MAINTENANCE OF THE TESTIS OF

ACHETA DOMESTICUS (L.) *IN VITRO*.

by

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ABSTRACT

The *in vitro* maintenance of the testis of *Acheta domesticus* (Orthoptera) was attempted. Oxygen consumption measurements of incubated testes showed that they could be maintained alive for up to 48h in Grace's insect tissue culture medium. Average oxygen consumption during incubation was highest under an atmosphere of balanced air and 5% CO_2. Addition of 20-0H ecdysone (10^{-6}M) resulted in limited development of germ cells from spermatid to early spermatozoa. Combinations of ecdysone and blood or 20-OH ecdysone and Juvenile Hormone III failed to elicit any response. No significant difference in oxygen consumption was found between organs incubated in Grace's medium and Modified Grace's medium.

M.Sc. Entomologie

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LE MAINTIEN *IN VITRO* DES TESTICULES

DE L'ACHETA DOMESTICUS (L.).

RESUME

Le maintien *in vitro* des testicules de l'*Acheta domesticus* (Orthoptera) a été tenté. Les mesures de la consommation d'oxygène des testicules ont démontrées qu'elles pouvaient facilement être maintenues en vie jusqu'à 48h dans le milieu d'incubation de Grace. La consommation moyenne d'oxygène durant l'incubation a été la plus élevée sous un atmosphère composé d'air et de 5% CO_2. L'addition de 20-OH ecdysone (10^{-6}M) a stimulé un développement partiel de cellules germinales passant du stade de spermatide à celui de spermatozoïde primitif. L'addition au milieu d'une combinaison d'ecdysone et de sang ou de 20-OH ecdysone et d'hormone Juvénile III n'a provoquée aucune réponse. Aucune différence significative dans la consommation d'oxygène entre les organes incubés dans le milieu de Grace ou dans sa version modifiée n'a été observée.

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INTRODUCTION

Copper and vitamin E have been shown to be essential for the process of spermiogenesis in crickets (Meikle and McFarlane, 1965; McFarlane 1974). There are indications that vitamin E plays an anti-oxidant role for poly-unsaturated fatty acids and that copper interacts with vitamin E in growth and reproduction. Although the effects are known, the story behind their specific relationships and mode of action has not been assembled yet. This project was an attempt to determine the nature of these interactions and to study the effects of the morphogenetic hormones 20-OH ecdysone and juvenile hormone on spermatogenesis.

An attempt was made to establish for the first time *in vitro* spermatogenesis in an orthopteran testis.

Such a system permits control of the chemical environment to which the testis is exposed. Improper response or failure of *in vitro* systems to respond could involve the hormone, the target or both. Use of such a system, along with highly purified hormones, reduces or eliminates this uncertainty in bioassays. Furthermore *in vitro* systems permit investigation of tissular compartmentation and the highly specific micro-environment required by actively differentiating germ cells.

LITERATURE REVIEW

Insect cell and tissue culture methods were pioneered nearly seventy years ago (Goldschmidt, 1915; Trager, 1935), but remained undeveloped until the early sixties. Advancements occurred more readily where specific biochemical features of insect body fluids were taken into consideration (Echalier, 1980). Marks (1980) has reviewed insect tissue culture for the period 1971-1978 and provides starting points for entry into the older literature.

The majority of cell and tissue cultures are from Lepidoptera and Diptera and secondarily, from Orthoptera. The maintenance of organs *in vitro* has been primarily for endocrinological purposes, including effects of hormones on target tissues and hormone production by cultured glands (Marks, 1976). The development and function of gonads are influenced by the moulting and juvenile hormones and have been studied in vitro in several insects.

Goldschmidt's (1915) initial attempts to observe male germ cells were done with hanging drop cultures of cysts. Similar preparations have subsequently been used to observe meiosis in isolated male germ cells (ruptured tubule preparation).

Lewis and Robertson	1916	*Chorthippus curtipennis*
Belar	1929	*Chorthippus lineatus*
Baumgartner and Payne	1930	unspecified grasshopper cricket and beetle
Ris	1949	unspecified grasshopper
Levine	1966	*Acheta domesticus*
	1972	*Acheta domesticus*
Shimuzi and Yagi	1978	*Mamestra brassicae*
Dumser	1980a	*Locusta migratoria*

However in all these instances the germ cells were observed for only a few hours after their isolation and the development *in vitro* was clearly a continuation of processes already underway in the intact insect. The culture of intact testes has almost exclusively been done with Lepidopteran species:

Lender and Duveau-Hagege	1962	*Galleria mellonella*
	1963a,b	*Galleria mellonella*
Yagi, Kondo and Fukaya	1969	*Chilo suppressalis*
Kambysellis and Williams	1971a,b	*Samia cynthia*
	1971b	*Hyalophora cecropia*
	1972	*Samia cynthia*
Takeda	1972	*Monema flavescens*
Fukushima and Yagi	1975	*Mamestra brassicae*
		Spodoptera litura

Work on dipteran species has been done by:

Fowler	1973	*Drosophila hydei*
Fowler and Johannisson	1976	*Drosophila hydei*
Gould-Somero and Holland	1974	*Drosophila hydei*
Kuroda	1974	*Drosophila melanogaster*
Leloup	1976	*Calliphora*

The culture of orthopteran testes as a tool for studying development has not been attempted until now (this thesis).

Culture Media Composition

An explanted cell or organ must survive a usually hostile *in vitro* environment. The first medium that permitted long-term insect cell growth and maintenance of a healthy condition was developed by Wyatt in 1956. The medium has subsequently been altered and new media developed by many workers. Grace's (1962) modification is probably the best known. Most media have been devised to duplicate the main physico-chemical features of the insect's body fluid, although

this has often proved to be a poor guide to the nutritional requirements of insect cells (Vaughn, 1973). At least 35 formulations of invertebrate tissue culture media, most for use with insect material, are known (Hink, 1976).

Media are minimally composed of inorganic salts, organic acids, sugars, amino acids and water soluble vitamins.. Cholesterol is usually provided in the form of sera and the phospholipid precursors choline and inositol are added. Specific cells and tissues are known to have particular requirements for extracellular factors for survival *in vitro*. These requirements are generally filled by added serum but are often made available by the addition of various supplements including:

lactalbumin hydrolysate

bacteriological peptone

yeast extract

whole egg ultrafiltrate

bovine plasma albumin

fetal bovine serum

insect haemolymph.

Fetal bovine serum is the most common additive and may constitute up to 20% of the final medium. Media have also been prepared by combining components used for the growth of other cells both vertebrate and invertebrate (Mitsuhashi, 1968). The antibiotics penicillin and streptomycin are also commonly added.

Supplements can be used as an alternative to haemolymph and eliminate the haemolymph phenoloxidase reactions that produce toxic quinones. The use of supplements can also be undesirable. Some lots of serum contain a factor toxic to insect cells

(McIntosh *et al.* 1976). Contaminants such as mycoplasma may also be present (Hirumi, 1976) and serum is expensive. Quantitfication of cell nutritional requirements is impossible due to unidentified components. Serum free media have been successfully developed (Mitsuhashi and Grace 1969; Sohi 1973; Brooks *et al.* 1980). Such media have been used in the investigation of phagocytosis and invertebrate cellular immunity in vitro (Goodwin, 1976; Hink *et al.* 1977).

Functions of Media Components

Glucose seems to suffice as an energy source in media and no benefit for growth or survival is gained from the addition of the primary haemolymph sugar trehalose (Vaughn, 1973). The proportion of amino acids does not seem to be critical. The same holds true for the internal salt balance of cells (Vaughn, 1973). However, cell cultures have been shown to flourish where inorganic cation proportions resemble those found in haemolymph (Wyatt and Wyatt, 1976). Presumably a medium with salts balanced to reflect haemolymph concentrations will be least harmful to any tissue or organ in culture. Osmolality appears to be more important in cell and tissue cultures than ionic balance (Vaughn, 1973). It can be adjusted by altering the level of relatively inert ingredients, such as sucrose, in the media and the most practical way of determining an optimum pressure is to test each cell line over a range of osmolalities.

The organic acids of the Kreb's cycle remain free in insect haemolymph, in contrast to vertebrate blood. *In vivo*, they

are important in binding large amounts of inorganic ions as well as in their metabolic roles. Their functions *in vitro* are presumably the same (Vaughn, 1973).

Vitamin requirements are usually for the water soluble types particularly the B group and may be added to culture media directly or as yeast hydrolysate.

Some insect cell cultures are very sensitive to pH changes in the culture medium whereas others are not. Sohi (1980) has found at least for one lepidopteran cell line, that reduced growth rate and viability at high pH are not due to loss of nutrient in precipitates but due to pH *per se*.

Developmental Hormones in Organ Culture

The development and function of the gonads are known to be influenced by moulting and juvenile hormones and in some cases other factors. Effects differ between sexes and between orders (Marks, 1980).

Macromolecular Factor

In the early fifties, while trying to develop a bioassay for growth and development hormone (now known to be ecdysone), Schmidt and Williams (1953) discovered a macromolecular factor (MF) in the blood of both pupating larvae and developing silkworm adults that was indispensable for germ cell differentiation.

Kambysellis and Williams (1971a,b) carried out *in vitro* experiments to determine the relationship between MF and ecdysone. They defined a permissive role for ecdysone in the

transport across the testis sheath of blood borne MF. These authors also demonstrated a limited capacity for various bovine sera to duplicate the activity of MF. Essentially the same results have been found for the slug moth *Monema flavescens* (Takeda, 1972).

Landureau and Szollosi (1974) have 'confirmed' the existence of MF proposed by Kambysellis and Williams (1971a,b) by showing that *Periplaneta americana* (Dictuoptera) hemocytes release into culture medium a factor which is capable of inducing *in vitro* spermatogenic differentiation of germinal cysts of several Saturnid moth species. The latter result demonstrates that MF is not species specific. Landureau (1976) was unable to find MF activity in mammalian sera which Kiss and Williams (1976) reported to be in the beta globulin Cohn fraction III.

How MF acts to maintain spermatids and how it affects other stages in spermatogenesis are questions that presently are unanswered. What stimulates and regulates MF production by haemocytes and whether or not MF plays a role in germ cell division are futher interesting research questions (Davey, 1980).

Ecdysone

Moulting hormone is produced by the prothoracic gland and has been shown to have predominantly two components (Karlson, 1956; Chino, 1976). The major component is ecdysone and the second 20-OH ecdysone (B-ecdysone). Several other ecdysteroids have been isolated. While ecdysone was long considered to be the active moulting hormone, 20-OH has proved to be more effective in most bioassays (Richards, 1981). Marks in his 1980 review of

insect tissue culture notes that 20-OH ecdysone concentrations of 10^{-6}M to 10^{-7}M are sufficient to stimulate evagination of imaginal discs in most culture systems. Levels of 10^{-3}M appear to inhibit development of imaginal discs.

Oxidation of steroid prohormones to hormones, specifically the formation of 20-OH ecdysone from ecdysone by various tissues, has been known since 1972 (King, 1972). Recently a mitochondrial cytochrome P450 dependent monooxygenase system responsible for this reaction has been characterized from fat body (Hodgson, 1983). Steroid hydroxylation is not always mitochondrial however. The hydroxylation of ecdysone in the fat body and Malpighian tubules of *Locusta migratoria* is microsomal (Feyereisen, 1977 and 1978; Feyereisen and Durst, 1978). A microsomal cytochrome P450 carries out the reaction and phenobarbital accelerates it *in vivo*, presumably by induction of P450 (Feyereisen and Hoffman, 1977).

The testis itself may also be a source of 20-OH ecdysone. Loeb *et al.* (1982) provide evidence for this by showing that the sheath of the testis of larval *Heliothis virescens* secreted ecdysteroids into the culture medium.

Hoffman and Behrens (1982) have found high concentrations of ecdysone and 20-OH ecdysone in the testis and other tissues of adult male crickets. Koolman *et al.* (1979) found large amounts of 20-OH ecdysone in the testis of *Calliphora vicina*.

