South Dakota State University

Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange

Electronic Theses and Dissertations

1977

In Vitro Analysis of Pre- and Early Postimplantation Development of Lethal Yellow (Ay/Ay) Mouse Embryos

Linda L. Johnson

Follow this and additional works at: <https://openprairie.sdstate.edu/etd>

Recommended Citation

Johnson, Linda L., "In Vitro Analysis of Pre- and Early Postimplantation Development of Lethal Yellow (Ay/Ay) Mouse Embryos" (1977). *Electronic Theses and Dissertations*. 5080.
<https://openprairie.sdstate.edu/etd/5080>

This Thesis - Open Access is brought to you for free and open access by Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of Open PRAIRIE: Open Public Research Access Institutional Repository and Information Exchange. For more information, please contact michael.biondo@sdstate.edu.

IN VITRO ANALYSIS OF PRE- AND EARLY POSTIMPLANTATION DEVELOPMENT
OF LETHAL YELLOW (A^y/A^y) MOUSE EMBRYOS

BY

LINDA L. JOHNSON

A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science, Major in
Biology, South Dakota
State University
1977

IN VITRO ANALYSIS OF PRE- AND EARLY POSTIMPLANTATION DEVELOPMENT
OF LETHAL YELLOW (A^Y/A^Y) MOUSE EMBRYOS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Date

Head, Biology Department

Date

ACKNOWLEDGMENTS

I am indebted to many people for their generosity, time and support which allowed the completion of this thesis. A special thank you is due:

To Dr. Nels H. Granholm, for providing financial support¹, endless encouragement and especially for spurring my interest in developmental biology.

To Dr. Gerald R. Cizadlo, for his patience in teaching me the techniques necessary to accomplish this work, for taking the time to answer my boundless questions and most importantly for communicating his enthusiasm for science to me.

To Gregory W. Bown, for technical assistance, help in maintaining the mouse colony, and above all for unwavering friendship.

To Phyllis M. Johnson, for advice and assistance willingly given.

To Dr. G. A. Myers and the Botany-Biology Department staff and to the Entomology-Zoology Department staff for sharing their facilities with me.

To my parents for actively encouraging my educational pursuits.

Finally, to my husband, Dick for accepting added responsibility and giving me the incentive to complete this work, and to my children, Jerusha, Claudine and Jared for making it all worthwhile.

¹ South Dakota State University Agricultural Experiment Station (SD737), National Institutes of Health (HD08631), and the Lalor Foundation.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	iii
LIST OF TABLES	v
LIST OF FIGURES AND PLATES	vi
INTRODUCTION	1
LITERATURE REVIEW	5
MATERIALS AND METHODS	21
RESULTS	27
A. Breeding Data	27
B. Embryo Data at Recovery (56-60 h.p.c.)	27
C. Embryo Data Generated During <u>In Vitro</u> Development (56-158 h.p.c.)	32
DISCUSSION	47
CONCLUSIONS	56
LITERATURE CITED	59

LIST OF TABLES

Table		Page
1	Summary of breeding data	28
2	Analysis of embryos at recovery (56-60 h.p.c.)	29
3	Development of embryos in culture (56-158 h.p.c.)	33
4	Characterization of abnormal embryos from recovery through five days of culture (56-158 h.p.c.)	35

LIST OF FIGURES AND PLATES

	Page
Figure 1. Histogram of developmental stage of experimental and control embryos from nulliparous female mice <100 days old at flushing (56-60 h.p.c.)	30
Plate 1. Normal embryo development	38
Plate 2. Abnormal embryo development	40
Plate 3. Abnormal embryo development from the AY/a x AY/a experimental mating	43

INTRODUCTION

The dominant A^Y gene of the agouti locus (linkage group V) in the house mouse (Mus musculus) is one of approximately eleven alleles specifying coat color. Homozygosity of the A^Y allele is thought to cause death of the embryo in utero around the time of implantation. For this reason the A^Y/A^Y genotype is called the lethal yellow mouse. The precise time that the primary gene defect is first expressed is not known. Attempts to pinpoint the phenocritical period in an effort to discover the basic biochemical alteration specified by the lethal gene have thus far been unsuccessful.

Mice heterozygous for the dominant A^Y allele are viable but present a diverse array of physiologic characteristics differing greatly from their homozygous recessive black (a/a) littermates and other non-yellow mice. Heterozygous (A^Y/a) mice have a yellow coat color, a longer body and tail length, greatly increased fat deposition, increased susceptibility to tumors, and reproductive inefficiency in females (Cizadlo, 1976). These phenotypic characteristics are reflections of an altered metabolism brought about by a single dose of the A^Y gene in the heterozygous animal. Neonates of yellow females (A^Y/a) have poorer survival rates to weaning than those of black (a/a) or other non-yellow females. Also, during gestation yellow uteri may provide a less suitable environment for embryonic and fetal development than black or non-yellow uteri. It may be that factors unique to yellow uteri act to accelerate or potentiate homozygous A^Y allele expression thus hastening mutant death.

Because the A^Y/A^Y embryonic lethality is expressed at the time of implantation, the yellow mouse system may lend itself to the investigation of implantation phenomena. It may provide information regarding: (1) hormonal control mechanisms operative during this time, (2) the uterine decidual response, and (3) trophoblast-endometrial interactions. However, careful characterization and documentation of the generation of the lethal syndrome should be carried out before knowing the full potential of the yellow mouse system as a model for investigating implantation phenomena.

In characterizing the lethal syndrome it is vital to know at what point in development the lethal gene shows its first effects (i.e., primary A^Y allele expression). Perhaps there is a definite stage in development when abnormalities first appear or perhaps the phenocritical period extends throughout preimplantation development. In either case, precise environmental factors triggering homozygous A^Y allele expression need to be known.

