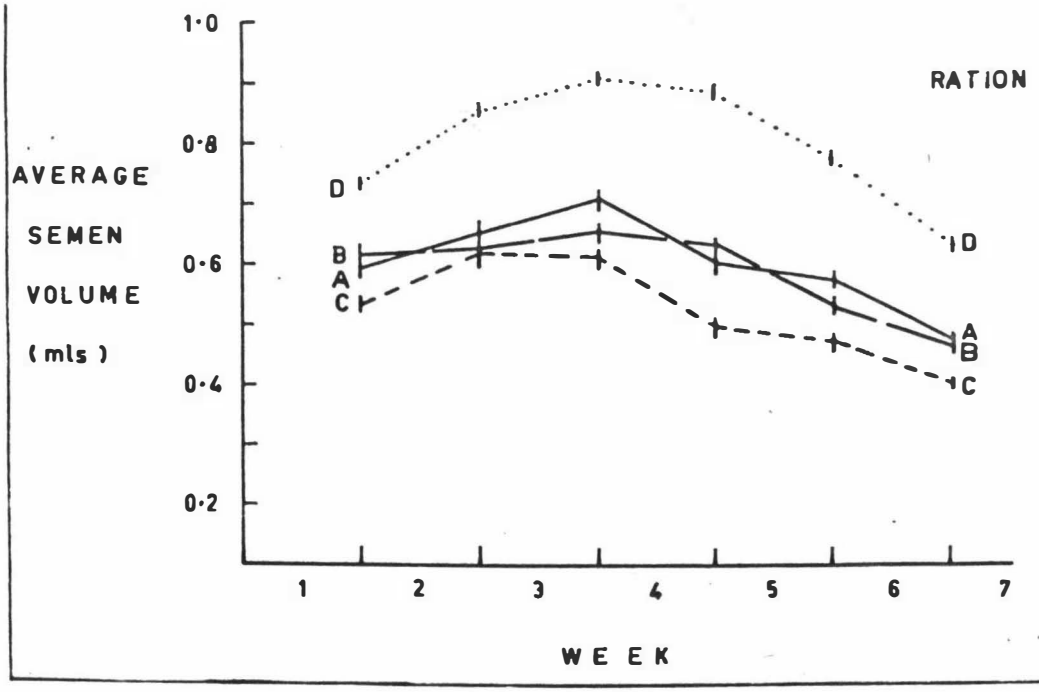


FIGURE 7

NUTRITIONAL EFFECTS ON SEMEN PRODUCTION



in Figure 7. The increases in the first three or four weeks were probably due to higher releases obtained from training with regular collections, and treatments only began to affect results during the last two or three weeks. This reveals the two main experimental deficiencies: the test was too short for mature stock with a comparitively unknown variable such as semen production, and equally important was the inadequate variation between the diets imposed.

With the meagre statistically useful results, Expt. 1 corroborates the rather negative attitude from the literature, that mature males are relatively tolerant to a wide range of protein consumption. Apparently caloric intake is more influential, as indicated by the higher correlation of energy than protein, with inter-group semen volumes.

A significant increase in semen volume was obtained from the light treatments in Expt. 2, in a period of general decline. This could be attributed to the beneficial effects of brighter illumination or extra intermittent light on semen production, hypothesised in the literature. However, it is very unlikely that spermatogenesis could be accelerated within a few days, via the oculohypothalamic-pituitary-testes pathway. A more likely explanation is that the cockerels quickly associated the lights, and activity of switching them on, with the apparently pleasant activity of semen collection. It is reasonable to assume that the extra psychological preparedness in some way favoured slightly larger ejaculations, possibly by a reduction in inhibition. This view could account for the notable carryover effect of light treatment on the group which reverted to control conditions. Although these cocks were not directly illuminated they could see the lights being switched on and some reflections off the ceiling.

Another result from the experiment, which will be discussed later, was the ability of the yearling cocks to maintain their previous levels of spermatozoa release with daily collections, at a time when most levels were decreasing.

This too, suggests that the cock is capable of a high level of spermatogenesis providing the psychological and physical treatments favour semen release.

From the general semen collection trends and shed lighting pattern as illustrated in Figure 4, it is impossible to isolate particular effects of lighting as being responsible for semen changes. Evidence from the literature indicates that ten hours daily light during rearing would delay sexual maturation and avoid precocity compared to more than twelve hours of daily light. The step up pattern from nineteen weeks of age would probably accelerate maturation and favour high spermatogenesis during the winter and spring months, in much the same way as female egg production was stimulated. However the rising summer temperatures coupled with sixteen hours daily light which were intended optimal for the last few weeks of egg production in December, were likely to favour the sexual refractoriness hypothesised by some workers. Unfortunately semen collections ceased during the early summer. Some of the experimental cocks were transferred to natural light conditions in small pens with about fifteen hens. Over 85% fertility from matural matings was obtained through the summer and into the autumn, when a sharp fall occurred. As this decline was coincident with a spate of avian encaphalomyelitis, the male contribution to fertility had not necessarily declined in the twelve months from puberty.

Semen volume records from cockerels maintained in natural light, indicated no significant correlations between semen release and light conditions at the time of collection.

Age and seasonal influences on semen production could not be differentiated in this study. Expt. 3 consisted of comparisons between semen records of males at different ages, or over different seasons. The conclusions are based on statistical differences in semen release; this does not necessarily mean differences in spermatogenesis or direct influence of light, temperature and age.

Nevertheless some results warrant comment. The July to October changes in semen volume illustrated in Figure 4, paralleled the changes in ambient temperature so closely that it is reasonable to suggest an association. In July and August virtually all cockerels exhibited a sharp and uniform decline in semen volumes. At this time daily light period was steadily increasing, but the lowest ambient temperatures and diurnal range were coincident with the semen decline. The changes in semen release were so clear cut that in the absence of any other known change in the environment or management, they appeared to be caused by temperature changes. July and October average semen volumes were significantly higher than August, but the latter values seldom fell by more than 30%.

The steady increases in volume during May and June conceal large variations between cockerels. Many responded with large uncontaminated semen releases to the first collections in May. Others, generally more difficult to handle, withheld from successful ejaculation well into June, giving the impression of a psychological aversion to handling. Undoubtedly virtually all the cockerels at six months of age were capable of spermatogenesis and ejaculation, thus the results suggest the aggregate volume trends in May and June were in response to training.

November and December, or the early summer months, constitute the third important phase in semen volume changes. These results are more difficult to explain as inconsistent trends between the strains suggested a genotypic-environmental interaction. ML and WC trends were so obviously different that the theory of superior adaptation to heat by Mediterranean strains must be eliminated. Likely alternatives are genetic variations in response to the temperature or multiple environmental influences, causing refractoriness, or possibly a psychological reaction to regular handling for semen collection. Some evidence for both theories, is reported in the review of literature. The range of potential factors which could reasonably be expected

to influence the persistence of semen production is enormous, and outside the scope of this discussion.

No notable correlations were derived between ambient temperature and semen characteristics. Most workers concluded that cockerels were tolerant to large temperature variarions, at least for a short time, to the extremes of heat prostration or frost bite; both unlikely in this environment. However it was noticed, but not proven, that cockerels in open sheds tended to be recalcitrant on days with a morning frost and frequently contaminated the ejaculate with caecal contents, possibly induced by the extra stimulation required.

In experiments comparing semen characteristics of cockerels in their juvenile and second years, the general impression obtained was a slight decline in volume and spermatogenesis. Apparently spermatozoa concentration was unchanged. The repeatability of ejaculate characteristics was high, particularly for spermatozoa concentrations.

Two reasons could be advanced for the decline in semen volumes: firstly because relatively less cloacal fluid is obtained from older birds; either because they are better adapted to handling for collection and the quick erection approximates a natural mating, or the superficial sinuses and epithelium releasing the fluid have dried with age or have reduced permeability from fibrous tissue which has developed in response to the abnormal pressures. Secondly, the testicular and vasa deferentia secretions could decline with age. Either of these changes could predispose the maintainence or even increase of spermatozoa concentration. However a comparison of depletion experiments conducted at nine and twentyone months on the same groups of males, suggested a decline in spermatozoa release caused by decreased spermatogenesis, not response to handling. A correlation of +0.976 was obtained between spermatozoa concentrations of successive collections in the depletion test reported in Expt.3.

Excepting a few rather safe conclusions drawn from the significant data, this study does not elucidate age or seasonal effects on semen production.

Genetic determination of semen volume was demonstrated by the consistently higher Australorp values. Although statistically unproven, striking differences in the psychological response to collection and experimental treatments between the strains, were clear throughout the study.

Australorp semen volumes were significantly higher than Leghorn from the earliest collections at six months age to those at twentyone months. As Fransen et al (1955) indicated, a significant correlation of testes and body weight, and spermatogenesis with testes weight, could account for some of the variation observed. For the two Leghorn strains, the average semen volume was not significantly different although the WC were generally about 16% higher. These impressions are illustrated in Figures 4, 5 and 8.

As previously mentioned, ML volumes reversed the general trend in early summer semen characteristics. Reports in the literature indicate this could be due to a superior physiological adaptation to high temperatures; better vitamin C utilization; or be related to the onset of spermatogenesis and relatively smaller and more consistent volumes during the previous six months. There is no evidence either way from this study.

The experimental treatments of the semen production studies; nutrition, light and age; did not interact significantly with the strain volumes. Even this limited range of treatments implies that the physical processes affecting semen production are similar in all strains, as would logically be expected.

Spermatozoa concentrations were not significantly different between the strains. Any hypotheses regarding genetic variation were invariably confused by the differential release of non-seminal fluids causing significant changes in concentration. Spermatozoa concentration and the tendency to release cloacal fluid appeared to be individual characteristics, where as in Expt. 7, the variation within strains tended to obscure the variation between them. In this respect as with other phenotypic characteristics, the ten ML cockerels were much more uniform.

Computed from all collections recorded between six and thirteen months of age the average semen volumes obtained were: AO, 0.713 ml.; WC, 0.411 ml.; and ML, 0.344 ml.

The aggregate average spermatozoa concentration estimated from haemacytometer counts, was 3.8×10^9 spermatozoa per ml.

A composite value which helps to reduce the inter-collection fluctuations, averaged at approximately 2000 million spermatozoa per ejaculate.

Individual ejaculations after one stimulation ranged from 0 to 2.3 ml., the latter very exceptional ejaculate was estimated to contain approximately 8000 million spermatozoa. During the depletion tests, 3.5 ml. of semen was ejaculated by one AO cockerel within three hours, but as the results in Expt. 7 show, the concentration declined. The highest concentration of any single ejaculate tested was 6.8×10^9 spermatozoa per ml., but only a small fraction of the collections were studied. Several aspermic ejaculates were obtained from yearling cocks during the autumn.

The psychological features distinguishing the strains in their response to most human activities, seem to apply for sexual stimulation as well. Specific reactions are noted in the discussion on semen release.

Australorp males after initial training, became visibly excited before a semen collection. Since the same pattern of human activity was maintained at approximately the same time during collection afternoons, the birds appeared to associate the routine and the handling for ejaculation. In most cases, a set sequence of collections was maintained, moving down one row of cages and up the other side. Cockerels became quite agitated when their neighbours were handled for ejaculation, probably by a combination of anticipation and the visual stimulation of another bird in an unnatural posture. Some Australorps proceeded to ejaculate almost spontaneously, a few in their cages, and the majority after one or two massages. Erection and ejaculation were rapid, and if the operator failed to control the engorged copulatory

organ, the semen was often forcibly expelled from the vent. Normally the engorged tissue could be gently retained by the thumb and forefinger and semen flowed from the white body into the tube. Post-collection excitement, assessed here as libido, was characteristically low in Australorp males.

M Line males exhibited relatively less pre-collection sexual excitement. This could be interpreted as: a more fearful response to the anticipated handling, which muted their sexual desire, or a genuinely different attitude to semen collection compared to the AO males. The latter may be that a higher threshold of sexual stimulation is required before the cockerels associate ejaculation with the pre-collection activity. In the handling phase, ML cockerels usually required several massages and often repeated stimulations, then a relatively slow and gentle application of pressure to the vent area. Erection and ejaculation were more prolonged with this strain, consequently the actual collection of semen was easier. In the post-collection phase most of the males were extremely excited, hence their libido score were high. The birds strutted around their cages, frequently sparring with a neighbour and occasionally crowing.

These differences between the ML and AO cockerels indicate that the former strain, more timid and alert, suffered a conflict of fear and sexual excitement prior to collection, but that the induced ejaculation promoted full expression of the latter. The less temperamental AO cockerels expressed their sexual excitement prior to collection and the emotion subsided rapidly after ejaculation. WC cockerel reactions varied considerably between these two extremes. Although no supporting data is available these observations suggest that AO semen collections approximate closer than ML, to natural mating. This could arise partly from anatomical differences determining the stress during handling, but apparently the response is influenced by the psychological state of the cockerel, which is part-genetically determined.

All comparisons were made using the one-man collection Method B, described in Section 2.5.

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Semen Release.

This section consists of a discussion of experiments 5, 6 and 7.

A useful confirmation of some of the opinions expressed in the previous paragraphs on the psychological control of semen release, is obtained from the results of Expt. 6. This experiment was originally intended to determine the merits of two collection methods applied locally, and in resolving this problem revealed some striking genotypic-environmental interactions. Of prime importance was the differential response of ML cockerels to a new collection technique; a significant increase in semen volumes was obtained by using Method B on group A cockerels, but virtually no response was obtained when Method A was applied to Group B cockerels. In contrast the AO cockerels were much less affected by the unaccustomed use of either new method. A second feature was that spermatozoa concentrations were far less affected by the collection techniques than volumes, suggesting that the ejaculations were approximately normal. This is important because it means that neither method sought to retrieve something at all costs - in fact an effort was made to avoid this; and that the differences arose from a failure to ejaculate and not the manipulation of copulatory tissue after ejaculation. Therefore the valuable results are: firstly that a response to stimulation was in part genetically determined; and secondly that the one-man Method B was superior to the two-man Method A illustrated in Figure 6.

Some explanations arise from a comparison of the methods. Method A employs two operators very close to the cages; in common with most domestic animals the fowl displays relatively more fear with the approach of two humans, especially from different directions, than one. Since the emotional capacity of the bird at any one time is limited, it is reasonable to expect a fear response to dominate the sexual excitement which is apparently necessary for ejaculation. If the degree of fear is low, the sexual excitement could prevail,

for instance in AO stock with their docile temperaments. The apparent pre-collection lethargy of ML cockerels could reflect a conflict between fear and libido. A third point is the differences in handling cockerels. Method A is fast, rough, and bears little resemblance to natural mating. As would be expected the training period is extended because each cockerel has first to overcome the fear of man-handling and then to respond to sacral-lumbar massage, which is the only sexually directed action. In contrast most modifications of Method B are slower and more gentle. The cockerel is approached by one operator as for most servicing activities, withdrawn entirely from its cage and held upright on his lap. Apart from the distortion caused by restraining his hocks, the cockerel is positioned naturally and comfortably and his keelbone rests on muscular tissue; a situation which approximates natural mating (see Plate 3). When sacral-lumbar massage was applied the cockerels could respond physically by basic mating movements without pain. The results indicated that 53% of the Leghorn cockerels and all Australorp cockerels, responded to their first artificial collection by an ejaculation. By the third collection 85% of the Leghorn cockerels responded and none remained recalcitrant by the fifth collection. The learned response to Method A developed more slowly and ejaculations were never elicited from a few cockerels. A fourth advantage of Method B was the better synchronisation of massage and vent pressure obtained, as the physical reactions of the bird could be clearly felt and the ejaculation anticipated which enabled the left hand and tube to be ready at the optimum moment.

After many months of training otherwise, the good response of Group A cockerels to Method B is rather surprising. Since the higher semen volumes maintained an equally high spermatozoa concentration, the extra response must have been obtained by a larger ejaculation. Two possible reasons for this could be the extra sexual arousal arising from the novelty of a new handling method, or the result of a better synchronised massage and semen collection procedure.

Figure 6 illustrates that the carryover effects on volume or concentration were negligible.

Generally these results confirm Lake's (1957b, 1967), conclusions on the psychological domination of ejaculation. They suggest that both the temperament and momentary mental state of the cockerel at the time of collection, determine what proportion of the ejaculatory ducts are evacuated and whether urinary or faecal products will contaminate the semen. Obviously the most important phase of semen collection is to elicit an ejaculation, recovery being a simple mechanical procedure.

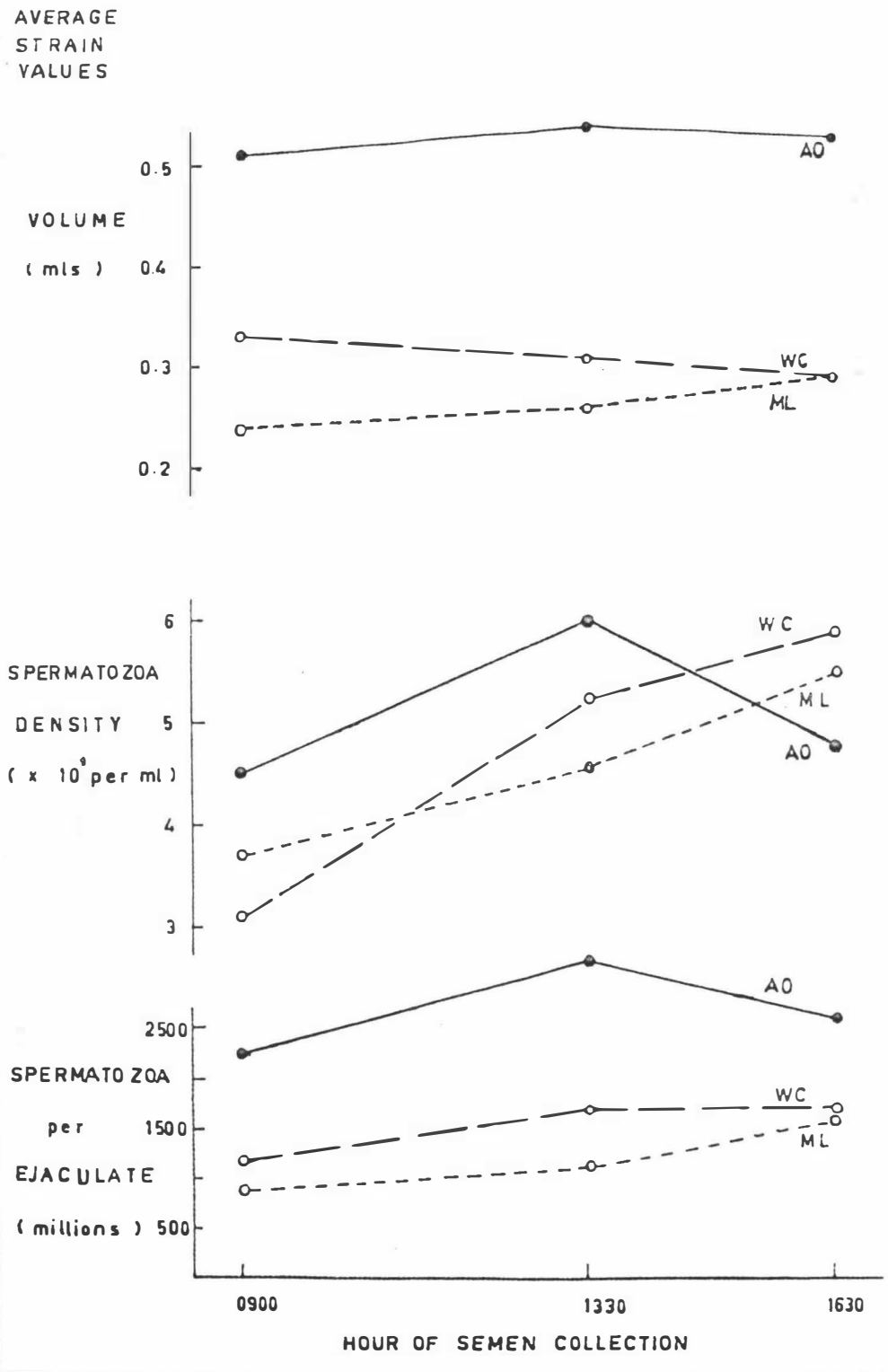
The significant increases in spermatozoa concentration during the day are rather surprising, as environmental conditions were apparently much the same at the 0855 and 1630 hours collections. Semen volumes, see Figure 8, were not significantly changed.

Two reasons for lower morning concentrations could be postulated: firstly because the contents of the vasa deferentia were more dilute; or secondly that a relatively higher proportion of cloacal transudate was released. There is no evidence for or against the first theory. The second is feasible, as cockerels appeared to be excited by handling at the unusually early hour and this could have induced correlated increases in engorgment of the copulatory tissue and the flow of transudates. Alternatively, greater pressure may have been applied to direct the semen, with the same effect on transudate flow. With similar reasoning to that used above, the greater uniformity in AO ejaculates is not unexpected.

Particularly surprising were the significant increases in the percentage live spermatozoa during the day, which were not wholly negated by the errors of estimation. It is unlikely that dead spermatozoa could be catabolised and readsorbed in the vasa deferentia during the short time involved. One possible explanation is that this phenomenon could be associated with changes in spermatozoa concentration. If a smaller proportion of the vasa deferentia

FIGURE 8

HOUR of COLLECTION & SEMEN RELEASE



contents are released in the morning collection and originate from the distal portion, this could contain relatively more dead and senile spermatozoa. In the afternoon collections, a relatively larger semen evacuation from the vasa deferentia, would modify the earlier characteristics.

The unexpectedly high AO spermatozoa concentrations of the midday collections illustrated in Figure 8, upset the general pattern. There is no logical explanation for them and they are probably insignificant.

Semen exhaustion results from Expt. 7 are illustrated in Figure 5.

The experiment, as originally intended, did not ascertain the stage at which either semen resources, or the ejaculatory response to stimulation ceased, but did produce useful data on partial semen depletion and recurrent sexual arousal.

The lower graph of Figure 5 illustrates the rather linear declines in spermatozoa release. This was due mainly to the significant decline in spermatozoa concentration observed with all cockerels. The apparent stability of volumes seemed to be an artifact of the collection technique, because the decline of seminal fluid was compensated by an increase in cloacal transudate inadvertently squeezed out as stimulation became more difficult. Presumably, if depletion had continued with ejaculations requiring more stimulation, at some stage the process would have become painful to the cockerel, causing a sudden fall-in libido and a resistance to further collections.

All the collections appeared to be genuine ejaculations as indicated by: the physical response of the cockerels; the linearity of concentration decline; and in particular, the insignificant changes in the fertilising capacity of each one. The results indicate that a cockerel does not release a large proportion of his mature spermatozoa during one artificial collection and that if sufficient time elapses for the ejaculatory ducts to refill and mental co-operation is forthcoming, a second collection is practicable. The

fifty minute interval was obviously adequate and probably far longer than necessary. Almquist and Hale (1956), reported similar depletion studies of bulls, where with twenty collections over several hours, 31% of the total spermatozoa were obtained in the first two, and 76% in the first ten ejaculations. It is unlikely that a cockerel could sustain twenty rapid ejaculations and they appear to release a relatively higher proportion of spermatozoa reserves in the initial ejaculations. As there is adequate evidence that most cockerels are capable of over twenty natural matings within a few hours, this highlights the important difference in artificial semen collection from the bull and cock; that the former's much more closely approximates a natural mating. Semen collection from a trained cock is largely determined by the operator's skill, while the initiation and maintainence of sexual desire are relatively more important in the bull.

In Figure 5, the results are expressed as strain averages and obscure the type classification used in the selection of cockerels. Both pairs of cockerels performed true to type; higher spermatozoa concentrations were maintained in Type 1 during successive collections, and Type 2 cockerels continued to release relatively more transudates with their ejaculations.

Stimulation grades gradually increased, particularly in the fourth collection, and libido decreased as ejaculations became more difficult to elicit. It is difficult to derive cause or effect for the latter phenomenon; whether only a certain amount of sexual arousal can be obtained in one afternoon and this controls ejaculation; or whether there was a feedback effect of semen depletion on sexual arousal. The well-documented advantages obtained from changing the teaser animal or visual stimuli to induce ejaculation in the bull, appears to be unimportant in the fowl, which in artificial collection, is aroused by massage and eventually a learned association with human activity.

No precise experiments were conducted to assess the long-term semen potential of cockerels. During the most intensive collection period, where WC

males ejaculated thirtyone times in seventyeight days during the late spring and summer, there was no indication of a decline in semen volume or concentration. In the most concentrated phase, with fifteen collections on consecutive days, semen volumes decreased very slightly, then seemed to settle to a uniform release, possibly geared to spermatozoa maturation. Over the main study period of seven months, cockerels ejaculated for an average fortyone collections, or approximately every fifth day, but all the evidence suggested they were capable of much more.

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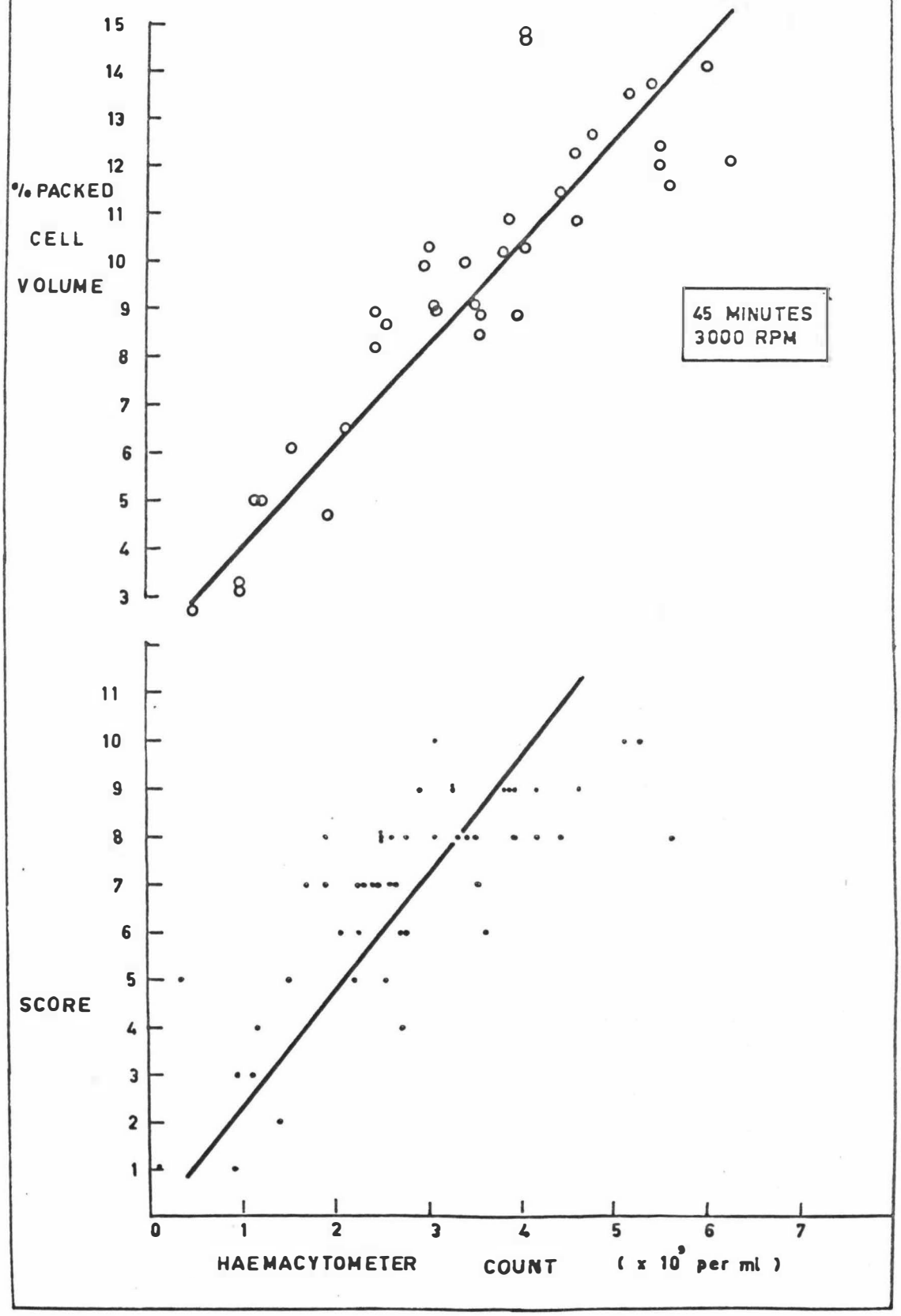
The subjective estimations of stimulation and libido were recorded for every collection, but were little use in analyses apart from semen exhaustion tests and the genetic determination of psychological response to collection.

Stimulation grades early in the experimental period served as a guide to the resistance of cockerels to training for collection; of temperament; and environmental disturbances at the time of semen collection. A reference to the stimulation grade throughout the study, helped to put the other results in perspective and indicate abnormalities. The main fault lay in the criterion used to grade the collections. If some measure of the massages or time involved in massaging could be taken, rather than the number of complete inducements to ejaculation, the grades would have indicated the variation in pre-collection responses which later occurred. As it was applied the grades varied little, apart from during the initial collections or in abnormal circumstances.

The negative correlation obtained between stimulation and semen volume is logical, as an ejaculation was progressively harder to elicit after each peak of libido during massage. More than three stimulations were rarely made and in none of these was an ejaculation obtained.

FIGURE 9

SPERMATOZOA COUNTING METHODS



Libido, as a guide to post-collection sexual arousal showed extremely high repeatability for individual males, but this too, was of little use in analyses. After several weeks of collection it was clear that this 'libido' score was actually a measure of the display of courting action in the first few seconds after ejaculation. This score served some purpose, but was inadequate either as a guide to sexual arousal throughout the collection, or acquiescence to stimulation. Probably the main value of libido as for stimulation grade, was that results from individual collections were kept in perspective by reference to these scores which reflected the particular genotypic-environmental interactions at the time.

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CHAPTER 3.

Semen Storage.