Yagi *et al.* (1969) were the first to demonstrate that diapausing *Hyalophora* spermatocysts could be made to undergo development in haemolymph free media with the addition of 20-OH ecdysone. In 1975, Fukushima and Yagi obtained results which

suggested that MF was not important for spermatogenesis in all insects. Testes of *Mamestra brassicae* and *Spodoptera litura* cultured in unsupplemented Grace's medium demonstrated initiation of spermatogenesis with addition of ecdysone but not 20-OH ecdysone. This suggested ecdysone alone could act to induce germ cell maturation.

The above discussion has not considered the mechanism of ecdysone action. This is examined below.

The testis maintains a level of differentiation consistent with the insect's somatic development; the testis neither continues its differentiation to inappropriate levels nor accumulates masses of spermatozoa. This argues strongly that there must exist rate regulating mechanisms or regulatory points within the developmental sequence.

Two control mechanisms in insect spermatogenesis have been theorized: control of sequential auto differentiation through division rate regulation and the 'stop-time' mechanism of specific autolysis of germ cells. Dumser (1980b) discusses these in detail and attempts to link them together.

The concept of sequential auto differentiation is one of progressive division linked differentiation of spermatogonia. It implies that the major hormones regulating somatic differentiation will be ineffective in abrupt reprogramming of spermatogonial differentiation. A further consequence of this hypothesis is that spermatocytes cannot be recruited from earlier stages in the spermatogonial division sequence. Dumser (1980b) cites observations on *Leucophaea maderae* and *Bombyx mori* in support of this concept. He also refers to direct

experimental evidence from work by himself and Davey (1975b) with *Rhodnius*. Allatectomy of 3rd instar larvae results in precocious adults with testes showing no germ cell more developed than the early spermatocyte stage. So it seems that while somatic elements of the testis differentiate to adult condition, spermatogonia remain tied to the division sequence.

Since the differentiation sequence appears to be defined and incapable of skipping steps, the size of the spermatocyte compartment at any given stage can be estimated mathematically (Dumser and Davey,1974). This requires a knowledge of cell cycle durations for spermatocytes and spermatogonial cells. Dumser (1980b) has calculated these values for *Acheta domesticus* from data obtained by Lyapunova and Zoslimovskaya (1973). There are 10 spermatogonial mitoses and the spermatogonial cell cycle duration is 24h. The spermatocyte cell cycle duration is 14 - 18 days. The large discrepancy between mitotic and meiotic durations is typical and reflects greater time spent in meiotic prophase. This filling of the spermatocyte compartment by the exponentially expanding spermatogonial population would occur at a level of 93-95% spermatocytes per total germ cells for *Acheta domesticus*. Thus the accumulation of spermatocytes during the pre-imaginal stadium is an unavoidable consequence of division kinetics and requires no specific block on the meiotic division (Dumser, 1980b).

Quantitative experimental evidence of increased division frequency in the presence of ecdysone has been obtained by Dumser and Davey (1975a) in *Rhodnius*. This suggests that sequential differentiation can be regulated by ecdysteroids. Dumser and

Hoffman (in preparation) are cited by Dumser (1980a) as having found acceleration of spermatogonial cells from G1 to S phase and G2 into M phase in cultured testes of *Locusta migratoria* with ecdysterone concentrations as low as 10^{-8}M. That is, ecdysterone effects are rate regulating rather than state regulating and result in an increase in division frequency and rate of differentiation. The rate of these divisions and hence the rate of the overall process can be accelerated by ecdysone but only in the relative absence of JH.

However, sequential differentiation will not account for static levels of differentiation during diapause. Evidence for a total block on germ cell divisions is lacking. Instead specific degeneration of the most advanced cohort of germ cells in the testis ensues. This is very common in all animals (Roosen-Runge, 1973) and in some insects can be clearly correlated with diapause or quiescence (names and references given on page 351 of Dumser, 1980b).

While ecdysteroids may stimulate division in a number of *in vivo* and *in vitro* situations in insect tissues, they may be equally inhibitory or inconsequential and should not be regarded as division hormones *per se*.

Higher Diptera, in contrast to Lepidoptera, have been shown to have cell differentiation and spermatogenesis proceed in cultured pupal and adult testes in the absence of ecdysteroids. In *Drosophila* and *Calliphora*, development of gonial cells and spermatocytes *in vitro* has been obtained, but RNA was not synthesized past the second meiotic division (Gould-Somero and Holland, 1974; Fowler, 1973; Fowler and Johannisson, 1976;

Kuroda, 1974; Leloup, 1976). However there is some contention with this supposed absence of ecdysteroids as in Diptera it has been shown that an ecdysone message can be retained even in the absence of ecdysone (Marks, 1976).

In vivo evidence favouring ecdysone stimulation in testicular development has been obtained by testis transplantation. Takeuchi (1969) showed maximum development of embryonic testes in last instar larvae of *Bombyx mori*, slight development in those implanted in newly pupated hosts and no development in the remainder of hosts, believed to be low in ecdysone titre.

Juvenile Hormone

The corpora allata was first confirmed as the source of juvenile hormone by detection of JH III in media used to culture this gland explanted from *Manduca sexta* (Judy *et al.* 1973).

Titre bioassays in hemimetabolous insects, such as those by Johnson and Hill (1973, 1975) with *Locusta*, have shown that in the penultimate larval instar there is an appreciable JH titre which declines from a peak about two-thirds of the way through the instar to a low level at larval-larval ecdysis. This low level occurs throughout most of the last instar. There is however a short peak preceding the larval-adult ecdysis when the titre levels reach about one-third of those of the preceding peak. Shaaya (1978) found a similar JH profile in the penultimate instar of *Periplaneta americana*.

JH has been shown to retard testicular growth. An

acceleration of testis development was demonstrated by Fukuda in 1944 with allatectomized larvae of *Bombyx mori*. A similar effect can be shown in *Leucophaea maderae*. On the other hand, implantation of active corpora allata in *Galleria mellonella* and *Bombyx mori* was shown to retard testicular growth. JH analogues have reduced testicular growth in *Ephestia kuhniella* when they are fed to the insect (Nowock, 1973) and when topically applied to *Rhodnius* (Dumser and Davey, 1974). Further work by Dumser and Davey (1975b) has shown that the natural hormone exerts its effect only on the accelerated division under ecdysterone influence and not upon baseline division activity maintained during quiescence in *Rhodnius*. Dumser (1980b) indicates that no other confirmations of this antagonistic effect are available, but similar results have been reported for variety of lepidopteran and dipteran larvae.

Dumser (1980b) also discusses three reports of the effect of JH on adult testes during reproductive diapause suggesting that the control mechanism may be different in the adult.

The Blood Germ Cell Barrier

Physiological evidence that a testicular barrier exists in insects first came from the finding that the luminal fluid of silkworm testis tubules differed in composition from the haemolymph (Michejda and Thiers, 1963). Szollosi and Marcaillou (1977) first demonstrated the presence of a blood germ cell barrier, analogous to the vertebrate blood testis barrier, in

Locusta migratoria.

They defined a barrier to be present along the sides and basal walls of the testis tubule by tracer (horseradish peroxidase) non-penetration. The tracer tight compartment (basal) in the *Locusta* tubule is located in the region where the germ cells enter into meiotic prophase down to where sperm are found. The basal compartment grows in size with developmental stage but in all cases the area containing dividing gonia and spermatocysts was found to be open (tracer penetrable).

Jones (1978) found that the formation of the insect blood testis barrier in *Schistocerca gregaria* was coincidental with moulting. Using advanced tracer techniques, Jones showed that testis incubation with 2×10^{-6}M ecdysone (threshold not investigated) produced premature barrier formation. The addition of JH had an antagonistic effect on the formation of the barrier. The germ cell barrier was found to appear in the third instar. The moult from 2nd to 3rd instar of course required an increase in ecdysone titre and a drop in JH titre.

Jones (1978) suggests that the balance of JH and ecdysone may be critical for the formation of the barrier and subsequent onset of meiosis. Given that macromolecular tracers do not enter the basal tubular areas, it is unlikely that hormones or other factors involved in the development of germ cells would do so either. They would probably have to pass through the cytoplasm of supporting cells. Jones hypothesizes that the permissive role of ecdysone defined by Kambysellis and Williams (1971a,b) is really the stimulation of formation of a membrane carrier molecule for macromolecular factor.

From the present evidence, it appears that the control of spermatogenesis is fairly complex. Testing of Jones' hypothesis is necessary before any definitive statement can be made.

Spermatogenesis

The form of the male reproductive system varies from species to species. Males of *Acheta domesticus* follow the basic plan. The spermatozoa are produced in the paired testes, containing clusters of tubules, each tubule delimited by its own epithelium and independently connected to the genital duct (Phillips, 1970). The spermatozoa leave the testis via the vas deferens and may be stored in the seminal vesicles, usually a simple dilation in each of the paired vasa efferentia.

Associated with the vas deferens in most species are mesodermal accessory glands (mesadenes) and their ducts. In crickets as well as in most other orthopteran males, the accessory glands become mature within 5-10 days after the imaginal moult (Hartman, 1971). They secrete proteinaceous substances used for spermatophore formation. Growth and function of the glands is usually controlled by the corpora allata and/or the neurosecretory cells of the pars intercerebralis (deWolfe and deLoof, 1973).

In *Acheta domesticus* the accessory glands are composed of blind tubules, the tubule cells each having well developed endoplasmic reticulum as might be expected for cells involved in protein synthesis (Kaulenas *et al*,. 1975). Ecdysteroids have been demonstrated in adult male *Gryllus bimaculatus* but

nothing is known of their effects on the accessory glands. Ecdysteroid peaks and spermatophore deposition do not correlate well, while ecdysteroid levels and oviposition appear to be linked in females (Hoffman and Behrens, 1982).

In a longitudinal section of a mature testis tubule, the entire process of spermatogenesis is visible, from the formation of the primary spermatogonia to the primary spermatocytes, through the first and second meiotic divisions which give rise to the spermatids which then differentiate to become spermatozoa (Engelmann, 1970). An illustration of this, prepared by the author, can be found in the results section (Figure 2).

In the apical region of the testis tubule are found embryonic tissues. The apex contains a single apical cell and one or more primary spermatogonia, the germ cells. The apical cell which is distinct from the germ cells, appears to have a nutritive role, as is suggested by its location and high metabolic rate.

The germ cells develop by synchronous division within a sac formed by a single layer of cells known as a cyst. The number of germ cells per tubule is determined during the course of embryonic development. The number of germ cells per tubule will have bearing upon total sperm productivity, as does the division frequency of such cells and the number of tubules per testis. The number of tubules per testis is species specific. The division frequency of germ cells is unknown for any species (Dumser, 1980a,b).

The number of spermatids per cyst is usually constant for a given species and generally equals 2^x where x is the number

of gonial divisions plus the two meiotic divisions (Phillips, 1974). As a result of incomplete cytokinesis, the products of late gonial and meiotic divisions in most male animals remain connected by a cytoplasmic bridge. Thus the cells of a cyst are functionally a synctium; they divide and develop in synchrony. In insects, the interconnected spermatids within a cyst remain aligned side by side in register throughout spermiogenesis so that a transverse section throughout the cyst cuts all the cells at the same level. In some insect species (a few Lepidoptera, the armored scale insect and the mealybug) the cyst is released from the testis intact and swims as a unit held together by a sheath of epithelial or extracellular material. In most species however, the cyst breaks open to release the sperm when it is mature (Phillips, 1974).

In most species of insects, gonial and meiotic divisions occur in pupal or larval stages of the life cycle. Therefore the imaginal testis contains only spermatids and spermatozoa.

Meiosis and much of spermatid differentiation occurs with the ordinary somatic histones present. In the very late stages of spermiogenesis in *Acheta domesticus* unusual proteins and their complements appear. Two of the proteins associated with late spermatids have been described by Tessier and Fallota (1973) and other protamine-like proteins are described by McMaster-Kaye and Kaye (1976). Elimination of all the histones must be accomplished at some stage before the sperm reaches the seminal vesicle. These protein changes seem to be able to occur despite the extreme condensation of chromatin.