It is equally important to discover whether or not the A^Y/A^Y homozygote is in fact an implantation lethal as has been reported in previous literature. Perhaps the lethality occurs at varying times prior to implantation or perhaps some A^Y homozygotes continue to be viable well after the time of implantation. Although breeding data show conclusively that A^Y/A^Y embryos are gestational lethals, the precise time of mutant death (i.e., phenolethal period) is not known.

Of primary importance to the documentation of the lethal syndrome is the ability to identify lethal mutants from their non-lethal littermates. This can only be accomplished by careful assessment of

the progress of development throughout the life of the embryo. Early investigations of the lethal yellow mouse system were carried out in utero using histological techniques. Current investigators have shown that development of embryos in vitro parallels that in vivo. Thus, the in vitro method is a valuable technique for characterization of developmental events. Advantages of embryo analysis in vitro are: (1) the embryo can be continually observed and assessed in a shorter time than is possible with histological studies, (2) embryos can be assessed by direct observation without the influence of uterine and other maternal factors, and (3) environmental and nutritional requirements can be controlled and defined (Pedersen and Spindle, 1976).

An in vitro analysis was therefore undertaken to meet the following objectives: (1) define morphological characteristics that will identify the class of A^Y/A^Y homozygotes in preimplantation embryo culture, (2) discover the extent of the phenocritical period of A^Y homozygotes, (3) discover if A^Y homozygotes are in fact implantation lethals and (4) test the background variability and abnormalities occurring in control crosses as compared to the experimental cross.

Expression of the homozygous A^Y allele occurs early in embryonic development causing lethality around the time of implantation. An in vitro analysis may provide new data on embryo preparation for attachment and trophoblast function. This system may also serve as a model to study the first cellular differentiation taking place in the embryo, i.e., that of inner cell mass and trophoblast cells. This initial differentiation occurs during the morula-to-blastocyst transformation and can be easily observed in culture. It is possible that

homozygous A^y expression somehow disturbs or modifies the first cellular differentiation. In turn, this modification may lead to a failure of the A^y/A^y mutant at a later developmental stage, i.e., implantation.

Any hypothesis attempting to explain the lethality of the homozygous yellow embryo must also account for effects seen in the heterozygote. An understanding of abnormal factors in either genotype may lead to the understanding of the other genotype. Improper cellular differentiation may also be a factor in the altered physiologic state of the A^y/a heterozygote. Finally, knowledge gained while investigating the lethal yellow mouse may have application in other mammalian systems ranging from molecular considerations (e.g., gene action), to hormonal (e.g., endocrine control of mammalian implantation), and others. Yellow mice (A^y/a), their dominant homozygous progeny (A^y/A^y), and their reciprocal interactions which are encountered during gestation represent a highly productive and provocative experimental system.

LITERATURE REVIEW

In a series of comprehensive genetic studies on coat color in mice Cuénot (1905) found that yellow ($A^Y/-$) was dominant to all other coat colors including the wild or agouti (A/A or $+/+$) condition. Because yellow mice would never breed true Cuénot (1905) concluded that yellows must be heterozygotes ($A^Y/-$). Upon breeding yellow heterozygotes ($A^Y/- \times A^Y/-$) the expected 3:1 yellow to non-yellow Mendelian ratio was not achieved; instead there were 72.4% (263/363) yellows to 27.6% (100/363) non-yellows. The Mendelian expectation has been missed by 2.6%.

In 1910, Castle and Little in more extensive genetic studies analyzed 1,235 progeny from yellow x yellow matings. Of these newborn mice, 64.8% (800/1235) were yellow and 35.2% (435/1235) were non-yellow. Castle and Little (1910) concluded,

"The result observed by us, 64.77 percent yellow in 1,235 young, is a wide deviation from 75 percent, but close enough to 66.66 percent to enable us to say with considerable certainty that the homozygous yellow class is entirely lacking and is not replaced by heterozygous animals of the same color."

Thus Cuénot (1905) and Castle and Little (1910) were the first to provide convincing data to confirm that A^Y homozygotes are gestational lethals.

Interestingly both Cuénot (1905) and Castle and Little (1910) observed that litters from yellow x yellow matings were smaller than yellow x non-yellow litters, but not as small as would be expected if the homozygous yellow zygotes simply perished without affecting the

rest of the litter. In an attempt to explain this observation Castle and Little (1910) offered two possibilities: (1) more eggs are ovulated than are young subsequently born, so that failure of some embryos to attach to the uterus may increase the chances for attachment of the surviving embryos, or (2) the production of a small litter at one birth may lead to the production of a larger litter at the next birth, through indirect stimulation of more free ovulation. Regarding the former possibility Castle and Little (1910) were the first investigators to suggest a relationship between homozygous A^Y allele expression and ". . .failure of some eggs to become attached to the uterus". This suggestion had a profound effect on subsequent research with the lethal yellow mouse system.

To test the assumption that homozygous yellow zygotes (embryos) are not viable, Kirkham (1917) serially sectioned ovaries, oviducts and uteri of yellow x yellow, yellow x white, and white x white mice from days 1 to 19 of pregnancy. Kirkham (1917) found the rate of cleavage and embryonic development to be the same for yellow and white mice and that the 2-cell stages of both yellow and white mice were normal. Criteria for assessing normalcy or for assessing cleavage and developmental rates were not elaborated. Degenerating morulae and blastocysts were found in yellow female tracts but not in white female tracts examined at the same stage of development. This last observation suggests two rather important phenomena associated with the lethal yellow mouse system, i.e., homozygous A^Y allele expression may begin during preimplantation development and yellow uteri may inherently be poorer environments for developing embryos than non-yellow uteri.

Kirkham (1917) also reported that degenerating embryos could induce a normal decidual reaction even though they arrested as blastocysts. In days 6 to 17 of gestation, 37.7% (26/69) of embryos from yellow x yellow crosses were degenerate, while only 2.4% (2/84) of control embryos were degenerate. Although the percentage of degenerate yellow embryos did not approximate the Mendelian expectation of 25%, Kirkham (1917) concluded that some of the 37.7% degenerates were the missing homozygous animals.

