

## STUDIES ON THE TRANSFER OF FERTILIZED MOUSE EGGS TO UTERINE FOSTER-MOTHERS

### I. FACTORS AFFECTING THE IMPLANTATION AND SURVIVAL OF NATIVE AND TRANSFERRED EGGS

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(Received 2 December 1955)

#### INTRODUCTION

In 1890 Walter Heape performed a classical experiment which he described as follows: 'On the 27th April, 1890, two ova were obtained from an Angora doe rabbit which had been fertilised by an Angora buck thirty-two hours previously. . . . These ova were immediately transferred into the upper end of the fallopian tube of a Belgian hare doe rabbit which had been fertilised three hours before by a buck of the same breed as herself. . . .

'In due course this Belgian hare doe gave birth to six young—four of these resembled herself and her mate, while two of them were undoubted Angoras.'

Heape thus demonstrated that a pregnancy could be artificially induced differing in two respects from a normal pregnancy. First, the Angora young had no blood relationship to their uterine foster-mother or to their foster-siblings. Secondly, their post-conception age differed by over a day from their foster-mother's post-coital phase, and hence from the post-conception age of their foster-siblings.

'The experiment. . .', wrote Heape, 'was undertaken to determine in the first place what effect, if any, a uterine foster-mother would have upon her foster-children, and whether or not the presence and development of foreign ova in the uterus of a mother would affect the offspring of that mother born at the same time.'

To this may be added another main category of inquiry opened up by Heape's achievement: the effect of the presence and development of foreign ova upon the uterine foster-mother herself. As a single example: was the duration of pregnancy in Heape's recipient rabbit influenced by her precocious fosterlings? For it is certain that either the fosterlings were born prematurely or their foster-siblings were born belatedly.

These three broad categories do not exhaust the range of problems in genetic's embryology, reproductive physiology, immunology and cancer research which can be attacked by the technique of egg transfer. A further reason for interest in the subject is the possibility of practical application to livestock farming. It has been shown that a sexually immature female can be induced by hormone treatment to ovulate an abnormally large number of eggs. These can be fertilized *in situ* and then transferred to a battery of sexually mature recipients. It therefore becomes possible in theory (1) to shorten the generation interval and thus to accelerate the improvement of livestock by selective breeding (see Adams, 1954), and (2) to multiply the

genetic contribution to the breed made by outstanding females, just as artificial insemination can be used to propagate the good qualities of outstanding males. Improvement of methods of storing mammalian eggs also holds out the possibility of transporting an entire herd or flock about the world within the confines of a vacuum flask.

In all the uses to which egg transfer has been put in a variety of mammalian species, the yield of living young obtained has been low. For its full exploitation as a research tool the technique will require to be brought to a high level of reliability in a cheap, fecund and rapidly maturing laboratory animal. As a step towards this end we have made a study of some of the factors affecting the implantation and survival of fertilized mouse eggs after transfer to uterine foster mothers.

The first successful transfer of mouse eggs to a uterine foster mother was performed in 1935 by Little (cited by Bittner & Little, 1937). Subsequent published work includes studies by Fekete & Little (1942), Fekete (1947), Beatty (1951), Runner (1951), Runner & Palm (1953) and Boot & Mühlbock (1953).

## MATERIALS AND METHODS

### *Donors and recipients*

As *donors* we used albino females supplied by 'The Mousery', Rayleigh, Essex. They were mated with albino males from the same source. As *recipients* we used miscellaneous adult females homozygous for full colour, mated with male  $F_1$  hybrids between the C<sub>3</sub>H and C<sub>57</sub>BL inbred strains. Embryos of donor origin were thus homozygous albinos and could be distinguished at autopsy from native embryos by their eye colour.

For egg transfers from immature donors, ovulation was induced according to the general procedure of Runner & Palm (1953). Priming doses of pregnant mare's serum were usually given on the twenty-seventh and twenty-eighth days of life, and chorionic gonadotrophin was injected at midday on the thirtieth day of life. According to Runner & Palm ovulation is expected to occur  $13 \pm 2$  hr. later, i.e. at about 1 a.m. This coincides with the time of night at which mice usually mate (Snell, Fekete, Hummel & Law, 1940).

The occurrence of mating in both donors and recipients was detected by the presence of a vaginal plug on the following morning. Operations were performed either 2 or 3 days later during the period 10 a.m.–4 p.m. Hence to an accuracy of a few hours either  $2\frac{1}{2}$  or  $3\frac{1}{2}$  days had elapsed between the mating of female mice and their use as donors and recipients.

### *Recovery of fertilized eggs*

The donors were killed and the gross appearance of the ovaries noted, although exact counts of corpora lutea were not made. Each uterine horn with attached oviduct was placed in a watch-glass in a few ml. of Ringer-phosphate saline (Pannett & Compton, 1924) at room temperature. The eggs were then recovered under a dissecting microscope.

In the case of  $2\frac{1}{2}$ -day donors, each oviduct was chopped with a mounted surgical needle into short lengths, and each length was stroked with the needle, so that all contained eggs emerged, together with epithelial debris.

In the case of  $3\frac{1}{2}$ -day donors the eggs were usually already in the uterus, but sometimes in the very last section of the oviduct where it enters the uterus. This terminal section was severed from the rest of the oviduct and emptied by stroking with the needle. The uterine horn was then cut through close to its junction with the oviduct, and the uterine fragment which remained attached to the oviduct was searched for eggs. Finally, a stream of saline was blown from a fine Pasteur pipette through the uterine horn from the vaginal towards the ovarian end. The entire field was then searched for eggs.

As the eggs were found they were immediately transferred in a very fine Pasteur pipette to a solid watch-glass containing a few drops of saline and covered with a glass cover-slip. When the search for eggs was complete, the eggs were marshalled together by gently directing streams of saline towards them. Finally, they were picked up together with a little fluid, using the capillary action of a Pasteur pipette having a very fine terminal section 2-3 cm. in length. The eggs were then ready for injection into the uterus of the recipient.

#### *Transfer of eggs to the host uterus*

The recipient was anaesthetized with ether and a dorsal transverse skin incision was made to the left of the mid-line. On retraction of the margins of the incision the left ovary could usually be seen through the semi-transparent abdominal wall, which was then incised at the point where it overlay the ovary. The ovarian fat pad was seized with forceps and pulled through the incision, carrying with it the ovary, the oviduct, and the upper part of the left uterine horn. The appearance of the ovary was recorded and the uterine horn was then punctured near the top with a hypodermic syringe fitted with a needle of 0.47 mm. external diameter. Through this puncture the end of the pipette containing eggs was introduced, with the tip pointing away from the oviduct. The saline and contained eggs were injected into the uterus by gently squeezing the bulb of the pipette.