Metabolic Aspects of Spermatogenesis

There is no information available on oxygen consumption of isolated insect testes. Consumption measurements are available for whole insects and some tissues, such as muscle, in handbooks of biological data. However the oxygen consumption of the mammalian testis has been examined in detail. The mammalian testis has an *in vivo* oxygen uptake that is comparable to other organs of the body but, as Setchell (1981) points out, is always on the verge of hypoxia due to its slow moving blood supply. No active transport of oxygen has been properly demonstrated and the seminiferous tubules are avascular, so developing germ cells within the tubules must rely upon oxygen diffusing through the walls of the tubules (Setchell, 1981). Germ cells in the insect testis tubule are likewise dependent upon the process of diffusion for their oxygen.

Table 1. Oxygen consumption rates for the testes of several mammals.

Animal	Method	Conditions	Rate µL O /mg/h	Author
Ram				
	Blood gas	in vivo	200	1
	Blood gas	in vivo	201	2
	Warburg	in vitro	40	3
Rabbit				
	Warburg	in vitro	51	4
	Warburg	in vitro	50	5
	Warburg	in vitro	31	6
Chicken				
	Warburg	in vitro	54	4
Rat				
	Warburg	in vitro	40	7

Author

1. Setchell and Waites 1964
2. Setchell and Hinks 1967
3. Zogg *et al.* 1968
4. Ewing *et al.* 1966
5. Ewing and Vandemark 1963
6. Ewing *et al.* 1964
7. Steinberger and Wagner 1961

[1]Original values (consumption per unit dry weight) have been converted to consumption per unit wet weight using using a 10% correction factor. Values have been truncated.

functional damage of subcellular membranes due to peroxidation of polyunsaturated fatty acids(PUFA).

A further effect of vitamin E deficiency in the cricket is reduction in size of the testis of last larval instars. It is in this developmental stage that spermiogenesis begins to occur (Meikle and McFarlane, 1965).

Many insects have a requirement for the PUFA linoleic acid. The house cricket is unusual in that it is capable of de novo synthesis of linoleic acid (Meikle and McFarlane, 1965; Blomquist *et al.* 1982). Males of *Acheta domesticus* reared on a diet deficient in PUFAs will grow well but without vitamin E in the diet, are sterile. Hence vitamin E is indispensable to spermatogenesis.

Linoleic acid supplementation (0.5- 0.8%) of the diet improves growth (Richtot and McFarlane, 1962; Meikle and McFarlane, 1965) but at 1% spermatogenesis is interfered with (Distler and McFarlane, 1981). This effect occurs in the presence of vitamin E at five times the minimum dietary requirement (17.2µg/g diet) for normal spermatogenesis on normal diets.

Copper has been shown to interact with vitamin E in growth and reproduction. Low levels of copper in the diet(0.66µg/g) result in preferential incorporation into the testis. Accumulation in the testis occurs to a level 16x that of the diet and 8x that found in the whole body (McFarlane, 1974). The penultimate testis apparently accumulates copper to a level of 26.5 +/- 3.2mg per 100g dry weight and the ultimate to a level of 32.5 +/- 0.4 mg per 100g dry weight (McFarlane, 1974). The

optimum concentration of copper in artificial diets was found to be between 2 and 10μg/g diet.

The oxidative enzymes leading to melanin formation contain copper. At low levels of dietary copper, last larval instar males do not form tyrosinase in their cuticle and become albino (but normally sclerotized) adults. The albino effect can be abolished by increasing the copper content of the diet (McFarlane, 1974).

Tri-o-cresyl phosphate is a vitamin E antagonist that has been found to alter the motility of spermathecal and spermatophoral sperm (Prevost and McFarlane, 1980). Adding vitamin E to the diet overcomes the effect indicating that vitamin E has a role in the maintenance of sperm motility. In this regard it is interesting to note that peroxidation of fowl sperm lipid has been shown to inhibit motility (Fujihari and Howarth, 1978).

Prostaglandins were first identified in insects by Destaphano *et al.* (1974) working with *Acheta domesticus*. They have since been found in *Bombyx mori* (Setty and Ramaiah, 1979) and *Teleogryllus commodus* (Loher *et al.* 1981).

A PG synthetase complex analogous to the vertebrate one has been found in the testes, seminal vesicles and spermatophore of male *Acheta domesticus* (rigorous proof lacking). It has not been detected in the bursa copulatrix, spermatheca, spermathecal canal or oviduct of virgin females, but is found in these same tissues from mated females (Destephano, 1978). Thus the female appears to receive the enzyme from the spermatophore. Loher *et al.* (1981) have found the same situation in the Australian

field cricket, *Teleogryllus commodus*.

The spermatophore of *T. commodus* contains in addition to the PG synthetase complex, substantial quantities of arachidonic acid. This prostaglandin precursor is presumably present in a form unavailable to the synthetase complex as no prostaglandins are detectable in the spermatophore (Stanley-Samuelson and Loher, 1983). Whether arachidonic acid is present in the spermatophore of *Acheta domesticus* has not yet been investigated.

Destephano and Brady (1977) found a PGE_2 like substance in the reproductive tissues of mated females, the testes and male reproductive tract of *Acheta domesticus*. These authors also showed PGE_2 to augment egg production in the female. PGE_2 has an effect on rabbit sperm motility (Spilman *et al*, 1973) and McFarlane (1983) believes it may also have such an effect in *Acheta domesticus*.

Murtaugh and Denlinger (1982) have confirmed Destephano and Brady's (1977) finding of PGE_2 at high levels in the testis of *Acheta domesticus*. The levels also appear to increase as the male develops from the penultimate instar to a mature adult. The levels of PGE_2 change in a manner that appears similar to the increasing proportion of mature germ cells in testis tubules (McMaster-Kaye and Kaye, 1976) found with more advanced developmental stages.

In summary, at this point in time vitamin E in the house cricket appears to be important because of its antioxidant effect on the PUFA's linoleic and (possibly) arachidonic. It also appears to enhance sperm motility. Copper, a promoter of peroxidation, accumulates in the testis and may serve to increase

availability of precursors for the synthesis of PGE_2 and other prostaglandins. In mammals, PG's seem to modulate the action of hormones rather than act as hormones. A modulating effect by PGE_2 on the insect morphogenetic hormones would not be surprising. However, the nature of the relationship between all of the above factors remains incompletely known.

MATERIALS AND METHODS

a) Stock Cricket Culture

Insects used in the experimental procedures were obtained from a permanent colony maintained in the Department of Entomology at Macdonald College. The procedures for maintaining the colony followed the methods of Ghouri and McFarlane (1958).

Crickets were reared on a diet of pulverized and non-pulverized Ralston Purina rabbit pellet chow. Fifty to one hundred insects per 3.8 L jar were incubated at 30 +/-2°C and 55 +/-5% r.h. Humidity was maintained using trays of saturated $Ca(NO_3)_2.4H_2O$ solution. A photoperiod of 14L:10D was used. Extra surface area within each jar was provided by crumpled paper towels and distilled water was supplied using 50mL glass vials plugged with absorbent cotton.

Eggs were obtained by placing paper serving portion cups (Lily brand) filled with wet sand in jars containing gravid females for a 24h period. During this period water vials were removed to prevent oviposition in the vials. Egg cups were removed at the end of the 24h period and wrapped loosely with a paper towel. A small pad of wet absorbent cotton was placed in a 475 mL ointment jar to maintain 100% humidity. The wrapped egg cup was added and the jar sealed with a tight fitting lid. Excess moisture condensed on the sides of the jar was removed along with the cotton pad on the seventh day of incubation. Eggs hatched on day 10-12 of incubation and larvae were transferred to the 3.8L jars as described above.

b) Experimental Insects

Male crickets of the desired developmental stage were removed from the stock culture as necessary. Penultimate crickets were identified by the presence of small wing pads and ultimate crickets by their intermediate size wing pads. Adults have unmistakable fully developed wings. Males are distinguished from females by lack of an ovipositor.

c) Dissection of Testes

The entire dissection procedure and transfer to culture vessel was carried out in a laminar flow hood (Pure Air, California). The insects were surface sterilized by submersion in 0.05% $HgCl_2$ in 50% ethanol for three minutes, followed by three rinses (20mL) in sterile distilled water (Kambysellis and Williams, 1971a). The insect was then transferred to a wax-filled dissecting tray that had been sterilized for 3 minutes with 70% ethanol and rinsed 3 times with sterile Belar's Ringer.

The insect was pinned, ventral side up, through both sides of the thorax at the level of the third coxae. With the aid of a stereo dissecting microscope, fine scissors, and No. 7 curved forceps (Irex, Toronto) a longitudinal slit was made from the fourth abdominal segment to the thorax. The testis was then squeezed out of the body with mild pressure on the ventral abdomen. The trachea, fat body and other elements binding the testis were severed. Oberlander (1976) has shown that even trace amounts of fat body cells or their products in culture media can modify hormonal response. The procedure was repeated for the other testis. The testes were then rinsed 3 times in sterile

Belar's Ringer. Finally they were transferred to a culture vessel containing selected media. One millilitre of media was used for incubation in all trials. All glassware used in the above procedure were steam sterilized and instruments were flame sterilized with 70% ethanol immediately prior to use.

d) Culture Vessels

Three types of culture vessels were used and are illustrated in Figure 1.

The first, referred to as a chamber, was constructed by cementing (Silicone Sealant, Dow Corning) 18 mm i.d. glass rings, in pairs, onto standard microscope slides. The glass rings were cut from glass tubing. After curing for at least 24h, the chambers were boiled for 20 minutes in a basic solution (10g $NaHCO_3$/100mL water) to neutralize the acetic acid component of the silicone seal. Chambers were then wrapped in aluminum foil and steam sterilized. Repeated use required only washing, re-wrapping and sterilizing.

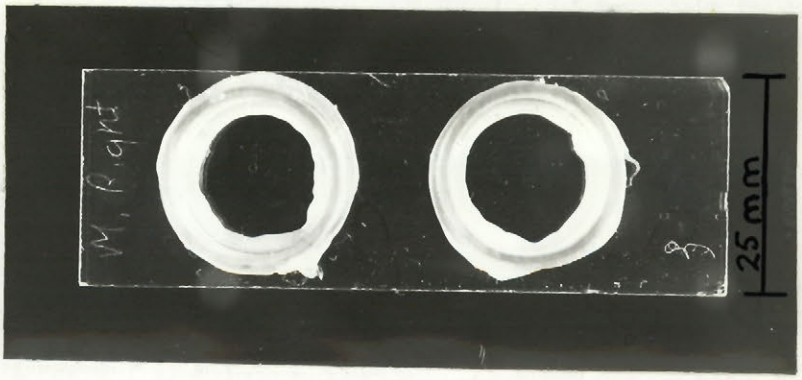
The second culture vessel, referred to as a flask, is similar to commercially available tissue culture flasks. Instead of a screw cap, a rubber stopper was employed. The stopper was drilled to accomodate glass capillary tubes, bent to the appropriate shaped with the aid of a Bunsen burner. The capillary tubes were installed to permit forced ventilation of the flask with air or gas mixtures. The apparatus for providing the ventilation is described below. The capillary tubes were fixed in place with Silicone Sealant. The capillary tubes were

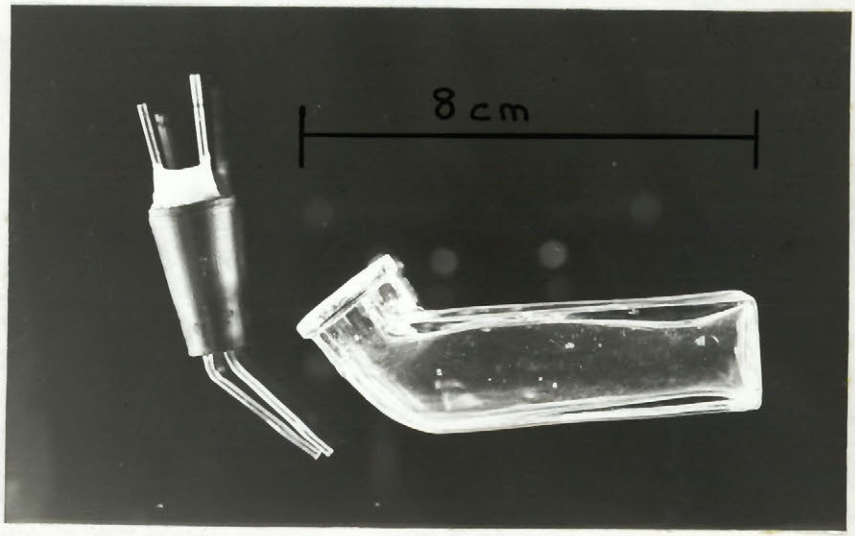
FIGURE 1. Vessels Used for the Incubation of Testes in Experiments