Kirkham (1919) published a further study of yellow mice in which he histologically examined ovaries, oviducts and uteri of yellow and white mice from days 1 to 20 of pregnancy. In agreement with his 1917 study Kirkham (1919) found no abnormalities in either group prior to the morula stage but observed abnormal morula and older stages from yellow x yellow crosses. Abnormalities reported included indistinct cell boundaries, shrunken appearance, small, crowded cells, and small blastocoeles. In addition Kirkham (1919) reported: (1) preimplantation embryos were alive at the time of preservation, (2) presumed homozygotes did not arrest until eliciting a uterine decidual reaction, and (3) abnormal control blastocysts were unable to cause the uterine response. Since 29% of embryos from yellow females 3 to 20 days pregnant were degenerate, while only 10% from white mice were degenerate, Kirkham (1919) concluded that the 29% degenerates included the A^Y/A^Y homozygotes.

Kirkham (1919) discussed the possibility that reproductive inefficiency may be correlated with yellow coat color and that the

yellow uterus may be an unfavorable environment for the developing embryo. In fact, Kirkham (1919) states that the unfavorable uterine environment, "... is actually a normal correlation with yellow coat color, an assumption further supported by the marked tendency in yellow mice of both sexes to fatness and sterility at a relatively early age." In order to test this hypothesis Kirkham (1919) suggested transplanting ovaries from yellow to non-yellow females and mating them with yellow ($A^Y/-$) males; in the more favorable uterine environment provided by a non-yellow mother, viable homozygous yellow mice might be produced.

In another study designed to provide evidence on the causes of death of homozygous yellow embryos in utero Ibsen and Steigleder (1917) dissected uterine crypts of yellow and non-yellow uteri from 13 to 19 days of pregnancy; apparently no embedding or histological analyses were conducted. Ibsen and Steigleder (1917) were the first investigators to utilize four crosses (one experimental and three controls) in their study. These were yellow x yellow, yellow female x non-yellow male (chocolate), non-yellow female (chocolate) x yellow male, and black x black. The experimental cross ($A^Y/-$ x $A^Y/-$) yielded 24.5% dead embryos, a close approximation to the Mendelian expectation. However, the yellow female x chocolate male cross produced 12.3% dead embryos, while the other two control groups produced 4.6% and 5.4% dead embryos, respectively. To explain the high percentage of dead embryos in the yellow female control cross, Ibsen and Steigleder (1917) suggested that the physiologic factor causing increased fat deposition in yellow females may in some way influence the increased occurrence

of dead embryos; Ibsen and Steigleder (1917) stated, "in mice there may be a 'lethal factor,' similar to those so well known in Drosophila, which is so closely linked to the factor for yellow that they are practically at the same locus and there is consequently no crossing over." Thus Ibsen and Steigleder (1917) as well as Kirkham (1917, 1919) provided evidence that A^Y homozygotes were in fact gestational lethals; they also suggested that yellow females may contribute in some way to additional embryonic losses.

Little (1919) examined 112 embryos from yellow x yellow crosses and found 18.7% (21/112) to be abnormal; however, he did not specify his method or the stage of pregnancy of mice at examination. In reciprocal yellow x non-yellow matings Little (1919) reported 2.4% (1/42) abnormalities. On the basis of his data and that of Kirkham (1917), Little (1919) concluded that ". . .the fate of the homozygous yellow mouse is known," i.e., A^Y/A^Y embryos die in utero.

Between 1919 and 1942 there do not appear to be published studies on the embryology of the lethal yellow mouse. The work of Robertson (1942) marks a new era in lethal yellow mouse research, because the ovary transplantation experiments represent the first experimental approach. Since 1942 there have been additional descriptive (Eaton and Green, 1962, 1963; Pedersen, 1974; Cizadlo, 1976) and experimental (Eaton, 1968; Pedersen and Spindle, 1976) investigations. However, both the timing and precise phenotypic effects of homozygous A^Y allele expression remain unknown.

In an effort to discover the precise timing of A^y allele expression, Robertson (1942) histologically analyzed embryos from 2 to 6.5 days of pregnancy in utero; ovaries, fallopian tubes and uteri were sectioned at 10 microns. All embryos from yellow x yellow crosses were judged normal through blastocoelic cavity formation. Of implanting embryos studied, 27.6% (8/29) were judged abnormal. Since these eight atypical embryos were similar, Robertson (1942) considered these to be the homozygous yellow group (A^y/A^y). He described them as very small, with no trophectoderm (trophoblast), no blastocoelic cavity and no differentiation of ICM into ectoderm and endoderm. Robertson (1942) concluded that the homozygous yellow embryo can effect a decidual response, but on contact with the uterine epithelium, the trophectoderm collapses and degenerates leaving the inner cell mass to become abnormal. At the egg cylinder stage, Robertson (1942) found 20 of 28 embryos normally implanted, but the remaining eight implantation sites (28.6%) were small and contained only a few scattered cells which were considered to be remnants of degenerated A^y homozygotes. Robertson (1942) concluded that the expression of the mutant gene does not begin until after blastocoele formation. Although this finding was in conflict with the observations of Kirkham (1917, 1919) who reported abnormalities occurring at the morula stage, conclusions drawn by Robertson (1942) were based on the examination of a rather small number of embryos (29 from 8-cell to early blastocyst stages).

To test the capacity for development of homozygous yellow embryos in the non-yellow uterine environment, as suggested by Kirkham (1919), Robertson (1942) transplanted ovaries from heterozygous yellow into

non-yellow females and mated them to heterozygous yellow males. The resulting embryos were preserved and studied from 2.5 to 7.5 days of pregnancy utilizing histological methods. Of embryos examined at the implantation stage, 29.4% (5/17) were judged abnormal. Through qualitative observations, this class of abnormal embryos was determined to be at a more advanced stage of development than the class of abnormal embryos found in the yellow uterus at the same stage. Although Robertson (1942) presents no convincing quantitative data to show the enhancement of embryo development in non-yellow uteri there are a number of qualitative observations to support this view. Robertson (1942) concluded that there are inherent problems in the homozygous yellow zygote and that the yellow uterine environment potentiates these problems.