After the ovary, oviduct and uterine horn had been pushed back with a probe into the abdominal cavity the skin incision was closed with cotton sutures. The incision in the abdominal wall was left unsutured, and was invariably found at autopsy to have healed, sometimes, however, with adhesion to the left ovarian fat pad.

#### THE PLAN OF THE EXPERIMENT

We have been concerned mainly to investigate three factors affecting the survival of transferred eggs: the relative stages post-coitum of donor and recipient, the trauma suffered by the recipient from the surgical procedure, and the sexual maturity or immaturity of the donor. The number of eggs injected was varied, so that we have been able to study this factor also.

Donors and recipients were used either  $2\frac{1}{2}$  or  $3\frac{1}{2}$  days post-coitum. All four combinations of donor and recipient stages were tested, using induced ovulation of

immature donors. The most successful combination (3½-day donor, 2½-day recipient) was repeated, using natural ovulation from mature donors. This combination was also repeated, together with the least successful (2½-day donor, 3½-day recipient), using recipients in which pseudo-pregnancy had been induced by mating to sterile vasectomized males. In addition, 'dummy' transfers (saline without eggs) were done on both 2½- and 3½-day recipients. Finally, intended recipients for which no donors were available were killed and examined after the same lapse of time as operated mice, thus providing a series of unoperated controls. The general plan of the experiment is set out in Table 1.

Table 1. *Plan of experiment*

Group	Post-coital stage (in days) of		State of donor	State of recipient
	Donor	Recipient		
Unoperated controls	—	—	—	—
0→3½	—	3½	—	Pregnant
0→2½	—	2½	—	Pregnant
2½→3½	2½	3½	Immature, induced ovulation	Pregnant
2½→2½	2½	2½	Immature, induced ovulation	Pregnant
3½→3½	3½	3½	Immature, induced ovulation	Pregnant
3½→2½	3½	2½	Immature, induced ovulation	Pregnant
Mature, 3½→2½	3½	2½	Mature, natural ovulation	Pregnant
2½→3½ (vas.)	2½	3½	Immature, induced ovulation	Pseudo-pregnant
3½→2½ (vas.)	3½	2½	Immature, induced ovulation	Pseudo-pregnant

Transfers were done into one uterine horn only (the left), so that its contents at autopsy could be assessed against those of the unoperated, or control, horn. 14 days after operation (16½ or 17½ days post-coitum) all recipients were killed and each implantation in both horns was classified as 'live alien embryo', 'live native embryo' or 'dead resorbing embryo'. On a few occasions several of the recipients were found to contain only dead resorbing embryos. Such pregnancies, numbering nine in all, were reckoned to be abnormal, and have been omitted from the data presented in this paper.

Embryos which die soon after implantation (i.e. after the fifth and before the tenth day) may be completely resorbed before autopsy and hence escape notice. Where necessary to avoid confusion we shall use the phrase 'successful implantations' to denote those which survive this period. Our later references to post-implantational mortality should be taken to mean 'post-implantational mortality detectable by our methods and criteria'.

## DESCRIPTION AND ANALYSIS OF RESULTS

*Control data*

As shown in Table 2, thirty-one out of fifty-four unoperated control females became pregnant. They were found to have 210 implantations distributed between the two horns, an average of 6.8 per female. Thirty-three of these were resorbing, making an overall resorption rate of 15.7%.

Table 2. *Control data on unoperated mice*

No. of mice showing vaginal plugs	No. of pregnancies		No. of embryos in						Grand total
			Right horn			Left horn			
			Live	Resorbing	Total	Live	Resorbing	Total	
54	31	Total	95	14	109	82	19	101	210
		Av. per pregnancy	3.1	0.5	3.5	2.6	0.6	3.3	6.8
		% resorbing		12.8			18.8		

Table 3. *Uninjected horns of operated mice compared with control data on unoperated mice*

	No. of mice showing vaginal plugs	No. of pregnancies		No. of implantations			% resorbing
				Alive	Resorbing	Total	
Unoperated mice	54	31	Total	177	33	210	15.7
			Av. per horn*	2.9	0.5	3.4	
Operated mice	202	129	Total	411	65	476	13.7
			Av. per horn†	3.2	0.5	3.7	

\* Both horns combined. † Uninjected horns only.

Considering the two sides separately, the right horns had an average of 3.5 implantations of which 12.8% were resorptions, while the left horns had an average of 3.3 implantations of which 18.8% were resorptions. Neither of these differences approaches statistical significance. There was no correlation between the two sides in the number of implantations ( $r = -0.025$ ,  $n = 31$ ). But there was a large and statistically highly significant positive correlation between the two sides in the number of resorptions ( $r = +0.651$ ,  $n = 31$ ).

*Systemic effects of surgical interference*

In Table 3 we give comparative data on operated and unoperated mice, from which we can inquire whether or not the operation has generalized effects upon the recipient's reproductive processes apart from the local effects upon the injected uterine horn.

(1) *Does the operation affect the pregnancy rate?*

The proportion of all operated mice which became pregnant (excluding those mated with vasectomized males) was  $129/202 = 64\%$  as compared with  $31/54 = 57\%$  in the unoperated controls. The difference is not significant. We may therefore exclude all non-pregnant mice from further consideration, since the pregnancy rate is evidently controlled by factors extraneous to the technique of egg transfer itself.

(2) *Does the operation affect implantation in the uninjected uterine horn?*

The mean number of implantations in the right (uninjected) horns of all operated mice (excluding those mated with vasectomized males) was  $3.69 \pm 0.16$  as compared with  $3.39 \pm 0.21$  for the unoperated controls (average of both horns). The former figure has received a trivial increment from the transmigration\* of four embryos (see Table 7) from the left into the right horn, and may possibly also include one or two resorptions derived from the same source. We can, however, conclude that the operation does not substantially reduce the implantation rate in the uninjected horn. We shall later come to an important, although quantitatively small, qualification of this conclusion, to the effect that when large numbers of eggs are transferred some inhibition of implantation in the uninjected horn appears to occur.

(3) *Does the operation affect post-implantational mortality in the uninjected horn?*

Of 476 implantations in the uninjected horns of the operated mice, sixty-five, or  $13.7\%$ , were resorbing, as compared with  $15.7\%$  in the unoperated controls (both horns combined).

Thus any effect on post-implantational mortality which may result from the operation must plainly be confined to the injected horn.

*Local effects of surgical interference*

(1) *Evidence from dummy transfers*

It was in order to assess the effects on the injected horn of the surgical trauma involved in egg transfer that we did the dummy transfers of saline without eggs. The relevant data are summarized in Table 4.

The left-hand side of the table shows that the injection of saline alone resulted in the loss before, during or shortly after implantation of about one-third of the recipient's own eggs, whether the operation was performed  $2\frac{1}{2}$  or  $3\frac{1}{2}$  days post-coitum. Since at the  $2\frac{1}{2}$ -day stage the recipient's eggs are still in her oviduct, the effect presumably acts, not directly upon the eggs, but upon the uterine horn, reducing the facility with which it subsequently forms or maintains implantation sites.