A. Review.

3.1 Introduction.

When the time between semen collection and insemination is extended spermatozoa must be stored in an environment favourable to the maintenance of their fertilising capacity. No particular method of storage has been proved superior for commercial conditions, and the most recent investigations show several markedly different approaches. Fowl semen may be retained without much preparation or loss in fertilising capacity for a few hours *in vitro*; fortunately this is usually adequate for insemination routine. Research into fowl semen long-term storage is concerned mainly with the development of a semen bank, where the genetic potential of males can be disseminated over a greater time or area. Unfortunately, most of the experience and techniques gained from research into mammalian semen storage can not be extrapolated to poultry. Fowl spermatozoa exhibit relatively good resistance to temperature shock *in-vitro* (Wales and White, 1959), and a long functional life *in-utero*, yet paradoxically they are much harder to store than mammalian spermatozoa. It is reasonable to ascribe this to a relative deficiency of knowledge on the biochemistry of fowl semen, as well as class differences.

The loss of fertilising capacity of stored spermatozoa has not been attributed specifically to reduced motility, but apparently reflects some degeneration in the genetic material in the head, and mitochondria in the mid-piece region. Lake (1954) suggested that the head and midpiece of the cell are initially covered by a thin cytoplasmic sheath, which disrupts in unfavourable conditions, and renders the genetic material vulnerable to physical and chemical damage. Other investigators have emphasised the need to conserve the endogenous reserves of the spermatozoa during storage, for

the effort required in traversing the lower oviduct immediately after insemination. This basic lack of agreement about the important factors in fowl spermatozoa storage, explains the diverse approaches in research.

Several groups of investigators have recently reported progress in extending semen storage time beyond one or two days. Freezing spermatozoa for long storage, probably remains the ultimate objective to most of the approaches.

Wilcox and Shaffner (1958) stored semen for one to three days at 10°C., in a sodium-phosphate buffer solution (see Appendix B). Their most successful method, involved a relatively high 1/11 dilution of semen in buffer plus antibiotics for storage, then a reconcentration of spermatozoa to their undiluted density in fresh buffer plus 2.5 mg/ml fructose, just prior to insemination. After twentyfour hours storage, the spermatozoa produced 69% fertility and 94% hatchability, from eggs collected on the 2nd to 8th day after insemination. Lake, Schindler and Wilcox (1959), from using the same method, achieved about 37% fertility from semen which had been collected in the United States, and flown to Scotland and Israel for insemination about thirty-eight hours later.

Some investigations into the inhibition of spermatozoal motility by 2-ethyl, 5-methyl benzimidazole (E.M.B.), have been reported by Harris, Wilcox and Shaffner (1961). Semen was diluted 1/2 in a sodium-phosphate buffer (see Wilcox etal 1958) containing 100 µg/ml EMB, for up to five days at 20°C., and the EMB was washed out just prior to insemination. Up to 75% fertility was achieved under the most favourable conditions, but the duration of fertility was not reported.

Another series of investigations involved storing semen in hypertonic carbonated diluents, also aimed at temporarily reducing the activity of the spermatozoa. Harris and Hobbs (1964), reported on varied gas to fluid ratios in sealed ampules with different concentrations of sodium citrate and carbon

dioxide in the diluent. They concluded that an optimum gas:fluid ratio of at least 3:1 for a 1/4 dilution, and higher for low dilutions, maintained good fertility in semen stored from twentyfour to fortyeight hours at 2°C.

For many years investigators have tried to produce a diluent similar to pure seminal plasma, which could be considered the most satisfactory fluid for spermatozoa to be stored in. Evidence against this reasoning is presented later in this review, however in some respects the plasma does indicate a fluid composition tolerable to spermatozoa. Lake (1960) devised a diluent which simulated fowl seminal plasma as collected from the vasa deferentia, with respect to phosphorus, sodium, magnesium, potassium and glutamate concentrations, and the osmotic pressure was adjusted by adding fructose. The semen was stored aerobically at about 2°C. and achieved 64% fertility after twentyfour hours, and 47% after fortyeight hours storage, from eggs layed on the 2nd to 7th day after insemination.

In contrast to the efforts to provide a carbon dioxide environment and temporarily reduce diluted spermatozoal activity, Proudfoot and Stewart (1967), experimented with an oxygen atmosphere for whole semen. They reported that 0.4 ml. samples of semen, stored in sealed cryovac enclosures at 10°C. for up to seventeen hours, produced better fertility in oxygen than in nitrogen, air or carbon dioxide gas.

The final and important aspect of research is storing semen at sub-zero temperatures. The main objective is to protect the genetic material from dehydration effects and mechanical stress, during freezing and thawing. Although mammalian semen has been successfully frozen and stored for many years (Mixner and Wiggin,1957), the methods have not been applicable to fowl semen. Polge, Smith and Parkes (1949), reported that 20% glycerol protected spermatozoa at -79°C., which retained their motility after thawing, provided that the glycerol concentration was reduced below 2%. They also suggested that freeze dryihg diluted semen avoided the salt concentration effects, as

the water was removed from the frozen material by sublimation. Semen was diluted 1/2 in Ringer's diluent containing 30% glycerol, vitrified in a thin layer at -79°C., and dried for three hours at -25°C. to a powder about 15% of the original weight. The dried spermatozoa were re-constituted by adding water and warmed to 40°C. for immediate insemination or testing; their fertilising capacity was lost, but up to 50% of the sperm were active. Tanaka, Chan and Okamoto (1967), investigated the effects of super-cooling temperatures on diluted spermatozoa motility, deformities and fertility, over two to four days storage. From 2°C. to -2°C. they observed no significant differences in any characteristics, but below -2°C., fertility was markedly reduced, although improved when a more hypertonic diluent was used.

3.2 Semen Characteristics.

Apparently, fowl seminal fluid is not a good medium for storing spermatozoa. Several reasons have been postulated:

1. seminal plasma could be an artifact of collection techniques, and chemically quite different to the small quantity of fluid bathing the spermatozoa while in the vasa deferentia.

2. the spermatozoa are in intimate contact with the epithelium lining in the male reproductive tract, and partly independent of the fluid around them. Owing to the common origin of the urino-genital systems in the fowl, some investigators consider the ejaculated fluid as partly excretory products.

3. seminal plasma is hypotonic with respect to the midpiece of the spermatozoa (Yamane 1962), which is irreversibly damaged by plasmolysis.

4. the poor buffering capacity of plasma.

5. physical and chemical changes occur in both the spermatozoa and plasma on exposure to the atmosphere. These effects are avoided in natural mating and internal fertilisation.

6. seminal plasma is not likely to be associated with the spermatozoa

PLATE 6.

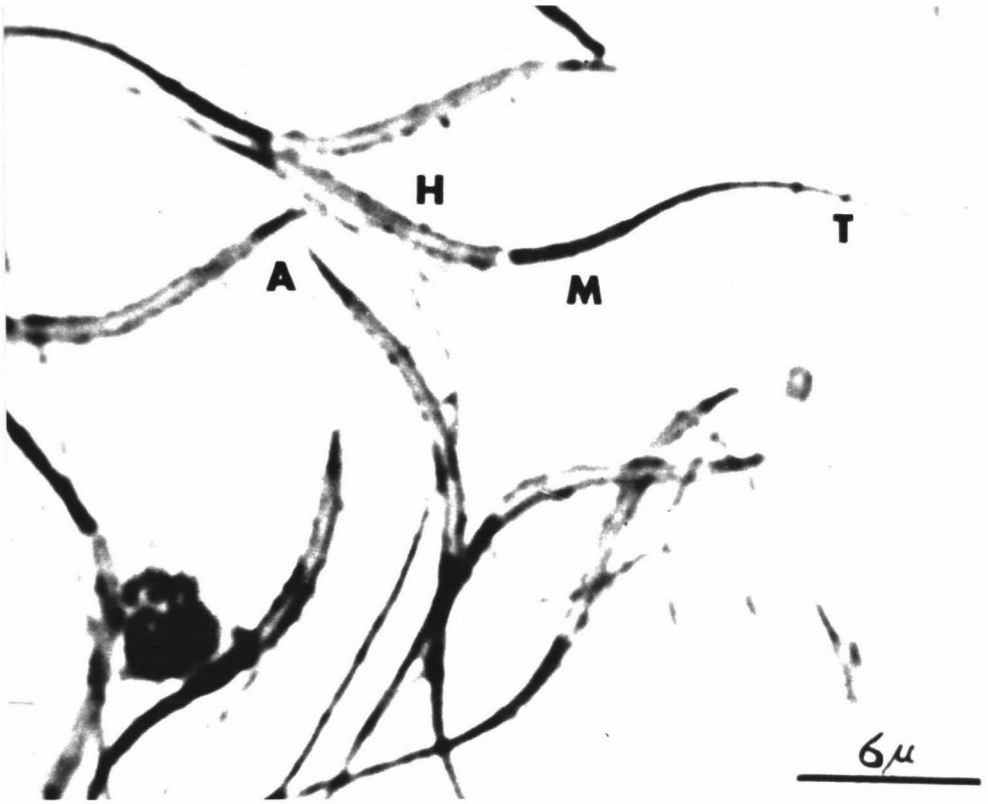


PLATE 6. Freshly ejaculated fowl spermatozoa stained with Nile Blue.

A : acrosome
H : head
M : midpiece
T : tail

(From: Lake, P.E., (1966). Res. vet. Sci. I, 121)

in the oviduct for more than a few hours, yet spermatozoa have been demonstrated to retain their fertilising capacity in that environment for up to thirtyfive days (Sturkie 1965).

Lake (1954) stressed the importance of an artificial environment which was favourable to the integrity of the thin sheath enclosing the spermatozoa. When the sheath is disrupted, the bulb at the head and midpiece junction, is rendered vulnerable to physical and chemical damage, leading to granulation of the mitochondrial components. If the spermatozoa are still motile, the impulses from the tail are likely to be transmitted over the ineffective midpiece and disrupt the fragile junction region which results in the head being bent backwards. As well as the damage to genetic material, the distorted spermatozoa are probably incapable of penetrating ova for fertilisation. Saeki (1960), using an electron microscope, found an increase in the percentage crooked-neck spermatozoa with time of storage and dilution. He found the incidence particularly high in the first ten hours after collection, and initially higher at low temperatures, over the range of 6^{o} to 15^{o}C. Saeki suggested that spermatozoa motility and viability were unaffected, but that penetration into the ovum germinal region was unlikely due to the displacement of the apical cap. These suggestions are usually corroborated by other investigators. Apparently, obnoxious substances in the semen, either as artifacts of collection or in the diluent, accelerate the disruption of the lipid-protein-polysaccharide sheath which is invested about fresh spermatoa. Lake (1954) considered that coiled spermatozoa, flattened heads, shrinking or swelling near the midpiece, and heads with refractile droplets along their length, were artifacts arising from the semen environment or handling methods. Saeki (1963), reported seasonal changes in semen characteristics, which may indicate differing effects of plasma on speratozoa, and a changing optima for semen storage.

Numerous investigations have demonstrated that fowl spermatozoa glycolyse and respire. Apparently, glucose, fructose and mannose are the

only carbohydrates metabolised (Wilcox 1959b, Yoshida and Masuda 1961, Harris and Wilcox 1962, Lake, Lorenz and Reiman 1962) in significant quantities, and glucose can be converted to fructose by the spermatozoa (Lorenz 1958, Lake et al 1962), causing a sparing effect on fructose if the carbohydrates are available in-vitro. The conversion is probably irreversible and not through glucose-6-phosphate isomerisation (Lake et al 1962). Harris and Wilcox (1962) suggest that carbohydrates are metabolised by the Meyerhof-Enden glycolytic pathway. Most investigators agree that carbohydrate metabolism proceeds at a higher rate under anaerobic conditions, and that where oxygen is available, the conversion of glucose to fructose is higher and glycolysis is more efficient (Lorenz 1958, Harris and Wilcox 1962, Proudfoot and Stewart 1967). Lactic acid is the main end product of anaerobic glycolysis of fowl spermatozoa, but apparently the accumulation is not responsible for the reduced motility, which would parallel muscular exhaustion in higher vertebrates (Nevo, Caplan and Schindler 1963 , cited by Lorenz 1964).

Spermatozoa respiration is highly correlated to motility over a range of 0.6% to 21% oxygen concentration (Nevo 1965), but below 0.6% there is a sharp decline until both respiration and movement cease under 0.13% oxygen. Yoshida and Masuda (1961, 1965), suggested that respiratory processes were more important than glycolysis in energy production for fowl spermatozoa. Unlike mammalian spermatozoa, relatively more endogenous energy contributes to the respiration of fowl spermatozoa (Scott, White and Annison 1962), particularly in the oviduct where the oxygen tension rises to 35 mm Hg (Nevo 1965). Yoshida and Masuda (1961) have reported that intermediates in the tricarboxylic acid cycle, especially succinate, stimulate respiration.

The favourable results achieved by storing semen in an oxygen atmosphere may indicate the importance of lipid oxidative metabolism to spermatozoa function (Lake 1966).

It appears that most of the reducing sugars in semen are due to the

method of collection, having arisen from the cloacal erectile tissues (Lake 1962 a). Research into the metabolism of stored semen and simulation of natural conditions, may have been misdirected into overemphasising glycolysis of extracellular carbohydrates.

3.3. Diluting Semen for Storage.

Fowl spermatozoa easily retain their fertilising capacity for from two to four hours after ejaculation, if they are stored at 10° to 12°C. with oxygen available. This time can be extended if the semen is diluted soon after ejaculation (Lorenz 1964). With the exception of a few investigations, like that of Proudfoot (1966), most research workers have emphasised the advantages of immediate dilution.

Three main aspects of semen dilution have been reported: 1. the rate of dilution during storage and at the time of insemination. 2. the chemical composition of the diluent and antibiotics. 3. the toxicity of the diluent.

When these factors are tested against the range light, temperature and gaseous influences throughout the storage period, there are numerous combinations possible. Over the last twenty years, the scientific literature has abounded with reports on some of the combinations, but unfortunately the factors are often so varied that they preclude comparisons. The most fundamental disagreement is whether the spermatozoa thrive best in an environment which suppresses their movement and metabolism, or whether the diluent should contain nutrient to maintain their activity.

Wilcox and his colleagues have presented a series of experiments which have indicated that seminal plasma is a relatively poor environment for spermatozoa in-vitro. Their two most successful methods have involved the use of a sodium-phosphate buffer to replace or dilute the plasma. In the first, fresh semen is centrifuged and the plasma supernatant is replaced by an equal volume of buffer plus antibiotics; this preparation is stored at about 10°C.

and augmented with fructose just prior to insemination. In the second and more successful method, fresh semen is diluted 1/11 in buffer plus antibiotics, then centrifuged and resuspended back to the original semen volume with fresh buffer plus fructose, just before insemination (Wilcox and Shaffner 1958). These results suggest that dilution during storage is not the critical factor; rather that avoidance of the plasma effects or the dilution rate at insemination, or a combination of both, is more important.

Semen which was stored in carbonated diluents with high carbon dioxide concentrations, for up to fortyeight hours at 2°C., produced significantly higher fertility when diluted 1/4 than 1/2 (Harris and Hobbs 1964).

Lorenz (1964) concluded that any dilution causes some damage to spermatozoa, but is necessary for storing semen. He proposed 1 to 2°C. as the optimum temperature and a moderate dilution of 1/2 to 1/4 in a compatible diluent.

The chemical interactions of semen and diluents have been studied more objectively than most aspects of dilution. Initial interest on simulating the composition of seminal plasma, has been replaced by more specific studies of the ions important to spermatozoa integrity, osmotic balance and the addition of antibiotics during storage. There is general agreement, that bicarbonate in a diluent increases spermatozoa respiration (Van Tienhoven 1960, Van Tienhoven and El Zayat 1960, Hobbs 1963); that phosphate decreases respiration (El Zayat and Van Tienhoven 1959, Ven Tienhoven 1960); that the effects of calcium, magnesium and potassium depend on the anions present (Van Tienhoven 1960); that monosodium glutamate which simulates the high glutamic acid content of fowl seminal plasma, enhances the motility and fertilising capacity of stored spermatozoa (Lake 1960, Bajpai and Brown 1963a, Auger and Wilcox 1964); that fructose added during storage increases the longevity of spermatozoa (Saeki 1960, Wilcox 1960); and that egg yolk reduces the fertilising

capacity of fowl spermatozoa (Wilcox 1960).

Tyrode solution (see Appendix B) has been considered relatively unsuccessful for storing semen by El Zayat and Van Tienhoven (1959), who reported a high initial respiration rate of the spermatozoa, followed by a marked decrease in respiration and fructolysis and a high incidence of deformed spermatozoa, twentyfour hours later.

Carbonated diluents have proved useful for short storage, and Hobbs (1963), attributes a beneficial effect to the suppression of spermatozoa motility by carbon dioxide.

Lake (1958 b, 1960) reported successful short storage of semen diluted in a seminal plasma-like fluid, of which the glutamate, sodium, potassium and magnesium concentrations, and the osmotic pressure, were similar to plasma.

Antibiotics have been found to protect spermatozoa during storage; it is not clear whether the entire benefit is antiseptic, or whether the 1000 µg dihydrostreptomycin plus 100 µg oxytetracylcline hydrochloride per ml. of fluid, used by Wilcox and Shaffner (1958), has a chelating action as well. Wales and White (1961) studied the toxicity of some antibacterials on fowl spermatozoa and found a considerable variation in the susceptibility of ejaculates to them. Seminal plasma apparently exhibited an unspecific mechanism to counter the spermicidal activity of many antibacterials at low concentrations.

A sodium-phosphate buffer (Wilcox and Shaffner 1958), has become widely used by many investigators for storing, washing and extending semen. The advantage of the buffer over Tyrode solution for storage is more clearly demonstrated by El Zayat and Van Tienhoven (1959), who compared 1/11 dilution of semen with Tyrode, the buffer, and a combination of the two. Initial respiration and fructose-disappearance rates were higher in the Tyrode and mixed solutions, however after twentyfour hours at 5°C. the Tyrode samples

exhibited much lower values than the buffer, and contained 87% abnormal spermatozoa versus 26% for the latter.

Whole milk has been successfully used as a diluent for storing semen. However, Van Tienhoven, Steel and Duchaine (1958), found the storage value of milk for turkey semen was depressed by the addition of antibiotics and glycine, and considered that repeated inseminations of milk into the oviduct may create an unfavourable environment for spermatozoa. Lake (1958 b) warned against using mammalian physiological salines, which could contain harmful concentrations of inorganic ions for fowl spermatozoa.

Ringer's and Locke's solutions have declined in popularity relative to the new buffered diluents for semen storage. Schindler, Weinstein, Moses and Gabriel (1955), diluted semen 1/4 with Ringer's, Locke's, pasteurised whole milk and a yolk-phosphate buffer, and held the samples at 4° and 10°C. for up to twentyfour hours. They reported that Ringer's and Locke's solutions could not maintain good fertilising capacity beyond about four hours of storage, while milk maintained spermatozoa best at 4°C., and undiluted semen proved best at 10°C. The yolk phosphate buffer was an inferior diluent at all temperatures, although Yamane, Tsukunaga and Takahashi (1962), reported the successful use of a yolk-citrate-glucose diluent for storing semen at 2° to 5°C.

Recently considerable emphasis has been placed on the optimum tonicity for stored fowl spermatozoa. This has caused a critical evaluation of the diluents in common use, and indicated that some of them are unsuccessful for this reason. Evidently hypertonic solutions maintain spermatozoa fertilising capacity and longevity, better than isotonic or hypotonic solutions (Yamane et al 1962, Saeki 1963, Tanaka and Okamoto 1966, Van Wambeke 1967 cited by Lake 1967), although Hobbs and Harris (1963), considered that an isotonic sodium citrate diluent maintained better fertilising capacity and motility of stored spermatozoa than a hypertonic condition. Yamane et al (1962),

considered that the spermatozoa midpiece was vulnerable to plasmolysis if the freezing point depression (FPD) was less than -0.93°C.; Tanaka and Okamoto (1966) reported an increase in the motility and percent deformed spermatozoa, when the FPD was less than -0.56°C.; Lake (1960) considered a medium of FPD -0.607° to -0.640°C. was optimal for fertility; and Saeki (1963) perhaps reconciled the differences in the degree of hypertonicity, when he reported seasonal variation in the optimum FPD for fertilising capacity.

3.4 Macroenvironmental Influences.

Ambient temperature is the most important single influence on all aspects of semen function and storage. Most reports confirm that fowl semen is relatively resistant to temperature changes, but can not be stored for long over 15°C. (Schindler et al 1955, Wales and White 1959, Bajpai and Brown 1964). The optimum temperature for storing whole semen is about 10°C.; diluted semen has been successfully held at temperatures of 0° to 5°C., or at 10° to 15°C., depending on the diluent and other factors involved. The special case of freezing semen is discussed in the next section.

Some effects of light on spermatozoa have been reported; Hunsaker and Aitken (1960), exposed spermatozoa to ultra violet-shielded light of 3,900 fc intensity, without a harmful effect on fertilising capacity. However Norman, Goldberg and Porterfield (1962), reported an immobilising effect of visible light on stored spermatozoa, which may have resulted from photosensitised oxidation. Williams and Hamner (1963), reported a dramatic increase in the respiration of spermatozoa suddenly exposed to light.

Fowl spermatozoa appear to be relatively tolerant to hydrogen ion concentration during storage. Bogdonoff and Shaffner (1954), considered pH 7 optimal for their metabolism and respiration, but that the fertilising capacity was not greatly affected within pH 6 to 8. Wilcox (1959 b) confirmed this tolerance, but suggested that slightly acid conditions were preferable as they

tended to suppress spermatozoal activity.

Contaminants arising from the ureters or cloaca of the male fowl, can induce spermatozoa abnormalities and render stored semen useless (Lake 1954). After a deliberate attempt to collect a high proportion of erectile tissue fluid in the semen, Lake (1956), reported a significant decline in fertilising capacity by as soon as twenty minutes after ejaculation, compared to the pure semen.

Little specific work has been reported on the influence of gaseous environment for stored semen. Recently, Nevo (1965), and Proudfoot (1966), have emphasised the importance of oxygen to spermatozoa motility, respiration and efficient carbohydrate metabolism. Proudfoot and Stewart (1967), have reported a correlated increase of fertilising capacity and oxygen concentration within a specified range, which they attribute to a sparing action on endogenous resources.

Indirect references to the effects of movement on stored spermatozoa, suggest that moderate centrifugation is acceptable (Wilcox and Shaffner 1958),and that slow vertical rotation to stir the semen during storage is advantageous (Harris, Hobbs and Peterson 1961).

A rather different set of factors influence the viability of frozen spermatozoa. Investigations have demonstrated methods of freezing, storing and thawing live spermatozoa (Polge, Smith and Parkes 1949, Smith and Polge 1950), but unlike the successes in freezing cattle semen, no significant breakthrough has led to a practical method of freezing and storing fowl semen for insemination. Glycerol has been used to protect the spermatozoa while frozen, but as fowl spermatozoa can not tolerate more than 2% when thawed, the glycerol must be centrifuged or dialysed out before insemination. Hendrikse, De Groot and Jansen (1956), suggested that the beneficial effect of glycerol arose from the reduction in ice crystal size, as well as the ability to transfer freely over the cell membrane and minimise salt concentration effects during freezing and thawing. Glycerol molecules dispersed throughout an equilibrated

sample of semen, attract unpolarised water molecules which subsequently are not available for polarisation into ice crystals, when the masses of water around the glycerol freeze. The dispersed plasma and intracellular ice crystals are much smaller, and impose less mechanical stress on the spermatozoa. Apparently the head and midpiece of the spermatozoa, are more susceptible to freezing damage than the tail region which determines the motility. In a recent project, Brown (1966), proposed that ethylene glycol would be a useful anti-freeze agent for turkey spermatozoa, but at present 10% to 20% final concentration of glycerol is the only sure protective agent for fowl spermatozoa. The toxic effects of glycerol on unfrozen semen can be avoided by immediate intrauterine insemination (Allen 1958), or intraperitoneal insemination of thawed or frozen semen (Brown, Harris and Hobbs 1963). Polge et al (1949) considered that the semen should be bulk frozen to avoid surface effects, although Clark and Shaffner (1960), favoured rapid freezing of small samples. The latter workers reported up to 40% fertility and normal hatchability from semen which had been frozen with $7\frac{1}{2}$% to $8\frac{1}{2}$% glycerol at -79°C., thawed quickly to 40°C., centrifuged at 1200 RCF for ten minutes to separate out most of the glycerol, and inseminated into the uterus. Recently Harris (1965) obtained fertile eggs from semen which had been stored at -196°C. in liquid nitrogen for seven days. He stored 0.4 ml. samples of semen diluted 1/4 in a saline diluent with a final concentration of 6% dimethyl sulphoxide (DMSO). The semen was thawed to 41°C. and the best results of up to 44% fertility were obtained from intraperitoneal insemination, without the necessity of removing the DMSO first.

A survey of the recent research, indicates that fowl spermatozoa can retain their functional capacity for several days under extremely diverse conditions. This suggests that the present difficulties may be attributed not to spermatozoa fragility, but possibly a fundamental misconception on the treatment of fowl semen.

B. Research.

3.5 Materials and Methods.

1. Stock.

Semen for each experiment was collected from the minimum number of males practicable, for use with pullets of the same breed. Unless otherwise specified the ejaculates were collected separately then quickly pooled for experimental use, irrespective of uneven semen volumes. The selection of males was arbitrary but only in exceptional cases where insufficient semen was available, were the ejaculates from two strains pooled for one experiment.

Fertility tests were made with pullets in batteries close to the cockerel cages. Genetically related pullets in adjacent cages with similar egg production rates, were allocated to one experiment. Many were used several times with a minimum four week lapse between two experimental inseminations. Most of the studies were arranged for the winter and spring months, coincident with the highest egg production rate, but a special reference to declining production is made for summer experiments.

2. Semen treatment.

Collection, evaluation and insemination methods have been described in Section 2.5.

When individual ejaculates had been measured they were drawn out by pipette and pooled without any precautions for temperature shock. The tubes containing semen were lightly sealed in the shed, but exposed to natural light and outside temperatures in transit to the laboratory.

Clean dry tubes were prepared for storing semen after mixing. Dilutions were made within twenty minutes of semen collection; normally the diluent was added from a pipette to a measured volume of semen in the tube and swirled by hand until the mixture was visibly uniform. Dilution rate was

expressed as a fraction : 1/3 meaning one part semen in three parts mixture. Undiluted semen, theoretically 1/1, was expressed as 0 dilution.

Storage temperatures are specified in the experiments. In nearly all cases semen was maintained at 10°C. in a dark cool-room. Refrigerated samples were transferred to the cool-room about thirty minutes prior to insemination. The stored samples were occasionally stirred if the semen had precipitated from the mixture.

3. Fertility tests.

The choice of adequate replications to make the fertility results statistically worthwhile was rather arbitrary at first. As the initial results were analysed and the factors were tested at more precise levels, the number of replicates increased from four to sixteen pullets. By mistake, a true block randomisation of pullets was not applied for the early experiments, but in most instances the results were clearly significant and the error was considered tolerable for the criterion of percentage fertile eggs. However, correctly randomised blocks were applied to most of the experiments reported here and the replication term appears in the analyses.

The pullets were inseminated from one semen sample at a time to avoid swapping and cleaning the cannulae between adjacent individuals. Although pullets for one treatment were all inseminated before starting on the next, the time lapse between first and last batches of semen rarely exceeded ten minutes.

3.6 Experiments.

A few aspects of semen storage were investigated as adjuncts to the evaluation of diluents, dilution rate and optimum temperatures, for maintaining the fertilising capacity of semen for up to twentyfour hours without freezing.

All the tables of results and analyses are presented in Appendix A.

Several references are made to the graphs, which are computed from untransformed percentages.

Figures within the tables present the true fertility percentages, however an arcsin transformation for analyses was made to reduce non-additive effects. Where comparisons between means were significant, the transformed treatments and significant differences between them are included in the text. Experimental methods and results are reported in this section and a discussion of the results follows in Section 3.7.

Experiment	9	Fertilising capacity of stored semen.
"	10	Dilution effect on semen fertilising capacity.
"	11	Dilution effect on stored semen fertilising capacity.
"	12	Temperature and dilution effects on semen storage.
"	13	Diluents for semen storage.
"	14	Diluents and dilution for semen storage.
"	15	Special treatments for semen storage.
"	16	Cloacal transudate concentration in stored semen.
"	17	Relationship between age of male and semen characteristics.

EXPERIMENT 9.

Methods.

Semen stored at 10°C. was evaluated hourly by insemination to observe the changes in spermatozoa fertilising capacity.

Semen was collected from twenty WC cockerels, pooled and diluted 1/2 with sodium-phosphate buffer for storage. Ten WC pullets were inseminated with 0.05 ml. from each hourly sample drawn from the stored semen.

The experiment was intended to show whether a significant decline in spermatozoa fertilising capacity occurred in the first 375 minutes of semen storage. Immediately after collection the semen was diluted 1/2 with sodium-phosphate buffer to a final volume of 8.8 ml. and held *in vitro* at 10°C. At

approximately hourly intervals the tube was mixed thoroughly and a small portion of the semen withdrawn for inseminations. Estimations of spermatozoa concentration, motility and percentage of live spermatozoa, were made between the inseminations.

Results.

Data treated as a single classification analysis of variance are presented in Table 20. Figures represent the percentage of fertile eggs from each of the ten pullet replicates, at the time maintaining 76% egg production, from the second to ninth day after insemination.

Average fertility.

Storage	Mean
135 minutes	78.2 %
80 "	64.7
255 "	59.3
375 "	58.7
315 "	50.2
195 "	47.3
30 "	43.0

(1) Moderately higher fertility ($P < 0.10$) was obtained from semen inseminated after 135 minutes *in vitro*, than that stored 30 minutes *in vitro*.

(2) There was no significant pattern between time of storage and fertility.

(3) There were no significant changes in the percentage of live spermatozoa or concentration effects from dessication.