Eaton and Green (1962) reported 40.0% (28/70) abnormal embryos from yellow x yellow crosses in uteri examined histologically between 5 days 7 hours and 6 days 12 hours of pregnancy. Control groups, yellow female x non-yellow male, and the reciprocal cross had 15.4% (6/39) and 2.9% (1/34) abnormal embryos, respectively. Numbers of corpora lutea and implantation sites were found to be absolutely correlated. Thus Eaton and Green (1962) concluded that homozygous yellow embryos are capable of implanting in the yellow uterus with death occurring shortly thereafter.

In a subsequent study, Eaton and Green (1963) examined histologically prepared uteri representing the time between primary implantation and the egg cylinder stage (i.e., 104-132 hours post coitum). Of 262

embryos examined from $A^Y/a \times A^Y/a$ crosses, 27.1% (71/262) were abnormal, a close approximation to the theoretical 25%. Control matings were not included in the study. All abnormal embryos were capable of implanting, with the stage of developmental arrest depending on the "degree of differentiation and attachment of equatorial giant cells to the endometrium." Some presumed A^Y/A^Y homozygotes were able to survive to the egg cylinder stage. Eaton and Green (1963) concluded that most A^Y/A^Y homozygotes die as partially implanted blastocysts, because they fail to remain in synchrony with the cycling uterine endometrium.

Eaton (1968) believing death of homozygous yellow embryos to be due to failure of trophoblast giant cell differentiation, attempted to enhance giant cell differentiation and subsequent implantation; he administered ovarian steroids to pregnant yellow females, thus delaying the timing of the A^Y/A^Y lethality and increasing the number of "escaper" embryos. Pregnant yellow females from the heterozygous yellow cross were assigned to seven different treatment groups and given progesterone and estradiol or progesterone alone at various stages of pregnancy. Yellow female controls were given no hormone injections. At the early egg cylinder stage (i.e., 5 days, 8-16 hours post coitum) all females were sacrificed, and their uteri dissected and prepared for serial sectioning.

Eaton (1968) reported that, in agreement with Mendelian expectation, 73.3% (99/135) of the control embryos implanted normally and 26.7% (36/135) made partial or no attachment to the uterus. Although

ovarian steroid treatments did not increase the number of normal implants, the percentage of viable embryos showing partial attachment to the uterus was increased (control = 2.2%; experimental groups 1, 2 and 3 = 5.8%, 7.4% and 13.3%, respectively). Eaton (1968) concluded that progesterone but not estradiol was necessary for giant cell differentiation and that a progesterone deficient yellow uterus may inhibit completion of implantation by the A^Y/A^Y mutants.

Wolff and Bartke (1966) analyzed extensive breeding data from the following crosses of the inbred YS/ChWf mouse strain: A^Y/a female x A^Y/a male, A^Y/a female x a/a male, and a/a female x A^Y/a male. The mean number of young born alive to A^Y/a females mated to a/a males was less ($P < 0.01$) than mean number of young born alive to a/a females mated to A^Y/a males. Also the mean litter size at weaning was significantly less ($P < 0.01$) in yellow female x black male versus the reciprocal mating. These observations on number of young born alive per litter and number of young weaned per litter in yellow x black reciprocal crosses support the notion suggested initially by Kirkham (1917, 1919) and Ibsen and Steigleder (1917) that the yellow female is a less efficient mother than the non-yellow female.

In order to generate further data on inherent reproductive problems in yellow versus black females, Wolff and Bartke (1966) conducted embryonic mortality studies on yellow females mated to black males and the reciprocal cross. No yellow x yellow matings were conducted in this study. Autopsy at various gestational stages indicated that about 25% of implanted embryos in both A^Y/a and a/a

females died prior to parturition. Interestingly, Wolff and Bartke (1966) stated, "Practically all deaths occurred on days 9, 10 and 11 of gestation." Although black and yellow females showed no differences in mean number of implanting embryos, survival at twelve days of pregnancy was higher ($P=0.02-0.05$) in black females. Mean number of weanlings from $A^Y/a \times A^Y/a$ matings was not different from that of A^Y/a female $\times a/a$ male matings. Since the yellow $\times$ yellow cross should theoretically produce only 75% as many young as the yellow $\times$ black cross, a difference in survival between birth and weaning is indicated.

Yellow $\times$ yellow crosses exhibited a greater incidence of sterile matings as well as a much shorter reproductive life than the other two crosses. In explanation of the deficiency of yellow heterozygotes produced, Wolff and Bartke (1966) suggest that the yellow A^Y/a uterus may in some way contribute to the failure of heterozygous embryos; they further suggest that metabolic differences responsible for the A^Y/A^Y lethality may contribute to the decreased viability of the A^Y/a embryo. Wolff and Bartke (1966) state, "whereas A^Y/A^Y embryos are below the survival threshold, most heterozygous $A^Y/-$ embryos develop successfully, and the proportion that dies may depend on the specific intrauterine milieu."

Upon comparison of YS/ChWf strain data with that of other mouse strains carrying the A^Y allele, Wolff and Bartke (1966) found a deficiency of A^Y/a young characteristic of all strains but increased uterine mortality of embryos in yellow females was unique to the

YS/ChWf strain. Because yellow females had smaller litters than black females and there was an apparent deficiency of yellow young born to yellow females, Wolff and Bartke (1966) were the first to provide experimental evidence that a modification of embryonic development and survival may occur in the yellow (A^Y/a) uterine environment.