\* In a publication on the transmigration of fertilized mouse eggs (McLaren & Michie, 1954) based on an earlier stage of the present work, we overlooked a previous finding by Runner (1951) of one transmigrant among eighty embryos examined. Runner's embryos were derived from eggs recovered from the donor immediately after ovulation and injected into the right ovarian capsule of the recipient.

Table 4. *The effects of dummy transfers*

Recipients		Average no. of successful implantations			Resorptions as percentage of successful implantations	
Stage	Pregnancies	Right horn (uninjected)	Left horn (injected)	Left horn as percentage of right	Right horn (uninjected)	Left horn (injected)
2½ days	21	3·6	2·5	68	17·1	15·4
3½ days	22	3·8	2·5	64	19·0	33·3

Such uterine damage could act in one of two possible ways. It could reduce the effective number of available implantation sites, thus lowering the upper limit to the number of implantations in the horn. This would decrease the frequency of large numbers of implantations, increase the frequency of intermediate numbers, and leave the frequency of small numbers unaffected, resulting in a skew frequency distribution. Or it could reduce impartially each egg's *chance* of successfully implanting, reducing the mean of the frequency distribution without materially affecting its shape. The observed distribution, shown in Table 5, indicates that the

Table 5. *Frequency distributions of the number of successful implantations in the right (uninjected) and left (injected) horns of recipients of dummy transfers. Results from 2½- and 3½-day recipients were in good agreement and have been combined.*

No. of implantations	0	1	2	3	4	5	6	7	Total
Frequency: Right horns	—	5	7	8	8	7	6	2	43
Left horns	5	10	7	8	7	6	—	—	43

second view is to be preferred. Further support for this view is lent by results from egg transfers to be described later; when large numbers of eggs were injected, the deficit caused by the surgical interference was more than made up, so that the average number of implantations in the left horn was actually greater than normal.

The right-hand side of Table 4 shows that the injection of saline had no effect on the resorption rate in 2½-day recipients, but that it substantially increased the rate in 3½-day recipients.

## (2) *Evidence from egg transfers*

We kept fairly detailed notes of all operations, and have consequently been able to make a rough classification of them as either 'traumatic' or 'non-traumatic'. 'Traumatic' operations included those in which air was injected into the uterine horn, or the uterine horn was punctured several times or seized with forceps. The various series of transfers differed in the incidence of 'traumatic' operations. This is because it was not possible to arrange for them to be exactly contemporaneous, although they overlapped in time. The work extended over a period of more than a year, during which time our surgical technique was continually improving.

Table 6 shows the recorded incidence of 'traumatic' operations, and the range of time occupied by each series.

In Table 7 we have set out the full results of each series, tabulating 'traumatic' and 'non-traumatic' operations separately. The results of the various series of egg transfers confirm and extend the conclusions derived from the dummy transfers.

Table 6. *Temporal distribution of the different types of transfer and of the incidence of 'traumatic' operations*

Type of transfer	1954									1955					
	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec.	Jan.	Feb.	Mar.	Apr.	May	June
0 → 3½	_____														
2½ → 3½	_____														
3½ → 3½	_____														
0 → 2½	_____														
2½ → 2½	_____														
3½ → 2½	_____														
3½ → 2½	_____														
(mature donors)															
'Traumatic' total	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡	‡

Table 7

Group	Type of operation	No. of pregnancies	Av. no. of eggs injected	Total no. of embryos in							
				Right horn (uninjected)				Left horn (injected)			
				Native	Alien	Re-sorbing	Total	Native	Alien	Re-sorbing	Total
0 → 3½	Traumatic	2	—	9	—	2	11	3	—	1	4
	Non-traumatic	20	—	59	—	14	73	33	—	17	50
2½ → 3½	Traumatic	6	16.7	18	0	2	20	7	2	9	18
	Non-traumatic	8	15.4	21	0	3	24	25	0	6	31
3½ → 3½	Traumatic	4	4.5	10	0	0	10	3	0	10	13
	Non-traumatic	15	7.2	57	1	1	59	24	10	11	45
0 → 2½	Traumatic	3	—	12	—	3	15	1	—	0	1
	Non-traumatic	18	—	51	—	10	61	43	—	8	51
2½ → 2½	Traumatic	7	10.6	27	0	1	28	10	1	6	17
	Non-traumatic	9	9.9	26	1	6	33	20	9	6	35
3½ → 2½	Traumatic	5	13.4	18	0	0	18	4	8	14	26
	Non-traumatic	15	9.2	42	2	10	54	25	28	12	65
Mature, 3½ → 2½	Traumatic	1	10.0	5	0	2	7	3	1	0	4
	Non-traumatic	16	9.2	52	0	11	63	23	34	15	72

Thus, even 'non-traumatic' transfers to 3½-day recipients caused a substantially increased resorption rate in the injected horn. The increase was still greater in 'traumatic' operations. The relative indifference of the 2½-day uterus to surgical trauma is also confirmed; 'non-traumatic' transfers to 2½-day recipients did not result in increased resorption rates in the injected horn. The infliction of more



serious injury on the  $2\frac{1}{2}$ -day uterus can, however, cause post-implantational mortality, as shown by the general tendency to increased resorption rates in 'traumatic' transfers to  $2\frac{1}{2}$ -day recipients.

The reduction in the average number of successful implantations, which was shown to result from the transfer of saline both to  $2\frac{1}{2}$ - and to  $3\frac{1}{2}$ -day recipients, is confirmed by the various series of egg transfers. In these series we must assess this reduction from the number of *native* embryos in the two horns, since the total number of implantations has received an increment from the transferred eggs. A deficit of native embryos in the injected horn is a general feature of the egg-transfer series, and it is evident that the deficit is greater in 'traumatic' operations; the yield of alien embryos is also reduced in these operations.

In addition it can be seen that, for each recipient stage, the reduction of the number of native embryos in the injected horn is greater in transfers of  $3\frac{1}{2}$ - than of  $2\frac{1}{2}$ -day eggs, i.e. in those series which give the higher yields of alien embryos. This suggests that there is competition between transferred and native eggs, a suggestion which is fully confirmed by facts which will be presented later. The effect is still apparent when attention is confined to the series which give no increased resorption rates in the injected horn ('non-traumatic' transfers to  $2\frac{1}{2}$ -day recipients), so most or all of the competition occurs before, during or shortly after implantation.

In order to make the various series more directly comparable, 'traumatic' operations will be excluded from further consideration, and subsequent analysis will be based solely on the results of 'non-traumatic' operations.

