In vitro fertilization – some comparative aspects

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Recently it seemed to me that if one could successfully carry out in vitro fertilization (IVF) in Man it might be possible also to do it in the Lepidoptera. Here the procedure would have great application, both for research and for breeding in butterfly farms. Since butterflies and Man are my two research animals they receive an undue proportion of attention in this review.

Before summarizing the Lepidoptera work it would be helpful to understand better the mechanism of fertilization and egg-laying in these insects (Figure 1). At mating spermatozoa are introduced in one or more little sacs (spermatophores) into the copulatory opening (a) of the female, from there they proceed to the bursa copulatrix (b). Here, after a few hours, the membrane of the spermatophore dissolves and the spermatozoa make their way (either through muscular contractions of the passages or by chemical stimuli) through the ductus seminalis (c) into the common oviduct (d) and thence by another fine duct into the receptaculum seminis (g).

When the mated female is about to lay, a muscular contraction occurs which pushes the egg towards the ovipore (h), and on its way causes a few spermatozoa to be squeezed out of the receptaculum seminis and into the oviduct (d) where one enters the micropyle of

the egg and fertilizes it as it is laid. A good female will continue to lay eggs very quickly, and if the eggs are fertile they can be seen to be 'browning' after about 24 h. When the female dies a single fertilized egg can be squeezed out through the ovipore or alternatively dissected out. The eggs still in the abdomen of the female, above this single fertile one, remain waiting in vain. While waiting, meiosis (the reduction division) does not take place, the eggs are still diploid. The stimulus for meiosis starts when the sperm enters the egg through the micropyle as (or just before) it is laid (cf. Man).

To study IVF in butterflies and moths we used many species, but an ideal one seemed to us to be the Egg Fly, *Hypolimnas bolina*, a tropical insect not unlike our Camberwell Beauty. Wild caught females are almost always mated, they travel well by standard air mail and on reaching England often live for several weeks, laying their eggs on stinging nettle and sweet potato leaves in a heated greenhouse. Fertility is confirmed when some caterpillars hatch, and then the laying female can be killed, and in the abdomen lie the unfertilized eggs glistening among the fat. The receptaculum is then slit and the sperm mixed with the eggs using various different solutions, and sometimes being injected through the micropyle – but no fertility has ever resulted¹. This is in spite of the fact that in the naturally mated female preliminaries such as additions from adjuvant glands and 'capacitation' of the sperm (see later) would presumably have already taken place in vivo.

An attempt to search the literature revealed our first mistake, which was a terminological one. For years² we had carried out 'artificial insemination' (AI) by hand pairing in swallowtails, but IVF was clearly different, though it might be thought of as a form of AI. We could find no IVF Lepidoptera references, but bees seemed a possibility, they are so important commercially and a great deal is known about them. However, the Bee Research Association denied all knowledge of IVF, but were interested in what we were doing and asked to be kept in touch. Much surprised, we changed our tack and asked them for AI bee references and back by return came about 200, among them two of 'real' IVF – one by John Hunter in 1792³ and the other by Barratt in 1919⁴.

Hunter's paper³ was primarily about bees, but as an aside he mentioned his success with the silk moth *Bombyx mori*:

'Exper. 1. I took a female moth, as soon as she escaped from her pod, and kept her carefully by herself, on a clean card, until she began to lay; I then took males that were ready for copulation, opened them, exposing the seminal ducts, and after cutting into these, collected their semen with a hair pencil; with this semen I covered the ova, as soon as they

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Figure 1. Reproductive system in the female Lepidoptera (see text)

passed out of the vagina. The card with these eggs having a written account of the experiment on it, I kept in a box by itself. In the ensuing season, 8 of the ova hatched at the same time with others naturally impregnated. Thus then I ascertained that the eggs could be impregnated by art, after they were laid . . .'

'Exper. 2. I took a female moth, as soon as she had escaped from the pod, and kept her on a card till she began to lay. I then took females that were fully impregnated before they began to lay, and dissected out that bag which I supposed to be the receptacle for the male semen; and wetting a camel hair pencil with this matter, covered the ova as soon as they passed out of the vagina. These ova were laid carefully on a clean card and kept till the ensuing season, when they all hatched at the same time with those naturally impregnated.'