The first attempt to investigate lethal yellow mouse embryos in vitro was that of Pedersen (1974) who identified presumed A^Y/A^Y embryos in culture and assessed their capacity for postblastocyst development. One experimental ($A^Y/a \times A^Y/a$) and one control (A^Y/a female $\times a/a$ male) cross were utilized. Eight-cell embryos were flushed from the reproductive tract on day three; morulae and blastocysts were flushed on day four. Eight-cell embryos from both crosses were judged normal prior to culture, but after 24 hours in culture, 16.8% (16/95) of experimental embryos and 5.6% (4/71) of control embryos became abnormal. Abnormalities in experimental embryos consisted of the arrest of one to three blastomeres or delayed cleavage. Abnormalities were found in 23.9% (16/67) of morulae and blastocysts flushed on day four, while controls were found to contain 8.8% (3/34) abnormalities. After five days in culture, 26.9% (18/67) of experimental embryos had failed to hatch from the zona pellucida while only 2.9% (1/34) of controls failed to hatch. The class of unhatched experimental embryos was presumed to be yellow homozygotes. Enzymatic removal of the zona pellucida allowed limited trophoblastic outgrowth in experimental embryos, but in contrast to normal outgrowths, the ICM's of presumed mutants disintegrated during outgrowth.

Pedersen (1974) reported that morphological abnormalities in embryos from $A^y/a \times A^y/a$ matings occur earlier in development than had previously been shown. He considered hatching failure to be a consequence of trophoblast malfunction or possibly a result of decreased blastocyst volume due to the earlier arrest of blastomeres.

Although a contemporary report, the work of Pedersen (1974) is a fundamental and perhaps classic paper in the understanding of the lethal yellow mouse system. It represents the first in vitro approach, and data generated provide new and significant information on homozygous A^y allele expression.

Breeding studies conducted in the mouse strain C57BL/6J by Cizadlo et al. (1975) support the findings of Wolff and Bartke (1966) that there exists a deficiency of newborn yellow mice from birth to weaning and that smaller litters are produced from the A^y/a female $\times$ a/a male versus the reciprocal cross. Cizadlo et al. (1975) suggested that metabolic alterations due to the presence of the A^y gene may decrease the viability of newborn yellow mice; also 17% fewer yellow females mated than black females suggesting a steroid hormone deficiency responsible for abnormal mating behavior in the A^y/a female.

Two ultrastructural studies dealing with the characterization of A^y/A^y mutants have been carried out. Calarco and Pedersen (1976) did ultrastructural and light microscope studies on seventeen preimplantation embryos presumed to be A^y/A^y mutants according to criteria developed by Pedersen (1974). Twenty-two percent of the embryos from $A^y/a \times A^y/a$ crosses were scored as A^y/A^y embryos because of the

presence of large excluded blastomeres. Embryos derived from control crosses were not analyzed in this study. Excluded blastomeres found on the periphery of the embryo had cellular characteristics similar to earlier developmental stages, perhaps having arrested as early as the four to eight cell stage. Characteristics of excluded blastomeres included the presence of intracisternal A particles (IAP), fibrous inclusions not found in non-excluded blastomeres, smaller mitochondria, fewer polysomes and decreased amounts of rough endoplasmic reticulum. Non-excluded cells of the presumed mutants remained normal until the late blastocyst stage and were able to form a small blastocyst. Presumed A^Y/A^Y embryos were found to be capable of ICM and trophoblast differentiation even though defects were present in the embryo as early as the 8-cell stage. However, A^Y/A^Y embryos had fewer interior cells than did control embryos. Calarco and Pedersen (1976) concluded that the expression of the A^Y allele occurs at varying times between early cleavage and implantation resulting in the ultimate death of the embryo at implantation.

In a subsequent study designed to define the developmental defect of yellow homozygotes, Cizadlo (1976) examined the ultrastructure of preimplantation embryos flushed from the reproductive tracts of yellow (A^Y/a) and black (a/a) female mice mated to yellow males at 62 and 80 hours p.c.. Of 24 embryos from the yellow x yellow cross, 25% (6/24) had some morphological defect, but no single abnormality was consistently found which would identify the class of A^Y/A^Y mutants. Of six abnormal embryos from the experimental cross, two contained an isolated

blastomere, a characteristic that Pedersen (1974) relied on for identification of lethal yellow mutants. Two abnormal embryos had unusual concentrations of IAP and large nucleoli. Another abnormal embryo had a vacuolated trophoblast cell; and one embryo was in an advanced stage of degeneration.

In a quantitative in utero approach to the generation of the homozygous lethality, Cizadlo (1976) serially sectioned through 105 hour p.c. implantation chambers in yellow and black females previously mated to yellow males. Embryos from $A^y/a \times A^y/a$ matings possessed a mean ($\pm$ SE) of 30.2 ± 2.4 nuclei per ICM component, while embryos from the control cross contained 41.7 ± 4.5 nuclei within the ICM. Thus ICM cell numbers were significantly different ($P < 0.05$) between genotypes. This finding supports the suggestion of Calarco and Pedersen (1976) that mutant embryos have fewer ICM cells and that ICM cells succumb more quickly to the effects of A^y homozygosity than do trophoblast cells. Embryos in both experimental and control groups were equally competent to elicit the uterine decidual response, indicating that the failure of the A^y/A^y homozygote is due to factors other than initiation of the implantation response (Cizadlo, 1976).