*The effect of the relative post-coital stages of donor and recipient upon the yield of live embryos from transferred eggs*

In Table 8 we have abstracted from Table 7 the yields of alien embryos from 'non-traumatic' transfers in the different series. It can be seen that the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  combination gave about twice the yield obtained from the  $3\frac{1}{2} \rightarrow 3\frac{1}{2}$  and  $2\frac{1}{2} \rightarrow 2\frac{1}{2}$

Table 8. *The yields of alien embryos obtained with different post-coital stages of donor and recipient ('traumatic' operations excluded)*

State and stage of donor	Stage of recipient	Av. no. of eggs injected	No. of pregnancies	No. of alien embryos	Average no. of alien embryos per pregnancy
Immature $2\frac{1}{2}$ -day	$3\frac{1}{2}$ -day	15.4	8	0	0.0
Immature $3\frac{1}{2}$ -day	$3\frac{1}{2}$ -day	7.2	15	11	0.7
Immature $2\frac{1}{2}$ -day	$2\frac{1}{2}$ -day	9.9	9	10	1.1
Immature $3\frac{1}{2}$ -day	$2\frac{1}{2}$ -day	9.2	15	30	2.0
Mature $3\frac{1}{2}$ -day	$2\frac{1}{2}$ -day	9.2	16	34	2.1

combinations; the  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  combination yielded no live embryos from the transfers summarized in the table in spite of the much larger average number of eggs injected. It is, however, possible to obtain live embryos from this combination, since two such were found in a single recipient of a 'traumatic' transfer (see Table 7).

Part of the explanation of these differences in yield can be sought in the occurrence, mentioned above, of competition between native and transferred eggs. On this view, where transferred and native eggs differ in developmental stage, the implantation of the precocious group hinders the subsequent implantation of their backward foster-siblings. Competition of this type would bear most heavily upon  $2\frac{1}{2}$ -day eggs transferred to  $3\frac{1}{2}$ -day recipients.

The occurrence of competition of this type can be independently demonstrated, and the stage at which it operates established, as follows:

In the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  series we calculate the coefficient of correlation between alien and native embryos in the injected horn. We find  $r = -0.375$ ,  $n = 31$ ,  $P < 0.05$ , which indicates the occurrence of competition. To determine the stage at which it mainly occurs, we exclude all post-implantational death by recalculating the correlation solely from those females with no resorptions in the injected horn. We find  $r = -0.468$ ,  $n = 19$ ,  $P < 0.05$ . We therefore conclude that the greater the number of precocious alien implantations, the fewer native eggs are able successfully to implant in the same horn. The possibility that some post-implantational competition occurs is, of course, not excluded. Fawcett, Wislocki & Waldo (1947) transplanted fertilized mouse eggs to the anterior chamber of the eye and concluded from their results that 'ova are capable of developing in close proximity only until the most precocious among them begins to implant when it sets up a sphere of influence which renders the immediate vicinity inimical to the further development of other eggs'.

The two synchronous combinations show no effect of this kind, nor does the material of Runner (1951), who performed synchronous egg transfers to the ovarian capsule immediately after ovulation. It is possible that competition is only important under conditions of crowding. Alternatively, the implantation of mouse eggs may render the uterus refractory to the *subsequent*, but not to the simultaneous, implantation of other eggs.

*Is competition sufficient to explain the whole of the observed differences in yield?*

In order to discover whether the relative stages of transferred eggs and *recipient uterus*, in addition to the relative stages of transferred and native eggs, was playing a part, we made further tests of the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  and  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  combinations, using recipients which had been mated with vasectomized males. The results were thus uncomplicated by the presence of native embryos, all eggs being of donor origin. They were also uncomplicated by undue surgical trauma, for it so happened that all operations of this type were 'non-traumatic'.

The results, set out in Table 9, show a large superiority of the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  over the  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  combination. The superiority is not connected with the greater average number of eggs injected in the former series, since the eight implantations were derived from three successful transfers averaging only eight eggs per transfer. Hence part of the difference in yield between  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  and  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  transfers is independent of the presence of competing native eggs.

Table 9. *Comparison of two combinations of donor and recipient post-coital stages, using recipients mated with vasectomized males*

Av. no. of eggs	Donor	Recipient	No. of recipients	Right horn		Left horn		Total implantations	Live embryos per recipient
				Live embryos	Resorptions	Live embryos	Resorptions		
8.9	2½-day	3½-day	14	0	0	1	0	1	0.07
10.0	3½-day	2½-day	7	1	1	5	1	8	0.86

*Comparison of artificially ovulated eggs from immature donors with naturally ovulated eggs from mature donors*

Table 10 gives comparative data on the results from mature and immature donors (excluding 'traumatic' operations). It is clear that the viability of artificially ovulated eggs from immature donors was not inferior to that of naturally ovulated eggs from mature donors. The two 3½ → 2½ series gave results which were similar in all respects, and in subsequent sections they will be combined.

Table 10. *Comparison of transfers from immature and mature donors (3½ days → 2½ days)*

State of donor	No. of pregnancies	Av. no. of eggs injected		Number of embryos in							
				Right horn (uninjected)				Left horn (injected)			
				Native	Alien	Re-sorbing	Total	Native	Alien	Re-sorbing	Total
Immature (induced ovulation)	15	9.2	Total Av. per pregnancy	42 2.8	2 0.13	10 0.67	54 3.6	25 1.7	28 1.9	12 0.80	65 4.3
Mature (natural ovulation)	16	9.2	Total Av. per pregnancy	52 3.3	0 0.00	11 0.69	63 3.9	23 1.4	34 2.1	15 0.93	72 4.5

*The frequency distribution of alien embryos*

Fig. 1 shows the thirty-one pregnancies of the 3½ → 2½ series arranged in a frequency diagram according to the yields of alien embryos obtained from them. By far the commonest class is that of zero yield, suggesting that over and above the random loss of individual eggs there is a second and distinct phenomenon of whole inoculum loss. In order to make the frequency of zero yield fit a smoothed curve drawn through the rest of the distribution on the assumption of random loss, we should have to discard about ten of the observations in the zero class, assigning them to the category of whole inoculum loss. We conclude therefore that something in the region of one-third of all transfers fail through loss of the whole inoculum of eggs as a unit.

*The effect of the number of eggs injected*

In Table 11 the results summarized in Table 10 are tabulated according to the number of eggs injected. The same information, together with data from the  $0 \rightarrow 2\frac{1}{2}$  series, is presented graphically after appropriate grouping in Fig. 2. The following points stand out:

(1) The average number of successful implantations of all sorts in the injected horn is less than that for unoperated controls by about 1, when *no* eggs are injected (dummy transfers). From this level it rises linearly by increments of about 0.2 for each additional egg injected, finally attaining a value of about 2 more than that found in unoperated controls. Thus, over the range which we tested, there is no evidence of a 'ceiling' to the number of eggs which can implant in one uterine horn.