Average motility grade of stored semen

Storage	Mean grade	Mean product.
60 minutes	5 : 4	18.3
120 "	5 : 4	20.0
180 "	5 : 4	18.3
240 "	5 : 3	16.7
300 "	5 : 3	16.7
360 "	5 : 3	16.7
420 "	4 : 2	7.3
480 "	4 : 2	9.3
540 "	4 : 2	8.7

Motility grade was computed from the average of three determinations hourly and the two scales multiplied to derive the mean product. The grade was composed of two subjective 0 to 5 scales; the first for general swarming motion, and the second estimating the proportion of spermatozoa displaying independent movement.

(4) An abrupt decline in motility occurred in the seventh hour of storage in vitro, without accompanying changes in the percentage live spermatozoa.

EXPERIMENT 10.

Methods.

This experiment was intended to assess the relationship between semen dilution in vitro and the persistence of fertilising capacity in utero.

Ten WC cockerels provided semen for each of the two collections and ten AO pullet replicates were inseminated with 0.05 ml. for each treatment.

Half of the pooled semen was stored unchanged and the other half diluted 1/2 with sodium-phosphate buffer. One insemination series was made 20 minutes after semen collection and the other after 240 minutes storage in vitro at 10°C. The time of egg collection was extended from the second to twelfth day in order to observe differences in the persistence of fertility arising from the dilution and time of storage. Approximately 2 mls. of fluid were stored for each treatment.

Results.

Fertility data analysed as for a 2^2 factorial, are presented in Table 14A. Figures represent the percentage of fertile eggs from each of the ten pullet replicates from the second to seventh day after insemination, with egg production at 83%.

Average fertility. (Table 14A)

Time	Mean	Rate	Mean
20 minutes	80.3%	0	74.0%
240 "	54.7	$\frac{1}{2}$	61.1

$D_{.01}$ = 20.20%

(1) Higher fertility ($P < 0.01$) was obtained from the fresh semen than that stored for 240 minutes, in the first six days after insemination. These results are illustrates in the lower graph of Figure 11.

(2) No significant dilution rate effect, or interaction between the main factors was observed.

Fertility data from eggs layed on the eighth to twelfth day after insemination are presented in Table 14B.

Average fertility (Table 14B)

Time	Mean	Rate	Mean
20 minutes	54.6%	$\frac{1}{2}$	50.1%
240 "	39.3%	0	44.8

(3) Moderately higher fertility ($P < 0.10$) was obtained from fresh semen than that stored for 240 minutes, in the second week after insemination.

(4) The effect of dilution rate on fertility was insignificant, however the diluted semen maintained higher levels than whole semen; an interesting crossover effect is illustrated in the lower graph of Figure 11.

(5) An analysis of fertility over the total egg collection period confirmed the superiority of fresh semen ($P < 0.01$), but revealed no significant dilution effects or interactions.

Daily fertility levels averaged over the ten pullets per treatment are analysed, untransformed, in Table 14C.

Average daily fertility (Table 14C)

Day	Mean
2	86.8%
3	82.8
6	78.3
5	77.5
1	68.0
7	64.3
8	52.5
9	52.0
10	50.8
11	16.8

$D_{.05} = 39.9\%$

$D_{.01} = 47.5$

Fertility recorded on the twelth day was lower ($P < 0.01$) than that

obtained on the second to seventh days inclusive after insemination. The trends in fertility of the averaged values for 0 and $\frac{1}{2}$ dilutions, are illustrated in the upper graph of Figure 11. An abrupt decline in fertility observed on the eleventh day after insemination, is similar to that illustrated for fresh semen in Figure 13.

EXPERIMENT 11.

Methods.

Semen was stored for up to twentyfour hours at 10°C. with varied dilution rates, to indicate the effect on spermatozoa fertilising capacity.

Three collections were made from twenty A0 cockerels within twentyone hours, to have semen available for varyihg lengths of *in vitro* storage, and so that all inseminations could be made at once. Six A0 pullets replicates per treatment were inseminated with 0.2 ml. of semen in order to reduce the spermatozoa number effect on fertility.

The pooled semen was split threeways immediately after each collection and one sample was diluted 1/2 with Tyrode solution, another diluted 1/10, and the third remained as an undiluted control. The periods of storage were three, six and twentyfour hours, and all inseminations took place at 1610 hours, with egg collections for fertility tests from the second to eighth day.

Results.

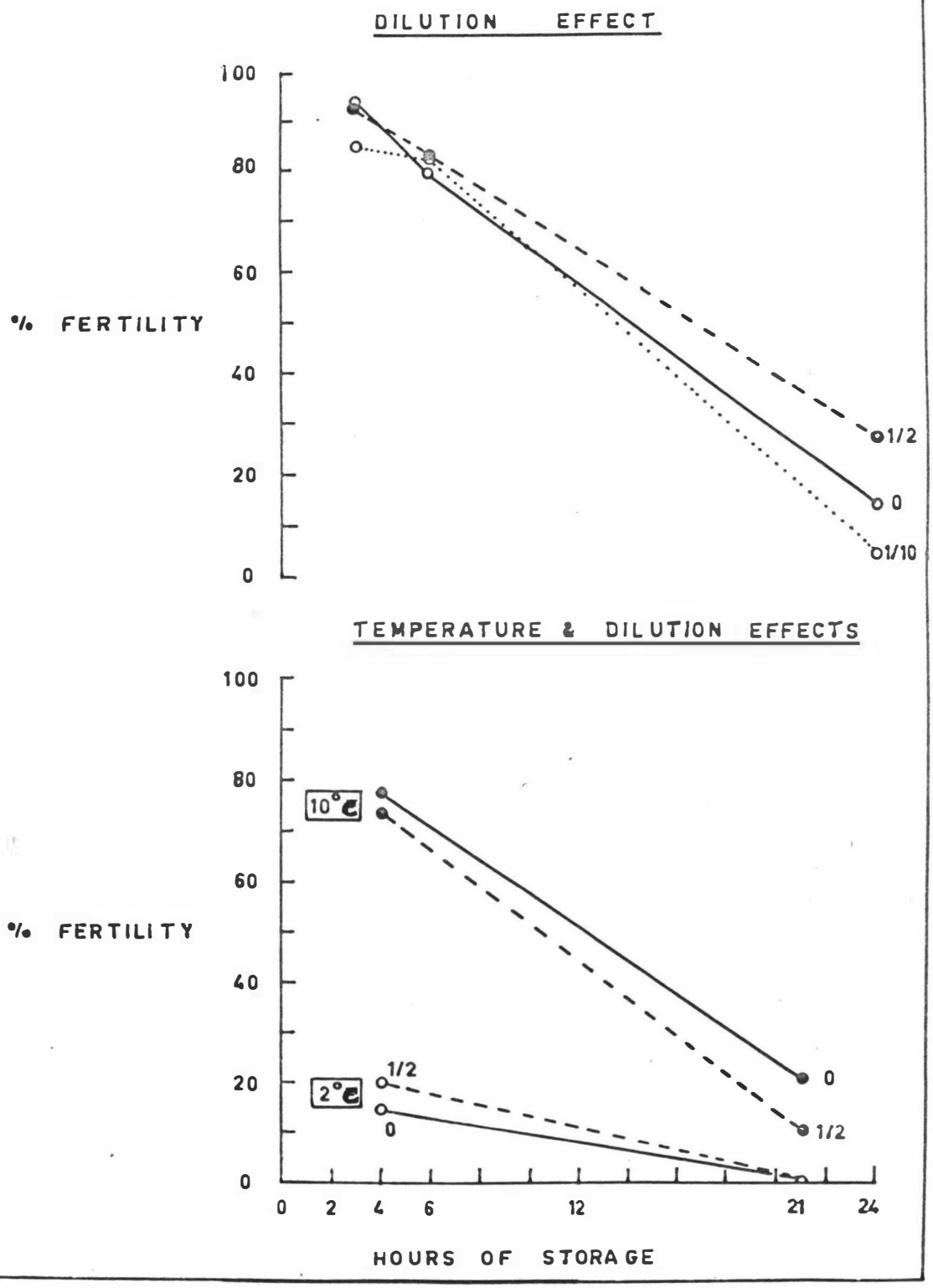
Data analysed as for a 3^2 factorial are presented in Table 15. Figures represent the percentage of fertile eggs from each of the six pullet replicates, maintaining 74% egg production.

Average fertility

Time	Mean	Dil. Rate	Mean
3 hours	77.7%	1/2	59.2%
6 "	70.1	0	55.1
24 "	16.1	1/10	49.6
$D_{.05}$ =	14.39%		
$D_{.01}$ =	18.28		

FIGURE 10

SEMEN STORAGE



(1) Fertility from semen stored six or less hours, was higher ($P < 0.01$) than that from semen stored twentyfour hours.

(2) There were no significant effects of dilution rate, although the influences of dilution on semen held twentyfour hours *in vitro* are illustrated in the upper graph of Figure 10.

(3) High fertility with fresh semen was obtained with a 1/10 dilution and 0.2 ml. inseminations.

(4) There was no significant interaction between time of storage and dilution rate; a 1/2 dilution was relatively more successful than undiluted semen for extended periods of storage.

EXPERIMENT 12.

Methods.

Temperature and dilution effects on the fertilising capacity of stored semen were examined.

Semen was collected from the same twenty Leghorn cockerels for both periods of storage, pooled, diluted, and later used to inseminate six WC pullet replicates per treatment with 0.1 ml. doses.

One batch of samples was stored at 10°C. in the usual manner and the other held at 2°C. in a refrigerator; the latter samples were transferred to the 10°C. coolroom thirty minutes prior to insemination. Two other factors were tested: storage, for four and twentyfour hours; and the effect of 1/2 dilution with pasteurised whole milk compared to an undiluted control during storage. Approximately 1.5 ml. of whole semen was allocated to each treatment, and the control batches were diluted 1/2 with milk kept at the same temperature, just prior to insemination.

Results.

Data analysed for a 2^3 factorial are presented in Table 16. Figures represent the percentage of fertile eggs from each of the six pullet replicates, maintaining 70% egg production.

Average fertility.

Time	Mean	Temperature	Mean
4 hours	42.3%	10°C.	40.3
21 hours	7.0	2°C.	9.0
	$D_{.01}$ =	16.56%	

(1) Higher fertility ($P < 0.01$) was obtained from semen stored four hours.

(2) Higher fertility ($P < 0.01$) was obtained from semen stored at 10°C.

(3) There was an insignificant effect from dilution rate but the relative advantages of dilution at low temperatures, within four hours of storage, are illustrated in the lower graph of Figure 10.

Average fertility; 2-factor interaction

Time-Temperature		Mean
4 hours	10°C.	66.6%
4 "	2°C.	17.9
21 "	10°C.	14.0
21 "	2°C.	0
	$D_{.05}$ =	23.12%
	$D_{.01}$ =	28.67

(4) A significant two-factor interaction indicated that semen storage at 2°C. was unsuccessful compared to 10°C., and that no fertility was obtained after twentyone hours at 2°C. Semen stored at 10°C. for four hours produced higher fertility ($P < 0.01$) than any other batches.

EXPERIMENT 13.

Methods.

A comparison was conducted of the fertilising capacity of semen stored for twentyfour hours with four diluents.

Unfortunately the semen collected from twenty AO cockerels was inadequate and had to be pooled and mixed with semen from ten WC cockerels. Seven WC pullet replicates per treatment were inseminated with 0.1 ml. doses.

The pullets were maintaining 78% egg production.

The four diluents applied were: a sodium-phosphate buffer; buffer plus the antibiotics, 90 µg oxytetracycline hydrochloride and 90 µg streptomycin sulphate, per ml.; pasteurised whole milk; and Tyrode solution. The inorganic diluents are described in Appendix B. All the batches were diluted 1/2 and held *in vitro* at 10°C. for twentyfour hours.

Results.

Data reported as a single classification analysis of variance are presented in Table 17. Figures represent the percentage of fertile eggs from each of the seven pullet replicates, from the second to eighth day after insemination.

Average fertility.

Diluent	Mean	
Buffer	60.8%	$D_{.05}$ = 31.2%
Buffer + abiots	46.0	$D_{.01}$ = 39.3
Milk	20.1	
Tyrode	3.8	

(1) The buffer diluents produced higher fertility ($P < 0.01$) than Tyrode solution.

(2) Buffer without antibiotics was the most successful diluent, producing higher fertility than with Tyrode solution ($P < 0.01$), milk ($P < 0.05$), or the presence of antibiotics

(3) Milk was a more successful storage diluent than Tyrode solution.

(4) An actual 69% fertility was obtained from the use of the sodium-phosphate buffer with semen stored *in vitro* for twentyfour hours at 10°C.

EXPERIMENT 14.

Methods.

The effects of two diluents at varied dilution rates were assessed by the fertilising potential of semen stored for twentyfour hours.

Semen was collected from twenty WC cockerels, pooled and split for

storage providing 1.0 ml. of undiluted semen per treatment. Fifteen WC pullet replicates, at 64% egg production, were inseminated with 0.1 ml. doses.

The two diluents, sodium-phosphate buffer and pasteurised whole milk were applied at 1/2 and 1/4 dilution within forty minutes of semen collection, and the preparations were stored at 10°C.

Results.

Data analysed as for a 2^2 factorial are presented in Table 18. Figures represent the percentage of fertile eggs layed by the fifteen pullet replicates from the second to eighth day after insemination.

Average fertility

Diluent	Mean	Dil. Rate	Mean
Buffer	14.0%	$\frac{1}{2}$	14.7%
Milk	6.8	$\frac{1}{4}$	6.2

(1) A higher fertility (NSD) was obtained from the buffer diluent.

(2)A higher fertility (NSD) was obtained at the 1/2 dilution rate.

(3) There was negligible interaction between the diluent and dilution rate.

(4) This experiment was conducted during early summer when both semen volumes and egg production were declining.

EXPERIMENT 15.

Methods.

This experiment was concerned with testing the beneficial effects of adding a metabolisable substrate to stored semen just prior to insemination.

Semen collected from sixteen WC cockerels was pooled, divided for storage and diluted to final volumes of 4.0 ml. Fifteen WC pullet replicates at 76% egg production, were inseminated with 0.1 ml. doses.

The main treatment effect was the addition of fructose solution at a final concentration of 2.5 mg./ml. to the semen mixture just prior to insemination. One batch of semen was diluted 1/2 and the other 1/4 with sodium-phosphate buffer and after twentyfour hours storage at 10°C. the former

was split, with fructose added to one half and the other serving as a control. The fructose which was also added to the 1/4 diluted batch, was prepared in a 60 mg./ml. H_2O stock solution and maintained at 10°C. The egg collection period was extended from the second to twelfth day after insemination, to assess the effects on the persistence of semen fertilising capacity.

Results.

Fertility data treated as a single classification analysis of variance are presented in Table 21A. Figures represent the percentage of fertile eggs produced by fifteen pullet replicates on the second to twelfth day after insemination.

Average fertility (Table 21A)

Treatment	Mean
½ Buffer + fructose	41.4%
½ Buffer	37.6
¼ Buffer + fructose	32.8

(1) No significant dilution effects were observed from the fertility of eggs collected over the total eleven days.

Fertility data obtained from eggs layed during the seventh to twelfth days after insemination are presented in Table 21B.

Average fertility (Table 21B).

Treatment	Mean
½ Buffer + fructose	26.0%
¼ Buffer + fructose	18.7
½ Buffer	15.3

(2) In this period higher fertility (NSD) was obtained from the treatments with fructose. The crossover effect of the two lower treatments is illustrated in Figure 13.

Fertility data obtained from eggs layed during the sixth to tenth days after insemination are presented in Table 21D.

Average fertility (Table 21D).

Treatment	Mean
½ Buffer + fructose	38.7%
½ Buffer	25.1
¼ Buffer + fructose	24.4

(3) A moderately higher fertility ($P < 0.10$) was obtained by the 1/2 buffer plus fructose treatment, in the second week after insemination; this superiority is illustrated in Figure 13.

The percentage fertility obtained over the eleven days after insemination is presented in Table 21C. A significant treatment effect was obtained with the untransformed data.

Average daily fertility (Table 21C).

Day	Mean
1	76.0%
3	69.0
2	66.0
4	65.0
5	52.7
6	37.7
7	37.0
8	19.0
9	6.7
10	5.3
11	5.3

$D_{.05} = 27.85\%$

$D_{.01} = 33.79$

(4) A significant decline in fertility occurred during the first week after insemination. Fertility from the second to fifth day was higher ($P < 0.01$) than that obtained from the ninth day after insemination, or during the seventh and eighth days ($P < 0.05$).

(5) One storage effect was the more rapid decline in fertility than was observed with fresh semen; comparable results are illustrated in Figure 13.

(6) The fructose effect appeared most obvious from the fourth to ninth day after insemination when the sharpest declines in fertility occurred.

EXPERIMENT 16.

Methods.

The effects of abundant cloacal transudate on undiluted semen, were assessed by the fertilising capacity of semen stored several hours at 10°C.

Ten AO cockerels were specially selected for their tendency to release a high or low proportion of cloacal transudate during artificial semen collection. This trait was particularly constant once the cockerels were accustomed to handling for collection and the flow of transudate was obtained without conscious modification of the technique or extra stimulation. Fifteen AO pullet replicates at 57% egg production, were inseminated with 0.05 ml. doses.

After a preliminary collection, semen was obtained from the two groups of five cockerels; 1.65 ml. from the 'low' transudate group and 5.10 ml. from the 'high' transudate group. When each batch had been thoroughly mixed, samples of approximately 1.5 ml. were withdrawn for storage at 10°C., and inseminated unchanged 6¼ hours later.

Results.

Fertility data treated as a single classification analysis of variance are presented in Table 19. Figures represent the percentage of fertile eggs obtained from fifteen pullet replicates on the second to eighth day after insemination.

Average fertility.

Treatment	Mean
High transudate	42.6%
Low transudate	35.7

(1) The concentration of cloacal transudate during storage had no significant effect on semen fertilising capacity.

(2) There were no significant changes in the percentage of dead spermatozoa of either semen batch after seven hours storage.

EXPERIMENT 17.

Methods.

The fertilising capacities of fresh and stored semen were

compared with ejaculates collected from cockerels and yearling cocks.

Five WC cockerels aged seven months and five WC yearling cocks aged nineteen months were used to obtain normal ejaculates after a preliminary settling period. Each batch of ejaculates was pooled, diluted 1/2 buffer for storage and ten WC pullet replicates at 74% egg production, were inseminated with 0.05 ml. doses.

The semen obtained from yearling cocks was relatively dilute; 1.76×10^9 spermatozoa per ml., compared to the cockerel concentration of 3.76×10^9 spermatozoa per ml., and this may have caused a variation in fertility from the number and concentration of spermatozoa inseminated. Approximately 1.0 ml. of each batch was stored at 10°C., with the first inseminations $2\frac{3}{4}$ hours after collection and the second 22 hours after insemination.

Results.

Fertility data analysed as a 2^2 factorial are presented in Table 31. Figures represent the percentage fertile eggs obtained from ten pullet replicates on the second to eighth day after insemination.

Average fertility

Storage	Mean	Males	Mean
$2\frac{3}{4}$ hours	65.1%	Cockerel	49.3%
22 "	11.0	Cock	26.8
	$D_{.01}$ = 14.02%		

(1) Higher fertility ($P < 0.01$) was obtained from semen stored $2\frac{3}{4}$ hours than semen stored 22 hours in vitro.

(2) Higher fertility ($P < 0.01$) was obtained using cockerel semen.

Average fertility; 2-factor interaction

Storage - Males		Mean
$2\frac{3}{4}$ hours	Cockerel	82.3%
"	Cock	47.8
22 "	Cockerel	16.3
" "	Cock	5.7
	$D_{.05}$ = 19.54%	

(3) A significant interaction ($P < 0.05$) between storage time and age of male was obtained.

3.7 Discussion.

Experimental results are split into three categories: firstly time and temperature influences on *in vitro* semen storage; secondly, dilution for storage; and finally the apparent relationship between gamete quality and fertility.

Several experiments verified the decline in fertilising capacity of stored semen. However the causative factor responsible for the decline was not resolved; whether it occurred from a significant proportion of dead spermatozoa, or reflected general spermatozoa senescence and reduce viability *in utero*.

Dilution rate and diluent trials were more precise as they were associated with comprehensive insemination tests which are reported in Chapter 4. The overall conclusions were that low dilution with a sodium-phosphate buffer diluent, provided an environment beneficial to spermatozoa integrity *in vitro* at $10^{\circ}C$. A longer duration of fertility was obtained from adding fructose to stored semen just prior to insemination, but no significant benefit was gained from using antibiotics with semen *in vitro*.

The association between gamete quality and fertility was derived by comparing the storing qualities of semen, at times when the male semen characteristics and female egg production varied.

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Time of Storage.

The length of semen storage *in vitro* was demonstrated to influence subsequent fertilising capacity, and to be modified by dilution, storage

temperature and gamete quality.

In considering the significant time effects obtained in several experiments, it is clear that large variations arose from the modifying factors. In Expt. 9, negligible differences in fertility were obtained with hourly inseminations from thirty minutes to up to seven hours - in fact there was no indication of a deterioration of semen in this time; yet a highly significant fall in fertility after four hours in vitro was recorded in Expt. 10. Highly significant drops in fertility were obtained between six and twentyfour hours in vitro in Expt. 11, and four and twentyone hours, in Expt. 12. The results from Expt. 11 are illustrated in the upper graph, and Expt. 12 in the lower graph, of Figure 10. Egg collection in Expt.10 was extended to the twelfth day after insemination and fertility in the first six and last five days is illustrated in the lower graph of Figure 11. In conclusion, the fertilising capacity of spermatozoa held at 10°C., significantly declined sometime between four and twentyone hours of storage in vitro. Considering that many trials were conducted with high spermatozoa concentrations, the decline in fertility could be attributed to a widespread senescence of spermatozoa. This opinion is reinforced by the results from Expt. 15 illustrated in the lower graph of Figure 13, where the sharp decline in fertility occurred about five days earlier than that recorded for fresh semen in the upper graph. Although 80% fertility was obtained in the first few days after insemination with stored semen, the rapid decline suggested a reduced ability of the spermatozoa to survive or to retain their fertilising capacity, in utero.

The modifying factors are considered briefly at this stage by reference to examples of their effects.

Dilution was shown to affect fertility, although insignificantly, in Expts. 10, 11 and 12, which are illustrated in Figures 10 and 11. The upper graph of Figure 10 showed that the minor dilution effects in up to six hours storage, became important after twentyfour hours. A striking effect of dilution

on the persistence of fertility from semen stored 240 minutes was illustrated in Figure 11, in the crossover of 0 and $\frac{1}{2}$ dilution during the first and second weeks after insemination. Highly significant differences between diluents were obtained in Expt. 13.

The influence of gamete quality on storing potential was derived from comparing the relevant results of Expts. 13, 14 and 15. All included a 1/2 sodium-phosphate buffer treatment of semen stored for twentyfour hours at 10°C., and an 0.1 ml. insemination dose. Experiments 13 and 15 in the winter, produced 69% and 54% fertility respectively, with 78% and 76% egg production from the pullets used. However only 20% fertility was obtained from Expt. 14 during the summer, with 64% egg production from the pullets.

The superiority of 10°C. for semen storage in vitro compared to 2°C., was highly significant within four hours from collection. The poor results at 2°C. were surprisingly low compared to values reported in the literature and implied that milk dilution was partly responsible. Lorenz (1964), concluded that 1 to 2°C. approximated the optimum temperature for storage of diluted semen, and 10 to 15°C. for undiluted semen; and the lower graph of Figure 10 confirmed his views. However the superiority of undiluted semen after twentyone hours storage at 10°C. contrasted with the results from Expt. 11 shown in the upper graph. Possibly the differences are resolved by the changing relationship between dilution and the persistence of fertility, illustrated in Figure 11.

Dilution for Storage.

This section is discussed from four aspects : firstly the effects of dilution compared to unmodified semen; secondly the rate of dilution; thirdly the diluents studied; and finally a brief discussion of special treatments imposed on stored semen.

From Expt. 10; dilution was shown to increase the persistence of fertility, particularly when the semen had been held in vitro for four hours. In the second to seventh days after insemination, undiluted semen proved superior by approximately 12% higher fertility, however the results indicated a delayed dilution effect durihg the eighth to twelfth days following insemination. In this period the dilution effect must have occurred within as little as twenty minutes storage in vitro, as both treatments produced 62% fertility; however as Figure 11 illustrates, the diluted semen held four hours in vitro decreased by only 6% fertility whereas the undiluted semen decreased by 31%. These results are also illustrated in the upper graph of Figure 11, but unfortunately the values are averaged for the two times of storage and do not feature the advantages of dilution on the persistence of fertility in the four hour treatment.

From Expt 11, dilution with Tyrode solution had negligible effects for the first six hours of storage in vitro, but caused some divergence after twentyfour hours. However the contrary results from Expt. 12, where milk was used, queried the significance of either. The advantages of dilution for 2°C. storage appear to be significant from Expt. 12 and reports in the literature.

A rather novel dilution of approximately 1/3 with cloacal transudate, was obtained in Expt. 16. Similar levels of fertility were obtained from high and low transudate samples, which invalidate either a dilution effect occurring after six hours of storage, or a detrimental effect from the transudate at 10°C.

Obviously the effects arising from dilution rate are proportional to

the original spermatozoa concentration and the insemination dose. From Expt. 11, which is illustrated in the upper graph of Figure 10, a 1/10 dilution was not significantly inferior to a 1/2 dilution at any stage although the fertility levels diverged after twentyfour hours storage. However in Expt 14, markedly different fertility levels were obtained with rates varying as little as 1/2 to 1/4. The variations were insignificant due to low absolute values, but the more sophisticated experimental design of Expt. 14, together with similar results presented in Chapter 4, imply that the rate of semen dilution influences the fertilising potential of fresh and stored semen.

Three diluents were tested at low dilution rates, for their ability to maintain the integrity of stored spermatozoa. From Expt. 14, the sodium-phosphate buffer with an average 16% fertility was clearly superior to milk at 6%. The larger differences at 1/4 dilution, suggested that the buffer was relatively more successful because the influence of a diluent would increase with its concentration in a sample. Similar significant variations occurred in Expt. 13. A most successful 69% fertility was obtained from semen diluted 1/2 in buffer and held *in vitro* for twentyfour hours, and this was significantly higher than the fertility obtained with milk or Tyrode dilution. Fertility with milk dilution, at 20%, was markedly higher than 3% for Tyrode solution in this experiment, but can not be validly compared in Expts. 11 and 12. These results coincide with the theories and conclusions expressed by several authors in the review. The general approach for semen storage diluents is changing from a simulation of seminal plasma, to criteria of buffering capacity and optimum ionic balances. Whole milk and Tyrode solution were inadequate for these reasons. El Zayat and Van Tienhoven (1959) showed that the buffer had an initial dampening effect on spermatozoa respiration and fructolysis compared to Tyrode solution, and indicated that spermatozoa exhausted *in vitro* were inferior *in utero*. Once again however, the problem of whether reduced

fertility was caused by a significant reduction of functional spermatozoa, or a general senescence, was unresolved.

From Expt. 16, the cloacal transudate which could be considered as a diluent, produced no significant changes in fertilising capacity after six hours storage at 10°C. This conflicted with Lake's (1956) report where he observed a decline in fertilising capacity after only twenty minutes, of semen plus cloacal fluid at 21°C.

Two special treatments reported by Wilcox and his colleagues, were compared with the simple dilution and storage at 10°C. method. Both involved the use of the sodium-phosphate buffer:

16.34 g Na_2HPO_4 + 5.16 g $NaH_2PO_4 \cdot H_2O$ per litre H_2O,

which was first applied in their laboratory, and are reported by Wilcox and Shaffner (1958).

The first experiment obtained negative results and was not reported in Section 3.6. In one treatment fresh semen was diluted 1/11 in buffer solution plus:

90 µg oxytetracycline hydrochloride + 90 µg streptomycin sulphate per millilitre, for twentyfour hours storage at 10°C. Just prior to insemination the mixture was centrifuged at 1200 RPM for ten minutes and resuspended in fresh buffer to the original undiluted volume. In the second treatment, the fresh semen was centrifuged at 1200 RPM for 10 minutes and resuspended in buffer plus antibiotics to the original undiluted volume, remaining this way for insemination. The blame for infertility was tentatively attributed to the antibiotic preparations, as the buffer diluent and centrifuging procedure were vindicated in later trials.

In Expt. 15, the fructose treatment was tested without the centrifuging and dilution procedures described by Wilcox and Shaffner (1958). All the fertility levels were extremely high for semen stored twentyfour hours. Two important results are illustrated in Figure 13: firstly that the fructose

effect was to prolong the persistance of fertility; and secondly, the reduced fertility attributable to extra dilution in spite of double the fructose concentration per spermatozoa. This suggests that the stored spermatozoa are responsive to extracellular nutrient when deposited in the female genital tract, and that they remain in contact with the diluent long enough to utilise some of the fructose.

From the experiments and review, the maintainence of spermatozoa resources by a depression of activity in vitro, apparently enables the spermatozoa to persist in utero or to reach the glands which are thought to harbour them.

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Gamete Quality.

Although there is no statistical verification the semen storage experiments suggest an association of gamete quality and fertility. Female egg production and male spermatogenesis are used as criteria of gamete quality; both factors obviously affected by the seasonal and aging effects discussed in Chapter 2. Fertility was assessed from the fertilising potential of semen after storage in vitro.

During the winter months, when egg production was high, fertility from stored semen was high. For example: in Expt. 9, no decline in fertility was observed during six hours storage; in Expt. 10, approximately 63% fertility was obtained after four hours storage; in Expt. 11, 70% after six hours storage; in Expt. 13, 69% after twentyfour hours storage; and in Expt. 15, 54% after twentyfour hours. Although there were a few exceptions like Expt. 11 where Tyrode diluent was used; the success of storage with sodium-phosphate buffer seemed to be related to egg production and possibly semen quality as indicated by volume and spermatozoa concentration.