Pedersen and Spindle (1976) obtained 2-cell embryos from yellow heterozygous parents and cultured all embryos flushed to the blastocyst stage. Time lapse cinematographic observations during the preimplantation period revealed a two to four hour cleavage delay as early as the 2- to 4-cell stage and a lag in development of the presumed A^y/A^y mutants throughout preimplantation development. Abnormal blastocysts,

presumed to be A^y/A^y homozygotes, were denuded with pronase and studied over a period of postblastocyst development. Presumed mutant blastocysts underwent normal cycles of expansion and collapse but before attachment extended blunt cytoplasmic protrusions unlike the slender filopodia of normal embryos. Inner cell masses present in mutant blastocysts were not observed at the time of trophoblast outgrowth. The authors thought it possible that delayed cleavage and blastomere arrest in the yellow homozygotes could indicate improper DNA replication differentially affecting inner cell mass cells. This possibility was evaluated by labelling abnormal blastocysts with [^3H] thymidine and comparing labelling indices with those of normal littermates. However, no significant differences in labelling between the two groups were observed. Pedersen and Spindle (1976) suggest that expression of A^y homozygosity leads to an accumulation of deficiencies throughout the preimplantation period and is subsequently reflected in hatching failure, ICM loss, and ultimate death to the A^y/A^y embryo.

Studies by Cizadlo (1976), Calarco and Pedersen (1976) and Pedersen and Spindle (1976) provide the first evidence that A^y homozygosity may differentially affect inner cell mass cells causing a decrease in numbers and ultimate disappearance of ICM's after attachment to the uterus. Also, 13% of the presumed mutant population identified as Class II embryos by Eaton and Green (1963) show selective ICM versus trophoblast cell deterioration.

In summary, Cuénot (1905) and Castle and Little (1910) through classical Mendelian genetics established the fact that A^y/A^y individuals

did not exist; therefore A^y/A^y progeny were never born and died as gestational lethals. Kirkham (1917, 1919) and Ibsen and Steigleder (1919) pinpointed the A^y homozygous lethal period at the time of implantation; these investigators also reported that the yellow female ($A^y/-$) may in some way contribute to embryonic lethality. Continued descriptive methods (Robertson, 1942; Eaton and Green, 1962, 1963; Wolff and Bartke, 1966; Calarco and Pedersen, 1976; Cizadlo, 1976) and experimental studies (Robertson, 1942; Eaton, 1968; Pedersen, 1974; Pedersen and Spindle, 1976) have allowed us to fix the period of homozygous A^y allele expression (phenocritical period) in vitro as early as the 2- to 4-cell stage with mutants undergoing death during implantation. Although at present there exists virtually no information on the precise biochemical lesion in A^y/A^y embryos, studies by Pedersen (1974) and Pedersen and Spindle (1976) do describe morphological correlates of homozygous A^y allele expression. In addition, studies on yellow heterozygotes (Cizadlo, 1976) are important in the understanding of the yellow mouse system.

pertinent information regarding the production colony. The record keeping system was adapted from Wolff (1967).

Twenty mating cages housing three to four A^y/a or a/a females and one A^y/a or a/a proven male were maintained continuously to provide animals for experimentation. Information recorded on mating cage cards included identification number, genotype, birth date, date that the male was originally put with females, and the date of vaginal plug detection.

All females in mating cages were examined for the presence of a vaginal plug at 8:00 a.m. daily. Females having vaginal plugs were assumed to have copulated at the mid-point of the dark cycle (0200 hours) and hours post coitum (h.p.c.) were calculated from that time. If no plugs were detected within two weeks after a male had been housed with females, the male was discarded and a new male was added. Non-productive females and obese yellow females were discarded if, after 30 days, they had not mated.

Pregnant females of all crosses ($A^y/a \times A^y/a$, $A^y/a \times a/a$, $a/a \times A^y/a$, $a/a \times a/a$) were sacrificed by cervical dislocation, and embryos were flushed from reproductive tracts at 56 to 60 h.p.c. using methods outlined by Rafferty (1970). All embryos obtained were transferred to 35 mm plastic culture dishes (Falcon) containing 2 ml of Brinster's BMOC-3 medium (GIBCO) and scored for developmental stage and morphology with phase-contrast microscopy (Nikon Inverted Phase Microscope) using 10X or 20X phase contrast objectives. Embryos were then cultured for 44 to 48 hours in an atmosphere of 5% CO_2 in air, 85% humidity, and

37° C. At 104 h.p.c., embryos were transferred to dishes containing Eagle's Minimum Essential Medium (GIBCO) with 10% Fetal Calf Serum (GIBCO) and cultured for an additional 54 hours. Embryos were observed, assessed at 6 and 18 hour intervals and photographs of normal and abnormal embryos were taken throughout the five day culture period. Photographs were taken using 10X and 20X phase contrast objectives (DDL, Nikon) with a 10X ocular in the photo tube yielding final magnifications on Kodak Plus X 35mm film of 50X and 100X, respectively (photo tube factor is one half). Contact prints were made for analysis, and prints for final plates (Plates 1, 2 and 3) were selected from contact prints.

The following criteria were used to assess developmental stage at flushing and throughout the culture period:

- (a) 4-cell -- embryo containing 3 to 4 countable blastomeres
- (b) 8-cell -- embryo containing 5 to 8 countable blastomeres
- (c) morula -- individual blastomeres can not be counted, surface contour smooth, and no blastocoelic spaces can be observed
- (d) early blastocyst -- when a definite blastocoelic cavity can be seen
- (e) mid-blastocyst -- when the blastocoelic cavity occupies approximately one half of the total embryo volume
- (f) definitive blastocyst -- when the blastocoelic cavity occupies at least four-fifths of the total embryo volume
- (g) expanded blastocyst -- when the blastocyst is one to two times larger than the definitive blastocyst, trophoblast

Breeding data from black (a/a) females mated to black (a/a) and yellow (A^Y/a) males were analyzed for number of litters, litter size, number of weanlings per litter, percent survival to weaning, sex and phenotype. Student's t-test was used for breeding data analysis. No breeding data were available for yellow female crosses, as all yellow females were used for experimental crosses.

Table 2 shows the developmental stages of embryos from all crosses at the time of flushing (56-60 h.p.c.). Number of females, number of embryos and average number of embryos per female were recorded for each cross. The percentage of abnormal embryos at flushing for each cross was computed. Table 2 includes only data from nulliparous females less than 100 days old. Figure 1 is a histogram representing the data on developmental stage at flushing from Table 2.