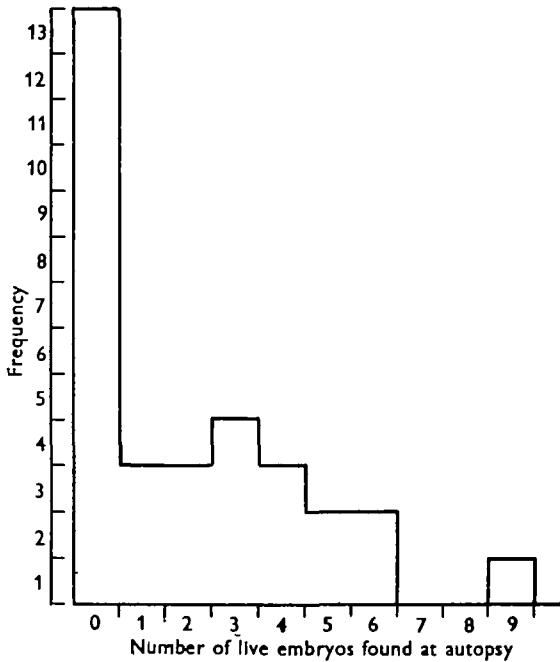


Fig. 1. Frequency distribution of the number of alien embryos in the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  series.

(2) But the average number of successful implantations in the two horns together does appear to reach a 'ceiling'. When large numbers of eggs are injected, the number of implantations in the *uninjected* horn decreases, so that the average number of implantations for the two horns together does not rise above 8.5. This effect will be further discussed in the next section.

(3) The average number of alien embryos in the injected horn, starting from zero in the dummy transfers, rises linearly by increments of 0.225 for each additional egg injected. This is what we should expect if each alien egg had a 22½%

Table 11. *Data on 'non-traumatic'  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  operations, arranged according to number of eggs injected*

No. of eggs injected	Donor (I=immature M=mature)	No. of embryos in							
		Right horn (uninjected)				Left horn (injected)			
		Native	Alien	Re-sorbing	Total	Native	Alien	Re-sorbing	Total
5	I	4	0	2	6	3	0	0	3
5	I	4	0	2	6	0	0	2	2
5	I	2	0	1	3	1	3	1	5
5	M	1	0	1	2	5	0	0	5
6	I	1	0	1	2	3	1	0	4
6	I	4	0	0	4	1	4	0	5
6	M	2	0	0	2	1	1	1	3
7	I	4	0	1	5	0	0	0	0
7	M	2	0	1	3	0	0	0	0
7	M	5	0	0	5	3	0	0	3
8	I	4	0	0	4	4	1	0	5
8	I	2	1	0	3	2	4	1	7
8	M	6	0	0	6	4	0	0	4
8	M	7	0	2	9	0	4	0	4
8	M	3	0	1	4	2	4	0	6
9	I	3	0	2	5	1	3	0	4
9	M	3	0	3	6	1	0	3	4
9	M	6	0	1	7	0	0	0	0
10	I	3	1	0	4	0	5	0	5
10	I	2	0	0	2	4	0	0	4
10	M	2	0	0	2	1	6	0	7
10	M	3	0	0	3	0	2	3	5
10	M	2	0	0	2	3	2	3	8
11	I	3	0	0	3	2	0	0	2
11	M	3	0	1	4	1	0	2	3
11	M	3	0	0	3	1	3	0	4
12	M	2	0	1	3	1	3	0	4
14	I	2	0	0	2	1	2	1	4
16	I	3	0	1	4	2	0	2	4
16	M	2	0	0	2	0	9	3	12
18	I	1	0	0	1	1	5	5	11

chance of survival irrespective of the number injected. Over the range which we tested there is no tendency for the yield per injected egg to fall off when large numbers are injected, i.e. no evidence of a 'ceiling' to the yield of alien embryos that can be obtained from one horn. In Table 12 a regression analysis of these data is shown.

(4) The average number of native embryos in the injected horn shows a concomitant decline at an overall rate of about 0.09 per additional egg injected. The corresponding regression analysis, set out in Table 13, shows that the effect is significant, thus confirming that the transferred eggs hinder the successful implantation or subsequent survival of the native eggs in the same horn.

(5) The total number of live embryos (natives + aliens) in the injected horn shows a steep initial rise from the level found in recipients of dummy transfers to a level somewhat higher than that found in unoperated mice, but thereafter rises slowly.

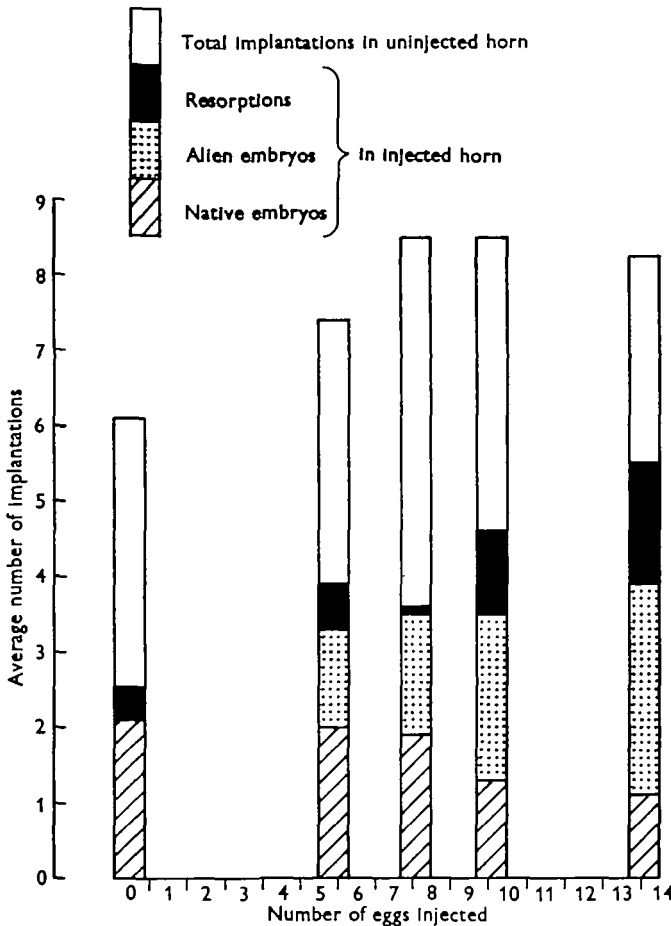


Fig. 2. The results of injecting different numbers of eggs in  $3\frac{1}{2}$ -day  $\rightarrow$   $2\frac{1}{2}$ -day transfers.

Table 12. Regression analysis of the relation in the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  series between the number of eggs injected and the number of live alien embryos found at autopsy

	Sums of squares	Degrees of freedom	Mean square	Mean square ratio
Regression	29.3250	1	29.3251	6.1 $P < 0.05$
Residual	140.5460	29	4.8461	
Total	169.8710	30		

(6) When the average number of implantations in the injected horn begins markedly to exceed the normal level, the resorption rate increases.