In contrast, only 41% fertility was obtained from semen stored six hours in Expt. 16, when egg production had fallen to 57%; and in Expt. 14, 20% fertility was obtained after twentyfour hours with 64% egg production. In Expt. 17 significantly higher fertility was obtained from fresh and stored cockerel semen, than semen from yearling cocks. The difference was accentuated by storage, where 16% fertility from cockerel semen was markedly higher than 4% from the older males. This experiment was conducted at a time of high egg production from the pullets, therefore male semen quality has been implicated. In spite of the relatively low spermatozoa concentration of cock semen and the interaction between males and storage time, spermatozoa from the aged males were functionally inferior within three hours storage in vitro. Unfortunately the laboratory evaluations of semen were inadequate to show which aspect of semen quality was associated with the decline in fertilising ability; in fact this summarises the main problem of semen storage, and it is unlikely that any important progress will be made until it is resolved.

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CHAPTER 4.

Artificial Insemination and Fertility.

A. Review.

Insemination is the final phase of an artificial breeding scheme, concerned with the preparation of spermatozoa for optimum fertility and an insemination to simulate natural mating.

This review of literature, is limited to the preparation of fresh semen and a few insemination techniques employed under unusual conditions which have not already been described in previous chapters. As well, reference is made to three aspects of fertility which have not been considered in the experimental work yet complement a discussion of the results. They are: laboratory predictions of fertility from tests of semen *in vitro*; spermatozoa in the female; and the female contribution to fertility.

Unfortunately fertility is reported, often vaguely, in several different ways by groups of workers and this sometimes precludes a sensible comparison of results. None of the methods are completely free of significant influences, particularly the variation in egg production. As Lorenz (1964) pointed out, the influences although uncontrollable are reasonable, as basically fertility is the probability that fertilised ova will be produced; thus involving the gamut of probabilities from semen production to a successful mating and ovulation. In this thesis fertility is limited to the probability that embryonic development will be detected in slightly incubated eggs, after a particular semen insemination. As percentages are computed for individual pullets and averaged over all replications, this method avoids some biases of uneven egg production between replicates. Fertility estimated by the duration of fertile egg production also avoids some objections, but is potentially more laborious in that eggs must be collected and tested until it is reasonably certain that pullets are infertile. After initial experiments it was practical

to limit the period of egg collection yet observe the duration of fertility, as each egg was identified to the day after insemination as well as the pullet.

4.1 Artificial Insemination.

Preparation of semen.

Macroenvironmental influences pertaining to stored semen have been discussed in Section 3.4, and apply equally to fresh semen. However fowl semen has proved relatively easy to retain *in vitro* for one or two hours in clean apparatus, without the precautions required for storage. Possibly temperature shock has small cumulative effects on spermatozoa integrity, but workers almost unanimously emphasise the temperature tolerance of fowl spermatozoa compared to domestic mammals. Wales and White (1959) suggested the property may be related to the relatively high temperature of spermatozoa in the fowl. A holding temperature of 10°C. to 15°C. appeared to be the optimum for fowl semen, once the sample had been allowed to cool naturally after ejaculation (Allen 1957, Wales and White 1959, Bajpai and Brown 1964, and Proudfoot and Stewart 1967). Schindler and Hurwitz (1966) recorded that spermatozoa kept at 39°C. *in vitro* were non-motile within four hours and within twentyfour hours at 18°C., and noted a higher maintenance of motility of spermatozoa recovered from the oviduct *in vivo*, which were subject to similar temperatures.

Dilution is an important step in the preparation of semen for insemination and perhaps the most controversial. The decision of whether and how to dilute semen is dependent on the objective of insemination: the desired ratio of progeny to one parent; the frequency of insemination; and duration of fertility expected. Although dilution is a useful method of extending fresh semen, most workers consider that spermatozoa integrity is affected by the mechanical damage in mixing, the introduction of toxic substances, the dilution of plasma nutritive or protective substances, or metabolic changes arising from a decrease in spermatozoa density. The severity of these

influences would seem to be dependent on the time that semen is retained *in vitro* or associated with the diluent *in utero*.

Characteristics of Tyrode solution, carbonated and plasma-like diluents, milk and a sodium-phosphate buffer, together with several ions and additives for dilution, have been reported in Section 3.3. In general the preference of diluents for semen storage has centred on buffered solutions with greater control over gas exchange and spermatozoa activity, and away from imitations of seminal plasma. Many choices of diluent for an immediate insemination are proposed in the literature: Ringer's solution by Bonnier and Trulsson (1939); glycine diluents and seminal plasma by Rowell and Cooper (1957); Tyrode solution by Allen and Skaller (1958); Kushner *et al* (1966) and Van Tienhoven (1958); and sodium-phosphate buffer by Wilcox (1958). This suggests that the precise content of many diluents does not significantly affect the fertilising potential of fresh semen.

The extent of dilution is usually dependent on the demand for semen, with a compromise between the practical limit of a minimum volume for handling and decreased fertility from high dilutions. There is considerable disagreement in the literature over the optimum rate of dilution, which probably reflects the methods of describing fertility. Bonnier and Trulsson (1939) obtained no significant changes in fertility with whole semen or a ten-fold dilution and 0.05 ml. inseminations; Munro (1938 c) suggested that extremely high dilution rates were satisfactory providing a threshold of one hundred million spermatozoa per insemination was achieved; Weakley and Shaffner (1952) considered that dilution up to 1/11 with fowl semen serum did not reduce fertility; and more recently Taneja and Gowe (1961 b) reported that dilution up to 1/11 increased the duration of fertility obtained from inseminations of less than 0.01 ml. volume. However the majority of recent reports endorse the advantages of low dilution rates up to approximately 1/4, coupled with low insemination doses if necessary (Allen 1957, Rowell and Cooper 1957 and 1960, Wilcox 1958, and Saeki 1964).

Insemination dose is inversely proportional to the rate of dilution where the spermatozoa number is limited. Once again there is considerable variation between reports for the preferred number of spermatozoa required to produce high fertility. The recommended minimum spermatozoa number for one insemination has steadily decreased over the years from one hundred million (Munro 1938 c) to sixtytwo million (Van Duijn 1964 cited by Lake 1967); and as the counting and inseminating techniques improve the number would be expected to continue falling. Some extremely small semen doses have produced moderate fertility; Taneja and Gowe (1960) inseminated an estimated 1.12 million spermatozoa in 0.0002 ml. of undiluted semen and obtained fertile eggs for an average 2.3 days, and 8.0 days with 0.001 ml., and only slight increases in the duration of fertility with more than 0.01 ml. or fiftysix million spermatozoa. Saeki (1964) obtained 20% fertility over seven days with a 0.00125 ml. dose of undiluted pooled semen, and maximum fertility with a 0.02 ml. dose; he noted increasingly high positive correlations of decreased dose with decreased fertility. which implied a threshold of spermatozoa number for fertility. Saeki et al (1965 a) reported that a 0.005 ml. dose of undiluted semen every fourth day produced higher fertility over one laying year, than 0.04 ml. every sixth day, or even higher volumes. The general trend appears to be toward reduced doses of relatively concentrated semen, particularly in some Japanese laboratories (Morimoto 1966 pers. comm.).

Insemination technique.

Intra-vaginal or intra-uterine inseminations are usually applied in commercial breeding programms. The intra-vaginal technique has been described in Section 2.5, but it is worthwhile to note that Gabriel (1957) devised a one-man insemination technique for caged hens and reported similar advantages in synchronisation and mental response to those obtained with one-man semen collection. Intra-uterine insemination has been applied in conjunction with frozen or glycerolised semen to place the spermatozoa beyond the utero-vaginal

junction, but in some cases this technique caused an inhibition of ovulation and great pain to the birds. Bobr, Lake, Lorenz, Ogasawara and Krzanowska (1965) reported a method of everting the vagina until the junction was exposed and quick careful penetration with a cannula; this caused less irritation and decline of egg production than the previous method of penetrating the utero-vaginal junction in situ.

Intra-peritoneal insemination has more severe effects on the birds and is normally restricted to research with frozen or glycerolised semen or attempts to place spermatozoa near the infundibular glands. Brown, Harris and Hobbs (1963b) described intra-peritoneal insemination with a 1" needle inserted 1.5 cm. posterior to the last rib and caudalward at 45° angle to the body wall. They suggested that the intra-peritoneal technique was superior to intra-uterine insemination, and that the use of small volumes of diluted fresh semen containing antibiotics reduced the detrimental effects on egg production. However both methods invite the ris of introducing microorganisms to the site of fertilisation. Harry (1963) reported that excepting the vagina, the reproductive tract of a healthy pullet contained few bacteria and that deposition of contaminated semen above the vagina could introduce bacteria before albumen deposition protected the ovum.

Most variations of handling techniques are modifications of Burrows and Quinn's (1937) technique, suited to the housing and labour conditions.

4.2 Fertility.

Spermatozoa in the oviduct.

After intra-vaginal insemination or copulation spermatozoa are thought to move through the vagina and utero-vaginal junction primarily by their own motility. For this reason the junction acts as a selective barrier to spermatozoa, although the significance and extent of selection is not clear. Once over the junction and in the uterus lumen, spermatozoa and similar inert substances apparently travel by the peristaltic or ciliary action of the oviduct.

Saeki, Tanabe, Katsuragi and Miyage (1963) using a tracer technique with ^{32}P - labelled cells, found living spermatozoa in the infundibulum within six teen minutes of intra-vaginal insemination; this extremely short interval necessitated external aid for the spermatozoa and rapid passage through the vagina. Inert substances deposited in the uterus moved at a similar rate especially in laying birds. Within twentyfour hours of intra-vaginal insemination more than 50% of the radioactivity was recorded in the infundibulum and some near the ovary, but the distribution was partly influenced by the stage of ovulation at the time. Allen and Grigg (1957) employing similar methods reached the same conclusions and suggested that the proportion of spermatozoa which reached the infundibulum was determined by the site of deposition.

Once near the uterus most of the living spermatozoa apparently move into a group of glands in the utero-vaginal junction, which are considered to be residence sites acting as a revervoir of spermatozoa required for sequential ovulations. Van Drimmelen (1949) first emphasised the maintenance role of 'sperm nests' glands in the caudal infundibulum and assumed the bulk of the stored spermatozoa were retained in close proximity to the site of fertilisation. Recent studies (Bobr et al 1964 a, b; Lorenz 1964) have discounted the biological significance of the infundibular glands and ascribed the main storage role to the histologically similar utero-vaginal glands. These glands are mainly in vaginal tissue where a thick ring of constricted folds delineating the uterus, are massed in the sphincter lumen (see Plate 10). Bobr et al (1964 a) detected abundant spermatozoa within the glands ten minutes after copulation and less than ninety minutes after an intra-uterine insemination whereas the infundibular glands were more slowly and sparsely filled.

Comparisons have been reported on the storage potential and biological significance of the two gland sites following a variety of insemination techniques, but do not necessarily apply to spermatozoa distribution after natural matings. Bobr et al (1964 a) considered the infundibular glands

unimportant spermatozoa residence sites, as albumen secretion or an inflated developing egg effectively blocked spermatozoa passage, and the average duration of spermatozoa in the infundibulum was less than that at the utero-vaginal junction. By applying intra-vaginal, intra-uterine and intra-peritoneal inseminations at different times in the ovulatory cycle, they observed an increasing proportion of spermatozoa in the infundibular glands as the semen was deposited deeper, and virtually no caudal migration after an intra-magnal insemination. Natural mating was apparently equivalent to penetration between the vaginal and uterine depositions, with a few spermatozoa achieving the infundibular region. In contrast, Takeda (1966) observed that the infundibular glands had a higher preservation capacity than the utero-vaginal area after an insemination of 100 million spermatozoa at the two sites. He obtained an average duration of fertility of 17.2 days at the infundibular site and 12.2 days from the utero-vaginal junction site spermatozoa, and the situation was unchanged by larger insemination doses. Van Krey, Ogasawara and Lorenz (1964) also obtained a longer duration of fertility from spermatozoa deposited intra-magnally after laparotomy, but in later tests Van Krey et al (1966) detected a high pre-oviposital embryonic mortality. This has been tentatively ascribed to several features of infundibular storage: firstly that inferior spermatozoa successfully competing for fertilisation would normally have been impeded in passage through the oviduct; that the blastoderm could have been disrupted by intense polyspermy after a fertilisation near to the storage site; or that the spermatozoa are rendered inneffective by severe overcrowding. These hypotheses elucidate some of the apparent advantages obtained with frozen or glycerolised semen after deposition above the vagina, and the increased fertility with artificial insemination of semen from low-fecundity males.

Less information is available on the spermatozoa release mechanism. Grigg (1957) reported the presence of living spermatozoa in the infundibulum lumen soon after egg passage had been simulated by an artificial ovum, and postulated a strong influence of mechanical distension. Subsequent experiments

have questioned these conclusions and although the mechamism is still unknown, it is thought to be synchronised with ovulatory cycles. Lorenz (1966) observed some spermatozoa releases after mock ovulations and hormone treatments but noted that 'normal' releases occurred only after a genuine ovulation and that spermatozoa had disappeared from the lumen again within two or three hours of ovulation. Most of the spermatozoa were released from the utero-vaginal glands. Other recent papers by Verma and Cherms (1965), Lake (1966 a, 1967) and Van Krey, Ogasawara and Pangborn (1967), help to establish a few points: spermatozoa inseminated in or near the uterus rapidly assemble head-first in the utero-vaginal glands and become fairly quiescent; during and after ovulation some spermatozoa - possibly the first to enter the glands, are released by an unknown mechanism and move up the oviduct to the site of fertilisation in the magnum or infundibulum; and that spermatozoa residence in the utero-vaginal glands is an integral part of avian sequential ovulation. Parker and Arscott (1965) clearly demonstrated the longer duration of fertility obtained with inseminations made from 1200 to 2100 hours compared to 0800 hours, and most workers attribute this to the stage of ovulation, assuming that an egg *in utero* blocks the ascending spermatozoa or prohibits a satisfactory eversion of the cloaca.

Competitive fertilisation has been demonstrated with old and fresh spermatozoa (Payne and Kahrs 1961, Smyth 1963 and Kushner *et al* 1966), defective spermatozoa (Allen and Champion 1955), and heteroplasmic unions (Atkinson, Kropp, Bradley and Quisenberry 1966, and Itagaki, Tsubokura, Inomata and Nakano 1966). The fertilising potential of aged versus fresh spermatozoa has been well documented and is useful for predicting the change in paternity, but the apparent disadvantages of heteroplasmic matings are unresolved.

Female contribution to fertility.

The evidence for genetic differences between females in their contribution to fertility, is rather fragmentary and as yet unrelated to physiological or psychological variation. Bonnier and Trulsson (1939) report-

ed significant variations in the ability of females to produce fertile eggs and Howes and Gowe (1960) after similar results suggested that the duration of fertility was at least partly determined by several non-dominant autosomal genes. Hays (1950) reported some evidence that female fertility was determined as a sex-linked trait by the sire. Maw (1956) and Allen and Skaller (1958), obtained heteroplasmic and strain variations in fertility and noted interacting male and female contributions. Recently Arora and Kosin (1966 a, b) reported an association of the stage of gastrulation of turkey eggs at oviposition with the ability of the blastoderm to survive pre-incubation storage. This confirmation of genetic differences in hatchability helps to explain the apparent differences in fecundity which were not detected by early fertility tests, and highlights the problem of measuring avian fertility.

More information is available on environmental effects on fertility. Apart from the nutritional, pathological and management influences discussed in Section 2.1, which obviously affect female gamete maturation as well as spermatozoa, most research has been concerned with the relationship of ovulation rate and fertility. Lamoreux (1940) reported that infertility was inversely proportional to clutch size or egg production over six weeks, for naturally mating fowls. Nalbandov and Card (1943) with similar results, suggested that low-producing females were mated less frequently and that the reduced hatchability reflected early embryonic deaths due to fertilisation by stale spermatozoa. Taneja and Gowe (1960, 1961 cited by Lorenz 1964) reported strain variations in the duration of fertility, which were associated with egg production rate and inversely related to the frequency of infertile eggs. Temperature influences were reported by Long and Godfrey (1952) relating lower fertility in cold periods to reduced mating; and Hafez and Kamar (1955) who considered winter ambient temperatures were most favourable for fertility with natural matings in the subtropics. McCluskey and Parker (1963) subjected pullets to different light regimen and observed a positive correlation of fertility with increasing light period, but showed that this was due to mating frequency and could be rectified

by artificial breeding. Sunde and Bird (1957, 1959) noted initially low hatchability due to early embryonic deaths in the first three to six weeks after the onset of lay; and Tomhave (1958) reported highest hatchability during the first two hundred days of egg production and an accelerated decline thereafter.

The significance of female immuno-reaction to repeated insemination or the infertility syndrome, is a controversial topic at present. The presence of circulating antibodies to spermatozoa has been established (Wentworth and Mellen 1963 b; Hosoda, Abe and Otsuka 1964; Saeki et al 1965 a, b; and Lake 1966 a), but many workers are sceptical of their relationship with declining fertility. Abe et al (1965) and Itagaki et al (1966) were unable to incriminate antibodies and Kushner et al (1966) suggested that the insemination technique and equipment could be responsible for declining fertility results. Some reports have indicated a higher immuno-reaction to artificial insemination than natural mating, and Lake (1966 a) has pointed out that this may be due to the higher proportion of male blood transudate obtained with artificial collection.

Laboratory predictions.

The data obtained from laboratory evaluations of semen for predicting fertilising capacity, are briefly mentioned in this review, in part to justify their ommission from the thesis research. Cooper and Rowell (1957) considered that a measurement of resazurin reduction time to a pink end-point (RRT), or initial pH, or percentage dead spermatozoa, were sufficient to identify cocks with an abnormally low fertilising capacity. They computed that 80.7% of the variation in fertility between cocks could be explained by a combination of RRT and percentage dead spermatozoa data. In a later paper (1958) they obtained significant correlations between RRT and percentage dead (+0.82***); motility and RRT (-0.76***); and motility with percentage dead (-0.95***). The most useful single criteria for laboratory prediction was the percentage of dead spermatozoa which accounted for 77.7% of the variation in fertility and this prediction was not significantly improved by multiple analyses with

other criteria, however the authors cautioned against the wide confidence intervals in predictions. As no association of fertility with spermatozoa concentration, semen pH or volume was obtained, the results imply that spermatozoa quality measurements are most valuable in predictions.

Other reports emphasise similar criteria : Sacki (1960) obtained a negative association between the proportion of crooked neck spermatozoa and fertilising capacity; and McDaniel and Craig (1962) reported that the combined criteria of motility, opacity score and RRT accounted for 64% of the variation in fertility of a subfertile strain but only 27% in a highly selected Leghorn strain. However as Lake (1967) points out, no precise parameters of spermatozoa morphology or metabolism have yet been sufficiently associated with their fertilising potential to form the basis of simple semen quality tests.

B. Research.

4.3 Material and Methods.

Materials.

Pooled semen obtained from cockerels of one strain was collected by the one-man technique described in Section 2.5.

Diluent chemical compositions are presented in Appendix B.

Artificial inseminations were made using a syringe with affixed cannula or one of two micropipettes. The 2 ml. capacity syringes were modified by a return spring on the plunger and a brass retaining tube around the bore which limited the dose to a specified fraction of a millilitre. Micropipettes and attached rubber blow tubes were applied for small dose inseminations as pictured in Plate 9. The larger pipette with a 0.10 ml. capacity was calibrated to 0.005 ml. and the bore diameter was approximately equal to the cannula bore. The smaller pipette, pictured in Plate 2, had a 0.025 ml. capacity and calibrations to 0.0025 ml.

Pullets required for fertility tests were randomly chosen from batteries

PLATE 7.

PLATE 7. Artificial insemination.

A : cage floor of third tier.
B : feed trough.
W : water trough of second tier.

The usual method of inseminating trained pullets. At the stage illustrated, the cloaca has been forcibly everted to expose the distal vaginal opening. The cannula has penetrated the vagina $1\frac{1}{2}$" to 2", and immediately following this, pressure was relaxed, the cloaca reverted, and semen was released into the proximal vagina.

PLATE 8.

PLATE 8. Artificial insemination.

R : distal rectum.

At the stage shown the cannula has penetrated the distal vagina. After the pullet was relaxed, the cloaca reverted and the semen expelled. Recalcitrant pullets were removed from their cages, where more directional pressure could be applied for eversion. The left hand pressed up the soft contents of the abdomen, while the right hand forced the upper vent and pygostyle anteriorly and exposed the area for insemination.

conveniently near the cockerel cages. Usually groups of pullets in contiguous cages or rows were allocated to one experiment and inseminated with semen from males of the same strain. Non-laying birds were omitted and a minimum period of four weeks was allowed between experimental inseminations. Pullets were subdivided into randomised blocks for each treatment, however the insemination sequence by treatments rather than replicates, was adopted for convenience and was unlikely to influence the fertility obtained with fresh semen.

Methods.

Semen required fresh for insemination, was collected between 1500 and 1630 hours and transferred to a laboratory for dilution and mixing.

Dilutions were made within fifteen minutes of collection when both diluent and semen had attained room temperature. The diluent was slowly added to the tube of semen from a pipette and swirled by hand until the mixture appeared uniform.

Insemination techniques have been described in Section 2.5. The use of syringes inside and outside the cages are pictured in Plates 7 and 8, and a micropipette insemination in Plate 9. During a series of inseminations for one treatment, the tube of semen was continually agitated to ensure a fair sample for every pullet.

Egg collection proceeded from the second day after insemination over the period prescribed in each experiment. Eggs were collected daily between 1400 and 1600 hours, with the date and pullet cage number marked on each one. Individual eggs were identified on the analysis sheet to both the pullet and the date of oviposition.

Fertility tests were conducted after twentyfour hours of incubation at 100°F. Eggs were broken out onto an illuminated glass platform with the blastoderm appearing on the top surface. Most decisions were made after a rapid macroscopic examination which classified eggs as fertile on the basis of

embryonic development. Unfertilised eggs were those exhibiting no embryonic development and the characteristic white opaque blastodisc centre with peripheral lacunae. Approximately 7% of eggs were classified as doubtful and examined under magnification and compared with diagrams made at various stages of incubation of unfertilised and fertile blastodiscs. The undetermined errors which could have arisen from pre-ovipositol mortality or parthenogenetic development were appreciated but not seriously considered, as the results were intended to be comparitave and relfect the fertility which would have been obtained with candling. A more serious omission arose from the lack of hatchability results; particularly in the semen storage experiments.

Fertility was expressed at two levels: pullet fertility was computed as the percentage of fertile eggs from individuals, and treatment fertility as the averaged percentages of all pullet replicates. Although raw data are presented in the Appendix A tables, most of the percentage fertility results were transformed for analysis and comparisons between means; however original percentages are illustrated in the graphs.

4.4 Experiments.

Artificial insemination and fertility were investigated from three aspects: diluents for extending fresh semen; dilution rate and insemination dose; and the repeatability of fertility.

In many experiments doses were varied in order to obtain a certain number of spermatozoa per insemination; often inversely proportional to the dilution rate. It was considered that dilution was relatively more important to fertility than dose, consequently the results are grouped with respect to dilution rate.

Experimental methods and results are presented here from tables in Appendix A, and a discussion of the results follows in Section 4.5

Diluents:

Experiment 18 Locke's, Tyrode and milk diluents with two strains.

" 19 Tyrode, milk and sodium-phosphate buffer diluents.

" 20 Tyrode and milk at two dilution rates.

" 21 Tyrode and milk at four dilution rates.

Dilution Rate:

Experiment 22 Three doses at three dilution rates with milk.

" 23 Three doses at two dilution rates with milk.

" 24 Two doses at three dilution rates with buffer.

" 25 Three doses at two dilution rates with milk.

" 26 Four dilution rates with milk.

" 27 Four dilution rates with milk for two strains.

Fertility:

Experiment 28 Duration of fertility after one insemination.

" 29 Repeatability of fertility.

Research methods:

Experiment 30 Centrifugation of semen.

" 31 Pullet training for artificial insemination.

" 32 Fertility determinations.

EXPERIMENT 18.

Methods.

Locke's and Tyrode solutions and pasteurised whole milk, were evaluated with an undiluted control, for dilution of fresh semen.

Semen was obtained from twenty AO and twenty WC cockerels, pooled within each strain, diluted 1/3 at 10°C., and inseminated within fortyfive minutes. Eight WC pullet replicates per treatment at 78% egg production, were inseminated with 0.1 ml. doses.

Fertility was computed using eggs obtained from the second to fifth days after insemination.

Results.

Data on the fertility results and analysis are presented in Table 3. Transformed percentages and significant differences are used for comparisons in this section.

Average fertility.

Diluent	Mean	Strain	Mean
Milk	78.6%	WL	75.6%
Control	78.1	AO	74.4
Locke's	72.5		
Tyrode	70.6		

(1) No significant differences between diluents were obtained.

(2) Undiluted semen produced fertility similar to that from a 1/3 dilution.

(3) Heteroplasmic and homoplasmic semen produced negligible fertility differences with WC pullets.

EXPERIMENT 19.

Methods.

Tyrode solution, milk and a sodium-phosphate buffer were evaluated for their dilution of fresh semen.

Semen was obtained from eight WC cockerels, pooled, diluted 1/2 and inseminated within fifty minutes. Fifteen WC pullet replicates per treatment, at 82% egg production, were inseminated with 0.05 ml. doses.

Results.

Data on the percentage of fertile eggs obtained from the second to eight day after insemination, are presented in Table 10.

Average fertility.

Diluent	Mean
Buffer	79.1%
Tyrode	67.7
Milk	53.6
$D_{.05}$ =	23.39

(1) Higher fertility ($P < 0.05$) was obtained from buffer than milk dilution.

(2) A longer duration of fertility was produced with buffer dilution than milk or Tyrode solution: reductions of 16% and 10% respectively in fertility up to and after the fifth day were obtained in the latter, but there was no decline over the eight day period with buffer.

EXPERIMENT 20.

Methods.

The dilution properties of Tyrode solution and milk were compared at high and low rates.

Semen was collected from ten WC cockerels, pooled, diluted 1/2 or 1/10 with milk or Tyrode solution, and used within sixty minutes. Eight AO pullet replicates per treatment, at 82% egg production, were inseminated with 0.05 ml. doses.

Results.

Data on the percentage of fertile eggs obtained from the second to ninth day after insemination, are presented in Table 12.

Average fertility.

Diluent	Mean	Rate	Mean
Milk	54.5%	$\frac{1}{2}$	66.7%
Tyrode	52.9	1/10	40.7
		$D_{.05}$	= 22.41

(1) Average fertility from milk and Tyrose solution were not significantly different, although milk was 18% higher at the 1/2 rate and Tyrode 15% higher at the 1/10 rate. The interaction was not significant.

(2) Higher fertility ($P < 0.05$) was obtained at the 1/2 rate.

EXPERIMENT 21.

Methods.

Tyrode solution and milk were compared at four dilution rates.

Semen was obtained from ten WC cockerels, pooled, diluted to 1/3, 1/6, 1/12 or 1/18 with milk or Tyrode solution, and used within fifty minutes. Five pullet replicates per treatment, at 80% egg production, were inseminated with 0.1 ml. doses.

Results.

Data on the percentage of fertile eggs layed from the second to eighth day after insemination, are presented in Table 4.

Average fertility.

Diluent	Mean	Rate	Mean
Milk	64.6%	1/3	80.1%
Tyrode	62.2	1/12	64.7
		1/6	58.0
		1/18	50.8
		$D_{.05}$ =	28.26

(1) Milk and Tyrode solution were not significantly different over a wide range of dilution rates.

(2) Higher fertility ($P < 0.05$) was obtained from a 1/3 dilution than 1/18, but no other significant differences from dilution rate occurred.

(3) In contrast to Expt. 20, milk proved a more successful solution at high dilution rates.

(4) One infertile pullet in the 1/6 milk treatment influenced the low fertility at that rate.

EXPERIMENT 22.

Method.

Three levels of semen dilution with milk were compared at three insemination dose levels.

Semen was collected from twenty AO cockerels, pooled, diluted 1/3, 1/6 or 1/10 in milk and utilised within seventy minutes. The original

spermatozoa concentration was estimated at 5.1×10^9 per ml., producing 1700 million spermatozoa per millilitre at the 1/3 rate, 850 million at the 1/6 rate, and 510 million at the 1/10 rate. Eight WC pullet replicates per treatment, at 69% egg production, were inseminated with 0.05 ml., 0.10 ml. or 0.22 ml. doses.

The estimated spermatozoa number per insemination in millions, with the true percentage fertility in brackets is tabled below.

Dose	0.05	0.10	0.22
Rate 1/3	85 (70)	170 (87)	370 (86)
1/6	42 (57)	85 (63)	187 (71)
1/10	25 (22)	51 (35)	112 (40)

Results.

Data on the percentage of fertile eggs layed from the second to ninth day after insemination, are presented in Table 7 and illustrated in the lower graph of Figure 12.

Average fertility

Rate	Mean	Dose	Mean
1/3	69.7%	0.22	56.4%
1/6	55.2	0.10	54.1
1/10	30.5	0.05	44.9
$D_{.05}$ =	15.52		
$D_{.01}$ =	19.59		

(1) Higher fertility was obtained with a 1/3 dilution rate ($P < 0.10$) than 1/6, and lower fertility ($P < 0.01$) with 1/10 dilution.

(2) No significant insemination dose or interaction effects were recorded.

(3) From the table of spermatozoa number and fertility it is noted that a large insemination dose did not negate the decline in fertility from dilution, and that for a given number of spermatozoa and concentrated semen samples were more effective.

PLATE 9.

PLATE 9. Artificial insemination.

A 0.025 ml. capacity micropipette and rubber blow tube are illustrated for a 0.005 ml. experimental insemination.

EXPERIMENT 23.

Methods.

Three small semen insemination dose levels were examined at different dilution rates.

Semen was collected from twenty AO cockerels, pooled, with one batch diluted 1/3 with milk and the other retained as an undiluted control. The original spermatozoa concentration of 5.1×10^9/ml. produced 1700 million spermatozoa per ml. at the 1/3 rate. Eight and sixteen AO pullet replicates per diluted and control treatments respectively, at 65% egg production, were inseminated with 0.010 ml., 0.025 ml. or 0.050 ml. doses within seventy minutes.