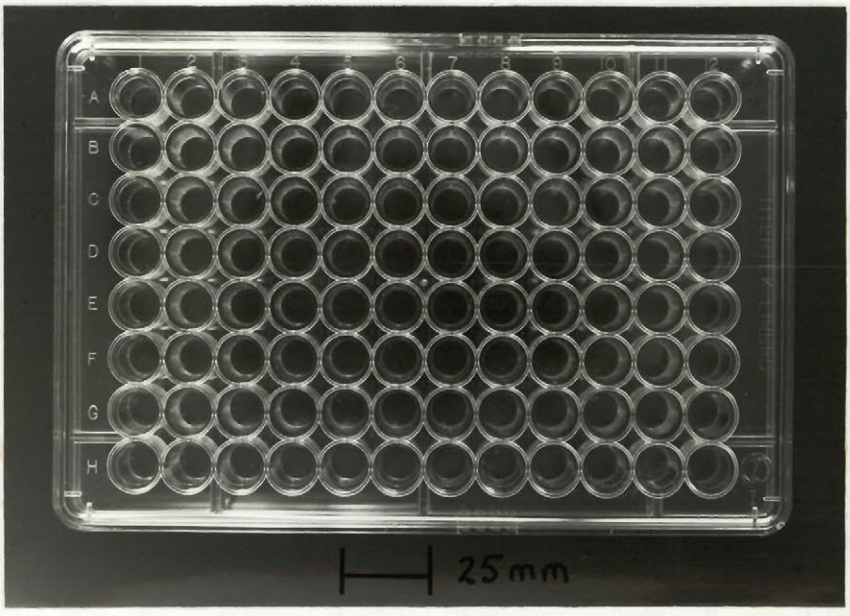
i) Chamber

ii) Flask

iii) Multi-well plate


W. Right
25 mm


8 cm


A
B
C
D
E
F
G
H
25 mm

cut short enough to permit insertion of the assembled stopper unit into the canted neck of the flask. With the stopper inserted loosely, the flask assembly was wrapped in aluminum foil and autoclaved.

It was anticipated that organs incubated in the first type of vessel (foil wrapped chambers) might be subject to anoxic conditions. Therefore flasks were constructed to permit replenishment of the contained air while minimizing the possibility for contamination of the incubation fluid. The large surface area inside the flask maximized diffusion of oxygen into the incubation medium. One millilitre of incubation fluid was used and this marginally covered the testis thereby minimizing the diffusion distance. A gas mixture of balanced air and 5% CO_2 was used for ventilation. Bowers (1961) found a similar mixture to improve the development of isolated spermatocysts in culture. The gas mixture was supplied from a tank with a two stage regulator at a delivery pressure of 35 kPa. Gas flowed through a sterile glass wool filter and then, in order to humidify it , was bubbled from an aquarium stone immersed in sterile distilled water. Subsequently gas was routed through a water trap to prevent blockage by condensation and finally into a Matheson Type 602 flow regulator. The regulator outflow was divided twice to provide four outlets, composed of No. 21 gauge sterile hypodermic needles attached to rubber hosing. The needles were inserted into the capillary tubes for ventilation of the flasks. The flow regulator was adjusted to provide a rate of flow resulting in one volume change every 2 minutes per flask.

The third type of chamber was a commercially available multi-well microtitration plate from Flow Laboratories (Inc.). Wells were 0.25 mL capacity and the plates were supplied pre-sterilized. The cover fitted in such a manner as to allow air circulation over the wells.

e) Preparation of Media

Several different media were employed in the incubation of the testes. They are briefly discussed below while complete formulations can be found in the appendix.

i) Mitsuhashi's CSM-2F Medium

This medium was developed for the culture of haemocytes of the rice stem borer, *Chilo suppressalis* (Lepidoptera: Pyralidae). It has subsequently been used for studies of spermatogenesis in this insect and *Monema flavescens* (Takeda, 1972a). The medium was prepared as described by Mitsuhashi (1968) and contains several supplements as well as TC-199, a defined artificial medium used in vertebrate cell culture. Use of this medium was curtailed early on due to time consuming preparation and difficulty in maintaining sterility with the various powdered components.

ii) Marks' M-20 Medium

This medium was developed by Marks (1973) for the long term (90 days plus) maintenance and growth of grasshopper and cockroach embryonic cells. The M-20 medium lacks fructose, phenol red, sodium acetate and ascorbic acid as compared with its forerunner M-14. This author prepared

the M-20 medium as described in the Grand Island Biological Company (Gibco) Technical Brochure (1980). This medium was also abandoned as testes incubated in it ruptured within 24h or became extremely fragile. This effect is believed to have been due to osmotic shock. The medium had an osmolar potential of 295 mOsm as measured with a Osmette S machine (Precision Systems Inc.). This potential is considerably lower than any value recorded by Woodring and Blakeney (1980) in their work on cricket blood. Blood osmolality ranged from 348 to 378 mOsm for penultimate females and 364 to 374 mOsm for ultimate females during the first six days after moulting to the respective stage. No figures are available for male crickets.

iii) Grace's Insect Tissue Culture Medium

This medium was purchased commercially (Gibco) with and without supplements added. Medium with supplements added by the manufacturer is referred to as Modified Grace's. Grace's medium was originally described in 1962 (Grace, 1962) and was used in the establishment of four strains of cells from ovarian tissue of the lepidopteran *Antherea eucalypti*.

iv) Sterilization of Media

Media prepared by the author with proteinaceous supplements proved impossible to filter sterilize (0.45 u pore size) under vacuum or pressure due to foaming or an excessive pressure requirement respectively. Therefore

media were sterilized and the supplements added aseptically afterwards. All media were dated and stored under refrigeration until use.

v) Antibiotics

Penicillin and streptomycin were added to provide 5,000 units/100mL media and 5mg/100mL media respectively. These antibiotics were either redissolved from a lyophilized preparation (Gibco) or weighed out and added as powder (Sigma Chemcials). These levels are at or below levels indicated as typical in vertebrate cell and tissue culture work as stated in the Gibco Technical Brochure.

vi) Additives

Fetal Bovine Serum

Fetal bovine serum (FBS) was purchased from Gibco. The heat inactivated, mycoplasma and virus screened product was chosen (Lot No. 19K3522). The stock was aseptically divided into aliquots and kept frozen until use.

Ecdysteroids

Ecdysone (2,3,14,22,25 penthydroxy-cholest-7-en-6-one) (Lot No. 102F-0286, Sigma Chemicals) or 20-OH ecdysone (Lot No. 102F-0777, Sigma Chemicals) was added using the solvent propanediol (1,2 propylene glycol). A stock solution of 10^{-4}M concentration was made in each case. The stock solution was vigorously agitated prior to use. Final solvent concentration in media was

maintained below 1:200. Kambysellis and Williams (1971b) found propanediol to have no effect on spermatogenesis in intact testes of *Samia cynthia* (Lepidoptera) after six days of culture. Fukushima and Yagi (1975) used 99% ethanol and found no deleterious effects with a final dilution of 1:1000.

Juvenile Hormone

Juvenile Hormone III (cis 10,11 epoxy-3,7,11 trimethyl trans,trans-2,6 dodecadienoic acid methyl ester) (Lot No. 12F-03591, Sigma Chemicals) was dissolved in media using propanediol as described for the ecdysteroids. JH III is a C16 (MW 256) compound and is the principal juvenile hormone detected in the Orthoptera (Gilbert *et al.* 1977). JH III is considered to be the 'primitive' juvenile hormone because of its simpler carbon skeleton and its predominance in the primitive Orthoptera.

Vitamin E

Vitamin E (D-alpha tocopherol, USBC[1]) was dissolved (1:1 v/v) in chloroform and acetone and the appropriate volume added directly to the incubation media. Upon addition to moderate volumes (15-30 mL) of medium the vitamin E- solvent mixture formed globules. Vigorous agitation was required to break up the globules. Where microlitre amounts of the mixture were added to small volumes of medium (e.g. 1mL) globule formation did not occur and dissolution was rapid.

[1]United States Biochemical Corporation

Copper

Copper was made available by direct addition of aqueous copper sulfate. A final concentration in the incubation medium of 1 μg/mL was generally used although concentrations above and below this level were tried. This concentration was thought to be adequate given that the dietary requirement is 2-10 μg/g diet.

Blood

Blood was obtained either by centrifugation of crickets or by collecting oozing blood after decapitation or abdominal incision. Centrifugation was done with a Sorval SS-1 centrifuge at a rotor speed of approximately 3,100 rpm and an R.C.F. of 1,230g. Crickets with their heads pierced were installed in plastic cylinders within centrifuge tubes (after Lipsitz, 1969) and then spun for 5 minutes. It was found to be more expedient to simply bleed the crickets to obtain blood. About 10 μL of blood could be obtained from an ultimate male.

f) Examination of Organs

Experimental and fresh organs were examined in squash preparations or as paraffin sections. In some cases measurement of oxygen consumption of the organ was also carried out.

Initially organs were examined as paraffin sections. Organs were fixed in Bouin's fluid in the usual manner and embedded in Tissue Prep (Fisher Scientific Co.) and

then sectioned at 3um on a Sorval JB4 microtome. Sections were stained with Heidenhain's Iron Haematoxylin or Periodic Acid Schiff stain. This method was abandoned in favour of the equally informative but faster and easier squash preparations.

Squash preparations were made by teasing approximately 30 tubules from each testis in a few drops of Belar's Ringer. A coverslip was then added and pressure applied to squash the tubules. Eosin-fast green FCF stain (see Appendix C for composition) was then drawn under the coverslip by raising it slightly with an insect pin. Excess fluid was wiped off and the squash examined under the light microscope at 100-200x magnification. Mammalian sperm heads appear as pink stained or clear structures against a green background (Sorenson, 1979; Mayer *et al.*, 1951). The stained heads are dead, the clear ones alive at the time of staining. Most cricket sperm appear stained while only some are clear. This is true for testicular and spermathecal sperm. It would appear that this does not distinguish live from dead cricket sperm as it does mammalian sperm. It is also unreliable when used with boar sperm.

Oxygen consumption measurements of single testes were made using a Rank Brothers (Cambridge, England) Oxygen Electrode. The electrode was designed for use with cell suspensions, subcellular particles or enzyme systems. Measurements taken with a whole testis in the electrode often show a fine flutter on the chart

recording. Relative to the apparatus and volume of incubate, the testis is rather large. The respiratory activity of the testis would produce local depletion effects that would be rapidly but not instantaneously smoothed out by stirring. Large fluctuations occurred if the testis became stuck under the magnetic stirring bar. A recording time of 5 to 10 minutes was sufficient to determine the nature of the oxygen consumption by the organ. Only linear recordings were used as they represent a constant rate of oxygen consumption. All measurements were done on the same instrument and carried out at 30°C, the organ incubation temperature.

A sample calculation of the rate of oxygen consumption is given in Appendix B. Organ weight is required in the calculations. Wet weight was used. Testes were touched ten times to a dry glass surface before weighing on an analytical balance. Filter paper was not used to absorb excess fluid as the organ often stuck to the paper particularly if the testis sheath had been broken by the stirring bar while in the electrode.

RESULTS

Data is presented in tabular form. In each experiment, one testis from a given animal was used as the control and the other testis was subjected to experimental conditions. The symbols (+) and (O) each represent a light microscope examination of a group of 30 to 40 tubules teased from each testis. A hollow box indicates that the experimental testis showed no germ cell in a developmentally more advanced stage than could be found in the control testes. A plus sign indicates the presence of germ cells (in the experimental testis) more advanced in development than the late spermatid stage and concurrent absence from the control testis.