Our next step was to repeat the Hunter experiment. Initially we used the silk moth *Cricula andrei* and had no success, but later using *Bombyx mori* neither did our colleagues in the Insect House at the London Zoo¹. What had gone wrong?

A visit to the Hunterian Museum at the Royal College of Surgeons gave me the opportunity to look at Hunter's 200-year-old eggs and with a hand lens I thought they had collapsed, not hatched. Could Hunter have been wrong? Female *mori* mate instantly if a male is around and was it possible that John could have been deceived? Our editor, Professor Harding Rains, had a splendid explanation - we were being too scientific. He thought Hunter would certainly have licked his paintbrush before dabbing on the sperm and who knows what capacitating enzymes that famous saliva may have contained? However, subsequently our inferior spit was useless and so sadly, but most interestingly, we think that the Lepidoptera still pose a problem.

Bees (*Hymenoptera*)

Here there is more to tell. Most people know about the extraordinary habits of queen bees. First they need mating to produce some diploid eggs, but they then continue to be able to lay either diploid eggs which produce workers (and sometimes queens) or haploid ones which produce drones. The two types of egg are laid in the three different types of cell of the hive, which the queen identifies with her forelegs⁵.

Barratt⁴ decided to try to fertilize the haploid drone eggs laid by a golden Italian queen, but he used sperm obtained from pure Punic (dark coloured) drones, so that he could recognize hybrid offspring. He writes: ' . . . Pure Punic drones, just arriving in the hive after a flight, were squeezed over the eggs in the hope that a spermatozoon could enter the micropyle of the egg and thus fertilise it . . . ' They did, the ensuing insects being hybrid-like. Barratt had triumphed.

Curiously, others were less successful. Reinhardt⁶ (who does not mention Barratt) had dipped haploid eggs into sperm diluted in Ringer solution, and observed that in some cases the nucleus of the egg fused with that of the spermatozoon, but he does not report any larval hatchings. Khmara⁷ thought that his own failure was due to a membrane forming on the surface of the sperm, the result of desiccation.

Could Barratt also have erred? Do fertile queens ever make mistakes and lay diploid eggs in drone cells, and if so had the yellow Italian queen had an earlier Punic mate - a not uncommon occurrence? Experts may be able to help here. Barratt's experi-

ments looked excellent to us, but why have they never been successfully repeated?

Flies (*Diptera*)

In the housefly Leopold and Degrugillier⁸ showed that female accessory secretions were needed to facilitate penetration of the egg by the sperm but even so they reported failure with IVF. The same was true of *Drosophila*, though here Degrugillier⁹ pointed out that fertilization probably involved the precise placement of gametes so that the sperm could enter the egg.

Crickets (*Orthoptera*)

Here the sequence of events in natural fertilization is similar to the Lepidoptera, though McFarlane and McFarlane¹⁰ had more success with the house cricket *Acheta domesticus* (but again there is a caveat, this time concerning parthenogenesis).

Eggs from a mated female and the contents of the receptaculum seminis were incubated together in insect Ringer's solution and later in distilled water, and 59% of the eggs showed evidence of fertilization, but only reached the four-day stage. As a control, eggs from a virgin female but no sperm were used and some development took place even here, but parthenogenesis is not uncommon in *A. domesticus*.

A point of interest is that the McFarlanes¹⁰ showed, using fluorescent microscopy, that sperm penetration was not restricted to the micropyle.

Vertebrates

Fish (*Pisces*)

Since in most species IVF is the rule, fish should have much to tell us. The male simply spawns on top of the female's eggs, but the timing is most important and this has to be borne in mind, particularly in hybrid studies. Given this their technique is most efficient, either in fresh or salt water, but sperm capacitation, using small concentrations of potassium or magnesium ions, is necessary to facilitate the acrosome reaction (see Man).