Estimated number of spermatozoa per insemination in millions and the true percentage fertility, are recorded in the table below.

Dose	0.010	0.025	0.050
Rate 0	51 (86)	127 (95)	255 (92)
1/3	17 (17)	42 (60)	85 (64)

Results.

Data on the percentage of fertile eggs layed from the second to ninth day after insemination are presented in Table 8 and illustrated in the lower graph of Figure 12.

Average fertility

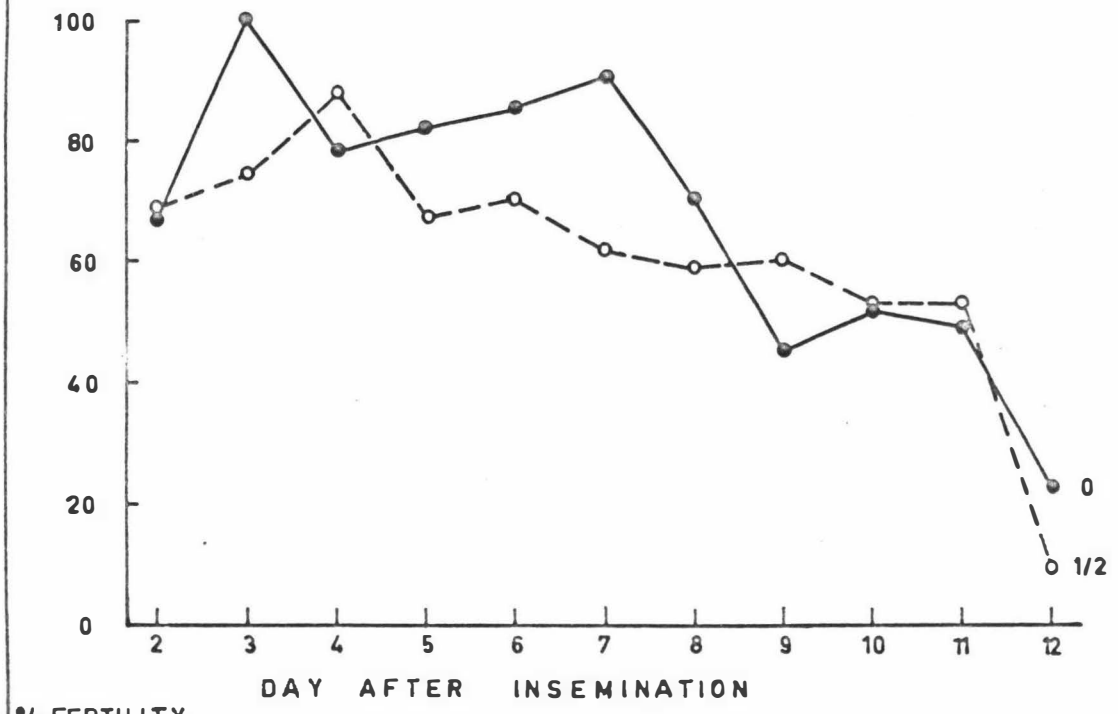
Rate	Mean	Dose	Mean
0	79.8%	0.050	68.4%
1/3	41.9	0.025	67.9
$D_{.01}$ =	12.28	0.010	46.3
		$D_{.01}$ =	17.03

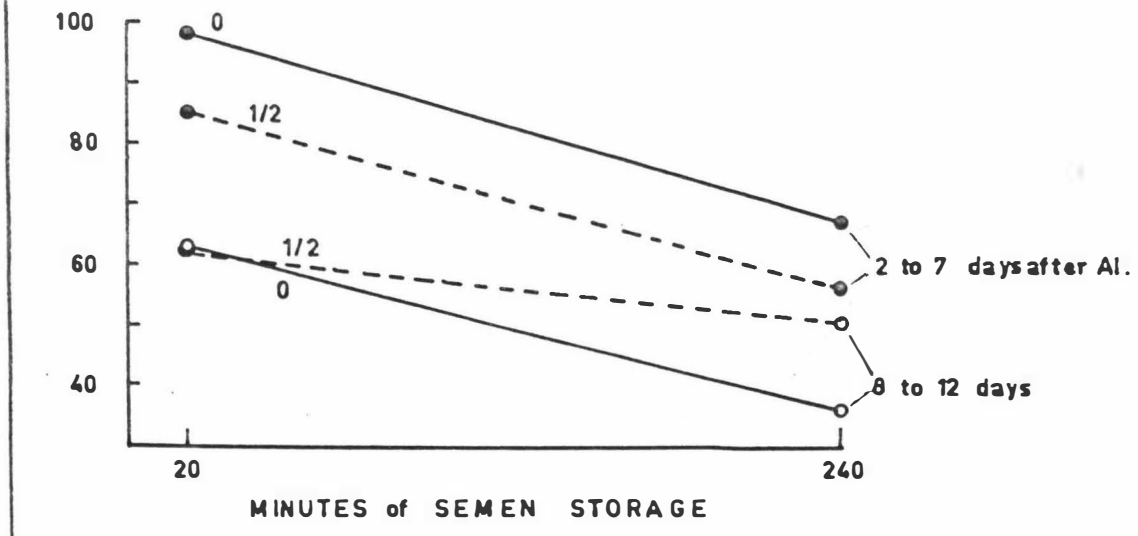
(1) Higher fertility ($P < 0.01$) was obtained from undiluted semen.

(2) Higher fertility ($P < 0.01$) was obtained with the 0.025 ml. or larger dose compared to 0.010 ml.

FIGURE 11

DILUTION EFFECT ON PERSISTENCE OF FERTILITY





Average fertility.

Rate - Dose		Mean
0	0.025	83.3%
	0.050	81.0
	0.010	75.1
1/3	0.050	55.7
	0.025	52.5
	0.010	17.4
	$D_{.05}$ =	23.56

(3) A significant interaction ($P<0.05$) was obtained; by which the fertility of a 1/3 dilution was significantly lower at the 0.010 ml. dose level.

(4) An insemination of fiftyone million spermatozoa was sufficient to produce 86% fertility over eight days of egg collection.

EXPERIMENT 24.

Methods.

Three rates of dilution with a buffer diluent were compared at two dose levels.

Semen was collected from eighteen WC cockerels, pooled and diluted to 1/3 or 1/6 or retained as an undiluted control batch and utilised within fifty-five minutes. Ten AO pullet replicates per treatment, at 83% egg production, were inseminated with 0.05 ml. or 0.10 ml. doses.

Results.

Data on the percentage of fertile eggs layed from the second to seventh day after insemination, are presented in Table 13A. Fertility obtained from the second to ninth day, is illustrated in the Figure 12 top graph.

Average fertility.

Rate	Mean	Dose	Mean
0	72.9%	0.10	57.7%
1/3	59.2	0.05	54.5
1/6	36.3		
$D_{.05}$ =	24.03		
$D_{.01}$ =	30.41		

(1) Highest fertility was obtained using undiluted semen, and that from 1/6 dilution was significantly lower ($P < 0.01$).

(2) There were negligible fertility differences between the dose rates at any level of dilution.

Data on the percentage of fertile eggs layed from the eight to twelfth day after insemination are presented in Table 13B.

Average fertility.

Rate	Mean	Dose	Mean
0	43.3%	0.05	36.5%
1/3	40.4	0.10	35.0
1/6	23.1		

(3) The fertility differences arising from dilution rate were insignificant in the last five days of egg collection.

(4) No significant dose effect was obtained, which indicated that halving the total number of spermatozoa inseminated, did not at these dilutions rates affect the duration of fertility.

(5) Over the whole eleven day collection period the only significant fertility differences were those between 0 and 1/6 dilution rate ($P < 0.05$).

EXPERIMENT 25.

Methods.

Two rates of semen dilution were compared at three insemination dose levels.

Semen was obtained from twelve AO cockerels and pooled; one batch was held undiluted and a second diluted 1/2 with milk. The original spermatozoa concentration of 3.84×10^9/ml., produced 1920 spermatozoa per ml. at the 1/2 rate. Ten AO pullet replicates per treatment, at 78% egg production, were inseminated with 0.005 ml., 0.010 ml. or 0.050 ml. doses within thirtyfive minutes.

The estimated spermatozoa number per insemination and subsequant fertility in brackets, are presented in the table below.

Dose	0.005	0.010	0.050
Rate 0	19 (62)	38 (65)	192 (78)
1/2	9.5 (26)	19 (65)	96 (72)

Results.

Data on the percentage of fertile eggs layed from the second to eighth day after insemination, are presented in Table 9 and illustrated in the upper graph of Figure 12.

Average fertility.

Rate	Mean	Dose	Mean
0	59.7%	0.050	66.9%
1/2	49.8	0.010	57.7
		0.005	39.7
		$D_{.05}$ =	24.88

(1) There were no significant dilution effects on average fertility, although a sharp decline occurred at the 0.005 ml. dose level.

(2) The average fertility obtained from a 0.050 ml. dose was higher ($P < 0.05$) than that from a 0.005 ml. dose.

(3) An estimated 19 million spermatozoa produced 65% fertility over a seven day collection period.

EXPERIMENT 26.

Methods.

The use of pasteurised whole milk at low dilution rates.

Semen was collected from eighteen WC cockerels, pooled, and three batches diluted to 1/2, 1/3 or 1/4 with a fourth retained as undiluted control. Sixteen AO pullet replicates per treatment, at 86% egg production, were inseminated with 0.025 ml. doses within forty minutes of collection.

Results.

Data on the percentage of fertile eggs layed from the second to eighth day after insemination, are presented in Table 11 and illustrated in the upper graph of Figure 12.

Average fertility.

Rate		Mean
0		65.8%
1/2		47.0
1/3		45.5
1/4		36.7
$D_{.05}$	=	21.20
$D_{.01}$	=	26.41

(1) Higher fertility ($P<0.01$) was obtained from undiluted than 1/4 milk diluted semen, but no other significant rate effects were obtained.

EXPERIMENT 27.

Methods.

Four dose and dilution rate combinations using a constant number of spermatozoa per insemination, were tested with semen from two cockerels.

One WC and one AO cockerel provided 0.8 ml. of semen each of which a portion was kept undiluted and the rest diluted to 1/2, 1/10 or 1/20, with milk. The WC semen concentration was estimated at 1.71×10^9 spermatozoa per ml., and the AO at 3.10×10^9/ml. An estimated 41 million spermatozoa were inseminated with each WC dose, and 71 million for the AO dose. Five WC pullet replicates per treatment, at 75% egg production, were inseminated with 0.024 ml., 0.048 ml. 0.24 ml. and 0.48 ml. doses for decreasing concentrations respectively, within seventy minutes of collection.

The estimated spermatozoa concentration per millilitre, in millions, is shown with the true fertility obtained in brackets, in the table below.

Rate	0	1/2	1/10	1/20
Male WC	1710 (85)	855 (86)	171 (0)	86 (4)
AO	3100(96)	1550 (93)	310 (7)	155 (18)

Results.

Data on the percentage of fertile eggs obtained from the second to eighth day after insemination, are presented in Table 5.

Average fertility.

Rate	Mean	Male	Mean
0	79.9%	AO	47.9%
1/2	78.0	WC	39.0
1/10	4.9		
1/20	11.1		
$D_{.05}$ =	19.58		
$D_{.01}$ =	24.48		

(1) Higher fertility ($P < 0.01$) was obtained from the 0 and 1/2 dilution rates than 1/10 or 1/20; conversely, lower fertility ($P < 0.01$) was obtained from the doses greater than 0.24 ml. than those less than 0.048 ml.

(2) There were no significant differences obtained between males, tested, although AO values with a higher spermatozoa concentration were notably higher at high dilution rates.

(3) A significant fall in fertility occurred with concentrations between 855 million and 310 million spermatozoa per millilitre.

EXPERIMENT 28.

Methods.

The duration of fertility after one semen insemination was recorded for two strains of pullets.

Semen was obtained from twenty WC cockerels, pooled and diluted 1/3 with milk for use within forty minutes. Fifteen WC pullets at 70% egg production and fifteen AO pullets at 67% egg production, per treatment, were inseminated with 0.1 ml. doses. Five pullets from each strain were inseminated weekly, to serve as a control.

Results.

Data on the percentage of fertile eggs obtained from the second

to twentyfirst day after insemination are presented in Table 27.

(1) A similar decline in fertility was observed with each strain; the average test and control fertility is illustrated in Figure 13. Over 80% fertility was maintained for the first week after insemination, and thereafter the average fertility fell by approximately 10% per day.

(2) The average fertility obtained from the WC pullets was higher ($P<0.01$) than that from the AO pullets.

(3) A high correlation (+0.926**) was obtained between the daily fertility levels of the two strains.

(4) The control fertility did not change significantly over the experimental period.

EXPERIMENT 29.

Methods.

Individual matings which produced extremes in fertility, were repeated twleve weeks later under similar conditions.

Two WC cockerels (No's. 114, 117), two ML cockerels (No's. 67, 109), and two AO cockerels (No's. 30, 108), were mated during September 1966 to 11, 14, 10, 11, 1 and 1 pullets respectively, of the same strain. Semen from Males 30, 67 and 108, was inseminated undiluted, but semen from Males 109, 114 and 117 was diluted 1/2 with milk. During September the average egg production was 70%. Twelve weeks later in December, 1966, with average egg production at 54%, the matings were repeated using the same semen treatments and stock.

Results.

Data on the percentage of fertile eggs obtained with two inseminations over a seventeen day period, are presented in Table 28.

(1) Higher fertility ($P < 0.10$) was obtained from the original matings in September.

(2) There was a correlation of +0.3614* of individual pullet fertility results in the two tests.

The average fertility of untransformed data is recorded in the table below.

Trial	Sept.	Dec.
Male 67	84.7%	77.6%
109	62.1	50.7
114	72.9	84.1
117	65.0	41.9
108	0	0
30	100.0	100.0
Mean	64.1	59.1

(3) The single matings of the two AO cockerels produced exactly the same fertility at both trials. The pullet mated to Male 108 was considered infertile.

(4) There were no significant differences in fertility of eggs collected after the first or second insemination in each trial.

EXPERIMENT 30.

Method.

This experiment was conducted to test possible detrimental effects of centrifugation on the fertilising ability of spermatozoa.

Semen was collected from twelve WC cockerels, pooled, then split into three batches, of which two were centrifuged at 1800 RPM (460 RCF) for ten minutes and the third held stationary at 10°C. as a control. The supernatant of the first centrifuged sample was withdrawn and replaced by buffer diluent up to the original volume; The second centrifuged sample was remixed by gentle agitation until the spermatozoa were uniformly distributed. Then WC pullet replicates per treatment, at 76% egg production, were inseminated with 0.05 ml. doses within seventy minutes from collection.

Results.

Data on the percentage of fertile eggs layed on the second to eighth day after insemination, are presented in Table 34.

Average fertility

Method	Mean
Buffer	81.6%
Remix	74.1
Control	63.0

(1) There were no significant method effects on fertility.

(2) Centrifugation at 460 RCF for ten minutes did not damage the fertilising potential of freshly inseminated semen.

EXPERIMENT 31.

Methods.

This experiment was conducted to determine the measurable response of two strains of pullets to their first artificial insemination.

Semen was obtained from ten ML cockerels, pooled and diluted 1/2 with milk. A group of twenty WC and twenty AO pullets which had not been handled for insemination previously, were treated in one of four ways prior to the insemination proper. The first group, referred to as 'semen' were inseminated normally with 0.1 ml. of 1/2 milk diluted semen. The second group, referred to as 'water' were inseminated with 0.1 ml. doses of distilled water. The third group, 'sham', were removed from their cages and everted, but not inseminated. The fourth group 'nil' were untouched. Two days later, five pullet replicates per method, at 79% egg production, were inseminated with 0.1 ml. doses of 1/2 milk diluted semen.

Results.

Data on the percentage of fertile eggs produced from the second to eighth day after insemination, are presented in Table 6.

Average fertility

Method	Mean	Strain	Mean
Semen	85.2%	WL	79.0%
Sham	81.9	AO	78.9
Nil	79.6		
Water	69.1		

(1) Pre-insemination handling produced no significant advantage for subsequent fertility, although it did make the pullets more tractable for the test insemination.

(2) There were no fertility differences between strains.

EXPERIMENT 32.

Methods.

Estimations of fertility were compared using a twentyfour hour incubation egg breakout technique and a five day incubation egg candling technique.

Semen was obtained from twelve AO cockerels, pooled and diluted 1/2 with milk. Sixteen AO pullet replicates at 84% egg production, were inseminated with 0.05 ml. doses within thirty minutes of semen collection. Eggs collected from the second to ninth day after insemination were subject to a candling or breakout fertility test; alternate eggs from each pullet were allocated to each method of testing. Candled eggs which were considered doubtful were broken out, and examined macroscopically for enbryonic development up to the twentyfour hour incubation stage.

Results.

Data are presented in Table 32.

Average fertility.

Method	Mean
Breakout	91.7
Candling	83.2

(1) There was no significant difference between the method of detecting

fertility. The higher fertility at twentyfour hours of incubation is attributed to a particular type of embryo which subsequently died and degenerated.

4.5 Discussion.

Most research effort was directed toward finding the best method to prepare spermatozoa for maximum fertilising capacity in the first week after insemination. Particular emphasis was laid on semen dilution rate.

The dose of semen inseminated was frequently analysed, in the sense of being inversely proportional to the dilution rate. However the small amount of research into dose was mainly concerned with the accuracy and ease of insemination with the syringes and pipettes used.

Excepting minor modifications in positioning the females during handling, no effort was made to improve the in-cage method of insemination. The choice of equipment was extended to include two sizes of pipettes and interchangeable bore tubes which determined the syringe fluid capacity. A brief study of pullet responses to handling for insemination was intended to complement similar experiments on handling cockerels for semen collection.

Four wellknown diluents were tested for their diluting properties of semen briefly retained *in vitro*, but no effort was made to isolate their precise effects on spermatozoa, their value for extending the duration of fertility, or their long-term effects on the female genital tract.

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Diluents.

It was difficult to determine the importance of dilution or the choice of diluent, in extending fresh semen. Where semen was diluted for convenience of handling or extending a cockerel's spermatozoa over many matings, the only

criterion of success, as in this study, was the level and duration of fertility obtained. The relatively low dilution rates used and the short periods of storage *in vitro*, an average thirty minutes; reduced the opportunity for variations in fertility which could be attributed to the diluent. The storage potential of the four diluents tested and discussed in Section 3.7, was not necessarily the dilution potential which was tested in the experiments with fresh semen.

As there have been no objective studies reported on the immediate or *in utero* influence of diluents on spermatozoa, speculation on their value with fresh semen is limited to the criteria considered for stored semen. They include: the chemical compatability of the diluent; the ionic composition; buffering and chelating properties; nutritive capacity; and the interaction of diluent with spermatozoa metabolism.

All of the diluents studied; Locke's and Tyrode solutions, pasteurised whole milk and sodium-phosphate buffer; had been extensively used by research groups and were apparently chemically compatible to spermatozoa. Of the other criteria, only the influence of a diluent-spermatozoa interaction would seem to be significant during the short period *in vitro*. This effect could work through a reaction *in vitro* which rendered the spermatozoa responsive to diluent in the oviduct.

Considering the wide ranges in chemical and carbohydrate content of the diluents studied, it appeared unlikely that either of these factors strongly influenced the results obtained.

In agreement with results obtained from stored semen, the sodium-phosphate buffer proved a superior diluent. Once again the higher fertility was manifested by a longer period of fertile egg production, which suggested that at least some of the benefits from the buffer applied with both stored and fresh semen, and possibly while in the oviduct. Locke's and Tyrode solutions and milk, did not produce significant variations in fertility or differ

systematically at low or high dilution rates. Unfortunately there were no studies on the total duration of fertility or the oviduct reaction to repeated doses of diluents; either of which could have clarified the main dilution effect.

The evaluation of diluents relied mainly on the results of semen storage experiments and to less extent on differences observed in diluting fresh semen. As there were no striking advantages in the fertility from fresh semen, the choice of diluent would partly depend on their availability and ease of preparation. Pasteurised milk is a physiological solution which is generally available at an appropriate temperature, however milk was susceptible to microbial and temperature induced changes which could affect spermatozoa function. Other problems in concentration estimates and dilution arose from the similarity in colour and consistency of milk and semen. Tyrode and Locke's solutions were transparent and easily stored at 2°C., but lacked buffering capacity and were laborious to mix with several inorganic salts and a carbohydrate.

Within the limitations noted above the sodium-phosphate buffer had proved superior for semen storage. It was easily mixed from two inorganic salts and stored at 2°C. without a deterioration in diluting qualities.

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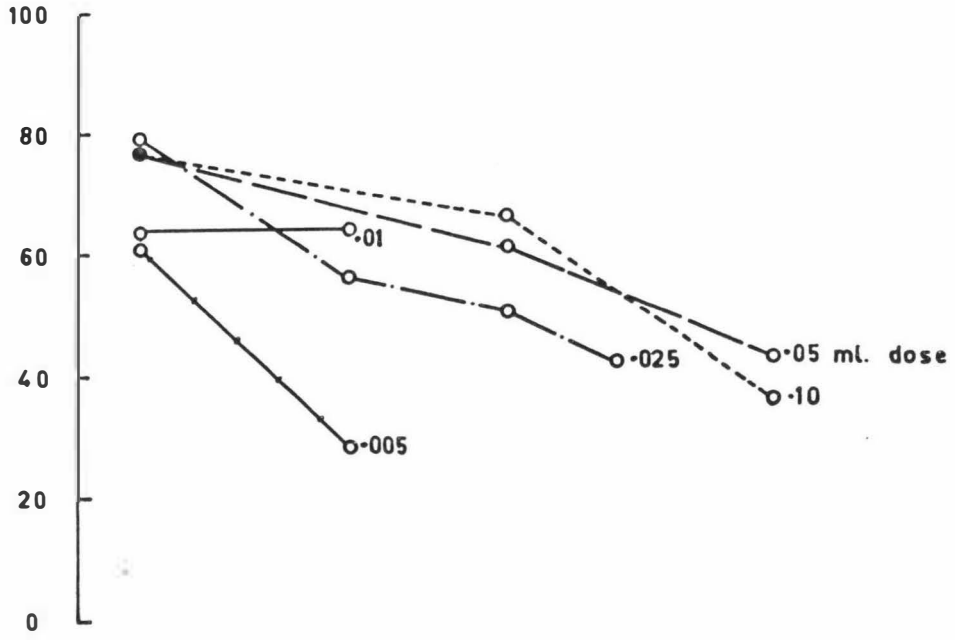
Optimal Utilisation of Semen.

This section is concerned with the preparation of semen for insemination. It is often necessary to dilute semen in order to obtain a volume sufficient for use in several inseminations, particularly where the equipment can not cope with small dose levels. However dilution has been shown to affect fertility and this discussion outlines factors involved in compromising dose level and dilution rate.

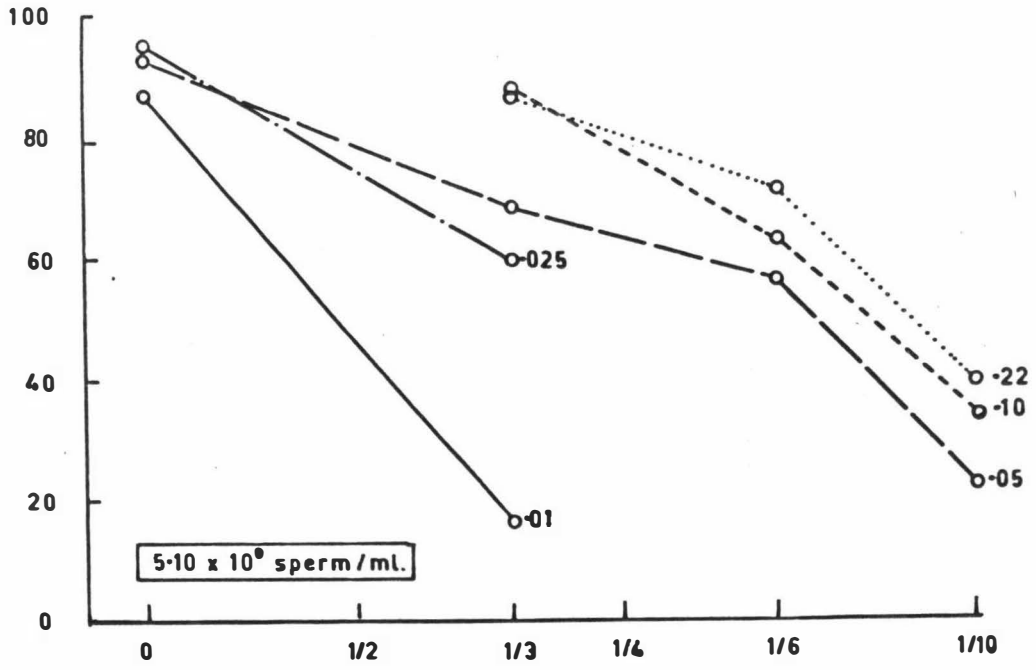
Three related aspects of semen modification have been considered:

FIGURE 12

SEMEN DILUTION & DOSE for INSEMINATION



% FERTILITY 2nd to 9th DAY AFTER INSEMINATION



DILUTION RATE (log scale)

dilution rate; insemination dose; and spermatozoa number.

The decline in fertility which accompanied increasing semen dilution, is clearly illustrated in Figure 12.

There are two obvious features shown in the graphs: firstly that fertility decreased more rapidly at the lower dose levels; and secondly, that little advantage was obtained at any dilution rate of a dose larger than 0.1 ml.

In all experiments an average dilution rate of 1/6 or greater, produced significantly lower fertility than the more concentrated samples. Undiluted semen produced the highest fertility at all dose levels but the dilution effect was not important until very small doses were tested.

The 'medium' dilutions including 1/3 to 1/6 proved to be relatively important at the dose levels commonly applied in insemination. Higher fertility was obtained from a 1/3 dilution than a 1/6 dilution in Expts. 22 and 24, however the difference was not modified by doses ranging from 0.05 ml. to 0.22 ml. This therefore, could be considered a dilution effect. In Expt. 26 where a 0.025 ml. dose was used, the same gradual decline in fertility from increasing dilution was observed.

More comprehensive data has been accumulated on the consequences of dilution. In Expts. 23, 24, 25, 26 and 27, undiluted semen produced higher average fertility than any dilution of semen at the same dose level. Where the dose level was less than 0.025 ml., the dilution effect was most obvious. In Expt. 23, a dilution of 1/3 caused a highly significant drop in fertility at the 0.01 ml. dose level; however in Expt. 25 where the same dose was tested at a 1/2 dilution, the fertility was maintained at the undiluted level. The latter result is illustrated in the upper graph of Figure 12, and the former in the lower graph. When the dose was reduced to 0.005 ml., a significant decline in fertility occurred with a dilution as low as 1/2.

In some cases the results could be interpreted with respect to spermatozoa concentration. From Expt. 22, with doses ranging from 0.05 ml. to

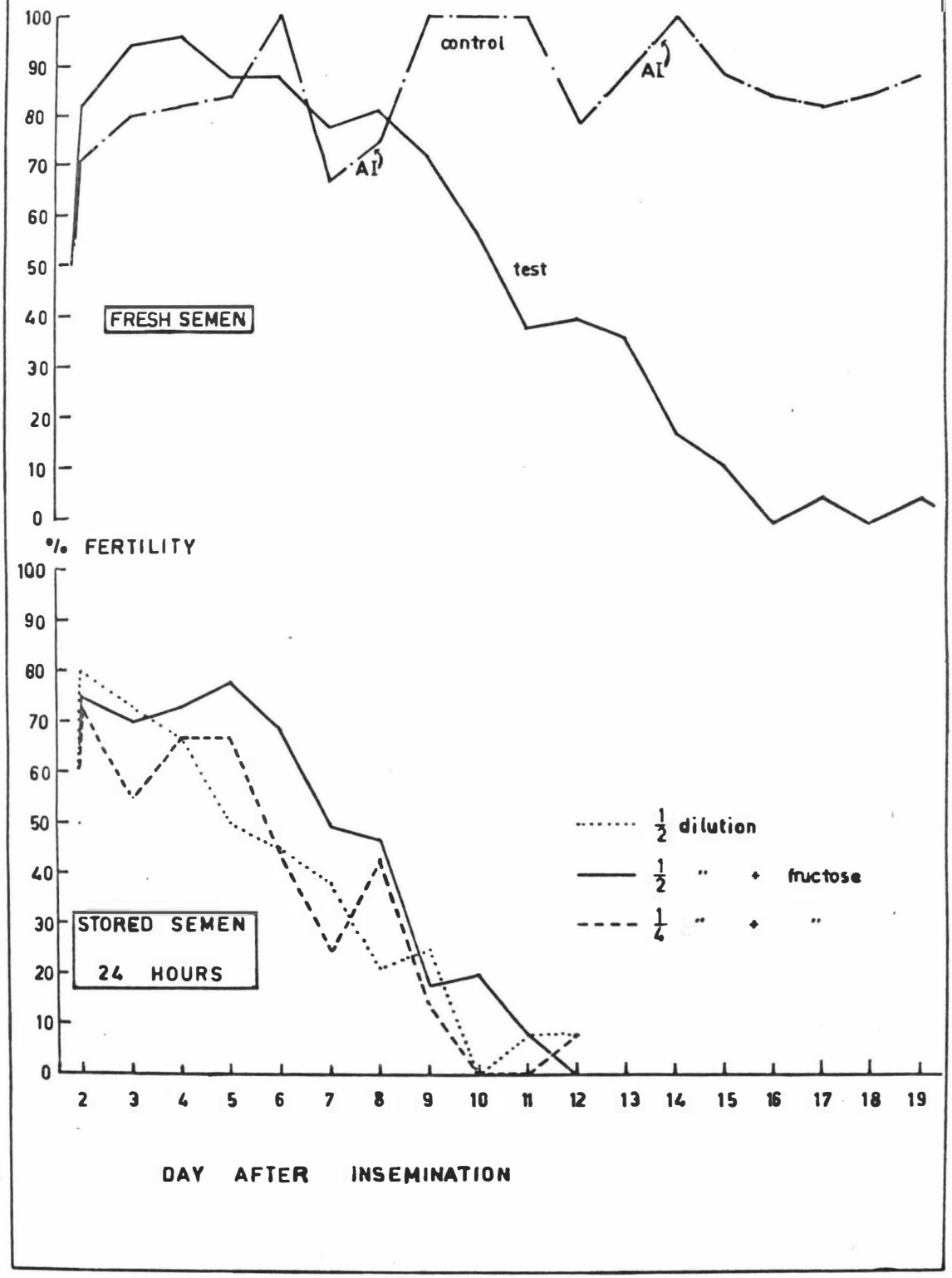
0.22 ml., the significant decline in fertility occurred between 850 million and 510 million spermatozoa per millilitre. For the lower dose levels from 0.01 ml. to 0.05 ml. in Expt 23, the drop occurred between 5,100 and 1,700 million spermatozoa per millilitre. In the 0.005 ml. dose of Expt. 25, the threshold lay between 3,840 and 1,920 million spermatozoa per millilitre. Possibly the most valuable result was obtained in Expt. 27, where concentrations varied while the spermatozoa number inseminated remained constant. Here the significant decline in fertility occurred between 855 and 310 million spermatozoa per millilitre. Apparently some of the detrimental effects of dilution with small inseminations can be countered by increasing spermatozoa number with a larger dose.