In Experiments D through M the entries in the left hand column are components of the media used in the experimental incubation. The entries in this column do not correspond to entries in the other columns.

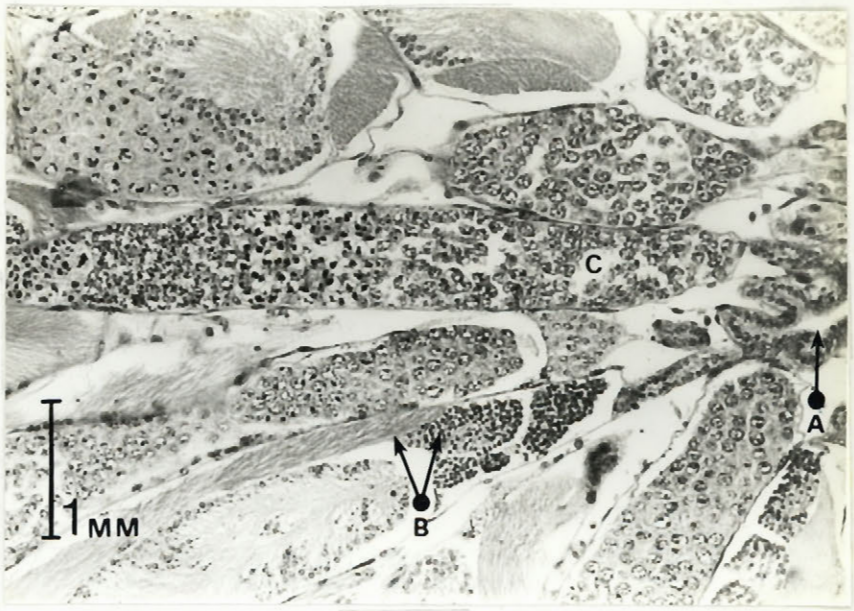
Oxygen consumption values are in nanograms of oxygen per milligram organ weight. Sample calculations for these values can be found in Appendix B. The symbol NR is used to indicate that no oxygen consumption could be detected.

Figures 2 illustrates germ cells at various stages of development as seen in a paraffin section. Figure 3 is an example of a stained tubule squash as used in microscopic examination of incubated testes.

FIGURE 2. Paraffin Sections of Testes

i) Section (3μM) of a penultimate testis (1 week old) stained with Heidenhain's Iron Haematoxylin showing the lumen of the vas deferens (A) and the heads and tails of spermatids (B). A tubule can be seen in longitudinal section lying horizontally near the centre of the photograph. Cysts containing germ cells at different stages of development are visible (C). Progression is from left to right.

ii) Section (3μM) of an ultimate testis (1 week old) showing early spermatids (A), late spermatids (B) and sperm (C).


C
A
B
1мм

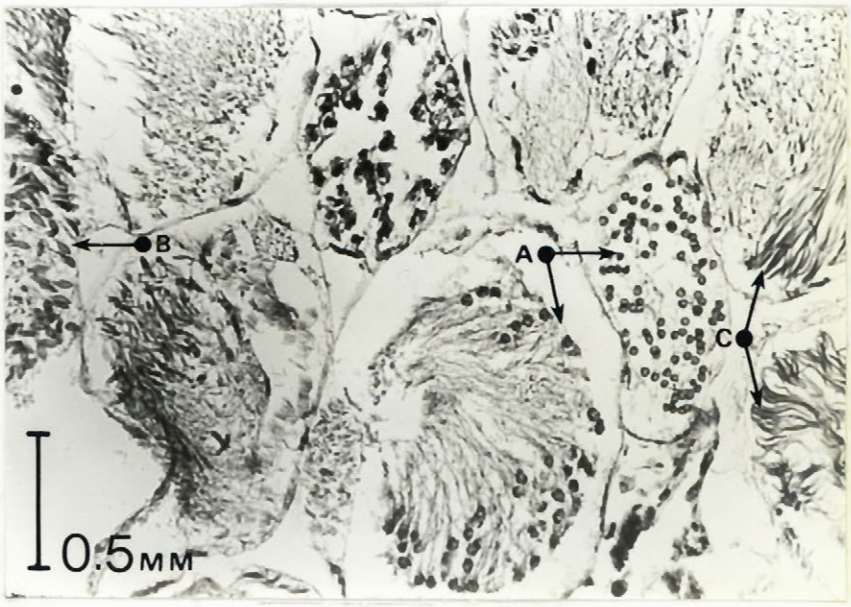

B
A
C
0.5мм

FIGURE 3. Tubule Squash Preparation (Stained) Viewed under the light microscope.

i) Numerous sperm are visible in this squash of several ultimate testis tubules. Head and tail are easily distinguished in the identified sperm (A).


A
1мм

EXPERIMENT A

Intact Penultimate Testes
Without Exogenous Hormonal Influence

Experimental Medium: Grace's medium supplemented with
(1) Vitamin E
(2) Copper

Control Medium: Grace's medium

24h INCUBATION

	Vitamin E 10 µg/mL	Vitamin E 1 µg/mL
Copper		
10 µg/mL	0 0 0 0 0	0 0 0 0 0
1 µg/mL	0 0 0 0 0	0 0 0 0 0
0.1 µg/mL	0 0 0 0 0	0 0 0 0 0
0.01 µg/mL	0 0 0 0 0	0 0 0 0 0

48h INCUBATION

	Vitamin E 10 µg/mL	Vitamin E 1 µg/mL
Copper		
10 µg/mL	0 0 0 0 0	0 0 0 0 0
1 µg/mL	0 0 0 0 0	0 0 0 0 0
0.1 µg/mL	0 0 0 0 0	0 0 0 0 0
0.01 µg/mL	0 0 0 0 0	0 0 0 0 0

EXPERIMENT B

Intact Penultimate Testes
With Exogenous 20-OH Ecdysone

Experimental Medium: Grace's medium supplemented with
(1) 20-OH Ecdysone
or
(2) 20-OH Ecdysone
and FBS 10%

Control Medium: Grace's medium

24h INCUBATION

	with FBS	without FBS
Ecdysone 10^{-6}M		
Copper		
1.0 µg/mL	O O O O O	O O O O O
0.1 µg/mL	O O O O O	O O O O O
0.01 µg/mL	O O O O O	O O O O O

48h INCUBATION

	with FBS	without FBS
Ecdysone 10^{-6}M		
Copper		
1.0 µg/mL	O O O O O	O O O O O
0.1 µg/mL	O O O O O	O O O O O
0.01 µg/mL	O O O O O	O O O O O

EXPERIMENT B cont'd

24h INCUBATION

	with FBS	without FBS
Ecdysone 10^{-7}M		
Copper		
1.0 µg/mL	0 0 0 0 0	0 0 0 0 0
0.1 µg/mL	0 0 0 0 0	0 0 0 0 0

24h INCUBATION

	with FBS	without FBS
Ecdysone 10^{-8}M		
Copper		
1.0 µg/mL	0 0 0 0 0	0 0 0 0 0
0.1 µg/mL	0 0 0 0 0	0 0 0 0 0

Experiment C

Intact Penultimate Testes with
20-OH Ecdysone, Vitamin E and Copper

Experimental Medium: Grace's with added (1) 20-OH Ecdysone
(2) Vitamin E
(3) Copper
(4) FBS 10%

Control Medium:Grace's medium

24h INCUBATION

20-OH Ecdysone 10^{-6}M	Copper 1 µg/mL	Copper 0.1 µg/mL
Vitamin E 100 µg/mL	0 0 0 0 0	0 0 0 0 0
10 µg/mL	0 0 0 0 0	0 0 0 0 0
1 µg/mL	0 0 0 0 0	0 0 0 0 0

48h INCUBATION

20-OH Ecdysone 10^{-6}M	Copper 1 µg/mL	Copper 0.1 µg/mL
Vitamin E 100 µg/mL	0 0 0 0 0	0 0 0 0 0
10 µg/mL	0 0 0 0 0	0 0 0 0 0
1 µg/mL	0 0 0 0 0	0 0 0 0 0

EXPERIMENT D

Ruptured and Intact Penultimate Testes
High Titre 20-OH Ecdysone

Experimental Medium: Grace's with added (1) 20-OH Ecdysone
(2) Vitamin E
(3) Copper
(4) FBS 10%

Control Medium: Grace's

24h INCUBATION

20-OH Ecdysone 10^{-4}M	without FBS		with FBS	
	Intact	Ruptured	Intact	Ruptured
	0	0	0	0
Vitamin E 10 µg/mL	0	0	0	0
	0	0	0	0
Copper 1 µg/mL	0	0	0	0

48h INCUBATION

20-OH Ecdysone 10^{-4}M	without FBS		with FBS	
	Intact	Ruptured	Intact	Ruptured
	0	0	0	0
Vitamin E 10 µg/mL	0	0	0	0
	0	0	0	0
Copper 1 µg/mL	0	0	0	0

EXPERIMENT E

Intact Penultimate Testes
with Ecdysone and Ultimate Male Blood

Experimental Medium: Grace's with added blood from ultimate males and
(1) Ecdysone
(2) Vitamin E
(3) Copper
(4) FBS 10%

Control Medium: Grace's medium

24h INCUBATION

0.3 mL blood	Visual	Oxygen Consumption	
		Control	Experimental
Ecdysone	0	NR	NR
10^{-6}M	0	NR	NR
	0	356	356
Vitamin E	0	NR	NR
10 µg/mL	0	NR	NR
	0	NR	NR
Copper	0	NR	NR
1 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR

EXPERIMENT F

Intact Penultimate Testes
with Hormones and Ultimate Male Blood

Experimental Medium: Grace's or Modified Grace's with added blood from ultimate males and

(1) Ecdysone
(2) Juvenile Hormone
(3) Vitamin E
(4) Copper

Control Medium: Grace's medium or Modified Grace's respectively

24h INCUBATION

Grace's Medium	Visual	Oxygen Consumption	
		Control	Experimental
Ø.3mL blood			
Ecdysone	0	NR	NR
10^{-6}M	0	NR	NR
	0	597	710
Vitamin E	0	187	336
10 µg/mL	0	305	336
	0	NR	283
Copper	0	NR	NR
1 µg/mL	0	NR	NR
	0	NR	NR
JH III	0	NR	NR
10^{-11}M			

24h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
Ø.3mL blood			
Ecdysone	0	NR	283
10^{-6}M	0	442	332
	0	442	497
Vitamin E	0	507	502
10 µg/mL	0	753	604
	0	NR	NR
Copper	0	NR	NR
1 µg/mL	0	NR	NR
	0	NR	NR
JH III	0	NR	NR
10^{-11}M			

EXPERIMENT G

Intact Penultimate Testes
with 20-OH Ecdysone and Juvenile Hormone

Experimental Medium: Modified Grace's with (1) 20-OH Ecdysone
(2) Juvenile Hormone
(3) Vitamin E
(4) Copper

Control Medium: Modified Grace's

24h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	345
	0	NR	621
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	491
	0	NR	NR
	0	NR	NR
Copper 1 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
JH III 10^{-11}M	0	NR	NR

48h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
	0	NR	NR
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	430
	0	387	332
Copper 1 µg/mL	0	NR	NR
	0	NR	NR
	0	502	753
JH III 10^{-11}M	0	NR	NR

EXPERIMENT H

Intact Ultimate Testes of Selected Ages
with 20-OH Ecdysone

Experimental Medium: Modified Grace's with (1) 20-OH Ecdysone
(2) Vitamin E
(3) Copper

Control Medium: Modified Grace's

12h Old Ultimate Testes 24h Incubation

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	NR
Copper 1 µg/mL	0	NR	NR

24h Old Ultimate Testes 24h Incubation

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	460	806
Vitamin E 10 µg/mL	0	354	398
	0	412	NR
	0	452	452
Copper 1 µg/mL	0	NR	NR

EXPERIMENT H cont'd

72h Old Ultimate Testes 24h Incubation

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	282	282
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	292
	0	326	326
Copper 1 µg/mL	0	NR	NR