The last two points suggest that, although we have found no limit to the number of eggs which can implant in a single uterine horn, we are beginning to approach a limit to the number of implantations which a single horn can keep alive.

Table 13. *Regression analysis of the relation in the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  series between the number of eggs injected and the number of live native embryos found in the injected horn (dummy transfers to  $2\frac{1}{2}$ -day recipients included)*

	Sums of squares	Degrees of freedom	Mean square	Mean square ratio
Regression	10.9509	1	10.951	4.9 $P < 0.05$
Residual	107.0491	48	2.230	
Total	118.0000	49		

*Possible interaction between uterine horns when the number of implantations in one horn is artificially raised by egg transfer*

We have seen that in unoperated controls there was no correlation between the number of implantations in the two uterine horns. This is in accord with observations by Danforth & de Aberle (1928) upon 500 pregnant mice. They interpret the lack of correlation as the resultant of (1) an initial negative correlation between the number of eggs on the two sides, and (2) heterogeneity between the females in factors affecting pre-implantational loss of eggs, causing a superimposed positive correlation. The negative correlation found by Runner (1951) in his unoperated controls may be due to the use of more homogeneous stocks than those used by Danforth & de Aberle and by ourselves, since his control females were taken from a single inbred strain. On the other hand, Hollander & Strong (1950) also found a negative correlation in 1080 females of very diverse origin.

Transfer of eggs weakens or removes the initial negative correlation between the number of eggs on the two sides, and should therefore result in a positive correlation between the number of implantations in the two horns. Runner found a positive correlation between the number of live embryos in the two horns (data on dead and resorbing embryos were not published) in females to which unfertilized eggs had been transferred. These females were taken from four different inbred strains, and so were probably less homogeneous than his controls. This factor, as we have seen above, would tend to increase the positive correlation.

In our own data we found a large and statistically significant *negative* correlation in recipients of  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  transfers between the number of successful implantations in the two horns ( $r = -0.483$ ,  $n = 31$ ,  $P < 0.01$ ). The greater part of the correlation arose from those females which received large numbers of eggs. In Table 14 the recipients of  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  transfers are divided into those receiving 5-8 eggs and those receiving 9-18 eggs, and are compared with the  $0 \rightarrow 2\frac{1}{2}$  transfers and with the unoperated controls. It will be seen that, as the average number of implantations increases, the degree of negative correlation also increases, and is especially pronounced in the group in which the average number of implantations in the left horn was markedly raised above the normal level (transfers of 9-18 eggs).

The simplest interpretation of these results is that the implantation of eggs in one horn hinders the implantation of other eggs, not only in the same horn, but also to some extent in the other horn; but that the process only operates to a

substantial extent when the number of implantations is raised above the normal level. It can be seen from Table 11 that as the number of eggs injected increases, so the number of implantations in the uninjected horn decreases; this trend is statistically significant ( $b = -0.16, P < 0.05$ ). As we have seen, this leads to a 'ceiling' for the average number of successful implantations in the two horns together.

Table 14. *The correlation between the number of implantations in the two horns in various groups of mice arranged according to the average number of implantations ('traumatic' operations excluded)*

Group	No. of eggs injected	No. of pregnancies	Correlation	Average no. of successful implantations		
				Right horn	Left horn	Total
0 → 2½ (dummy transfers)	0	18	+0.075	3.4	2.6	5.9
Unoperated controls	0	31	-0.025	3.5	3.3	6.8
	5-8	15	-0.184	4.3	3.7	8.0
	9-18	16	-0.670	3.3	5.1	8.4

This interpretation, however, fails to account for the contradiction with Runner's result. The contradiction would be largely explained if the hindrance to implantation in the uninjected horn were exerted solely by the *precociously* implanting (i.e. alien) eggs; in Runner's work alien and native eggs were contemporary. However, an analysis of our data by the method of partial correlation (Fisher, 1925-50) showed that implantation in the right (uninjected) horn is hindered to the same extent whether the number of precocious alien implantations in the left horn increases while that of natives in that horn stays constant, or vice versa. In either case the effect is very much more marked when large numbers of eggs are injected.

Since the precocity of our aliens does not account for the contradiction between our results and Runner's, we presume either that his experimental females were more heterogeneous than ours (he drew them from four different inbred strains), or that his mice were such that no 'ceiling' to the average number of implantations was reached in the range tested. Fig. 3 suggests that both factors are operating; the frequency distribution for his data shows a greater spread than ours, and, unlike ours, it is roughly symmetrical, showing no evidence of an upper limit to the number of implantations. It should be remembered that his figures refer to living embryos only.

#### DISCUSSION

##### *Relative stages of donor and recipient*

It is possible to gain the impression from the literature on fertilized egg transfer that the best results are obtained by synchronizing the post-coital stages of donor and recipient. Fekete & Little (1942) found that the 52 hr. → 52 hr. combination was more successful than the 52 → 28 and 52 → 76 combinations. The 52 → 28 combination labours under the special disadvantage that the recipient uterus is required to accommodate eggs 2 days earlier than it would normally receive



them. Beatty (1951) obtained his five successes from more or less synchronous combinations. Both Fekete & Little and Beatty also made transfers of 3-day eggs, but in neither case was the stage of the recipient stated. It is therefore not clear whether or not the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  combination, which we found to be superior to the synchronous  $3\frac{1}{2} \rightarrow 3\frac{1}{2}$  and  $2\frac{1}{2} \rightarrow 2\frac{1}{2}$  combinations, was also tested by these authors.

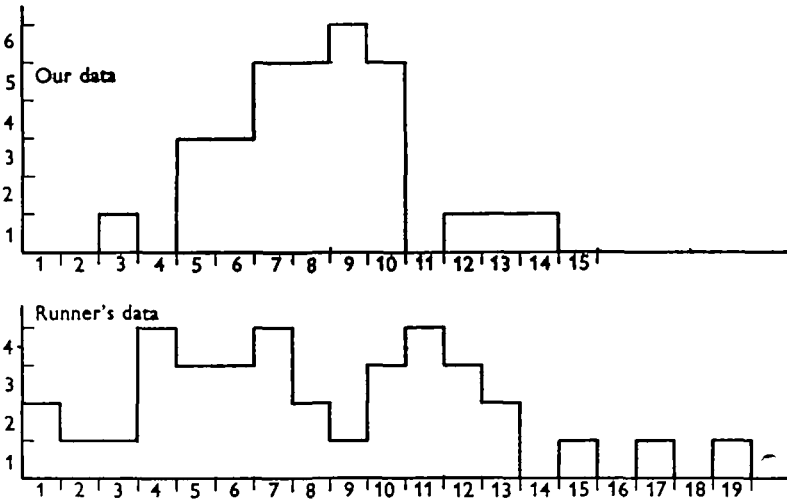


Fig. 3. Frequency distributions, for our data and for Runner's, of the total numbers of implantations and of live embryos respectively in unoperated control females (both horns combined).