Unlike the rate of dilution, the insemination dose is restricted by the practical limits to using small volumes of semen. Doses below 0.05 ml. were inseminated relatively easily with a micropipette but needed at least 15% more time and required much more skill in both drawing in the correct dose and in penetrating the vagina. However the method would not be required for the majority of inseminations, but valuable for the few cases of low volume ejaculations and high female to male mating ratios. For example in Expt. 23, 86% fertility was obtained from an undiluted dose of 0.01 ml., but only 64% fertility from an 0.05 ml. dose of 1/3 dilution, although the latter contained more spermatozoa. In Expt. 25, 62% fertility was obtained from 0.005 ml. of undiluted semen, versus 72% from a 0.05 ml. of 1/2 diluted semen which required five times the number of spermatozoa.

Within the 0.05 ml. to 0.22 ml. range of doses applied by syringe, the fluctuation of results obscured clear fertility effects. In Expt. 22 higher fertility was obtained by doubling the 0.05 ml. dose, but there was no advantage from this in Expt. 24. Dilution with milk in the first and buffer in the second experiment, could have affected the results. However the variation in pullet egg production would seem to be relatively more important; 69% in Expt. 22, and 83% in Expt. 24. It is possible that the oviduct was better

FIGURE 13

PERSISTENCE of FERTILITY AFTER ONE INSEMINATION



equipped to sustain spermatozoa at the time of maximum ovulation rate, and that the number of spermatozoa inseminated was not so critical. Considering the fluctuations in ejaculate quality and ovulation rate, it would be worthwhile to inseminate a maximum 0.10 ml. dose, if the semen is diluted.

The significance of using the best dilution and dose ratio possible, is clearly illustrated by the negligible association of spermatozoa number and fertility. For example in Expt. 22, 87% fertility was produced over eight days with an estimated 170 million of 1/3 diluted spermatozoa, but only 71% from 187 million 1/6 diluted spermatozoa. In Expt. 23, 86% fertility was obtained from 51 million undiluted spermatozoa over eight days, versus 64% fertility from 85 million spermatozoa diluted to 1/3. With the very low insemination doses applied in Expt. 25, as few as 19 million spermatozoa, either undiluted or diluted to 1/2, produced more than 60% fertility over a seven day egg collection period. The sharp decline in fertility by a reduction to 9.5 million spermatozoa, indicated that either a dilution or volume effect could have been responsible because the halving of the 0.01 ml. dose, or diluting 1/2 of the 0.005 ml. dose, both halved the spermatozoa number.

These results confirm the negligible relationship between spermatozoa number and fertility, which is frequently expressed in the literature. However the situations with extremely low spermatozoa numbers or long periods of fertility testing, have not been resolved by this study.

No attempt was made to study the frequency of insemination and the maintenance of fertility, apart from ensuring that weekly inseminations were satisfactory with 0.05 ml. or 0.10 ml. doses. Expt. 28 was intended to show the decline in fertility after one 0.1 ml. insemination, and as illustrated in Figure 13, the fertility was held above 80% in the first eight days. Although fertility in the control group fluctuated due to the small number of replicates used, weekly inseminations maintained a level between 80% and 100%. Reports

PLATE 10.

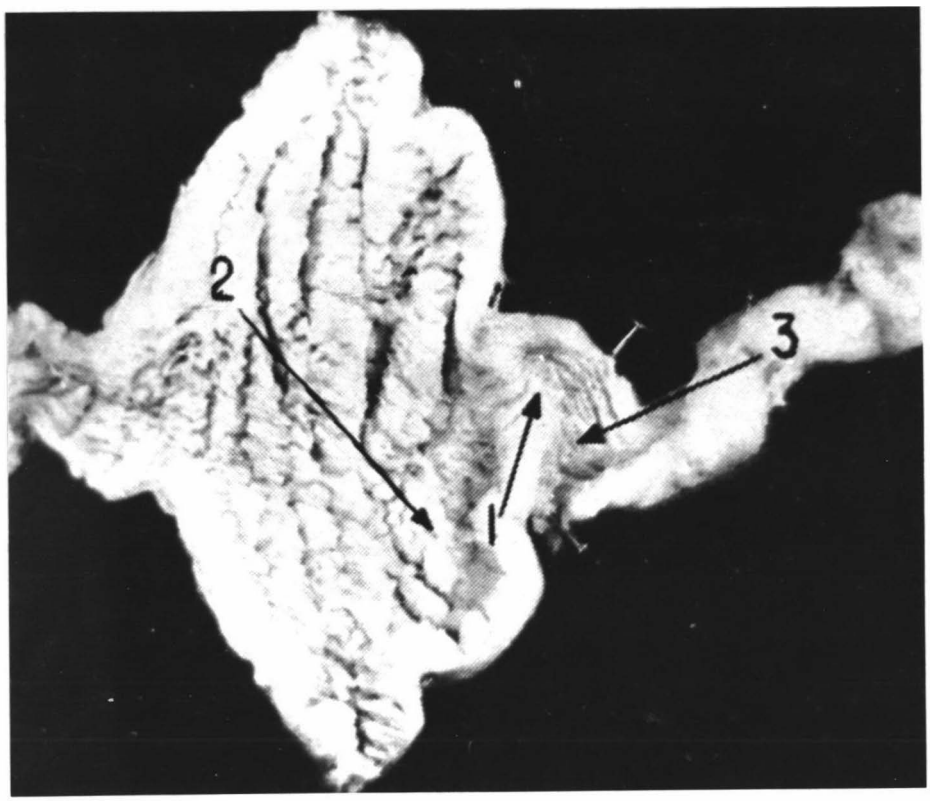


PLATE 10. Dissected uterovaginal region of fowl.

1. parallel glandular vaginal folds surmounting the sphincter.
2. uterus epithelium.
3. non-glandular vaginal folds.

(From: Bobr, L.W., Lorenz, F.W. and Ogasawara, F.X., (1964). J. Reprod. Fertil. 8, 39). Scale x 0.3

in the literature indicate that more frequent inseminations are only necessary when small doses are used or at a time of low semen or egg production.

Throughout this study, the absolute level of fertility was positively correlated to the rate of egg production. Reference has been made to this association in the review; but although most of the fertility trials and Expt.29 in particular, confirmed the relationship, there was no justification for determining causes and effects.

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Methods.

Artificial insemination with the use of a syringe, deposited semen intra-vaginally approximately 5 cm. from the cloaca. The average insemination, which included catching the pullet, deposition and release; occupied two operators for thirty seconds.

Preparing the female was not difficult and the results from Expt. 31 indicated that fertility was unaffected by pre-insemination training. It would have been possible for the females to reduce fecundity in two ways: firstly by resisting pressure for eversion which would prohibit penetration; or secondly by a decline in ovulation rate which occurred as a result of being frightened or damaged in handling. Continued resistance to pressure for eversion rarely occurred with birds which were laying regularly, but the decline in egg production could have been an important, if occasional response to insemination.

Intra-vaginal deposition of semen was probably one of the most critical phases of artificial breeding which was not studied. The cannulae were inserted gently with a pronounced lateral searching action until a firm resistance encountered signified the constricted utero-vaginal junction, and the semen was

PLATE 11.

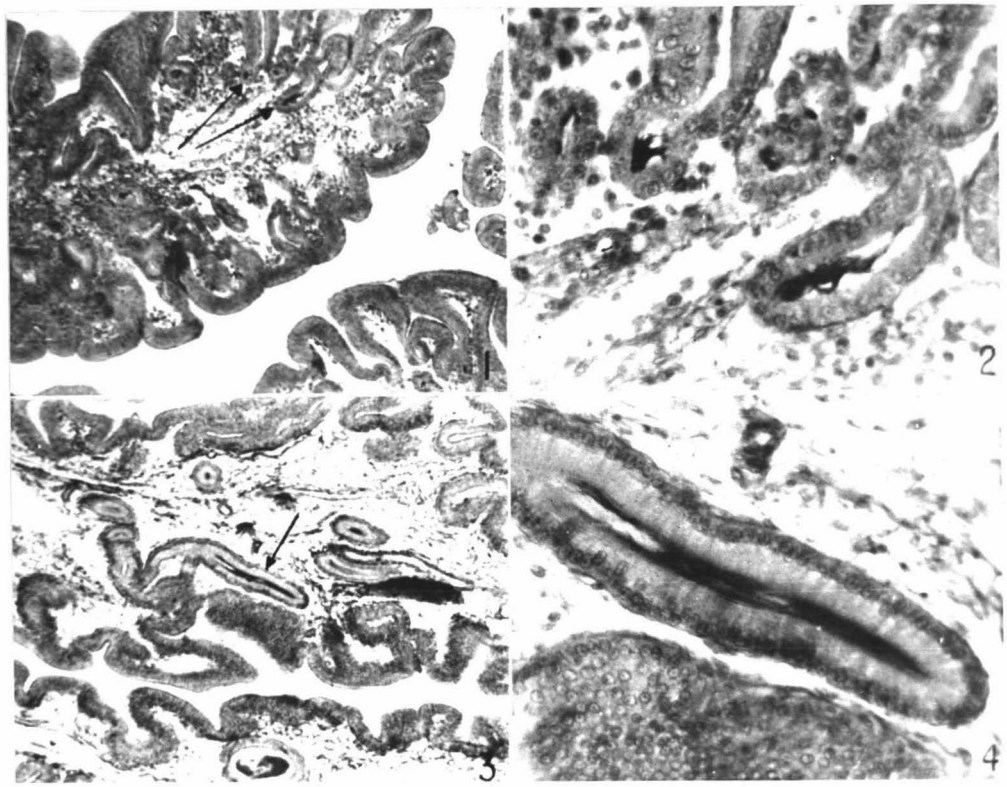

PLATE 11. Infundibulal and uterovaginal glands.

11.1 mid-chalaziferous region of infundibulum with spermatozoa-containing glands in the corium. Scale x 75

11.2 a magnified area, indicated by arrows, showing spermatozoa in the glands. Scale x 325

11.3 glandular vaginal folds with spermatozoa-containing glands in longitudinal section and cross-section. Scale x 75

11.4 glands in longitudinal section showing the parallel arrangement of spermatozoa. Scale x 325

From: Bobr, L.W., Lorenz, F.W., and Ogasawara, F.X., J. Reprod. Fertil. 8 39).

expelled after the bird has been relaxed. It is doubtful whether the cannula always reached the proximal vagina and whether semen was partially sucked back when the tube was withdrawn. As spermatozoa apparently propel themselves through the vagina and junction, it is reasonable to expect that a relatively larger proportion are liable to destruction or malfunction as the length of passage increases. This may be a biologically significant phase of spermatozoa selection however, as semen is thought to be deposited in the mid-vaginal region during natural mating.

Semen which adhered to the outside wall of the cannula in being wiped off in the vagina, was a constant but fluctuating source of dose error. The extent of adhesion depended on the viscosity of the semen, the depth to which the cannula was plunged while being refilled with semen, and the length of cannula which penetrated in close contact with the vaginal wall. This problem was estimated to increase small doses by up to 100%, therefore the micro-pipettes were wiped prior to insemination in the dose experiments.

A check on spermatozoa functional capacity following centrifugation at 460 RCF for ten minutes was considered necessary following unsuccessful attempts at centrifuge and dilution techniques for semen storage, which were described in Section 3.3. As centrifugation did not produce a change in fertilising ability, it was assumed that the techniques failed due to an unsatisfactory preparation of antibiotics.

Macroscopic examination for embryonic development of twentyfour hour incubated blastoderms, obtained very similar results to those of a five day incubation and candling method in Expt. 32. The slightly higher fertility from the former was anticipated from the small proportion of embryonic deaths between the first and fifth days of incubation. It was appreciated that neither method detected all the fertilised eggs and that the theoretical fertility was probably higher. This was of no concern in this study unless the frequency

of pre-oviposital or early embryonic deaths, was related to the treatments imposed on the semen. Recent reports suggest that fertilisation can occur in extremely unfavourable conditions for both gametes, but that development is often transient and abnormal and the detection of fertility requires a sophisticated staining procedure.

The method adopted for this study served adequately in comparing the functional capacity of different batches of semen.

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CHAPTER 5.

CONCLUSIONS.

The Study.

Research has been planned and undertaken with very close attention to the literature. The experiments were either a check of research materials and methods or an extrapolation of the published work for practical application in New Zealand.

In the field of semen release and collection, the study has progressed beyond the largely mechanical avian approach and drawn from experiences in mammalian artificial breeding and avian psychology. However the two remaining fields of semen storage and preparation for insemination, have been restricted by limitations of facilities and specialised knowledge.

Conclusions are based almost entirely on analyses of results from fixed parameters, at the 1% and 5% probability levels, with occasional reference to less significant results which have contributed to the general conclusion. This approach was at once safe and inadequate: statistical significance did allow the results to be interpreted with some certainty and revealed interactions between the main factors, yet some analyses, particularly correlations, were nonsensical and liable to detract value from other results. The points labelled in each experimental section were almost entirely significant results, but this rigid approach was modified by illustrating the main findings graphically.

Some experiments suffered from a lack of objectivity. Semen production studies were assessed by a few ejaculation responses, themselves liable to other environmental influences which could not be measured or always recognised. The same fault applied to some semen storage experiments: the percentage of fertile eggs produced was not necessarily a measurable effect

wholly caused by a certain treatment of semen *in vitro*.

In retrospect, the choice of factors to be tested in the semen production experiments was inadequate. In the nutrition trial some overwhelming environmental influences diluted down the interpretation of the results until they could only vaguely confirm some conclusions in the literature. Light, temperature and age of stock studies foundered for similar reasons.

Semen storage could have become an advanced study on its own, therefore a compromise was adopted whereby this aspect was studied at an elementary level concerned with commercial situations and materials, and the results interpreted from published work.

Insemination experiments avoided the main faults of semen production and storage studies, because the treatments were generally precise and relevant, and the results clearly demonstrated by the main criterion of fertility.

The Interpretation.

Cockerel spermatogenesis proceeds at a high rate and functional spermatozoa can be collected with very little effort compared to domestic mammals. In the absence of any information to the contrary, apparently the lighting pattern, rearing conditions and diet which successfully bring females to egg production, suffice for male semen production. Dietary, disease, light and temperature levels are thought to influence the persistence of both semen production and release.

Where cockerels are individually caged, semen can be collected in the following manner. One operator dressed in familiar apparel should approach the cages from the usual direction and proceed to work through the cockerels in the usual sequence. The bird can be taken completely out of his cage, massaged with one hand and the ejaculated semen caught in a tube by the other, and then gently returned to his cage. The ejaculate need not be protected from cold shock or light, but contamination from dirty equipment or atmospheric dust should be

avoided.

The functional capacity of normal spermatozoa is retained with stationary storage at approximately 10°C. in a dark room; the main objective is to depress the light or thermal activation of spermatozoa metabolism. If the semen is to be diluted for insemination, it is preferable to dilute immediately after collection. The retention of fertilising capacity is partly dependent on the inherent quality of the spermatozoa and seminal plasma, but where the insemination is expected to produce one week's fertile eggs, semen can be satisfactorily held in vitro for one to four hours at 10°C. A low dilution with sodium-phosphate buffer apparently reduces the deterioration of spermatozoa function.

In the rather unusual circumstances of over-night storage, semen can be diluted to between 1/2 and 1/4 in buffer, mixed thoroughly, then allowed to remain at 10°C. with free gas exchange.

Prior to insemination semen is prepared in a form commensurate with the main objective of the fertilisation; this could differ according to the frequency of insemination, the availability and characteristics of semen, or the in vitro age of the spermatozoa. The performance of stored semen is enhanced by the addition of 2.5 mg./ml. of fructose just prior to insemination. In general it is beneficial to inseminate fresh semen in the most concentrated form practicable. For dilution rates of up to 1/6, an insemination dose between 0.05 ml. and 0.10 ml. deposited intra-vaginally every seventh day, has proved sufficient to avoid significant daily fluctuation in fertility. However the preparation of semen and frequency of insemination must be adapted to the special characteristics of the stock; in particular the state of egg production and spermatogenesis, the age and artificial breeding history of the stock, and the spermatozoa fertilising potential.

Semen is deposited from a clean glass cannula or pipette to a depth of three to six centimetres, in the everted distal vagina. A longer duration

of fertility can be obtained if spermatozoa are deposited soon after oviposition; in practice this generally means insemination between 1300 and 1700 hours, which is advantageous from the semen collection and insemination aspects.

Fertile egg production can be expected from the first ovulation following artificial insemination, and under the many conditions prescribed above, the fertility from fresh semen should not decline significantly in the first eight days.

The Philosophy.

Artificial breeding is an unavoidable consequence of the caged housing of poultry. Considering the expanding role of artificial breeding with farm animals and the accrued advantages and knowledge, it is reasonable to expect benefits far greater than the technical success of semen transfer, with poultry as well. Research into avian reproductive physiology has been remarkably meagre and incohesive, but the commercial demand for artificial breeding has necessitated an objective study of fowl spermatozoa and their requirements in transfer from the male to female.

Some problems in artificial breeding are peculiar to the fowl and in the absence of comparable results from mammals, research into spermatozoa requirements *in vitro* and in the oviduct, is becoming increasingly vital. Of prime importance is the question of whether to deliberately sustain or depress spermatozoa metabolism during storage above freezing temperatures. With the discoveries of spermatozoa storage glands in the oviduct, many of the procedures in semen storage and the site of insemination, need to be critically re-examined. The divergence of approaches in the recent literature suggests that this is happening already; research has been directed toward the sites, and capacity of the utero-vaginal glands to accept, support and release spermatozoa.

The apparently satisfactory methods of semen collection described in

this thesis could also require a revaluation, as a more consistent fluid fraction will almost certainly be required with precisely controlled storage environments. It is doubtful at present whether this problem should be tackled by a change in collection techniques or by separating the spermatozoa and fluid fractions prior to storage. As avian semen is collected relatively easily yet does not closely resemble a natural ejaculate, there is obvious potential in the latter method. The methods of housing and feeding cockerels for maximum spermatogenesis have not been adequately investigated, but generally improvised from the methods adopted for caged pullets.

All research in avian artificial breeding must be related to the significance of any phase of the procedure. Considering the relatively rapid genetic progress possible with the fowl and the multiplicity of breeding objectives, it may be uneconomic to establish semen banks in a commercial organisation. However there is a need for satisfactory methods of storing and transporting avian semen for several days *in vitro*.

The homogametic status of avian spermatozoa has prohibited the experimental modification of sex ratios, which is a potentially important characteristic of domestic mammals.

There is little doubt that research will eventually clarify the relationship between spermatozoa morphology and function. When this occurs, all three phases of artificial breeding could well be redirected from a pre-occupation with semen *per se* to the unit spermatozoon.

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APPENDIX A

TABLE 1. Nutritional effects on semen production.

Averaged volume of two semen collections in millilitres
Factors: ration (4) x breed (2) x period (6); 5 cockerel replications

Ration	Period	Breed	Replications 1	2	3	4	5	Treatment Sum	Mean
A	1	WL	.35	.65	.53	.18	.20	1.91	.382
		AO	.97	1.32	.68	.43	.18	3.58	.716
	2	WL	.58	.85	.68	.25	.25	2.61	.522
		AO	1.00	1.35	.95	.55	.05	3.90	.780
	3	WL	.50	.83	.70	.43	.23	2.69	.538
		AO	1.00	1.73	.98	.60	.13	4.44	.888
	4	WL	.25	.68	.73	.23	.15	2.04	.408
		AO	.98	1.35	.90	.55	.23	4.01	.802
	5	WL	.28	.58	.63	.33	.20	2.02	.404
		AO	1.03	1.28	.83	.45	.18	3.77	.754
	6	WL	.18	.68	.53	.33	.15	1.87	.374
		AO	.73	1.10	.65	.23	.20	2.91	.582
B	1	WL	.47	.62	.43	.50	.25	2.27	.454
		AO	1.18	.70	.45	.88	.70	3.91	.782
	2	WL	.60	.68	.38	.50	.23	2.39	.478
		AO	1.55	.75	.30	.85	.40	3.85	.770
	3	WL	.50	.53	.35	.58	.20	2.16	.432
		AO	1.45	.78	.50	.80	.88	4.41	.882
	4	WL	.70	.53	.30	.58	.30	2.41	.482
		AO	1.45	.68	.48	.83	.45	3.89	.778
	5	WL	.50	.63	.38	.48	.20	2.19	.438
		AO	1.10	.58	.35	.73	.35	3.11	.622
	6	WL	.40	.55	.23	.38	.20	1.76	.358
		AO	.95	.40	.30	.75	.53	2.93	.586
C	1	WL	.18	.20	.58	.68	.35	1.99	.398
		AO	.60	.87	.70	.58	.62	3.37	.674
	2	WL	.25	.23	.73	.80	.43	2.44	.488
		AO	.75	.68	.83	.78	.78	3.82	.764
	3	WL	.30	.15	.53	.88	.43	2.29	.458
		AO	.70	.75	.85	.70	.85	3.85	.770
	4	WL	.10	.08	.45	.68	.30	1.61	.322
		AO	.20	.68	.90	.75	.85	3.38	.676
	5	WL	.15	.10	.53	.60	.23	1.61	.322
		AO	.55	.60	.60	.60	.83	3.18	.636
	6	WL	.08	.23	.30	.65	.40	1.66	.332
		AO	.53	.58	.28	.48	.50	2.37	.474
D	1	WL	.47	.43	1.15	.50	.20	2.75	.550
		AO	.98	.72	.87	.95	1.10	4.62	.924
	2	WL	.55	.48	1.20	.65	.40	3.28	.656
		AO	1.10	.83	1.15	1.25	.95	5.28	1.056
	3	WL	.53	.43	1.30	.60	.25	3.11	.622
		AO	1.23	.78	1.03	1.75	1.15	5.94	1.188
	4	WL	.50	.40	1.23	.63	.83	3.59	.718
		AO	1.25	.80	.85	1.15	1.20	5.25	1.050
	5	WL	.50	.35	1.13	.48	.20	2.66	.532
		AO	1.38	.73	.85	1.00	1.15	5.11	1.022
	6	WL	.35	.40	1.10	.30	.23	2.38	.476
		AO	.83	.65	.73	.80	.95	3.96	.792
			32.76	31.98	33.11	30.66	22.02	150.53	

Continued....

TABLE 1 Continued....

Analysis of Variance.

Source	df	Sum square	Mean square	F test
Breed (B)	1	6.39	6.39	81.92**
Period (P)	5	1.30	0.260	3.33**
Ration (R)	3	2.53	0.843	10.81**
RP	15	0.19	0.013	NS
BP	5	0.19	0.038	NS
RB	3	0.16	0.053	NS
RBP	15	0.15	0.010	NS
Replications	4	1.78		
Error	188	14.60	0.078	
Total	239	27.29		

Comparison between means.

Ration	$D_{.05}$	=	0.1307
	$D_{.01}$	=	0.1584
Breed	$D_{.05}$	=	0.0706
	$D_{.01}$	=	0.0928
Period	$D_{.05}$	=	0.1773
	$D_{.01}$	=	0.2094

TABLE 1.

B. Feed consumption and live weight, averaged over five cockerels per treatment.

Ration	A		B		C		D	
Breed	WL	AO	WL	AO	WL	AO	WL	AO
lb Feed cons.	15.5	17.5	14.4	17.2	17.7	16.9	14.7	16.3
Calorie cons.	14,625	16,536	18,665	22,352	16,725	16,025	19,107	21,105
lb Prot.cons.	1.61	1.82	1.50	1.79	2.88	2.76	2.40	2.65
lb Prot./day	.0328	.0372	.0306	.0366	.0588	.0564	.0490	.0541
lb initial L Wt.	5.72	7.88	5.50	8.04	5.74	8.36	6.04	8.38
lb final L Wt.	5.84	8.10	5.66	8.64	5.88	8.38	6.08	8.92
% chng L Wt.	+ 2.1	+ 2.5	+ 2.9	+ 7.5	+ 2.4	+ 0.2	+ 0.7	+ 6.4
Cal/lb L Wt.	2,524	2,098	3,346	2,689	2,873	1,927	3,173	2,456
lb Prot/lb L Wt.	.278	.230	.263	.216	.495	.332	.398	.307
% cr Prot.	10.35		10.35		16.30		16.30	
Cal ME/lb	946		1298		946		1298	
% casein	1		1		8		8	
% sugar	7		27		0		20	
% sawdust	20		0		20		0	
C/P	90		125		58		80	

TABLE 1.

C. Spermatozoa per ejaculate in millions, of the first and last collections.
Factors: ration (4) x breed (2) x collection (2); 5 cockerel replications.

Ration	Colln.	Breed	REPLICATIONS 1	2	3	4	5	Sum	Mean
A	1	WL	1560	3465	2365	1350	1775	10515	2103
		AO	3100	4950	3500	1450	210	13210	2642
	2	WL	693	1837	2871	1533	1020	7954	1591
		AO	3705	2090	1250	575	350	7970	1594
B	1	WL	1755	1430	1360	2385	480	7410	1482
		AO	5900	2380	2380	4000	2380	17040	3408
	2	WL	1930	1554	620	1141	544	5789	1158
		AO	4560	780	600	2730	495	9165	1833
C	1	WL	440	885	1710	2170	1200	6405	1281
		AO	1500	3100	2430	2800	1740	11570	2314
	2	WL	18	378	1242	470	876	2984	597
		AO	675	3640	315	1925	1680	8235	1647
D	1	WL	900	2380	4050	760	150	8240	1648
		AO	4000	3480	2700	490	3930	14600	2920
	2	WL	992	1520	3608	912	288	7320	1464
		AO	3230	2160	2700	900	2600	11590	2318
		Sum :	34958	36029	33701	25591	19718		

Analysis of Variance.

Source.	df.	SS.	MS.	F test
Breed (B)	1	16,893,977	16,893,977	12.78 **
Collection (C)	1	9,788,104	9,788,104	7.41 **
Ration (R)	3	4,764,768	1,588,256	N.S.
R C	3	817,808	272,603	N.S.
B C	1	1,495,224	1,495,224	N.S.
R.B	3	3,005,808	1,001,936	N.S.
R B C	3	1,038,020	346,007	N.S.
Replications	4	12,486,676	3,121,669	N.S.
Error	60	79,299,710	1,321,662	
Total	79	129,590,095		

TABLE 2. Extra artificial light on semen release.

Semen volumes in millilitres summed over five males per subgroup.
Factors: light regimen (2) x group (2); 6 replications per treatment.

Group	I	II	Sum
Reps	A	B	
1	1.34	1.18	2.52
2	1.45	.99	2.44
3	1.07	.90	1.97
4	1.39	.94	2.33
5	1.45	1.32	2.77
6	1.50	1.13	2.63
	B	A	
7	1.23	1.28	2.51
8	1.39	1.37	2.76
9	1.75	1.13	2.88
10	1.12	1.61	2.73
11	.96	1.13	2.09
12	1.18	1.23	2.41

Light Treatment
Sum A = 15.95
Sum B = 14.09

Analysis of Variance.

Source	df	Sum square	Mean square	F test
Light treatment	1	0.144	0.144	NS
Group	1	0.109	0.109	NS
Replications	11	0.422	0.038	
Error	10	0.388	0.039	
Total	23	1.063		

TABLE 3 Comparing diluents.

Percent fertile eggs layed on 2nd to 5th day after insemination.
Factors: diluent (4) x Breed (2); 8 pullet replications.

Trt.	1	2	3	4	5	6	7	8
Diluent	Locke's		Tyrode		Milk		Undiluted.	
Breed	WL	AO	WL	AO	WL	AO	WL	AO
1	100	100	100	0	100	67	100	75
2	75	100	67	100	100	100	75	67
3	67	100	75	100	-	100	100	100
4	100	100	33	100	100	67	100	75
5	100	100	67	100	100	100	100	67
6	100	33	100	75	67	100	100	100
7	67	100	100	67	50	100	75	100
8	67	0	100	100	-	100	100	100
Sum	676	633	642	642	517	733	750	683
Mean	84.5	79.1	80.3	80.3	86.2	91.6	93.8	85.4
Fert. eggs	21	19	20	21	16	25	25	25
Total eggs	25	23	25	25	18	27	27	29
Trfd. mean	73.1	71.9	70.6	70.6	76.0	81.2	82.5	73.7

Analysis of Variance. (percentages transformed to angles).

Source.	df.	Sum square.	Mean square.	F test.
Diluent	3	773	258	NS
Breed	1	23	23	NS
Interaction	3	400	133	NS
Error	56	30,443	544	
Total	63	31,639		

TABLE 4. Dilution rates.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: diluent (2) x dilution rate (4); 5 pullet replications.