EXPERIMENT I

Intact Penultimate Testes
with 20-OH Ecdysone and Juvenile Hormone

Experimental Medium: Modified Grace's with (1) 20-OH Ecdysone
(2) Juvenile Hormone
(3) Vitamin E
(4) Copper

Control Medium: Modified Grace's

Incubation Fluid Volume: 0.25 mL
Multi-well plate incubation chamber

24h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
	0	NR	NR
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
Copper 1 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
JH III 10^{-11}M	0		

48h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
	0	NR	NR
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
Copper 1 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
JH III 10^{-11}M	0		

EXPERIMENT J

Intact Ultimate Testes
with 20-OH Ecdysone

Experimental Medium: Modified Grace's with (1) 20-OH Ecdysone
(2) Vitamin E
(3) Copper

Control Medium: Modified Grace's

Incubation Fluid Volume:0.25 mL
Medium replaced every 12h
Multi-well plate incubation chamber

24h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
	0	NR	NR
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR
Copper 1 µg/mL	0	NR	NR
	0	NR	NR
	0	NR	NR

48h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone 10^{-6}M	0	NR	NR
	0	NR	NR
	0	NR	NR
Vitamin E 10 µg/mL	0	NR	NR
	0	NR	NR
Copper 1 µg/mL			

EXPERIMENT K

Intact Ultimate Testes
with 20-OH Ecdysone

Experimental Medium: Modified Grace's with (1) 20-OH Ecdysone
(2) Vitamin E
(3) Copper

Control Medium: Modified Grace's

Incubation Fluid Volume: 0.25 mL
Multi-well plate incubation chamber

24h INCUBATION

Modified Grace's	Visual	Oxygen Consumption	
		Control	Experimental
20-OH Ecdysone			
10^{-4}M	0	NR	NR
	+	263	236
	0	NR	184
Vitamin E	0	133	113
10 µg/mL	0	NR	245
	+	266	262
Copper	+	281	242
1 µg/mL			
	+	NR	197
	0	156	272
	+	256	306
	0	230	213
	+	345	355
	+	363	483
	0	247	NR
	+	NR	161
	0	125	166
	+	272	211
	0	265	184
	0	215	215
	+	239	303
	0	141	172
	+	240	276
AVERAGE[1]		236.87	250.56

[1]Significant difference between rows (crickets) at the 0.01 level.
No significant difference between control and experimental (treatments) at the 0.01 level.

EXPERIMENT L

Intact Ultimate Testes
Oxygen Consumption in Different Containers

Incubation Medium: Modified Grace's

24h INCUBATION

	Old Chamber	Ventilated Flask
Modified Grace's		
	307	372
	295	421
No	584	622
Supplements	397	476
	161	194
AVERAGE[1]	348.80	417.00

48h INCUBATION

	Old Chamber	Ventilated Flask
Modified Grace's		
	607	590
	620	743
No	737	729
Supplements	153	184
	463	536
AVERAGE[2]	258.00	278.20

[1]Significant difference between old chamber and ventilated flask at the 0.01 level.
Significant difference between rows (crickets) at the 0.01 level.
[2]Significant difference between rows (crickets) at the 0.01 level.

EXPERIMENT L cont'd

24h INCUBATION

	Multi-well Plate	Ventilated Flask
Modified Grace's		
	NR	193
	121	247
No	299	292
Supplements	333	300
	288	316
	271	274
	178	276
	NR	NR
AVERAGE[1]	248.30	284.10

48h INCUBATION

	Multi-well Plate	Ventilated Flask
Modified Grace's	NR	NR
	NR	NR
	NR	NR
	NR	NR
	NR	NR
No	NR	NR
Supplements	NR	NR
	NR	NR

[1]Significant difference between plate and flask at the 0.01 level.
Significant difference between rows (crickets) at the 0.01 level.

EXPERIMENT M

Freshly Dissected Testes
Oxygen Consumption

Dissection Medium:Belar's Ringer

Incubation Medium:Modified Grace's and Grace's medium

Penultimate Testes

	Grace's Medium	Modified Grace's
	859	859
	767	585
	886	650
No	613	633
Supplements	745	726
	779	760
	834	667
	682	682
	698	781
	691	766
AVERAGE[1]	755.40	710.90

Ultimate Testes

	Grace's Medium	Modified Grace's
	552	368
	427	559
No	463	482
Supplements	490	514
	552	518
	794	631
	599	491
	553	645
	618	520
	614	609
AVERAGE[2]	566.20	533.70

[1,2] No significant difference between treatments at the 0.01 level.

DISCUSSION

The formation of spermatids and spermiogenesis are generally delayed until the last larval or pupal stadium. In the house cricket, spermatids appear in the penultimate testis and spermiogenesis begins in the ultimate. The guiding undercurrent for these morphogenetic changes are the hormones ecdysone, 20-OH ecdysone and juvenile hormone.

Experiment A was an attempt to induce development by removing any endogenous hormonal influence that might restrict development. Of course, residual hormone content in the organ is not eliminated. The known nutritional requirements for copper and Vitamin E were met with supplements. Final concentrations of the supplements ranged from 10x above to 100x below normal physiological levels based on determinations made by McFarlane (1974). The absence of response in all testes indicates that simple removal from circulating hormonal influence is insufficient to permit or promote the occurrence of any large step in the process of spermatogenesis.

An apparent lack of development upon explantation has been encountered by other workers. Shimuzi and Yagi (1978) using Grace's medium found that naked spermatocysts of *Mamestra brassicae* did not undergo spermiogenesis *in vitro* when cultured alone. Changes were observed when spermatocysts were co-cultured with an intact or ruptured testis or when incubated in medium that had been conditioned by preculture for 5 days.

Failure of spermiogenesis to occur in ruptured tubule preparations was observed in the majority of liberated

spermatocysts from the testis of the rice stem borer, *Chilo suppresalis* (Lepidoptera) (Yagi *et al.*, 1969). In this case, testes were cultured in medium CSM-2F. Development of abnormal appearing spermatocysts occurred in ruptured testes that were cultured for several days. Incubation had to be extended to 20 days before any normal elongate spermatocysts appeared.

Kambysellis and Williams (1971a) also found that isolated spermatocysts of *Hyalophora cecropia* were insensitive to both ecdysone and 20-OH ecdysone.

The next experimental condition to try was the addition of macromolecular factor (MF), considered critical for spermatogenesis and the existence of which was first described by Kambysellis and Williams (1971a,b).

In **Experiment B** , intact penultimate testes were incubated in Grace's medium containing 20-OH ecdysone with and without FBS (source of MF). Only copper was added. No noticeable development was seen in any case. It was expected that development at least might proceed to a very late spermatid stage.

Other workers have encountered lack of response with exposure to 20-OH despite the presence of FBS. Fukushima and Yagi (1975) subjected testes of both *Mamestra brassicae* and *Spodoptera litura* to 20-OH at 6 x 10^{-6}M and 6 x 10^{-8}M and found no sign of differentiation. These authors cite an unpublished report by Yagi *et al.* (no date given) that little spermatid formation was observed when *Mamestra* testes from six day old last instar larvae destined for diapause were cultivated in Grace's medium with ecdysone.

Using medium containing 15% FBS, Kuroda (1974) was only able to obtain further development in explanted testis fragments containing germ cells in the beginning stages of spermiogenesis. Explanted fragments containing earlier stages showed little or no development. The testicular fragments were taken from 48h pharate adults of *Drosophila melanogaster*.

A third attempt to stimulate spermiogensis was made (**Experiment C**) using Modified Grace's medium, 20-OH ecdysone and both the nutritional requirements vitamin E and copper. Modified Grace's contains two protein supplements, whole egg ultrafiltrate and bovine albumin fraction V in addition to FBS. Vitamin E was made available in low, medium and high amounts. Again no specific response could be elicited even after a lengthy 72h incubation period.

Results from studies where FBS has been present have been mixed. Yagi *et al.* (1969) were actually the first to show germ cell response to ecdysteroids. They obtained spermiogenesis in response to 4 x 10^{-7}M 20-OH ecdysone with testes of the rice stem borer (*Chilo suppressalis*) cultured for 7 days. The significance of MF and FBS was unknown at the time.

The prime example of a response to 20-OH ecdysone is the work of Kambysellis and Williams (1971a,b) with *Hyalophora cecropia*. They demonstrated an absolute requirement for MF and determined that the threshold of effect for 20-OH ecdysone was 10^{-7}M. However, examination of the incubated organs was not carried out until the seventh day. Therefore it is not possible to estimate the minimum time required for differentiation.

In 1972 Kambysellis and Williams found development in the

intact testis of *Samia cynthia* when either ecdysone or 20-OH ecdysone was used. The same threshold (10^{-7}M) was also found. The incubation medium contained cell free plasma from 5 to 6 month old pupae which was known to contain a high titre of MF.

In contrast Leloup (1976) found 10^{-7}M and 0.5 x 10^{-7}M ecdysone to have no effect on testes of 9 day prepupae and 17 day old pupae of *Calliphora erythrocephala*. Incubation was for less than 72h and FBS (10%) was present.

20-OH ecdysone is believed to modify the permeability of the testis sheath to MF. **Experiment D** examined both the effect of a high dose (10^{-4}M) of 20-OH ecdysone and the effect of high ecdysteroid titre combined with rupture of the testis sheath to permit free access of MF to the tubules. Neither approach was effective. It was anticipated that with rupture of the testis sheath the majority of tubules would be exposed to 20-OH ecdysone and MF. With sufficient incubation time (48h), some development should have been obvious. Higher titres of 20-OH ecdysone were not tried as one of the objectives of this project was to establish developmental responses under physiological conditions. Furthermore Marks (1976) has recorded inhibition of development of imaginal disks at 10^{-3}M ecdysone.

It has been demonstrated in numerous species that tissues other than the prothoracic gland or homologous structures can convert ecdysone to 20-OH ecdysone. These tissues include the fat body, Malpighian tubules and oenocytes (Hoffman and Hetru, 1983) although quantitatively these conversion sites are probably much less important than the glands themselves.

The repeated lack of apparent difference between control and treated organs raised the question of whether or not the organs were viable for the duration of the incubation period. An attempt to demonstrate viability was made with the supra-vital stain pinacyanole (Humason, 1979) but the stain proved difficult to use. Oxygen consumption was the parameter finally chosen to monitor viability. Measurements were carried out with an electrode of nanogram (of oxygen consumed) precision.

Experiment E was set up with the consideration that the testis may require ecdysone or that conversion to 20-OH ecdysone be carried out by oenocytes. Blood was taken from ultimate males as more blood is available per cricket than from penultimate crickets. Also the JH titre would be lower reducing any restrictive effects the hormone may have on development.

The results of Experiment E show that with the exception of one pair of cultured testes, all were apparently dead. That is, no indication of sustained oxygen consumption could be obtained. Visual examination revealed no differentiation. Added blood was found to have clotted. At the time it appeared as if the addition of blood was the detrimental factor perhaps due to phenoloxidase reactions. Contamination occurred even with the use of antibiotics and made it very difficult to maintain sterility in chambers being incubated for more than 24h.

Experiment F was similar to Experiment E except that JH III was added to complement the ecdysone. Loher *et al.* (1983) have determined the JH III concentration for different developmental stages of another cricket *Teleogryllus commodus*. The corresponding information is not available for

Acheta domesticus so JH III was added to the incubation fluid to match blood concentrations in *T. commodus*.

Both Grace's and Modified Grace's medium were used. Survival was much higher than in Experiment E, 7 out of 20 (Grace's) and 9 out of 20 (Modified Grace's) organs being viable at the end of 24h. However no developmental changes were elicited with this combination of blood and hormones.

Some of the incubation chambers used in this experiment were new. Incubation chambers were made of glass and plastic. Broken units were replaced form a pool of unused ones, which had not previously been autoclaved. Some chambers were replaced prior to Experiment F. In retrospect, it is suspected that a toxic agent may have been released from the cement with repeated autoclaving. These new chambers were made at the same time and with the same cement as for the old ones. Hence if toxicity developed with ageing of the cement only, no organs should have survived.

The possibility that the media had become toxic with storage had not been ruled out. New media were ordered.