Since spawning is such a direct procedure, and differences in genitalia do not have to be overcome, fish might be expected to hybridize easily and this is in fact the case, both in nature, particularly in freshwater fish, and experimentally¹¹.

The majority of fish lay haploid eggs but parthenogenesis does sometimes occur, though here a sperm may be necessary to act as a stimulus to division, though there is no nuclear fusion.

Fish alert us to the importance of the exact constituents of the medium in which they spawn, ie the composition of the capacitation fluids.

Marsupials

Marsupials, too, have no placenta, but I have not been able to find any report of IVF. However, there is information about the male in opossums. Here, while undergoing maturation in the epididymis sperm join head-on and remain so until fertilization, when one of them enters the egg. Possibly 'pairing' protects the entering sperm in the region of the acrosome, a sensitive area¹².

Man

As in butterflies, it seems useful to start with a brief description of the reproductive processes.

Figure 2. Rupture of the ovarian follicle with extrusion of the oocyte in a human female (by courtesy Professor Geoffrey Chamberlain)

In the human female the oogonia (immature eggs and therefore still diploid) are formed during her very early embryonic life - up to a total of about a million, and these are not added to. The eggs are just visible to the naked eye when later the follicles rupture (Figure 2).

How human eggs become haploid and therefore ready for fertilization is remarkable. At the age of about 15 weeks the oogonia enter the first stage of meiosis, but strangely this is halted at or near delivery and the second meiotic phase is deferred for many years until in fact the girl reaches puberty. At this time the first meiotic division becomes complete and the egg haploid (an oocyte). Next, when ovulation occurs the second phase of meiotic division begins, but this is only completed at the time of fertilization, which takes place in the Fallopian tube. Then, after 3 to 5 days, the developing embryo reaches the uterus and there, at about the 8-cell stage, implantation begins.

Why there should be two phases to the first meiotic division is unknown, but one suggestion is that if meiosis had been completed eggs could not live for as long as 20 years.

In the male, the testes contain seminiferous tubules, the walls of which consist of the primitive diploid spermatogonia. The process of converting a spermatogonium into sperm includes the two meiotic cell divisions (sperm do not have to live long) so that each spermatogonium gives rise to four sperm cells. These gradually move through the vas deferens which drains the tubules and thence into the epididymis where they undergo further maturation and are stored. Unless sperm have been through the vas deferens and the epididymis they cannot fertilize eggs, but the details are different in different species.

Another extremely important feature which is essential before the sperm is capable of fertilization is 'capacitation', and this normally takes place in the female reproductive tract, so that in vitro it has to be done artificially. This is effected by mixing the sperm with a variety of chemicals, one ingredient being calcium. The necessity for capacitation probably arises because the head of the sperm (the acrosome) has to be prepared so that it can bind to and penetrate

into the tough outer covering of the oocyte. Furthermore, the particular part of the sperm membrane lying around its head must fuse with the egg membrane so that the sperm nucleus can incorporate itself into the cytoplasm of the egg and thence into the nucleus (the acrosome reaction). Another aspect of capacitation is that it initiates the whiplash movement of the tail of the sperm.

In Man the main use of IVF is to overcome infertility, either in the male or in the female, and it is now known that where there are problems in conceiving they are almost as likely to derive from the father as from the mother¹³.

IVF is also potentially of very great help in the prevention of the birth of abnormal babies. If both parents are known to be carriers of a disease - for instance cystic fibrosis - and are anxious to have a child, it may be possible by DNA tests on the pre-embryo (that is the fertilized egg before 14 days, when the primitive streak appears) to detect an abnormality and only to put back into the uterus a healthy pre-embryo.

In the standard use of IVF, whether there is obstruction of the Fallopian tubes can be determined by laparoscopy, enabling the viewer to inspect the abdominal cavity through a small incision below the umbilicus. After introduction of the laparoscope the cavity is inflated with carbon dioxide; a telescope and light source of optical fibres threaded through the instrument show up the ovaries and the condition of the Fallopian tubes. If the lesion is not curable surgically, IVF may be indicated. Eggs are aspirated from the follicles via the laparoscope and mixed with the previously capacitated sperm from the husband. Cell division is then watched for.