Trt.	1	2	3	4	5	6	7	8
Diluent	Milk				Tyrode			
Dil. rate	1/3	1/6	1/12	1/18	1/3	1/6	1/12	1/18
1	100	83	83	50	100	80	60	40
2	83	83	100	100	83	100	50	75
3	100	67	83	50	100	80	30	40
4	80	0	100	30	100	80	83	100
5	83	100	60	83	100	17	100	0
Sum	446	333	426	313	483	357	333	255
Mean	89.2	66.6	85.2	62.6	96.6	71.4	66.6	51.0
Fert. eggs	27	20	24	19	28	17	17	12
Total eggs	30	29	28	30	29	25	27	25
Trfd. mean	75.0	55.3	72.4	55.8	85.1	60.9	56.9	45.7

Analysis of Variance. (percentages transformed to angles).

Source	df.	Sum square.	Mean square.	F test.
Dilution rate	3	4,676	1,559	2.9*
Diluent	1	59	59	NS
Interaction	3	1,136	379	NS
Error	32	17,351	542	
Total	39	23,222		

Comparisons between transformed means.

$D_{.05}$ (dilution rate) = 28.26

TABLE 5. Dose and dilution rate combinations.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: male (2) x dilution rate (4); 5 pullet replications.

Trt.	1	2	3	4	5	6	7	8
Male			WC				AO	
Dil. Rate	0	1/2	1/10	1/20	0	1/2	1/10	1/20
1	100	60	0	0	100	80	0	50
2	100	71	0	0	100	86	17	40
3	100	100	0	0	80	100	17	0
4	83	100	0	0	100	100	0	0
5	40	100	0	20	100	100	0	0
Sum	423	431	0	20	480	466	34	90
Mean	84.6	86.2	0	4.0	96.0	93.2	6.8	18.0
Fert. eggs	22	25	0	1	28	22	2	5
Total eggs	26	29	27	24	29	24	23	28
Trfd. mean	75.0	75.6	0	5.3	84.7	80.3	9.8	16.8

Analysis of Variance. (percentages transformed to angles).

Source	df	Sum square	Mean square	F test
Dilution rate	3	50,499	16,833	64.7**
Male	1	793	793	NS
Interaction	3	66	22	NS
Error	32	8,321	260	
Total	39	59,679		

Comparisons amongst transformed means:

$D_{.05}$ (dilution rate) = 19.58

$D_{.01}$ (dilution rate) = 24.48

TABLE 6. Insemination techniques and training for pullets.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: breeds (2) x handling method (4); 5 pullet replicarions.

Trt.	1	2	3	4	5	6	7	8
Breed	WL				AO			
Method	Semen	Water	Sham	Nil	Semen	Water	Sham	Nil
1	83	83	100	100	100	80	67	100
2	83	100	100	71	100	14	100	80
3	100	100	100	100	100	100	86	100
4	100	0	83	100	100	100	100	100
5	100	100	100	100	100	100	100	50
Sum	466	383	483	471	500	394	453	430
Mean	93.2	76.6	96.6	94.2	100.0	78.8	90.6	86.0
Fert. eggs	31	21	22	28	26	18	26	23
Total eggs	33	28	23	30	26	25	29	26
Trfd. mean								

Analysis of Variance. (percentages transformed to angles).

Source	df	MS	SS	F test
Breed	1	0	0	NS
Method	3	1,522	507	NS
Interaction	3	437	146	NS
Error	32	81,622	2,551	
Total	39	83,581		

TABLE 7. Dose and dilution rate combinations.

Percent fertile eggs layed on 2nd to 9th day after insemination.
Factors: dilution rate (3) x dose inseminated (3); 8 pullet replications.

Trt.	1	2	3	4	5	6	7	8	9
Dose (ml)		0.05			0.10			0.22	
Dil. rate	1/3	1/6	1/10	1/3	1/6	1/10	1/3	1/6	1/10
1	83	0	20	88	40	50	100	100	25
2	33	75	25	86	100	43	100	100	17
3	80	100	50	100	83	0	86	67	0
4	88	67	0	100	0	0	80	86	83
5	67	14	14	100	50	17	100	83	43
6	100	57	29	57	50	33	71	67	67
7	83	40	0	100	100	67	100	0	83
8	29	100	40	67	83	67	50	67	0
Sum	563	453	178	698	506	277	687	570	318
Mean	70.4	56.6	22.3	87.3	63.3	34.6	85.9	71.3	39.8
Fert.eggs	34	22	10	39	23	17	29	31	20
Total eggs	48	45	46	45	37	49	33	42	53
Trfd.mean	59.6	50.6	24.4	75.2	55.1	31.9	74.2	59.8	35.2

Analysis of Variance. (transformed percentages).

Source.	df.	Sum square.	Mean square.	F test.
Dilution rate	2	18,822	9,411	16.69**
Dose	2	1,776	888	NS
Interaction	4	263	66	NS
Error	63	35,511	564	
Total	71	56,372		

Comparison between transformed means:

$D_{.05} = 15.52$

$D_{.01} = 19.59$

TABLE 8. Small dose and dilution rate combinations.

Percent fertile eggs layed on 2nd to 9th day after insemination.
Factors: dilution rate (2) x dose inseminated (3); 8 and 16 pullet replications.

Trt.	1	2	3	4	5	6
Dose (ml.)	0.010		0.025		0.05	
Dil. rate	0	1/3	0	1/3	0	1/3
1	50 100	-	86 80	100	80 100	83
2	83 100	0	100 100	6	60 100	30
3	67 100	0	83 100	20	- -	50
4	60 100	0	100 100	86	100 100	83
5	67 67	6	100 100	86	100 100	17
6	100 100	40	67 100	80	100 100	-
7	100 100	71	100 100	100	100 100	86
8	83 100	0	100 100	0	50 100	100
Sum	1,377	117	1,516	478	1,290	449
Mean	86.1	16.7	94.8	59.8	92.1	64.1
Fert.eggs	72	8	94	29	72	24
Total eggs	83	36	99	43	78	37
Trfd. mean.	75.1	17.4	83.3	52.5	81.0	55.7

Analysis of Variance. (transformed percentages).

Source	df.	Sum square.	Mean square.	F test.
Dilution rate	1	22,989	22,989	55.71**
Dose	2	4,568	2,284	5.54**
Interaction	2	3,270	1,635	3.97*
Error	62	25,542	412	
Total	67	56,369		

Comparisons between transformed means:

$D_{.05}$ (dilution rate) = 9.23
$D_{.01}$ (" ") = 12.28
$D_{.05}$ (dose) = 13.52
$D_{.01}$ (") = 17.03
$D_{.05}$ (interaction) = 23.56
$D_{.01}$ (") = 28.20

TABLE 9. Small dose and dilution rate combinations.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: dose (3) x dilution rate (2); 10 pullet replications.

Trt.	1	2	3	4	5	6
Dose (ml.)	0.005		0.010		0.050	
Dil. rate	0	$\frac{1}{2}$	0	$\frac{1}{2}$	0	$\frac{1}{2}$
1	100	71	100	43	100	100
2	100	0	60	100	100	60
3	0	0	86	43	0	86
4	86	17	100	100	80	100
5	-	33	-	100	100	100
6	0	-	57	0	100	100
7	0	20	17	86	100	43
8	86	50	100	33	100	33
9	100	14	0	-	100	0
10	83	29	67	83	0	100
Sum	555	234	587	588	780	722
Mean	61.7	26.0	65.2	65.3	78.0	72.2
Fert. eggs	35	13	36	34	50	39
Total eggs	54	51	55	55	61	56
Trfd. mean	52.4	27.0	57.5	57.9	69.3	64.5

Analysis of Variance. (transformed percentages).

Source	df.	Sum square.	Mean square	F test
Dose	2	7,172	3,586	3.67*
Dilution rate	1	1,335	1,335	NS
Interaction	2	1,687	824	NS
Blocks	9	13,090	1,454	NS
Error	41	40,057	977	
Total	55	63,341		

Comparison between transformed means:

$D_{.05} = 24.88$

$D_{.01} = 31.57$

TABLE 10. Comparing diluents.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factor: 3 diluents; 15 pullet replications.

Diluent	Tyrode	Milk	Buffer
1	100	67	100
2	100	29	100
3	0	71	83
4	57	57	100
5	57	83	0
6	83	83	100
7	67	67	83
8	60	100	100
9	100	80	100
10	100	100	100
11	100	17	100
12	60	100	100
13	100	83	83
14	83	0	100
15	100	0	100
Sum	1,167	937	1,349
Mean	77.8	62.5	89.9
Fert. eggs	69	54	78
Total eggs	90	90	87
Trfd. mean	67.7	53.6	79.1

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Between diluents	2	4,919	2,460	3.54
Error	42	29,198	695	NS
Total	44	34,117		

Comparison between transformed means:

$D_{.05} = 23.39$

TABLE 11. Dilution effect with small doses inseminated.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factor: 4 dilution rates; 16 pullet replications.

Dilution rate	0	1/2	1/3	1/4
1	50	63	43	40
2	85	88	67	75
3	57	71	100	67
4	83	43	29	0
5	85	25	100	50
6	88	75	14	33
7	83	63	50	0
8	100	83	63	43
9	0	80	38	67
10	86	0	50	0
11	83	63	50	63
12	83	75	40	0
13	100	88	0	83
14	80	0	0	43
15	100	20	85	57
16	100	60	83	57
Sum	1,263	897	812	678
Mean	78.9	56.1	50.8	42.2
Fert eggs	86	64	56	47
Total eggs	107	111	112	112
Trfd. mean	65.8	47.0	45.5	36.7

Analysis of Variance: (transformed percentages).

Source	df.	Sum square	Mean square	F test
Between treatments	3	7,172	2,391	4.65**
Blocks	7	5,574	796	NS
Error	53	27,255	514	
Total	63	40,001		

Comparison between transformed means:

$D_{.05}$ = 21.20

$D_{.01}$ = 26.41

TABLE 12. Diluents and dilution rate combinations.

Percent fertile eggs layed on 2nd to 9th day after insemination.
Factors: diluent (2) x dilution rate (2); 8 pullet replications.

Trt.	1	2	3	4
Diluent	Milk		Tyrode	
Dil. rate	1/2	1/10	1/2	1/10
1	43	0	67	14
2	100	71	0	0
3	100	50	100	29
4	100	0	100	14
5	86	63	33	86
6	88	100	43	100
7	86	12	75	100
8	100	0	100	75
Sum	703	296	518	418
Mean	87.9	37.0	64.8	52.3
Fert. eggs	48	21	34	21
Total eggs	55	56	53	49
Trfd. mean	75.8	33.2	57.6	48.1

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Diluent	1	22	22	NS
Dilution rate	1	5,458	5,458	5.89*
Interaction	1	2,196	2,196	NS
Blocks	7	5,739	820	NS
Error	21	19,461	927	
Total	31	32,876		

Comparison between transformed means:

$D_{.05} = 22.41$

$D_{.01} = 30.58$

TABLE 13. Dose by dilution rate combinations and the persistence of fertility.

A. Percent fertile eggs layed on 2nd to 7th day after insemination. Factors: dilution rate (3) x dose inseminated (2); 10 pullet replications.

Trt.	1	2	3	4	5	6
Dose (ml)		0.05			0.10	
Dil.rate	0	1/3	1/6	0	1/3	1/6
1	100	100	83	100	80	0
2	83	75	0	33	0	25
3	100	0	50	100	100	33
4	40	100	100	80	100	80
5	0	80	100	60	40	100
6	100	100	0	100	100	80
7	100	50	0	80	80	0
8	75	50	33	100	80	0
9	100	0	67	100	100	0
10	100	83	0	100	50	80
Sum	798	638	433	853	730	398
Mean	79.8	63.8	43.3	85.3	73.0	39.8
Fert. eggs	43	32	18	37	38	20
Total eggs	54	49	49	45	53	51
Trfd. mean	70.5	54.9	38.1	75.3	63.4	34.5

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Dose	1	159	159	NS
Dilution rate	2	13,661	6,831	6.94**
Interaction	2	382	191	NS
Blocks	9	8,445	938	NS
Error	45	44,286		
Total	59	66,933		

Comparison between transformed means.

$D_{.05}$ = 24.03

$D_{.01}$ = 30.41

TABLE 13

B. Percent fertile eggs layed on 8th to 12th day after insemination.

Trt.	1	2	3	4	5	6
Dose (ml.)		0.05			0.10	
Dil. rate	0	1/3	1/6	0	1/3	1/6
1	60	75	40	100	40	0
2	60	80	0	0	0	0
3	20	0	50	100	67	25
4	0	100	100	50	100	25
5	0	100	-	25	0	100
6	100	67	0	0	25	25
7	80	0	0	0	50	0
8	50	0	0	33	50	0
9	80	0	40	60	100	0
10	50	67	0	100	0	50
Sum	500	489	230	468	432	225
Mean	50.0	48.9	25.6	46.8	43.2	22.5
Fert. eggs	23	20	10	21	16	10
Total eggs	45	41	39	41	40	41
Trfd. means	43.5	41.3	23.7	43.1	39.4	22.5

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Dose	1	38	38	NS
Dilution rate	2	4,618	2,309	NS
Blocks	9	9,011	1,001	NS
Error	46	49,426	1,074	
Total	58	63,093		

TABLE 14. Dilution effect on short storage.

A. Percent fertile eggs layed on 2nd to 7th day after insemination. Factors: dilution rate (2) x storage time (2); 10 pullet replications.

Trt.	1	2	3	4
Time (min.)	20		240	
Dil. rate	0	$\frac{1}{2}$	0	$\frac{1}{2}$
1	100	100	60	75
2	75	60	25	100
3	100	80	100	83
4	100	100	80	60
5	100	60	17	50
6	100	67	100	-
7	100	83	100	17
8	100	100	100	0
9	100	100	67	83
10	100	100	20	33
Sum	975	850	669	501
Mean	97.5	85.0	66.9	55.7
Fert. eggs	51	44	35	25
Total eggs	52	52	52	42
Trfd. mean	87.0	73.6	60.9	48.5

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Storage time	1	6,201	6,201	11.81**
Dilution rate	1	1,465	1,465	NS
Interaction	1	167	167	NS
Blocks	9	1,701	300	NS
Error	26	13,637	525	

Comparisons between transformed means.

$D_{.05}$ = 14.93

$D_{.01}$ = 20.20

TABLE 14.

B. Percent fertile eggs layed on 8th to 12th day after insemination.

Trt.	1	2	3	4
Time (min.)	20		240	
Dil. rate	0	$\frac{1}{2}$	0	$\frac{1}{2}$
1	60	100	25	67
2	100	20	20	33
3	60	50	100	50
4	75	60	0	100
5	20	40	0	0
6	60	0	50	-
7	75	75	60	0
8	80	100	80	100
9	20	75	0	80
10	75	100	25	20
Sum	625	620	360	450
Mean	62.5	62.0	36.0	50.0
Fert. eggs	27	27	17	14
Total eggs	45	44	44	33
Trfd mean	53.9	55.2	33.6	45.0

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Storage time	1	2,353	2,353	NS
Dilution rate	1	425	425	NS
Interaction	1	201	201	NS
Blocks	9	9,341	1,038	NS
Error	26	18,536		
Total	38	30,856		

TABLE 14.

C. Percent fertile eggs layed daily.

Trt.	1	2	3	4		
Time (min.)	20		240			
Dil. rate	0	$\frac{1}{2}$	0	$\frac{1}{2}$	Sum	Mean
Day 1	90	75	44	63	272	68.0
2	100	67	100	80	347	86.8
3	100	89	56	86	331	82.8
4	100	83	63	50	296	74.0
5	100	100	70	40	310	77.5
6	100	90	80	43	313	78.3
7	70	60	70	57	257	64.3
8	50	100	40	20	210	52.5
9	70	55	33	50	208	52.0
10	75	50	22	56	203	50.8
11	33	20	14	0	67	16.8
Sum	888	789	592	545		
Mean	80.7	71.7	53.8	49.5		

Analysis of Variance.

Source	df.	Sum square	Mean square	F test
Treatments	3	7,173	2,391	9.06**
Days	10	16,220	1,622	6.14**
Error	30	7,911	264	
Total	43	31,304		

Comparisons between means.

$D_{.05}^{(day)} = 39.9$

$D_{.01}^{(day)} = 47.5$

$D_{.05}^{(treatment)} = 18.8$

$D_{.01}^{(treatment)} = 23.5$

TABLE 15. Dilution rate and storage of semen.

Percent fertile eggs layed on 2nd to 8th day after insemination
Factors: storage time (3) x dilution rate (3); 6 pullet replications.

Trt.	1	2	3	4	5	6	7	8	9
Time (hr.)		3			6			24	
Dil.rate.	0	1/2	1/10	0	1/2	1/10	0	1/2	1/10
1	100	67	-	67	50	67	0	43	0
2	100	100	-	100	100	100	0	0	0
3	100	100	-	83	80	100	50	0	-
4	100	100	83	60	100	100	17	50	17
5	80	100	71	100	83	60	0	20	0
6	83	86	100	67	-	67	20	50	-
Sum	563	553	254	477	413	494	87	163	17
Mean	93.8	92.2	84.7	79.5	82.6	82.3	14.5	27.2	4.3
Fert. eggs	30	33	15	24	24	25	5	10	1
Total eggs	32	36	18	30	29	31	32	32	21
Trfd. means	81.5	80.5	71.0	67.7	70.8	71.8	16.0	26.3	6.1

Analysis of Variance. (transformed percentages).

Source	df.	Sum Square	Mean square	F test
Dilution rate	2	375	188	NS
Storage time	2	35,146	17,573	49.6**
Interaction	4	914	229	NS
Error	39	12,252	314	
Total	47	48,687		

Comparison between transformed means.

$D_{.05}$ = 14.39

$D_{.01}$ = 18.28

TABLE 16. Temperature, dilution rate and time of semen storage.

Percent fertile eggs layed on 2nd to 7th day after insemination.
Factors: storage time (2) x temperature (2) x dilution rate (2);
6 pullet replications.

Trt.	1	2	3	4	5	6	7	8
Time (hr.)	4				21			
Temp.(°C.)	2		10		2		10	
Dil.rate	0	½	0	½	0	½	0	½
1	0	20	50	67	0	0	0	0
2	0	0	75	75	0	0	0	0
3	0	60	100	100	0	0	0	0
4	50	0	100	100	0	0	60	0
5	40	20	100	0	0	0	20	60
6	0	20	40	100	0	0	40	0
Sum	90	120	465	442	0	0	120	60
Mean	15.0	20.0	77.5	73.7	0	0	20.0	10.0
Fert.eggs	4	5	19	20	0	0	6	3
Total eggs	29	30	26	27	27	27	25	27
Trfd.mean	14.0	21.8	69.0	64.2	0	0	19.4	8.5

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test.
Replications	5	2,446	489	NS
Temperature (E)	1	11,772	11,772	26.395**
Time (T)	1	14,929	14,929	33.473**
Rate (R)	1	50	50	NS
TE	1	3,621	3,621	8.119**
TR	1	142	142	NS
ER	1	417	417	NS
ERT	1	2	2	NS
Error	35	15,595	446	
Total	47	48,974		

Comparison between transformed means.

$D_{.05}$ (time, temp.) = 12.39
$D_{.01}$ (" ") = 16.56
$D_{.05}$ (TE) = 21.92
$D_{.01}$ (TE) = 28.67

TABLE 17. Diluents for 24 hour semen storage.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factor: 4 diluents; 7 pullet replications.

Diluents	Buffer + antibiotics	Buffer	Milk	Tyrode
1	50	83	50	0
2	75	100	50	20
3	17	33	20	0
4	33	25	0	0
5	100	75	17	0
6	33	100	0	0
7	29	67	0	0
Sum	337	483	137	20
Mean	48.1	69.0	19.6	2.9
Fert. eggs	18	26	8	1
Total eggs	40	37	42	34
Trfd means	46.0	60.8	20.1	3.8

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test.
Diluents	3	13,658	4,553	10.32**
Error	24	10,585	441	
Total	27	24,243		

Comparison between transformed means.

$D_{.05}$ = 31.2

$D_{.01}$ = 39.34

TABLE 18. Comparing diluents and dilution rates in 24 hour storage of semen.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: diluent (2) x dilution rate (2); 15 pullet replications.

Trt.	1	2	3	4
Diluent	Buffer		Milk	
Dil. rate	$\frac{1}{2}$	$\frac{1}{4}$	$\frac{1}{2}$	$\frac{1}{4}$
1	0	0	0	0
2	80	0	0	0
3	0	0	0	0
4	0	0	0	0
5	20	20	0	0
6	-	0	-	0
7	-	0	0	0
8	0	0	33	25
9	60	20	50	0
10	0	0	0	0
11	0	0	20	0
12	0	0	0	0
13	67	100	20	0
14	33	25	-	0
15	0	0	0	0
Sum	260	165	123	25
Mean	20.0	11.0	9.5	1.7
Fert. eggs	14	4	6	1
Total eggs	64	72	58	75
Trfd. mean	17.75	11.55	10.25	2.00

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Diluent	1	1,035	1,035	NS
Dilution rate	1	728	728	NS
Interaction	1	14	14	NS
Error	52	20,017	385	
Total	55	21,794		

TABLE 19. Effect of abundant cloacal fluid on stored spermatozoa.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factor: Low and high cloacal fluid samples; 15 pullet replications.

Trt.	Low	High
1	0	75
2	50	46
3	67	0
4	50	0
5	0	80
6	0	50
7	0	100
8	60	0
9	50	60
10	0	20
11	0	17
12	80	25
13	100	100
14	60	100
15	100	20
Sum	617	693
Mean	41.1	46.2
Fert. eggs	26	27
Total eggs	62	58
Trfd. mean	35.7	42.6

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Treatments	1	364	364	NS
Error	28	29,391	1,049	
Total	29	29,755		

TABLE 20. Spermatozoa aging in-vitro and fertility.

A. Percent fertile eggs layed on 2nd to 9th day after insemination.
Factor: age of semen (7 tests); 10 pullet replications.

Age (min.)	30	80	135	195	255	315	375	Sum
1	0	100	100	86	71	14	67	438
2	0	71	71	71	86	57	67	423
3	100	67	86	83	67	-	0	403
4	43	50	57	57	0	100	80	387
5	83	100	100	57	86	83	83	592
6	67	86	100	57	88	100	75	573
7	17	75	100	50	100	57	-	399
8	86	14	100	86	71	43	100	500
9	-	88	100	14	-	0	100	302
10	-	100	86	0	86	50	57	379
Sum	396	751	900	561	655	504	629	
Mean	49.4	75.1	90.0	56.1	72.9	56.0	69.9	
Fert. eggs	21	47	55	38	47	37	42	
Total eggs	48	64	62	67	64	59	59	
Trfd. mean	43.0	64.7	78.2	47.3	59.3	50.2	58.7	

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Age	6	8,059	1,343	NS
Blocks	9	3,606	401	NS
Error	49	32,044	654	
Total	64	43,709		

TABLE 21. Fructose added to stored semen.

A. Percent fertile eggs layed on 2nd to 12th day after insemination.
Factor: Fructose treatments (3); 15 pullet replications.

Trt.	¼ Buffer + fructose	½ Buffer + fructose	½ Buffer	Blocks
1	29	60	20	109
2	44	67	40	151
3	33	44	33	110
4	67	50	63	180
5	38	71	33	142
6	63	38	44	145
7	0	50	0	50
8	75	0	29	104
9	0	57	38	95
10	83	86	60	229
11	30	44	100	174
12	50	50	55	155
13	30	0	33	63
14	0	50	0	50
15	0	40	40	80
Sum	542	707	588	1,837
Mean	36.1	47.2	39.2	
Fert. eggs	45	60	44	
Total eggs	128	130	120	
Trfd. mean	32.8	41.4	37.6	

Analysis of Variance. (transformed percentages).

Source	df.	Sum square.	Mean square.	F test.
Treatments	2	555	278	NS
Blocks	14	8,126	580	NS
Error	28	10,185	364	
Total	44	18,866		

TABLE 21.

B. Percent fertile eggs layed on 7th to 12th day after insemination.
Factor: fructose treatments (3); 15 pullet replications.

Trt.	¼ Buffer + fructose	½ Buffer + fructose	½ Buffer	Blocks
1	0	20	0	20
2	20	40	0	60
3	0	0	0	0
4	40	25	40	105
5	20	50	33	103
6	40	0	0	40
7	0	33	0	33
8	50	0	0	50
9	0	50	0	50
10	67	67	33	167
11	0	20	-	20
12	17	33	40	90
13	17	0	25	42
14	0	20	0	20
15	0	17	33	50
Sum	271	375	204	850
Mean	18.1	25.0	14.6	
Fert. eggs	12	17	11	
Total eggs	73	71	68	
Trfd. mean	18.7	26.0	15.3	

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test
Treatment	2	887	444	NS
Blocks	14	6,966	498	NS
Error	27	7,540		
Total	43	15,393		

TABLE 21.

C. Percent fertile eggs on 11 days of egg collection.
Factor: fructose treatments (3); 15 pullet replications.

Trt.	¼ Buffer + fructose	½ Buffer + fructose	½ Buffer	Days Sum	Mean
1	73	75	80	228	76
2	55	70	73	198	66
3	67	73	67	207	69
4	67	78	50	195	65
5	44	69	45	158	53
6	25	50	38	113	38
7	43	47	21	111	37
8	14	18	25	57	19
9	0	20	0	20	7
10	0	8	8	16	5
11	8	0	8	16	5
Sum	396	508	415	1,319	
Mean	36.0	46.2	37.7		

Analysis of Variance.

Source	df.	Sum square	Mean square	F test
Treatments	2	641	321	3.59*
Day	10	22,613	2,261	25.26**
Error	20	1,790	90	
Total	32	25,044		

Comparison between means.

$D_{.05}$ (treatments) = 10.20

$D_{.05}$ (day) = 27.85

$D_{.01}$ (day) = 33.79

TABLE 21.

D. Percent fertile eggs layed on 6th to 10th day after insemination. Factors: fructose treatments (3); 15 pullet replications.

Trt.	1	2	3	
	¼ Buffer + F.	½ Buffer + F.	½ Buffer	Sum
1	0	50	0	50
2	25	75	20	120
3	0	25	0	25
4	75	67	75	217
5	33	50	60	143
6	50	0	25	75
7	0	50	0	50
8	50	0	0	50
9	0	50	33	83
10	67	100	40	207
11	20	50	-	70
12	40	60	50	150
13	25	0	33	58
14	0	25	0	25
15	0	40	25	65
Sum	385	642	361	
Mean	25.67	42.80	25.79	
Fert.eggs	15	25	14	
Total eggs	59	59	54	
Trfd.means	24.38	38.66	25.13	

Analysis of Variance (transformed percentages).

Source	df.	Sum square	Mean square	F test
Treatment	2	1,924	962	2.54 (P 0.10)
Blocks	14	10,984	785	NS
Error	27	10,246		
Total	43	23,154		

TABLE 22. Male Exhaustion

Percent fertile eggs layed on 2nd to 5th day after insemination.
Factors: Male (4) x collection (4); 4 pullet replication.

Hour	1415				1509				1602				1656			
Strain	ML		AO		ML		AO		ML		AO		ML		AO	
Type	I	II	I	II	I	II	I	II	I	II	I	II	I	II	I	II
Male	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D
Reps 1	0	0	100	100	100	100	100	100	75	0	100	75	100	100	50	0
2	50	50	100	100	100	67	50	75	100	100	100	75	67	67	50	100
3	100	100	50	67	75	100	33	50	100	-	100	100	0	-	100	100
4	100	0	75	100	75	0	100	67	100	0	75	67	100	0	100	67
Sum	250	150	325	367	350	267	283	292	375	100	375	317	267	167	300	267
Mean	62.5	37.5	81.3	91.8	87.5	66.8	70.8	73.0	93.8	33.3	93.8	79.3	66.8	55.7	75.0	66.8
Fert. eggs	5	6	11	11	12	8	9	8	12	3	13	10	8	5	8	10
Total eggs	7	13	13	12	14	11	13	12	13	8	14	13	13	8	10	14
Trfd. mean	56.3	33.8	71.3	81.2	75.0	58.7	65.0	62.5	82.5	31.3	82.5	66.2	58.7	48.2	67.5	58.7

Analysis of Variance. (transformed percentages).

Source	df.	Sum square	Mean square	F test.
Replications	3	1,546	513	NS
Hour (H)	3	623	208	NS
Strain (S)	1	3,053	3,053	2.95 (P 0.10)
Type (T)	1	3,490	3,490	3.38 (P 0.10)
H S	3	2,843	828	NS
H T	3	1,950	650	NS
S T	1	1,718	1,718	NS
S T H	3	749	250	NS
Error	45	46,545	1,034	
Total	63	62,157		

TABLE 23. Semen volume exhaustion.

Semen released per collection in millilitres.
Factors: collection (4) x male (4).

Hour	Male				Sum	Mean
	A	B	C	D		
1407	0.40	0.45	0.80	0.65	2.30	0.575
1456	0.35	0.35	0.70	0.60	2.00	0.500
1556	0.35	0.30	0.60	0.65	1.90	0.475
1656	0.30	0.20	0.50	0.80	1.80	0.450
Sum	1.40	1.30	2.60	2.70	8.00	
Mean	0.350	0.325	0.650	0.675		

Analysis of Variance.

Source	df.	Sum square	Mean square	F test
Males	3	0.4250	0.1417	16.287**
Collection time	3	0.0350	0.0117	NS
Error	9	0.0785	0.0087	
Total	15	0.5350		

TABLE 24. Spermatozoa release exhaustion.

Estimated spermatozoa per ejaculate in millions.
Factors: collection time (4) x male (4).

Hour	Male				Sum	Mean
	A	B	C	D		
1407	2,192	594	4,512	1,456	8,754	2,189
1456	1,211	322	2,828	2,364	6,725	1,681
1556	1,176	138	2,196	1,729	5,239	1,310
1656	522	100	680	816	2,118	530
Sum	5,101	1,154	10,216	6,365	22,836	
Mean	1,278	289	2,554	1,591		

Analysis of Variance.