With the repeated incidences of no oxygen consumption by incubated organs the function of the measuring device was brought into question. This possibility was soon dismissed as the instrument behaved normally when used by other workers for bacterial studies and responded normally to handling between measurement periods.

Experiment G examined the combined effects of 20-OH ecdysone and JH III without the presence of blood. Survival was sporadic and decreased in comparison with Experiment F. No evidence of development was found in the treated (experimental)

organs. The failure of any organ to survive despite the use of fresh media pointed directly to the incubation chambers as the source of the problem. New incubation vessels were sought. Commercially available multi-well titration plates seemed suitable although the well volume was only 0.25mL, one quarter of that in the fabricated chambers.

Penultimate testes had shown no response even with a full complement of hormones and supplements. After a small trial run (unrecorded) in which ultimate testes were found to be consuming oxygen after 24h, the new plates were employed in **Experiment H** . Penultimate crickets were not available at the time this experiment was set up. Testes were selected from crickets of less than 12, 24 and 72h of age, under the presumption that there may be an energy saving decrease in testis metabolic activity during and shortly after the moulting process. The increase the youngest to the 72h testes favours this hypothesis. However, the inconsistent survival limits consideration of the results to no more than circumstantial evidence.

Experiment I was identical to Experiment G except for the use of multi-well plates. Of 38 organs incubated not one showed any signs of sustained respiration nor development. No reason for this outcome was immediately apparent. It was suspected that the small incubation volume (less than 2x the volume of a testis) might permit the accumulation of metabolic end products to toxic levels.

In Experiment J , the incubation fluid was replaced every 12h as an attempt to limit accumulation of waste products. Hence penultimate testes were replaced with ultimate testes.

This third attempt with the multi-well plates was also unproductive. No survival was apparent.

Airborne sources of interference had not been considered to this point. Cockroaches were being raised in the same environmental chamber in which the organs were being incubated. Cockroaches release substantial quantities of ammonia as an excretory product. The testes would be exposed to this ammonia, a highly toxic product as it circulated in the atmosphere of the enviromental chamber.

A separate incubator was set up for use in **Experiment K** . The first set (7 pairs) of testes examined not only showed significant oxygen consumption but in 3 pairs development of sperm in the experimental testis past the level in the control testis was observed. The sudden change in survival rate would seem to confirm the above hypothesis.

However the second set of organs was accidentally placed in the incubator containing the cockroaches and subsequent survival was equal to if not greater than the first set. The third set were deliberately placed in the incubator containing the cockroaches and survival was again excellent. There appears to be no rhyme or reason to the changes in organ survival.

As in set 1, sets 2 and 3 produced several pairs of testes with distinct differences between control and experimental organs. In these pairs, the experimental testis was found to contain sperm undergoing cytoplasmic sloughing in the head region. No stage more advanced than late spermatid could be found in the control testis. Some pairs were found to contain substantially more sperm (eg. 9 clusters of 50 or more) in the

experimental than in the control (eg. 2-3 clusters). These were not indicated under the visual column in the results tables. About 1 in every 8 pairs seemed to have a control testis with more advanced stages or greater numbers of sperm. This could not have been due to a mix-up in incubation fluids as the technique for filling multi-well plates made it impossible.

Statistically, no significant difference in oxygen consumption could be detected between treatments using a 1 way analysis of variance. A significant difference (0.01 level) between crickets was detected, which indicates that testes from one cricket will not consume oxygen at the same rate as testes from another. This difference was expected as the crickets were selected from the stock culture without regard to variation in age or weight. This finding also indicates that measurements from a much larger number of crickets would be desirable.

In mammalian cell and tissue culture work there are numerous reports where the atmosphere above the incubation fluid contains 5% CO_2 acting in conjunction with solution to maintain pH. Only two reports in the insect literature mention the use of 5% CO_2 in air for culture work. Berlot and Goodman (1984) used this gas mixture in studying embryogenic development of neurons in a grasshopper. Bowers (1961) found this gas mixture to improve the development of isolated spermatocysts in culture.

In Experiment L , testes were incubated under different atmospheres and their oxygen consumption examined after 24 and 48h. Testes in chambers were exposed to the air of the incubator while those in the ventilated flasks were incubated under balanced air and 5% CO_2. A significant difference (0.01

level) in oxygen consumption after 24h was found in favour of the ventilated flask. A significant difference between testes from different crickets was also found (0.01 level). Although the average oxygen consumption was still higher for testes incubated in the ventilated flasks at 48h, the difference was no longer significant.

Flasks ventilated with 5% CO_2 in air proved superior (significant at 0.05 level) to the multi-well plates after 24h. Two day incubations did not work even after a repeated attempt.

Irrespective of the incubation chamber used the average rate of oxygen consumption is much lower at 24 or 48h than for fresh organs (data in **Experiment M**).

The decrease in oxygen consumption upon incubation no doubt reflects the hostile nature of the *in vitro* environment. In cell culture work, metabolic data for respiration and glycolysis studies are often calculated on the basis of a total cell count rather than on the basis of viable cells, making the data somewhat less reliable. Such a count is impossible in organ culture so one cannot tell whether the decrease is an overall decrease in metabolic activity or death of part of the organ.

The average oxygen consumption rate for freshly dissected organs was about 700 ng oxygen per minute per mg wet weight, a rate equivalent to approximately 58 µL per mg per hour. Incubated organs have a consumption rate of about half this value, the decrease perhaps reflecting the hostile nature of the *in vitro* environment. The rates for freshly dissected organs fall within the range of rates for *in vitro* oxygen consumption in mammalian testes as presented in the literature

review.

The survival of organs for up to 48h in Experiments G and M indicate that apart from the toxicity problems that were encountered, the incubation systems employed will sustain cricket testes *in vitro*. Ideally organs should be incubated under a atmosphere of 5% CO_2 in air and with Modified Grace's medium. This combination will provide assistance to solution buffers to limit fluctuations of the critical factor, pH, maintain osmotic support and meet energy requirements. The *in vitro* culture system may appear to limit the efficiency of the organs as far as oxygen consumption and germ cell transformation go, but with refinement advanced studies should be possible. This is not to say the required refinements will be easy to determine or implement.

It is the complexity of an *in vivo* process that makes it so very difficult to mimic *in vitro* and was the basis for a great part of the problems encountered in this attempt to stimulate spermiogenesis *in vitro*. Although it is well established that 20-OH ecdysone promotes spermatogenesis in a number of insects, a culture system that supports the process in its entirety has not been achieved to date. This is also true for mammalian systems despite numerous attempts to maintain whole organs, tissue or testicular fragments.

In mammalian culture work spermatogonia have been maintained for 7 weeks, spermatocytes for 4 weeks and spermatids for about 1 week (Mann and Lutwak-Mann, 1981). Also, in mammalian culture systems, true transformation of a germinal cell has only been observed to occur to a limited extent. Pre-leptotene

spermatocytes from human testes have been found to differentiate through leptotene, zygotene and early pachytene stages. However, they do not develop any further and subsequently degenerate (A. Steinberger *et al.* 1964; E. Steinberger *et al.* 1964; Steinberger and Steinberger, 1970). This development occurred in a medium without additional testosterone or gonadotrophin as long as it was supplemented with glutamine, vitamins A,C and interestingly E. Subsequent to the degeneration, waves of primary spermatocytes fail to appear (Steinberger and Steinberger, 1967; 1970).

Should it become possible to complete differentiation in culture one can safely predict it will not occur without ecdysteroids, since information already suggest that development is closely bound up with hormonal stimulation. The apparent problem of lack of ecdysteroid to promote spermatogenesis in adult insects may be resolved if the testis is a source of 20-OH ecdysone. This has in fact been shown by Loeb *et al.* (1982). Hoffman and Behrens, (1982) have found high concentrations in the testis of adult crickets and Koolman *et al.* (1979) have found large amounts in the testis of *Calliphora vicina*. It is possible that both stimulatory and inhibitory factors are involved in the control of meiosis and spermiogenesis in insects. The negative effects of JH on spermatogenesis make it likely to be the prime inhibitor. The supporting cells of the testis may mediate in the effects of these factors in a way similar to that of Sertoli cells which are involved in the production of an androgen binding protein in mammals (French and Ritzen, 1973a,b; Hansson *et al.* 1973;

Steinberger, 1975). It is in this respect that the blood germ cell barrier appears critical. In the final analysis, testicular function must be understood in the context of all the relationships the testis may have with other organs of the body.

SUMMARY

In vitro culture techniques were used with isolated testes in an attempt to determine the specific relationships and mode of action of vitamin E, copper and the morphogenetic hormones in spermiogenesis in the insect *Acheta domesticus*. Numerous problems with viability of incubated organs were encountered most of which were due to the possible presence of a toxic agent(s) in incubation vessels. Apart from this, viability of whole testes *in vitro* was maintained for up to 48h in incubation fluid volumes of 1 mL or less. No development of germ cells was seen upon isolation from any restrictive effects of endogenous hormones. A partial stimulation of spermatogenesis using 10^{-6}M 20-OH ecdysone was achieved but completion of spermiogenesis was not seen. Combination of 20-OH ecdysone and JH III or ecdysone and blood failed to elicit any developmental response. No significant difference in oxygen consumption was found between testes incubated in Grace's medium and the protein supplemented, high osmolarity, Modified Grace's medium.

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APPENDIX A

CULTURE MEDIA COMPOSITION

i) Mitsuhashi's CSM-2F Medium

COMPONENT	mg/L
INORGANIC SALTS:	
$NaH_2PO_4.H_2O$	120.00
$MgCl_2.6H_2O$	300.00
$MgSO_4.7H_2O$	400.00
KCl	300.00
$CaCl_2.2H_2O$	100.00
OTHER COMPONENTS	
Glucose	200.00
Fructose	200.00
SUPPLEMENTS	
Lactalbumin hydrolysate[1]	1300.00
Bacteriological peptone[2]	1300.00
TC-Yeastolate[2]	500.00
Choline Chloride	100.00

The above are added to 100mL H_2O to constitute CSM-2 mixture.

To prepare CSM-2F medium, these further additions are required.

CSM-2 mixture	40.0mL
TC-199 medium[2]	20.0mL
Redistilled water	19.0mL
Fetal bovine serum	20.0mL
Antibiotics	1.0mL

[1] United States Biochemical Corporation Cleveland, Ohio

[2] Difco, Detroit, Michigan

CULTURE MEDIA COMPOSITION cont'd

ii) Marks M-20 Medium

COMPONENT	mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	450.00
KCl	400.00
$MgCl_2.6H2O$	200.00
NaCl	1500.00
$NaHCO_3$	200.00
$NaH_2PO_2.H_2O$	200.00
NaH_2PO_3	200.00
NaH_2PO_4	400.00
Na_2SO_4	100.00
OTHER COMPONENTS	
Citric acid	100.00
Fumaric acid	100.00
D-Glucose	15000.00
Alpha-Ketoglutaric acid	300.00
Malic acid	500.00
Succinic acid	200.00
Sucrose	10000.00
Trehalose	5000.00
AMINO ACIDS	
L-Alanine	100.00
B-Alanine	200.00
L-Arginine HCl	800.00
L-Asparagine	500.00
L-Aspartic acid	400.00
L-Cystine 2HCl	56.50
L-Glutamic acid	1000.00
L-Glutamine	1000.00
Glycine	400.00
L-Histidine $HCl.H_2O$	656.00
L-Isoleucine	100.00
L-Leucine	200.00
L-Lysine HCl	200.00
L-Methionine	300.00
Phenylalanine	200.00
L-Proline	500.00
L-Serine	200.00
Taurine	50.00
L-Threonine	100.00
L-Tryptophan	50.00
L-Tyrosine (HCl)	100.00
L-Valine	300.00