There are thus no published data which contradict our finding that, at least when in competition with native eggs,  $3\frac{1}{2}$ -day eggs transferred to  $2\frac{1}{2}$ -day recipients give strikingly better results than are obtained from the two synchronous combinations which we tested. Boot & Mühlbock (1953) also tested the 3-day  $\rightarrow$  2-day combination, and commented that 'None of the other time combinations gave better results'. Unfortunately, they did not reveal what other combinations they had studied, nor did they distinguish results obtained from recipients mated with fertile males from results obtained from pseudo-pregnant recipients which had been mated with vasectomized males. Chang (1950) transferred fertilized rabbit eggs of various ages to pseudo-pregnant recipients of various post-coital stages, and found that, for a given donor stage, the synchronous combination was always as good as the best non-synchronous combination. However, he overstated his findings in his summary, when he said: 'Thus there is little or no chance for a transferred ovum or blastocyst to develop into young when it is in the tracts one or two days before or after the corresponding corpus luteal stage.' His own results, in fact, showed that 2-day  $\rightarrow$  3-day transfers were as successful as 2  $\rightarrow$  2 transfers, 4  $\rightarrow$  3 as successful as 4  $\rightarrow$  4, and 6  $\rightarrow$  5 as successful as 6  $\rightarrow$  6.

*The viability of artificially ovulated eggs from sexually immature females*

In discussing their work on the transfer of unfertilized mouse eggs, Runner & Palm (1953) make the following comment: 'Ova artificially ovulated from pregnant mice and transplanted to recipient foster mothers were shown by one of us (Runner, '51) to have a survival of 20%. The present results based on ova artificially induced from prepuberal mice and transplanted at the time of ovulation provided a survival of 14.2%. Although the two experiments were of somewhat dissimilar design, available data indicate that unfertilized ova from prepuberal donors may survive less frequently than do ova from pregnant donors.' The difference was not in fact statistically significant ( $\chi^2=0.928$ ,  $P>0.3$ ).

Our results show no difference in viability after transfer at  $3\frac{1}{2}$  days between spontaneously ovulated eggs from mature donors and artificially ovulated eggs from immature (30-day-old) donors. Gates (1956) has obtained similar results from transfers of  $3\frac{1}{2}$ -day eggs to  $3\frac{1}{2}$ -day recipients.

This result, if generally valid, is important in connexion with agricultural applications of egg transfer. In the most promising of such applications the eggs are obtained by superovulation of immature females. By this method not only can large numbers of eggs be obtained on a single occasion from a single donor, but also the generation interval can be shortened, since it is not necessary to await the onset of puberty—the calf, for example, can be made to ovulate soon after birth. These advantages could be nullified if eggs obtained in this way possessed sub-normal viability, and it is encouraging to find that, in the mouse at least, there is no evidence of such an effect.

*Limits to implantation and subsequent survival of large numbers of eggs*

Our data show no evidence of a limit to the number of implantations which can occur in a single uterine horn of an adult female mouse; but they suggest that when the average number is increased much above normal, higher rates of embryonic mortality supervene. They also indicate a limit to the number of successful implantations *per female*, since increase above a certain level of the number in one horn is compensated by decrease of the number in the other horn. This might appear to conflict with the findings of Engle (1927), who induced superovulation in adult female mice with pituitary implants. On the ninth or tenth day after mating the number of implantations per female was found to range from nineteen to twenty-nine. At this stage of pregnancy all implantations can be counted. We, however, have only been able to count what we have called 'successful implantations', i.e. those which remain alive long enough to leave a recognizable trace at autopsy on the sixteenth day. It is therefore possible that the limit which we observed was not a limit to implantation but was imposed by mortality occurring immediately after implantation, due perhaps to insufficiency of corpora lutea to supply the progesterone requirements of the excessive number of implantations. Parkes (1942) found that the great majority of implanted embryos in superpregnant rabbits (average of eighteen implantations per female) were already dead and

resorbing by the end of the first third of pregnancy, so that the number of live young produced was actually less than in normal pregnancies. Parkes comments: 'The reason why the uterus is unable to support the growth of supernormal numbers of embryos is not clear, but in view of the stage of pregnancy at which regression seems to have taken place. . . it is unlikely to be primarily of mechanical or metabolic origin.'

Evans & Simpson (1940) reported similar results in rats, which, however, are not fully comparable since the animals used were sexually immature.

We wish to stress that the average number of live young which female mice are capable of bringing to term is not an absolute quantity but varies according to the genetic make-up of the stocks used. A remarkable colony of mice has been described (Hauschka, personal communication) in which the average litter size is 12.6. Environmental circumstances undoubtedly also play a part. For example, Searle (personal communication) has found that embryonic mortality approaches 100% if the females are not allowed to mate until they are 8 months old.

#### *One-horn versus two-horn operations*

A point of practical importance on which our results throw light is whether to inject all of the eggs intended for a given recipient into one horn of the uterus, or whether to distribute them equally between the two horns. Our data indicate that, at least so long as the expected number of alien embryos recovered does not exceed about three, nothing is gained in exchange for the time and trouble lost by splitting the inoculum. At what point above this level we would encounter diminishing returns due to overcrowding remains to be determined. Our evidence of increased mortality in the injected horn associated with large inocula suggests that we may have already reached the threshold of diminishing returns. Hollander & Strong (1950) obtained sixty-eight pregnancies after unilateral ovariectomy. This procedure raised the average number of implantations *per horn* to more than eight. They found no significant increase in embryonic mortality. However, our method of crowding a single horn differs from theirs in that the number of embryos carried by the female may exceed the number of her corpora lutea.

#### *Limits to the yield of live young from the transfer of fertilized mouse eggs*

The factors limiting the yield obtained from egg transfer are of two kinds: (1) The natural hazards causing loss between mating and parturition in unoperated females of the recipient stock; in operated females, the transferred eggs are presumably as much exposed to these hazards as are the native eggs. (2) The technical hazards arising from the procedure of transfer which cause additional loss of transferred eggs.

#### (1) *Natural hazards*

These can conveniently be divided into failure, for whatever reason, to become pregnant, and loss by females which become pregnant of some of their eggs between fertilization and birth.

Failure to become pregnant is the easier to eliminate. Our recipient females (including unoperated controls) gave a pregnancy rate of only 60%, but prior to mating they were kept in relatively bad environmental conditions, being stored in large stock cages containing about twenty to thirty mice in each cage. We find that in pair matings of cross-bred mice about 80% of post-partum heats are followed by pregnancy (Michie, 1955), unless mating occurs in every post-partum heat, this underestimates the pregnancy rate. The colony of mice which we mentioned previously as having an exceptionally large average litter size approaches a 100% pregnancy rate.