Source	df.	Sum square	Mean square	F test
Male	3	10,465,268	3,488,423	6.97*
Collection time	3	5,855,116	1,951,705	3.90*
Error	9	4,503,617	500,402	
Total	15	20,824,001		

Comparisons between means.

$D_{.05}$ = 1,558 (both factors).

TABLE 23b. Spermatozoa concentration exhaustion.

Estimated spermatozoa concentration (X 10^9/ml.)
Factors: collection time (4) x males (4).

	Male				
Hour	A	B	C	D	Mean
1407	5.48	1.32	5.64	2.24	3.67
1456	3.46	0.92	4.04	3.94	3.09
1556	3.36	0.46	3.66	2.66	2.54
1656	1.74	0.50	1.36	1.02	1.16
Mean	3.51	0.80	3.68	2.44	

Analysis of Variance.

Source	df.	Sum square	Mean square	F test.
Males	3	20.965	6.988	8.582**
Hour	3	13.907	4.636	5.693**
Error	9	7.329	0.814	
Total	15	42.201		

Comparisons between means.

$D_{.05}$ = 1.99 (both factors)

$D_{.01}$ = 2.69

TABLE 25. Time of collection and semen release.

A. Semen volume in millilitres summed over three cockerels per subgroup.
Factors: strain of fowl (3) x time of day (3); 3 day replications.

Time		0855			1335			1630			
Strain		WC	ML	AO	WC	ML	AO	WC	ML	AO	Sum
Day	1	1.30	.65	1.60	1.20	.90	1.70	.65	1.25	1.70	10.95
	2	1.40	.70	1.20	.40	.90	1.55	1.10	.75	1.45	9.45
	3	.30	.80	1.75	1.20	.50	1.60	.85	.60	1.65	9.25
Sum		3.00	2.15	4.55	2.80	2.30	4.85	2.60	2.60	4.80	
Mean		1.00	.72	1.52	.93	.77	1.62	.87	.87	1.60	

Analysis of Variance.

Source.	df	Sum square	Mean square	F test
Strain	2	3.21	1.605	15.58**
Time	2	0.01	0.005	NS
Days	2	0.18	0.090	NS
Interaction ST	4	0.07	0.018	NS
Error	16	1.64	0.103	
Total	26	5.11		

Comparison between means:

$D_{.05}$ (strain) = 0.383

$D_{.01}$ " = 0.502

B. Spermatozoa concentration in 10^9 cells/ml., averaged over 3 cockerels
Factors: strain (3) x time (3); 3 day replications.

Time		0855			1335			1630			
Strain		WC	ML	AO	WC	ML	AO	WC	ML	AO	Sum
Day	1	2.2	3.3	4.0	5.9	4.4	6.4	5.4	5.6	5.5	42.7
	2	5.0	3.8	5.1	5.1	4.1	5.6	6.5	5.6	4.3	45.1
	3	2.4	3.9	4.4	4.9	5.4	-	5.8	5.4	4.5	36.7
Sum		9.6	11.0	13.5	15.9	13.9	12.0	17.7	16.6	14.3	
Mean		3.2	3.7	4.5	5.3	4.6	6.0	5.9	5.5	4.8	

Analysis of Variance.

Source.	df	Sum square	Mean square	F test
Time	2	13.89	6.95	12.64**
Strain	2	0.57	0.29	NS
Interaction	4	6.30	1.58	NS
Days	2	0.79	0.40	NS
Error	15	8.20	0.55	
Total	25	29.75		

Comparison between means: $D_{.05}$ (time) = 0.906. $D_{.01}$ (time) = 1.192

TABLE 26. Semen collection techniques.

A. Semen volume in millilitres averaged over two collections per treatment. Factors: method (2) x strain (2) x phase (2); 10 cockerel replications.

Method	A				B			
Strain	ML		AO		ML		AO	
Phase	Norm.	Revd.	Norm.	Revd.	Norm.	Revd.	Norm.	Revd.
Reps 1	.38	0	.23	.50	.38	.83	.48	.73
2	.28	0	.75	.20	.30	.48	.33	.80
3	.35	0	.65	.45	.18	.83	.68	.85
4	.48	.03	.60	.95	.40	1.28	.50	.55
5	.45	.03	.50	.30	.63	.70	.43	.35
6	.35	.03	.55	.23	.35	.80	.43	.75
7	.40	.03	1.15	.60	.55	.90	.53	1.23
8	.50	.05	.53	.50	.45	.55	.58	.70
9	.43	.10	.63	.03	.30	.58	.38	1.00
10	.38	.18	.45	.63	.08	.55	.55	.48
Sum	4.00	0.45	6.04	4.39	3.62	7.50	4.89	7.44

Analysis of Variance.

Source	df	Sum square	Mean square	F test
Method (M)	1	0.92 ,	0.92	28.75**
Strain (S)	1	0.65	0.65	20.31**
Phase (P)	1	0.02	0.02	NS
MS	1	0.28	0.28	8.75**
MP	1	1.69	1.69	52.81**
SP	1	0		NS
SPM	1	0.14	0.14	4.38*
Replications	9	0.59		
Error	63	2.01	0.032	
Total	79	6.30		

Comparison between means.

Strain, method, or phase:	$D_{.05}$	=	0.080
	$D_{.01}$	=	0.106
MP, MS	$D_{.05}$	=	0.150
	$D_{.01}$	=	0.184
MPS	$D_{.05}$	=	0.251
	$D_{.01}$	=	0.332

Continued.....

TABLE 26 Continued.....

B. Average spermatozoa concentration in 10^9/ml. of ten cockerels per subgroup. Factors: method (2) x strain (2) x phase (2); 2 day replications.

Method	A				B			
Strain	ML		AO		ML		AO	
Phase	N	R	N	R	N	R	N	R
Rep 1	4.3	3.7	5.3	4.5	4.2	4.2	5.0	6.1
2	4.2	3.0	4.6	3.3	4.1	4.0	4.2	4.3
Sum	8.5	6.7	9.9	7.8	8.3	8.2	9.2	10.4

Analysis of Variance.

Source	df	Sum square	Mean square	F test
Method (M)	1	0.64	0.64	NS
Strain (S)	1	1.96	1.96	11.26*
Phase (P)	1	0.49	0.49	NS
MS	1	0.03	0.03	NS
MP	1	1.57	1.57	9.02*
SP	1	0.07	0.07	NS
SPM	1	0.14	0.14	NS
Replications	1	1.96		
Error	7	1.22	0.174	
Total	15	8.08		

Comparison between means:

$D_{.05}$ (strain) = 0.494

$D_{.05}$ (MP) = 0.978

TABLE 27. Duration of fertility from one insemination.

Percent fertile eggs lay daily; 15 pullets per strain, control 10 pullets, inseminated weekly.

Strain	WC	AO	Mixed Control
Day after AI			
1	0	0	0
2	82	82	71
3	100	86	80
4	100	96	82
5	93	80	83
6	100	78	100
7	89	75	67
8	82	80	75
9	92	38	100
10	68	38	100
11	55	20	100
12	40	40	80
13	46	18	89
14	23	0	100
15	9	13	88
16	0	0	85
17	10	0	83
18	0	0	85
19	10	0	88
20	0	0	88
Sum	999	744	1644
Mean	49.95	37.20	82.20

Correlation of daily fertility between strains

$r = +0.926^{**}$

Difference between strain fertility.

$t = 3.82^{**}$

TABLE 28. Repeatability of fertility

Percent fertile eggs layed over sixteen days with two inseminations.
Factors: 6 males; 2 fertility trials of the same mated cocks and hens.

Trial	1 (SEPT.)	2 (DEC.)
Male 67	100	71
No.	78	88
	83	58
	50	64
	67	63
	100	94
	80	81
	100	100
	100	86
	89	71
109	62	79
	64	29
	46	36
	70	50
	50	8
	75	75
	60	53
	67	36
	44	67
	100	50
	45	75
114	67	80
	100	100
	38	86
	67	94
	100	93
	92	93
	100	36
	67	50
	55	93
	90	100
	36	100
117	45	21
	50	27
	88	27
	100	67
	40	43
	67	0
	50	0
	100	70
	100	30
	20	55
	10	73
	82	71
	91	81
	67	21
108	0	0
30	100	100
Sum	3,352	2,945
Mean	69.83	61.35

Gross correlation of fertility in two trials; $r = + 0.3614^*$

Analysis of Variance (transformed percentages).

Source	df.	SS	MS	F test
Month	1	1,406	1,406	(P 0.10)
Error	94	46,871	499	
Total	95	48,277		

Average percentage fertility

		Sept.	Dec.
Male	67	84.7	77.6
	109	62.1	50.7
	114	72.9	84.1
	117	65.0	41.9
	108	0	0
	30	100	100

TABLE 29. Aging and seasonal effects on semen production.

A. Semen volume in millilitres of twelve males tested November 1966 and May 1967.

Year	1966	1967
Male 1	.10	.10
2	.90	.50
3	.55	.50
4	.25	.25
5	.25	.15
6	.55	.15
7	.15	.20
8	.30	.15
9	.65	.25
10	.45	.20
11	.85	.30
12	.70	.70
Sum	5.70	3.45
Mean	0.4750	0.2875

Correlation between semen volumes
r = + 0.646*

B. Spermatozoa concentration in 10^9/ml.

Year	1966	1967
Male 1	4.40	6.06
2	4.50	3.94
3	6.30	4.10
4	5.40	5.52
5	7.10	4.48
6	4.40	2.27
7	1.80	2.48
8	4.20	2.02
9	1.70	1.80
10	3.30	4.60
11	3.60	3.28
12	0.70	0.05
Sum	47.40	40.60
Mean	3.950	3.383

Correlation between spermatozoa concentrations.
r = + 0.684 *

TABLE 30. Ageing effects on semen production.

A. Semen volume in millilitres, of four collections from one male within three hours.

Year	1966	1967
Hour 1400	.45	.70
1500	.35	.35
1555	.30	.50
1650	.20	.30
Sum	1.30	1.85
Mean	0.3250	0.4625

Correlation of semen volumes
r = + 0.8242 (NS)

B. Spermatozoa concentration in 10^9/ml. of four collections from one male within three hours.

Year	1966	1967
Hour 1400	1.32	1.34
1500	.92	.51
1555	.46	.13
1650	.50	.05
Sum	3.20	2.03
Mean	0.80	0.51

Correlation of spermatozoa concentrations.
r = + 0.9762*
Significant ($P < 0.05$) decline in mean spermatozoa concentration.

TABLE 31. Age of male and semen fertilising capacity.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factors: age of male (2) x freshness of semen (2); 10 pullet replications.

Treatment	1	2	3	4
Storage (hours)	$2\frac{3}{4}$		22	
Males	Ckl.	Cock	Ckl.	Cock
Reps 1	100	40	17	0
2	100	38	20	0
3	100	67	0	0
4	100	100	60	0
5	100	33	-	0
6	100	50	0	0
7	80	0	0	0
8	100	67	0	17
9	80	83	50	-
10	83	67	0	20
Sum	943	545	147	37
Mean	94.3	54.5	16.3	4.1
Fert. eggs	47	28	8	2
Total eggs	50	55	51	51
Trfd. mean	82.3	47.8	16.3	5.7

CONTINUED.....

TABLE 31 Continued.....

Analysis of variance. (transformed percentages)

Source	df	Sum square	Mean square	F test
Storage	1	27,652	27,652	116.2**
Males	1	5,106	5,106	21.5**
Interaction	1	1,344	1,344	5.7*
Replications	9	4,850	539	NS
Error	25	5,956	238	
Total	37	44,909		

Comparison between means.

Storage and males ; $D_{.05}$ = 10.34

$D_{.01}$ = 14.02

S M ; $D_{.05}$ = 19.54

TABLE 32. Methods of detecting fertile eggs.

Percent fertile eggs layed on 2nd to 9th day after insemination.
Factor: methods (2); 16 pullet replications.

Method	Candling	Breakout
Pullet 1	100	100
2	50	33
3	50	100
4	50	100
5	67	100
6	67	100
7	100	67
8	100	100
9	100	100
10	67	67
11	100	100
12	100	100
13	100	100
14	100	100
15	100	100
16	80	100
Sum	1,331	1,467
Mean	83.2	91.7
Fert. eggs	43	44
Total eggs	54	52

No significant difference between the methods of detecting fertility, with transformed data.

TABLE 33. Accuracy of spermatozoa concentration estimates from haemacytometer counts.

Number of spermatozoa counted in two determinations from five randomly selected squares.
Factors: male (4) x samples from ejaculate (4); 10 determinations, each comprising the number of spermatozoa in four sub-squares.

Male No.		58 (ML)				61 (ML)				83 (AO)				84 (AO)			
Sample		1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Detn.	1	7	12	7	13	18	15	16	16	24	16	23	16	16	10	17	23
	2	15	4	12	17	24	16	22	15	27	16	33	19	20	14	12	24
	3	4	4	7	11	19	14	15	11	21	21	23	22	17	15	14	29
	4	9	11	7	12	16	24	17	18	21	13	26	23	5	15	22	24
	5	6	12	12	12	26	18	23	16	20	23	30	20	9	20	25	20
	6	12	15	10	14	30	16	17	17	20	17	28	21	14	19	14	22
	7	12	16	18	11	21	18	17	15	26	16	33	18	7	15	17	18
	8	9	12	15	10	22	28	20	43	20	21	29	20	19	18	10	29
	9	11	10	11	16	32	21	28	21	34	10	25	13	15	19	19	36
	10	8	13	6	15	18	20	14	18	17	22	31	21	26	18	14	22
Sum		93	109	105	131	226	190	189	190	230	175	281	193	148	163	164	247
Mean		9.3	10.9	10.5	13.1	22.6	19.0	18.9	19.0	23.0	17.5	28.1	19.3	14.8	16.3	16.4	24.7

Analysis of Variance.

Source.	df	Sum square	Mean square	F test
Males	3	2,748	916	7.63**
Samples (W. males)	12	1,438	120	5.45**
Determinations	144	3,205	22	
Total	159	7,391		

Comparison between sample means.

$D_{.05} = 5.46$
$D_{.01} = 6.66$

TABLE 34. Centrifugation effects on spermatozoa function.

Percent fertile eggs from 2nd to 8th day after insemination.
Factor: methods (3); 10 pullet replications.

Methods	Buffer	Remix	Control
Rep 1	83	83	100
2	100	100	33
3	100	80	100
4	100	100	71
5	80	83	100
6	83	80	100
7	100	100	80
8	-	86	0
9	100	100	60
10	100	67	80
Sum	846	879	724
Mean	94.0	87.9	72.4
Fert. eggs	37	45	41
Total eggs	40	52	57
Trfd mean	81.6	74.1	63.0

Analysis of Variance. (transformed percentages)

Source	df.	Sum square	Mean square	F test
Method	2	1,678	839	NS
Error	26	10,892	419	
Total	28	12,570		

TABLE 35. Comparing fresh and stored Tyrode solution for dilution.

Percent fertile eggs layed on 2nd to 8th day after insemination.
Factor: age of Tyrode (2); 10 pullet replications.

Storage	22 wks.	2 wks.
Rep 1	83	50
2	-	100
3	75	67
4	100	0
5	86	100
6	60	83
7	100	75
8	100	-
9	33	100
10	100	67
Sum	737	642
Mean	81.9	71.3
Fert. eggs	41	35
Total eggs	50	50
Trfd. mean	71.1	61.2

Analysis of Variance. (transformed percentages)

Source	df	Sum square	Mean square	F test
Storage	1	441	441	NS
Error	16	9,896	619	
Total	17	10,337		

TABLE 36. Semen volume (1966).

Semen volume in millilitres of each experimental cockerel, averaged per month.

Month		Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Avge.	Total	Col'ns.
Strain	Male										
WC	145	.33	.58	.36	.38	.49	.51	.29	0.405	22.65	56
	146	.33	.45	.27	.47	.46	.43	.37	0.391	21.50	55
	147	.30	.33	.27	.25	.23	.11	.12	0.208	11.65	56
	148	.25	.39	.22	.25	.44	.24	.23	0.291	15.70	54
	149	.20	.35	.22	.18	.20	.14	.17	0.211	11.40	54
	150	.60	.76	.43	.47	.61	.57	.53	0.572	25.35	44
	151	.30	.44	.36	.36	.45	.39	.30	0.385	16.55	43
	152	.15	.29	.18	.23	.39	.29	.33	0.275	11.10	40
	153	1.03	.83	.83	1.00	1.19	1.11	1.08	0.976	41.00	42
	154	.73	.67	.51	.58	.60	.41	.43	0.566	24.35	43
	155	.60	.61	.42	.44	-	-	-	0.495	13.85	28
	156	.65	.71	.59	.56	.73	.64	.53	0.638	26.15	41
	157	.41	.49	.23	.33	.51	.48	.58	0.424	17.80	42
	158	.48	.69	.50	.48	.64	.61	.50	0.572	23.45	41
	159	.30	.37	.14	.01	.25	.20	.20	0.220	9.00	41
	160	.49	.74	.46	.58	.80	.61	.40	0.558	31.25	56
	161	.28	.42	.15	.18	.28	.28	.41	0.307	16.90	55
	162	.10	.29	.18	.28	.38	.30	.19	0.254	13.45	53
	163	.20	.34	.19	.18	.24	.23	.15	0.211	10.95	52
	164	.14	.31	.18	.18	.23	.16	.18	0.197	10.80	55
	175	.70	.63	.38	.41	.53	.53	.20	0.462	13.85	30
	176	.15	.42	.23	.23	.35	.30	.07	0.257	7.70	30
	177	.75	.66	.61	.67	.65	.73	.43	0.637	20.45	32
	178	.50	.71	.51	.44	.72	.62	.62	0.578	19.65	34
	179	.30	.38	.31	.29	.36	.23	.17	0.303	10.60	35
	180	-	.28	.25	.21	.23	.17	.13	0.224	6.50	29
	181	.48	.52	.41	.34	.41	.33	.25	0.387	13.55	35
	182	.40	.49	.35	.41	.54	.50	.53	0.449	14.80	33
	183	.38	.56	.39	.46	.38	.38	.33	0.424	13.15	31
	184	-	.57	.43	.50	.45	.46	.38	0.467	17.75	38
Mean		.42	.51	.32	.38	.47	.41	.35	0.411	17.03	43
Col'ns.		68	207	230	182	201	167	207			1278
ML	165	.65	.44	.27	.42	.34	.33	.56	0.411	19.75	48
	166	.25	.28	.17	.30	.35	.32	.44	0.280	11.50	41
	167	.43	.31	.15	.17	.28	.12	.09	0.211	8.45	40
	168	.43	.49	.29	.36	.35	.48	.45	0.382	16.05	42
	169	.15	.63	.36	.34	.28	.32	.34	0.381	14.10	37
	170	.38	.43	.21	.22	.11	.18	.28	0.261	10.95	37
	171	.48	.58	.35	.41	.49	.37	.43	0.429	15.45	36
	172	.48	.40	.30	.45	.54	.57	.59	0.425	20.40	48
	173	.50	.49	.32	.35	.50	.58	.53	0.436	17.45	40
	174	.30	.28	.19	.18	.30	.22	.21	0.224	7.85	35
Mean		.41	.43	.26	.32	.35	.35	.39	0.344	14.19	41
Col'ns.		22	91	110	78	41	30	40			409

Continued.....

TABLE 36 Continued.....

Month		Jun.	Jul.	Aug.	Sep.	Oct.	Nov.	Dec.	Avge.	Total	Col'ns.
Strain Male											
AO	185	.91	1.08	.78	.80	1.08	.98	.85	0.924	40.65	44
	186	.66	.82	.49	.49	.69	.43	.53	0.588	25.85	44
	187	.91	.77	.73	.77	.98	.91	1.00	0.832	43.25	52
	188	1.21	1.33	1.07	1.14	1.43	1.24	1.05	1.168	52.55	45
	189	.54	.67	.47	.67	.76	.69	.82	0.641	28.20	44
	190	.45	.72	.55	.64	.78	.62	.55	0.630	27.70	44
	191	1.08	1.09	.74	.77	1.38	1.17	1.41	1.063	44.65	42
	192	.61	.84	.58	.73	.93	.81	.85	0.757	31.80	42
	193	.53	.59	.49	.61	.79	.59	.53	0.595	25.60	43
	194	.68	.68	.51	.65	.71	.55	.40	0.594	24.95	42
	195	1.06	.94	.81	.95	1.17	.98	1.35	0.955	47.75	50
	196	.80	.92	.79	.83	1.06	1.10	.94	0.926	37.05	40
	197	.41	.62	.41	.46	.37	.32	.36	0.399	16.75	42
	198	.66	.92	.70	.62	.81	.79	.70	0.755	31.70	42
	199	.70	.92	.53	.63	.70	.61	.65	0.663	26.50	40
	200	.49	.78	.42	.58	.48	.41	.51	0.496	20.35	41
	201	.93	1.36	.88	.91	.84	.77	.85	0.920	36.80	40
	202	.64	.60	.54	.53	.69	.73	.59	0.617	26.55	43
	203	.55	.64	.34	.59	.64	.44	.33	0.498	19.90	40
	204	.15	.36	.38	.30	.10	.18	.21	0.244	10.00	41
Mean		.73	.82	.63	.71	.89	.76	.77	0.713	30.93	43
Col'ns.		80	138	188	95	160	122	76			861
Aggregate											
Mean		.51	.60	.42	.47	.57	.50	.48	0.501	21.19	42
Col'ns.		170	436	528	355	402	319	321			2548

TABLE 37. Estimates of spermatozoa concentration.

Haemacytometer counts in 10^9 spermatozoa/ml. ; percent packed cell volumes in centrifuged semen ; and subjective opacity scores of fresh semen.

Detns	Count	% PCV	Count	Score
1	0.5	2.7	3.8	9
2	1.0	3.0	2.2	5
3	1.0	3.3	2.7	6
4	1.3	4.9	1.5	5
5	1.4	4.9	1.7	7
6	1.9	4.7	1.9	8
7	1.6	6.1	2.2	7
8	2.2	6.4	2.5	7
9	2.4	8.2	2.6	8
10	3.7	8.5	4.2	8
11	2.6	8.7	2.0	6
12	2.4	8.9	3.5	7
13	3.6	8.9	3.4	8
14	3.1	9.0	2.7	4
15	3.0	9.1	3.9	8
16	3.5	9.1	1.9	7
17	2.9	9.9	2.7	6
18	3.9	9.9	5.6	8
19	3.4	10.0	3.8	9
20	3.8	10.2	3.6	6
21	3.0	10.3	.9	1
22	4.0	10.3	2.6	7
23	3.8	10.9	.9	1
24	4.6	10.9	2.6	7
25	4.4	11.5	.1	1
26	5.6	11.7	2.5	8
27	6.3	12.2	3.5	8
28	4.6	12.3	2.3	7
29	5.5	12.5	3.9	9
30	4.7	12.7	2.4	7
31	5.2	13.6	1.1	3
32	5.4	13.8	1.4	2
33	6.0	14.2	3.7	9
34	4.1	14.7	2.5	8
35	4.0	14.9	.4	5
36			4.6	9
37			2.5	5
38			2.2	6
39			3.0	10
40			2.5	8
41			3.3	9
42			4.1	9
43			3.0	8
44			3.3	8
45			3.3	9
46			5.2	10

Continued.....

TABLE 37 Continued.....

Detns.	Count	% PCV	Count	Score
47			1.2	4
48			.9	3
49			4.4	8
50			2.9	9
51			2.7	8
52			5.1	10
Sum	120.4	332.9	143.4	355
Mean	3.44	9.51	2.76	6.83

b = 1.989 % PCV per 10^9 sp./ml.

t = 11.43**

Confidence limits ($P < 0.01$)

$1.5132 \leq \beta \leq 2.4646$ % PCV

b = 1.514 units score per 10^9 sp./ml.

t = 8.907**

Confidence limits ($P < 0.01$)

$1.0589 \leq \beta \leq 1.9695$ units score

TABLE 38. Relationship between semen release and collection characteristics.

Semen volume in millilitres; semen opacity score; stimulation grade and libido; of ten WC yearling cocks undergoing daily semen collection for two weeks.

Detn.	Volume	Score	Stimul'n.	Libido	Detn.	Volume	Score	Stimul'n.	Libido
1	.30	10	1	1	44	.05	7	2	3
2	.40	8	1	2	45	.15	10	1	3
3	.20	8	2	2	46	.35	4	1	3
4	.05	8	2	3	47	.25	7	1	3
5	.20	10	2	4	48	.20	8	2	4
6	.80	4	1	3	49	.15	7	2	3
7	.40	8	2	3	50	.10	10	1	3
8	.30	10	2	4	51	.35	4	1	3
9	.20	7	2	3	52	.60	7	1	3
10	.15	11	1	4	53	.15	6	1	4
11	.30	9	1	1	54	.15	7	1	2
12	.40	8	1	2	55	.15	9	1	3
13	.15	9	1	4	56	.30	6	1	3
14	.15	7	2	4	57	.70	8	1	3
15	.15	9	2	4	58	.10	9	2	4
16	.40	2	1	3	59	.10	9	2	3
17	.20	4	1	2	60	.15	11	1	4
18	.20	9	1	5	61	.20	10	1	2
19	.15	10	1	3	62	.45	9	1	3
20	.15	11	1	3	63	.15	9	2	4
21	.30	5	1	1	64	.15	7	1	4
22	.40	8	1	1	65	.20	9	1	4
23	.05	9	2	3	66	.20	9	1	1
24	.20	7	1	3	67	.20	8	1	3
25	.10	9	1	4	68	.20	8	1	4
26	.40	3	1	3	69	.25	8	2	3
27	.40	5	2	3	70.	.20	8	1	4
28	.10	6	2	4	71	.30	11	1	2
29	.20	6	2	2	72	.35	10	1	3
30	.15	9	1	3	73	.20	9	1	4
31	.40	5	1	3	74	.40	6	1	4
32	.40	9	1	3	75	.05	9	1	4
33	.15	9	1	4	76	.20	9	1	1
34	.10	8	1	2	77	.30	9	1	2
35	.20	10	1	4	78	.10	10	1	4
36	.30	3	1	3	79	.30	0	1	3
37	.35	7	1	3	80	.15	10	1	4
38	.15	8	2	4	81	.20	10	1	2
39	.10	9	2	3	82	.30	9	1	2
40	.15	10	1	1	83	.05	8	1	4
41	.40	4	1	3	84	.15	9	2	3
42	.20	8	1	3	85	.20	8	1	4
43	.15	8	2	4	Mean	.24	7.97	1.27	3.05

Correlations.

Vol x Score	=	-0.3714**
Vol x Stimul'n	=	-0.3065**
Vol x Libido	=	-0.2631
Stim x Libido	=	+0.2140

APPENDIX B

Diluent Composition.

-per litre H_2O-

LAKE'S:

19.20 gm monohydrous sodium glutamate.
1.28 gm monohydrous potassium citrate.
5.13 gm sodium acetate.
0.676 gm magnesium chloride.$6H_2O$.
10.00 gm fructose.

(FPD = -0.59°C)

* * *

LOCKE'S:

9.20 gm sodium chloride.
0.42 gm potassium chloride.
0.12 gm calcium chloride.
0.15 gm sodium bicarbonate.
1.00 gm glucose.

(FPD = -0.64°C ; pH = 8.2)

* * *

TYRODE:

8.00 gm sodium chloride.
0.20 gm anhydrous calcium chloride.
0.20 gm potassium chloride.
1.00 gm sodium bicarbonate.
0.10 gm magnesium chloride.
0.05 gm NaH_2PO_4 .

(FPD = -0.65°C)

* * *

SODIUM-PHOSPHATE BUFFER:

16.34 gm Na_2HPO_4 .
5.16 gm NaH_2PO_4 . H_2O .

(FPD = -0.607 to -0.639°C ; pH = 7.2)

* * *

MILK:

(FPD = -0.54°C ; pH = 6.6)

* * *

APPENDIX C

EXPERIMENT 1 - RATION ANALYSIS.

I BASAL:

55%	wheatmeal
12.5%	barleymeal
11.5%	wheat pollard
8%	bran
5%	lucerne
4.5%	oyster shell
1.5%	buttermilk powder
0.5%	salt
1.5%	stone grit
0.6	oz per 100lb selenium premix
2.0	oz per 100lb Trelcovite
10.0	mg per lb vitamin E
20.0	mg per lb $MnSO_4$
	Santoquin (antioxidant)

(C/P = 78 ; 13.1% crude protein ; 1150 Cal ME/lb)

II AMINO ACID ANALYSIS:

Acid	72% Basal	100% Casein	8% Casein	1% Casein
Arginine	0.58%	3.40%	0.85%	0.62%
Cystine	0.17	0.30	0.20	0.18
Glycine	0.56	1.50	0.68	0.57
Histidine	0.22	2.50	0.42	0.24
Isoleucine	0.43	5.70	0.89	0.48
Leucine	0.71	8.60	1.40	0.79
Lysine	0.39	7.00	0.95	0.46
Methionine	0.14	2.70	0.36	0.16
Phenylalanine	0.49	4.60	0.86	0.53
Threonine	0.31	3.80	0.61	0.36
Tryptophan	0.16	1.00	0.24	0.17
Tyrosine	0.30	4.70	0.68	0.34
Valine	0.47	6.80	1.01	0.54

III RATION COMPOSITION:

Treatment A;	1%	casein
	7%	sugar
	20%	sawdust
	72%	basal
Treatment B;	1%	casein
	27%	sugar
	72%	basal
Treatment C;	8%	casein
	20%	sawdust
	72%	basal

RATION COMPOSITION Continued.....

Treatment D; 8% casein
20% sugar
72% basal

IV CONSTITUENT ANALYSIS:

(per pound)

Wheat	14.0%	crude protein	1380	Calories ME
Barley	10.5%	"	1240	"
Pollard	16.0%	"	1060	"
Bran	25.0%	"	920	"
Lucerne	14.5%	"	500	"
Buttermilk	33.0%	"	1340	"

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