A more recent method of recovering oocytes is by transvaginal aspiration (now becoming a standard practice) but whichever method is used it may be necessary to stimulate the production of several oocytes by giving gonadotrophic hormones, eg human chorionic gonadotrophin or a menopausal gonadotrophin such as clomiphene, at the appropriate times in the menstrual cycle. A minority of workers is against such superovulation, thinking that it predisposes to congenital abnormalities.

If after egg recovery fertility is effected, the embryo is transferred to the uterus of the mother at the 8-cell stage and IVF is over. When successful, the sex ratio is normal (1.01 to 1, male to female).

Where there is no blockage of the Fallopian tubes the technique of GIFT (gamete intra-Fallopian transfer) is now usually employed. Here the sperm and the recovered egg(s) are put together in fluid and injected into the Fallopian tube; if fertilization occurs it is a moot point whether it has taken place in vitro or in vivo.

In the male, oligospermia from various causes is frequent and IVF can sometimes remedy this by concentrating the sperm. Also, there may be immunological reactions between the sperm and the egg (see section on rabbits) and occasionally the sperm cannot penetrate the hard outer covering of the egg and slitting this may enable it to enter.

Infertility may also be caused by chromosome abnormalities, for example translocations, and some of these only cause sterility in the male¹³. It would be of interest to look at the fertility of families where Down's syndrome is caused by 15/21 translocations, and there is a hint that this might be useful since two

men with translocations were ascertained by their sterility, though here the anomaly was 14/21, and Robertsonian¹⁴.

In the early days IVF was marred by the fact that after three or four days, when the developing embryos were thought to be ready for implanting in the uterus it was found that they did not survive, because the eggs were being taken out at the wrong time in relation to the steroid pattern in the cycle. Later, by checking the surges of luteal hormones it was possible to gauge the time when natural ovulation took place, and this was the time to carry out laparoscopy or GIFT. Success came in 1978 when an 8-cell embryo derived from an egg taken out at the time of ovulation was implanted in the uterus of a patient from Bristol, and at the end of nine months a perfect baby (Louise Brown) was produced¹⁵.

A point of great interest is that pre-implantation embryos stand freezing for long periods of time. The 'twins' Amy and Elizabeth, now aged four and two years, both spent several months in deep freeze before being implanted at intervals in their mother's uterus.

In spite of Louise Brown, and others later, the success of IVF as regards a take-home baby is not high, around 10–15%, or rather higher with recent developments. However, if the success rate is related to the number of cycles in which the mother is treated it is considerably lower.

A paper from WHO¹⁶ summarizes the risks of IVF. Ectopic pregnancies may occur, birthweights are low and Caesarean deliveries high (41.6% for singletons and 62.7% for multiple pregnancies in Australia and New Zealand). Furthermore, for laparoscopy general anaesthesia is required, which has its own problems, and if vaginal retrieval is used this may cause bleeding, infections and injury to blood vessels and viscera. WHO regard IVF as still being experimental, partly because, most surprisingly of all, many patients while waiting for IVF become pregnant in the normal way. Is the converse true? Does IVF sometimes work when normal copulation does not, when no cause can be found for the infertility?

This WHO paper¹⁶ received a lot of criticisms in the *Lancet* (2 December 1989)¹⁷, the most adverse comments being that WHO used old data and that with modern techniques the outlook was much better. The discussion continued in the *Lancet* (16 December 1989)¹⁸.

Nevertheless, *Homo sapiens* cannot stand comparison with fish, though successes of course hit the headlines.

Racehorses

These could be of considerable commercial importance, particularly in cases where a high-class mare has damaged tubes perhaps as a sequel to injury, and normal 'covering' would then be useless. However, IVF in mares is a complete failure, probably for two reasons: first, unlike most farm animals, mares are difficult to superovulate, and mature oocytes are therefore very limited. A way round this - though still ineffective - is to use follicular oocytes obtained by dissecting ovaries from the slaughterhouse when six mature oocytes can on average be obtained from each ovary (they can apparently live for several hours in a cadaver).