Table 3 shows the developmental success of embryos cultured from 56 to 158 h.p.c. for experimental and control crosses. Number of females used and number of embryos examined are listed for each cross. Number and percentage of total embryos from each cross that developed normally to the morula stage, blastocyst stage, hatched from the zona pellucida and outgrew are given.

Table 4 shows the various developmental stages between flushing (56 h.p.c.) and culture (158 h.p.c.) at which abnormal embryos were first judged abnormal. Table 4 gives an account of abnormal embryos that did not develop successfully throughout the five day culture period.

Results from reproductive tract flushings and in vitro experimentation were evaluated statistically using analysis of variance and the Chi-square test. Average number of embryos per female (Table 2) for each of the four mating groups were analyzed by analysis of variance to test the hypothesis that there were no differences in the populations represented by the four mating crosses. Differences between experimental versus control groups were evaluated by Chi-square for the following comparisons: developmental stage of embryos at recovery and percentage of abnormal embryos at recovery (Table 2), percentage of embryos that developed to morula, developed to blastocyst, hatched from the zona pellucida, and outgrew (Table 3), and developmental stage at which embryos first showed abnormalities (Table 4).

RESULTS

A. Breeding Data

Analyses of breeding data (Table 1) from 26 a/a female x a/a male matings and 72 a/a female x A^y/a male matings revealed a mean litter size of 5.8 for the black x black cross and 6.3 for the black x yellow cross, a non-significant difference. Black x black matings yielded a mean of 4.7 weanlings per litter (82.0% survival to weaning) which was not different than a mean of 5.1 weanlings per litter in the black x yellow cross (80.3% survival to weaning). At weaning the black x black cross produced 48.0% females and 52.0% males, while the black female x yellow male cross produced 48.8% females and 51.2% males. There were no significant differences in percentages of males and females within the two crosses or between the two crosses. The a/a female x A^y/a male mating yielded 51.8% yellow weanlings which was not different than 48.2% black weanlings. Because A^y/a females were used exclusively for in vitro experiments, no breeding data were available for comparison with black (a/a) female controls.

B. Embryo Data at Recovery (56-60 h.p.c.)

Data are summarized in Table 2 and Figure 1. Reproductive tract flushings at 56 to 60 h.p.c. yielded a total number of 212 embryos from 31 A^y/a females mated to A^y/a males (average number of embryos per female = 6.8). Of 22 yellow females from the yellow x black control cross, reproductive tract flushings yielded 148 total embryos (average

TABLE 1. SUMMARY OF BREEDING DATA.

	Matings	
	♀ a/a x ♂ a/a	♀ a/a x ♂ AY/a
Total number of matings	26	72
Mean litter size	5.8	6.3
Mean number of weanlings per litter	4.7	5.1
Percent survival to weaning	82.0% (123/150)	80.3% (367/457)
Percent females at weaning	48.0% (59/123)	48.8% (179/367)
Percent males at weaning	52.0% (64/123)	51.2% (188/367)
Percent yellows at weaning	0.0%	51.8%
Percent blacks at weaning	100.0%	48.2%

FIGURE 1. DEVELOPMENTAL STAGE OF EXPERIMENTAL AND CONTROL EMBRYOS
FROM NULLIPAROUS FEMALE MICE <100 DAYS OLD
AT FLUSHING (56-60 H.P.C.)

TABLE 2. ANALYSIS OF EMBRYOS AT RECOVERY (56-60 H.P.C.).

Parent Genotype ♀ ♂	Total number of females ¹	Total number of embryos	Average number of embryos/♀	% Abnormal at flushing	Stage at flushing ²				
					2-cell	4-cell	8-cell	Morula	Blasto- cyst
A ^y /a x A ^y /a	31	212	6.8	7.5 (16/212)	5 2.3%	57 26.9%	139 65.5%	4 1.9%	7 3.3%
A ^y /a x a/a	22	148	6.7	8.8 (13/148)	0 0.0%	25 16.9%	122 82.4%	1 0.7%	0 0.0%
a/a x A ^y /a	25	169	6.8	3.5 (6/169)	1 0.6%	16 9.5%	147 87.0%	2 1.2%	3 1.8%
a/a x a/a	15	109	7.3	5.5 (6/109)	1 0.9%	20 18.3%	86 78.9%	2 1.8%	0 0.0%

¹ All females were nulliparous and <100 days of age.

² The percentage figure represents the number of embryos in that class divided by the total number of embryos for that cross.

FIGURE 1. DEVELOPMENTAL STAGE OF EXPERIMENTAL AND CONTROL EMBRYOS
FROM NULLIPAROUS FEMALE MICE <100 DAYS OLD
AT FLUSHING (56-60 H.P.C.)

number of embryos per female = 6.7). Twenty-five black females mated to yellow males had a total of 169 embryos (average number of embryos per female = 6.8). The black x black cross yielded 109 embryos from reproductive tract flushings of 15 females (average number of embryos = 7.3). An analysis of variance revealed that mean numbers of embryos per female were not different among the four groups (computed F value = 0.315; critical value of F for $\alpha = 0.05$ is 2.72).

In the experimental cross ($A^Y/a \times A^Y/a$) 7.5% (16/212) of the embryos were abnormal at recovery (56 to 60 h.p.c.), while A^Y/a female x a/a male, a/a female x A^Y/a male, and a/a female x a/a male control crosses had 8.8% (13/148), 3.5% (6/169) and 5.5% (6/109) abnormal embryos at recovery, respectively (Table 2). A chi-square test revealed no differences among the four crosses (computed value = 4.29, critical value of chi-square for $P = 0.05$ and 3 degrees of freedom is 7.81).