CULTURE MEDIA COMPOSITION cont'd

ii) Marks M-20 Medium cont'd

COMPONENT	mg/L
VITAMINS:	
Biotin	0.01
D-Ca pantothenate	0.02
L-Carnitine HCl	0.01
Choline chloride	0.20
Folic acid	0.02
i-Inositol	0.02
Niacin	0.02
Para-aminobenzoic acid	0.02
Pyridoxine HCl	0.02
Riboflavin	0.02
Thiamine HCl	0.02

iii) Grace's Insect Tissue Culture Medium

COMPONENT	mg/L
INORGANIC SALTS:	
$CaCl_2$ (anhyd.)	750.00
KCl	4100.00
$MgCl_2.6H_2O$	2280.00
$MgSO_4.7H_2O$	2780.00
$NaHCO_3$	350.00
$NaH_2PO_4.H_2O$	1013.00
OTHER COMPONENTS	
Alpha-Ketoglutaric acid	370.00
Fructose	400.00
Fumaric acid	55.00
D-Glucose	700.00
Malic acid	670.00
Succinic acid	60.00
Sucrose	26680.00
AMINO ACIDS	
Beta-Alanine	200.00
L-Alanine	225.00
L-Arginine HCl	700.00
L-Asparagine	350.00
L-Aspartic acid	350.00
L-Cystine	22.00
L-Glutamic acid	600.00
L-Glutamine	600.00
Glycine	650.00
L-Histidine	2500.00
L-Isoleucine	50.00

CULTURE MEDIA COMPOSITION cont'd

iii) Grace's Insect Tissue Culture Medium cont'd

COMPONENT	mg/L
L-Leucine	75.00
L-Lysine	625.00
L-Methionine	50.00
L-Phenylalanine	150.00
L-Proline	350.00
DL-Serine	1100.00
L-Threonine	175.00
L-Tryptophan	100.00
L-Tyrosine	50.00
L-Valine	100.00
VITAMINS:	
Biotin	0.01
D-Ca pantothenate	0.02
Choline chloride	0.20
Folic acid	0.02
i-Inositol	0.02
Niacin	0.02
Para-aminobenzoic acid	0.02
Pyridoxine HCl	0.02
Riboflavin	0.02
Thiamine HCl	0.02

Modified Grace's contains the following additional serum supplements:

Bovine Albumin Fraction V	1.0mL
Fetal Bovine Serum	7.0mL
Whole Egg Ultrafiltrate	10.0mL

APPENDIX B

PRINCIPLE OF OPERATION OF OXYGEN ELECTRODE

The principle of operation of the electrode is that it measures the activity and not the concentration of oxygen present (Chappell, 1964) Oxygen diffuses through a thin (0.005mm) teflon membrane and is reduced at a platinum electrode surface in contact with the membrane.

$$O_2 + 2e^- + 2H^+ \longrightarrow H_2O_2$$

$$H_2O_2 + 2e^- + 2H^+ \longrightarrow 2H_2O$$

The other half cell is also included in the incubation vessel and is composed of a Ag-AgCl electrode. The platinum electrode is negatively polarized with respect to the AgCl electrode. The current flowing is proportional to the activity (partial pressure) of oxygen in solution over a wide range (Rank Brothers, Oxygen Electrode Instructions) and fed to an amplifier-chart recorder.

The calculation of oxygen consumption requires a knowledge of the solubility of oxygen in the incubation medium at a given temperature. This information is not available for Grace's and Modified Grace's medium. Chappell (1964) indicates that this value can be calculated for simple solutions but is not easy to determine for complex mixtures and that non-electrolytes affect the solubility of oxygen in water.

Chappell (1964) experimentally determined oxygen saturation

values for a medium[1] used in the study of isolated plant mitochondria. Members of the Department of Microbiology (to which the instrument belonged) routinely use these values in the calculation oxygen consumption rates. Chappell's oxygen saturation value for 30°C was used for the calculations in this project as no other value was available.

SAMPLE CALCULATION OF OXYGEN CONSUMPTION RATE

Oxygen saturation of medium[1] at 30°C.
442 ng O mL^{-1}

Chart recorder represents 0-100% saturation over 80 divisions on paper.

Therefore
442 ng O per 80 divisions

= 5.525 ng O $division^{-1}$

Paper speed = 1 cm min^{-1}

Measured slope of line on chart recording

16 divisions rise per 9.0 cm length

= 1.77 divisions min^{-1}

Therefore
1.77 divisions min^{-1}

X 5.525 ng O $division^{-1}$

= 9.77 ng O min^{-1}

The consumption rate is then divided by the organ weight to give a rate of consumption per unit weight per unit time.

5.93 ng O min^{-1} divided by 0.0151g

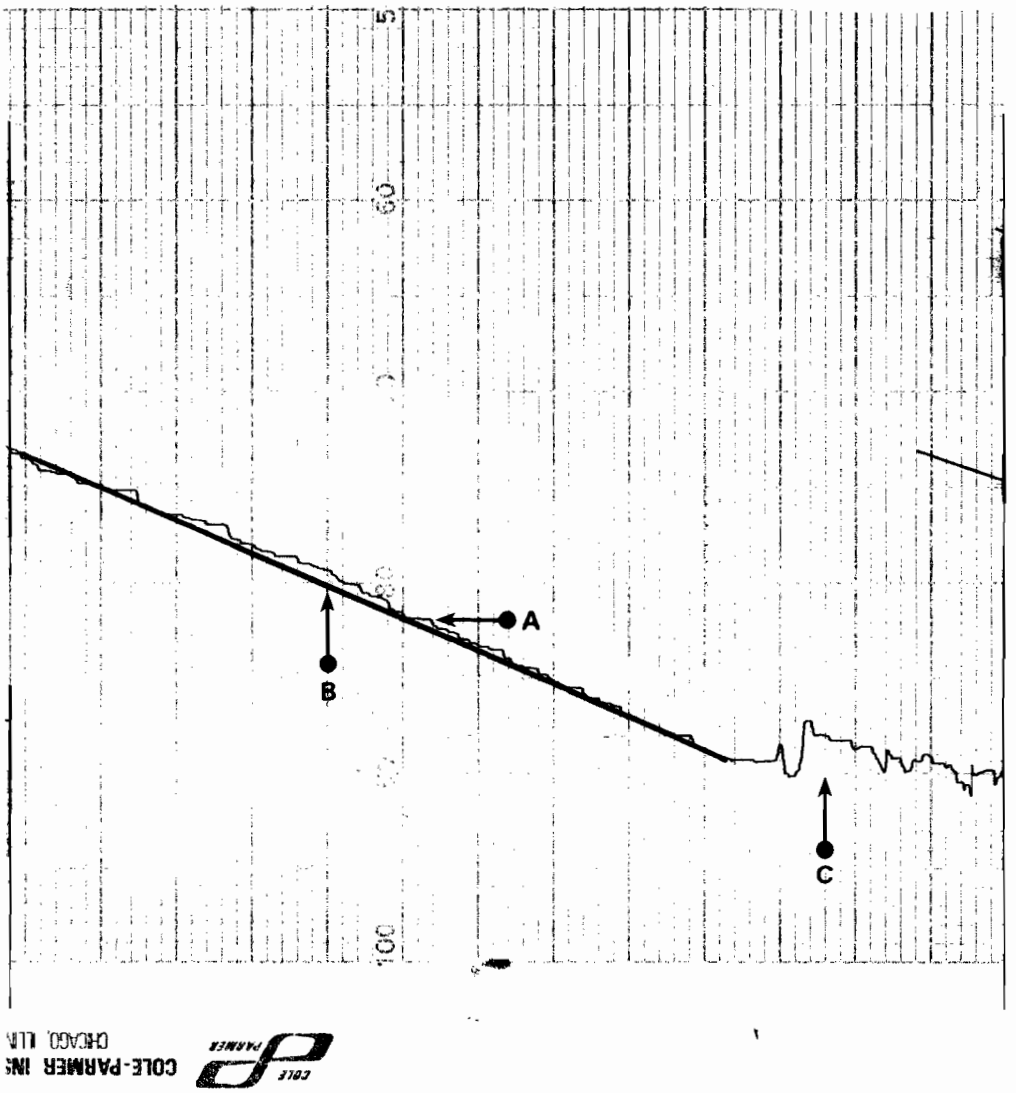
=647.18 ng O min^{-1} g^{-1}
which is truncated to an integer value

Figure 4 is a sample chart recording.

[1] Medium used by Chappell (1964) was composed of 80mM KCl, 15 mM Pi, 20mM triethanolamine hydro- chloride buffer, pH 7.2 and $1x10^{-6}$M cytochrome c.

FIGURE 4. Sample Oxygen Consumption Chart Recording.

i) A good oxygen consumption recording will show only a minor flutter (A) in the trace. The slope of the recording is approximated by a visual line of best fit (B). Large fluctuations in the recording (C) are usually caused by the testis getting stuck underneath the stir bar in the oxygen electrode.


A
B
C
COLE-PARMER INS
CHICAGO, ILLN

APPENDIX C

SPERM STAIN COMPOSITION AND PREPARATION

The following information was taken from p.127 of Sorenson (1979).

COMPONENTS

Na_2PO_4
KH_2PO_4
Fast Green FCF
Eosin Bluish
Distilled water

PREPARATION

1. A phosphate buffer is used in the stain and was prepared as follows:

   a) 2.2 g of Na_2PO_4 was dissolved in 50mL of distilled water

   b) 0.85 g of KH_2PO_4 was dissolved in 50 mL of distilled water

2. The phosphate solutions were combined to make a total of 100 mL of phosphate buffer

3. 2 g of Fast Green FCF was added to the buffer solution.

4. 0.4 g of Eosin Bluish was added to the Fast Green - buffer mixture.

5. The complete mixture was brought to a boil, cooled and filtered.

6. The solution was stored in a well stoppered container.

APPENDIX D

Analysis of Variance Tables

A complete randomized block design was used. In all cases blocks (crickets) were fixed and treatments were random.

Experiment K

Intact Ultimate Testes with and without 20-ØH Ecdysone

	DF	SS	MS	Fcal	Ftab Ø.Ø1
Total	31	187742.5			
BTcomb.	31	187742.5			
Blocks	15	161879.Ø	1Ø791.9	6.64	3.52
Treat	1	1498.8	1498.8	Ø.92	8.68
Exp. err.	15	24364.7	1624.6		

C.V. = 16.53%

Experiment L

24h Incubation, Ultimate Testes/Old Chamber vs. Ventilated Flask

	DF	SS	MS	Fcal	Ftab Ø.Ø1
Total	9	2Ø6456.9			
BTcomb.	9	2Ø6456.9			
Blocks	4	192289.4	48Ø72.3	75.73	6.39
Treat	1	11628.1	11628.1	18.31	15.98
Exp. err.	14	2539.4	634.7		

C.V. = 6.57%

Experiment L cont'd

48h Incubation, Ultimate Testes/Old Chamber vs. Ventilated Flask

	DF	SS	MS	Fcal	Ftab 0.01
Total	9	411433.6			
BTcomb.	9	411433.6			
Blocks	4	400547.6	100136.9	58.85	15.98
Treat	1	4080.4	4080.4	2.39	15.98
Exp. err.	4	6806.0	1701.5		

C.V. = 7.69%

Experiment L cont'd

24h Incubation, Ultimate Testes-Multiwell Plate/Ventilated Flask

	DF	SS	MS	Fcal	Ftab 0.01
Total	11	39712.2			
BTcomb.	11	39712.2			
Blocks	5	26006.7	5201.3	2.63	10.97
Treat	1	3852.0	3852.0	1.95	5764
Exp. err.	5	9853.4	1970.6		

C.V. = 16.67%

Experiment M

Penultimate Testes
Freshly dissected in Grace's and Modified Grace's

	DF	SS	MS	Fcal	Ftab 0.01
Total	19	182633.0			
BTcomb.	19	182633.0			
Blocks	9	117460.0	1305.1	0.0011	5.35
Treat	1	706348.5	706348.5	0.6283	10.56
Exp. err.	9	10117346.0	1124149.5		

C.V. = 14.46%

Experiment M cont'd

Penultimate Testes
Freshly dissected in Grace's and Modified Grace's

	DF	SS	MS	Fcal	Ftab 0.01
Total	19	160569.0			
BTcomb.	19	160569.0			
Blocks	9	105719.5	11746.6	0.0017	55.35
Treat	1	5281.3	5281.3	0.0007	6022.
Exp. err.	9	60984468.2	6776052.0		

C.V. = 22.45%