Second, and more important, there are probably difficulties in capacitating stallion sperm *in vitro*, since they cannot be made to bind to and penetrate

the outer zone of the horse oocyte (Allen, personal communication, 1989). Possibly endogenous opioids which have binding properties might help here.

AI is forbidden in racehorses - to be seen 'covering' is essential; the Jockey Club is wise.

Cattle

Cattle were of considerable use for experimental purposes in the early days of IVF. Thus they provided evidence regarding capacitation of the sperm, and showed that there was a difference of from 9% to 75% in fertilization success between individual bulls, even when pre-treatment of the sperm was the same. Furthermore, cattle research taught workers how to watch the development of the fertilized oocytes before transfer to recipient cows. At the present time IVF is not much used, and embryo transfer (which is not IVF) has taken its place. Here a valuable cow is superovulated, fertilized by artificial insemination from a super-bull; the embryos are later washed out, grown for a while in culture and then put back into several surrogate mothers. In this way an average cow can produce 100 calves in her life compared with the normal ten¹⁹. Is this ethical? I wonder what animal lovers say.

Sheep

Here the type of approach is similar to that in cattle and there is the added advantage that frozen sperm or eggs can be satisfactorily transported for very long distances¹⁹.

Pigs

There has been success here¹⁹, and in embryo transfer more than one embryo can be put back as sows give birth to litters. In both sheep and pigs it has been found that IVF, which used to be difficult to achieve, can now be carried out successfully by capacitation at 39°C rather than 37–38°C²⁰.

Rabbits

These have been used experimentally in immunological incompatibility where the outer coat of the egg may react unfavourably with the sperm, but this can be avoided by microinjection¹⁶.

Dogs and cats

In a recent publication²¹ there is frequent mention of AI in dogs but none of IVF so presumably this is not in general use. However, if there were a risk of an inherited recessive disease in a proposed mating, flushing out eggs and doing IVF might enable an assessment to be made (using DNA probes) as to which embryos were likely to be affected. Again, if the disease were sex-linked one could put back only female embryos (see also under Man).

Cats are of interest in that their spermatozoa are capable of penetrating eggs as early as 20 min after insemination, and there is some doubt whether they even require capacitation.

Mice

Sadly, unlike cattle, there is no demand for an increase in the mouse population. However, the animal has its uses in research, and as regards IVF (used in a rather broad sense) 'transfection' is the key word. For example, an autosomal recessive gene causing mouse dwarfism can be cured by manipulation of fertile eggs derived from a dwarf female mated

naturally to a dwarf male. First, using superovulation techniques, the many fertile eggs can be discharged from the vagina and microinjection (as in IVF) then used to add growth hormone DNA. This transmits normal growth to the embryo and is also incorporated in the genome so that further generations are no longer dwarfs. After the injection the eggs are replaced in the Fallopian tubes²².

The hamster

Here is a suitable finale to our stories. Egg and sperm nuclei usually fuse only if both are from the same or closely related species. However, in 1976 USA workers showed that hamster eggs denuded of their zona pellucida could be fertilized to several divisions by human sperm²³. This penetrates the cytoplasm and acts as a non-specific stimulus but only to a few cell divisions. It is probably not proper fertilization and has been criticized on this count, but apparently it gives useful results when testing for infertility in human males. Interestingly, sperm from marsupials and budgerigars will also fuse with the cytoplasm of the hamster.

Conclusions

It will be seen that IVF is a difficult proposition (except in fish) and although successful results are striking, failure must be regarded as the norm and, as has been said, the technique is really still in the experimental stage. This is surprising when one considers that capacitation of the sperm, their swimming velocity, the acrosome reaction and all the other preliminaries must be successfully happening in ordinary people millions of times every day, and the 'real thing' makes IVF look a very clumsy alternative.

Science is still nowhere near a match for the Almighty.

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Note added in proof

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