Things are different with inbred mice. In the C57BL and C3H inbred strains we find the post-partum pregnancy rate to be less than half the rate in inter-strain  $F_1$  hybrid females (Michie, 1955). But where the conditions of the experiment necessitate the use of inbred recipients, other means, which we did not use, are at hand for increasing the pregnancy rate in recipients. First, Fischberg & Beatty (personal communication) report a strong correlation between the size of the vaginal plug and the probability that pregnancy will ensue. Females with small plugs could be rejected. Secondly, all females could be rejected whose ovaries did not show signs of recent ovulation. In our operations only the left ovary was exposed to view, but if this ovary had not ovulated we found that the odds were against the recipient's becoming pregnant.

The natural loss of eggs between fertilization and birth in those females which become pregnant has been estimated by Fekete (1947) as 16% in the C57BL inbred strain and 42% in the DBA inbred strain. This quantity, which is evidently highly dependent upon the genetic make-up of the mice, is compounded of failure of fertilized eggs to implant, and failure of those eggs which implant to survive to birth. In our material at least 14% of all implanted embryos died, as judged by the observed resorption rate. We know of no direct estimate in the mouse of the first component, i.e. the natural loss of fertilized eggs through failure to implant. Danforth & de Aberle (1928) estimated that 24% of corpora lutea in their mice were not represented by implantation sites, but this quantity also included eggs which ripened but were not shed, and eggs which were shed but were not fertilized.

## (2) *Technical hazards*

These can be divided into whole-inoculum loss, partial loss of inocula through injury or escape of some of the eggs, and reduction of implantation rate due to surgical interference.

Whole-inoculum losses were estimated in our material as accounting for about 33% of all eggs injected. These losses must arise from technical errors which presumably could be eliminated, although at present we can only guess at their nature. Runner (1951) seems to have encountered a similar phenomenon in transfers of unfertilized eggs to the ovarian capsule; his results, when tabulated in the same way as we have tabulated ours in Fig. 1, suggest a frequency of whole-inoculum loss similar to ours.

Loss through reduction of the implantation rate by surgical interference was found by us to be about 33%. It was considerably greater in operations classified as traumatic. This suggests that the loss could be further reduced by improvement of surgical technique.

We can now make a very rough estimate of the remaining technical hazard—loss through injury or escape of individual eggs—by calculating the expected yield from eggs transferred to recipients which became pregnant, after allowing for the other factors which we have listed. The answer can then be compared with the yield actually obtained.

We assume that 33% of injected eggs were lost as whole inocula, that of the remainder a proportion, say not more than 20%, failed through natural causes to implant, that of the remainder 33% failed to implant owing to surgical injury to the uterus, and that of the remainder 14% died after implantation. This leads to an expected yield of about 30% as compared with 22% actually obtained. Hence injury or escape of parts of inocula probably accounts for about a third of all injected eggs. Here again it is reasonable to hope that technical improvement could reduce this source of loss.

If whole-inoculum loss could be eliminated and loss from the other two technical hazards could be reduced by, say, half, then yields around 50% of all eggs injected could be attained from moderately favourable recipient stocks, even allowing some failures to become pregnant. This represents, perhaps, a reasonable target for the immediate future. The nearest approach to date has been made by Gates (1956) with a yield of 40.5% of 925 eggs injected in transfers from 3½-day donors to 126 3½-day pregnant recipients.

#### SUMMARY

1. The origin and potential uses of the method of egg transfer in mammals are briefly surveyed.
2. An experiment is described in which genetically labelled fertilized mouse eggs were transferred to the left uterine horns of recipient female mice. Eggs were obtained both by induced ovulation of sexually immature donors and by spontaneous ovulation of adult donors. Both pregnant and pseudo-pregnant recipients were used. The post-coital stages of donors and recipients were independently varied. At 16½ or 17½ days post-coitum the recipients were killed and their uterine contents recorded.
3. The operation had no effect upon the recipients' chances of becoming pregnant, nor did it have substantial effects upon the implantation and subsequent survival of eggs in the *uninjected* horn of the uterus.
4. In the injected horn, the implantation rate was reduced by about one-third in recipients both at 2½ and 3½ days post-coitum. Post-implantational mortality in the injected horn was increased in 3½-day recipients, but not in 2½-day recipients, except when the operation was accompanied by gross surgical trauma.
5. The yield of live embryos from eggs transferred to recipients which had themselves been mated to fertile males was highest in the 3½ → 2½ days combination,

lowest in the  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  days combination, and intermediate in the two synchronous combinations. These differences may in part be attributable to competition between native and transferred eggs. Such competition was shown mainly to occur before, during or shortly after implantation, and to be a property of non-synchronous rather than synchronous donor-recipient combinations. But the differences were in part independent of the presence of competing native eggs, as shown by transfers to recipients mated to sterile males; the yield from the  $3\frac{1}{2} \rightarrow 2\frac{1}{2}$  combination was still very much greater than that obtained from the  $2\frac{1}{2} \rightarrow 3\frac{1}{2}$  combination.

6. Transfers of eggs artificially ovulated from sexually immature donors gave results in all respects similar to those obtained with eggs spontaneously ovulated by sexually mature donors.

7. The distribution of alien embryos among the recipients suggested that apart from the random loss of parts of inocula through escape or death of individual eggs, there was another and distinct process at work causing the loss of whole inocula as units.

8. Over the range tested (0-18 eggs) the number of alien embryos and the number of implantations of all sorts in the injected horn rose linearly with increasing numbers of eggs injected. The number of native embryos in the injected horn declined with increasing numbers of eggs injected.

9. When the number of implantations, with increasing numbers of eggs injected, began to exceed the normal quota for one horn, the number of live embryos in the injected horn (alien + native) increased less steeply and the proportion of dead and resorbing embryos began to rise.

10. The  $3\frac{1}{2}$ -day  $\rightarrow$   $2\frac{1}{2}$ -day series gave some evidence that when the number of implantations in the injected horn was raised above the normal level, successful implantation in the *uninjected* horn was reduced, so that the total number in the two horns combined never exceeded an average of about  $8\frac{1}{2}$ .

11. The experimental results are discussed in the light of previous work and of future application. We conclude that with reasonable control of natural and technical hazards a yield of about 50% of fertilized mouse eggs recovered as live young should be attainable.

This work was done in the Department of Zoology, University College, London. We wish to thank Prof. P. B. Medawar, F.R.S., Prof. F. W. Rogers Brambell, F.R.S., and Mr Allen Gates for valuable criticisms and comments. Our thanks are also due to the Agricultural Research Council for financial support.

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