For each cross, developmental stages of embryos at recovery (56 to 60 h.p.c.) were pooled into two categories for analysis: embryos less than or equal to the 4-cell stage, and embryos greater than or equal to the 8-cell stage. A chi-square test revealed that fewer embryos from the experimental group had reached the 8-cell stage at the time of recovery than embryos of control crosses ($P < 0.01$). The computed chi-square value is 22.9. The critical chi-square value for $P = 0.01$ and 3 degrees of freedom is 13.3. Differences in developmental stages of embryos among control groups were not significant (computed chi-square value = 5.3, critical value for $P = 0.05$ and 2 degrees of freedom is 5.9).

C. Embryo Data Generated During In Vitro Development (56-158 h.p.c.)

Data are summarized in Table 3. In order to statistically test for differences in developmental success in culture between experimental and control groups, a chi-square determination was conducted for each developmental stage (Table 3). Results showed all groups equally able to complete development to the morula stage (computed chi-square value = 6.9; critical value for $P = 0.05$ and 3 degrees of freedom is 7.8) and to the blastocyst stage (computed chi-square value = 1.1; critical value for $P = 0.05$ and 3 degrees of freedom is 7.8). However, fewer embryos from the $A^y/a \times A^y/a$ cross versus the control crosses successfully hatched from the zona pellucida (computed chi-square value = 7.0; critical value of chi-square for $P = 0.1$ and 3 degrees of freedom is 6.2). Of the experimental embryos which successfully developed to the blastocyst stage 75.0% (72/96) hatched from the zona pellucida. In control crosses the number of embryos that hatched after normal blastocyst development were 89.5% (85/95), 89.8% (129/145) and 92.6% (137/148) in yellow female x black male, black female x yellow male and black female x black male crosses, respectively (Table 3).

As a result of hatching failure in the $A^y/a \times A^y/a$ cross, fewer experimental embryos outgrew than did control embryos (computed chi-square value = 10.5; critical value of chi-square for $P = 0.05$ and 3 degrees of freedom is 7.8). In the experimental group, 95.8% (69/72) of the embryos that hatched from the zona pellucida successfully outgrew. In control crosses the number of embryos that outgrew after hatching from the zona pellucida were 90.6% (77/85), 96.9% (125/129) and

TABLE 3. DEVELOPMENT OF EMBRYOS IN CULTURE (56-158 H.P.C.).

Parent Genotype ♀ ♂	Total number of females	Total number of embryos	% Developed to morula ¹	% Developed to blastocyst ¹	% Hatched from zona pellucida ¹	% Outgrown ¹
A ^y /a x A ^y /a	16	113	93.8 (106/113)	85.0 (96/113)	63.7 (72/113)	61.1 (69/113)
A ^y /a x a/a	17	117	82.9 (97/117)	81.2 (95/117)	72.6 (85/117)	65.8 (77/117)
a/a x A ^y /a	23	172	86.0 (148/172)	84.3 (145/172)	75.0 (129/172)	72.7 (125/172)
a/a x a/a	21	173	88.4 (153/173)	85.5 (148/173)	79.2 (137/173)	77.5 (134/173)

¹ Percent figure in each category represents the percent of the total number of embryos in each cross.

97.8% (134/137) in yellow female x black male, black female x yellow male and black female x black male crosses, respectively (Table 3).

Table 4 summarizes data on abnormal embryos from experimental and control crosses according to the developmental stage at which they were first judged abnormal from recovery (56 h.p.c.) through five days of culture (158 h.p.c.). A chi-square test for independence indicates no differences in number of embryos judged abnormal in experimental and control crosses at the 2- to 4-cell stage (computed chi-square value = 2.7; critical value for $P = 0.05$ and 3 degrees of freedom is 7.8) or at the 8-cell stage (computed chi-square value = 3.9; critical value for $P = 0.05$ and 3 degrees of freedom is 7.8). However, fewer embryos from $A^y/a \times A^y/a$ crosses were first judged abnormal at the morula stage than control embryos at that stage (computed chi-square value = 9.9; critical value of chi-square for $P = 0.05$ and 3 degrees of freedom is 7.8). The number of embryos first revealing abnormalities at the blastocyst stage was higher in the experimental versus the control crosses (computed chi-square value = 9.4; critical value of chi-square for $P = 0.05$ and 3 degrees of freedom is 7.8). Also, abnormalities which first appeared during post-blastocyst development were more frequent in the experimental group than in control groups (computed chi-square value = 6.4; critical value of chi-square for $P = 0.1$ and 3 degrees of freedom is 6.3).

Morphological characterization of abnormal embryos from experimental ($A^y/a \times A^y/a$) matings from recovery throughout culture showed that 4.5% (2/44) abnormal embryos were first judged abnormal at the

TABLE 4. CHARACTERIZATION OF ABNORMAL EMBRYOS FROM RECOVERY THROUGH FIVE DAYS OF CULTURE (56-158 H.P.C.).

Parent Genotype ♀ ♂	Total number of abnormal ¹ embryos	2-4 cell stage ²	8-cell stage ²	Morula stage ²	Blastocyst stage ²	Post- blastocyst stage ²
A ^y /a x A ^y /a	44	4.5% (2/44)	9.1% (4/44)	2.3% (1/44)	22.7% (10/44)	61.4% (27/44)
A ^y a x a/a	40	10.0% (4/40)	17.5% (7/40)	25.0% (10/40)	2.5% (1/40)	45.0% (18/40)
a/a x A ^y /a	47	14.9% (7/47)	23.4% (11/47)	12.8% (6/47)	8.5% (4/47)	40.4% (19/47)
a/a x a/a	39	10.3% (4/39)	23.1% (9/39)	20.5% (8/39)	10.3% (4/39)	35.9% (14/39)

¹ Represents the number of embryos (from Table 2) that were abnormal at recovery (56 h.p.c.) or became abnormal during the five day culture period (from Table 3).

² Represents the stage at which embryos were first judged abnormal.

2- to 4-cell stage; one 2- to 4-cell embryo had a disintegrating blastomere (Plate 3, Figure a), and one 4-cell embryo had unequal size blastomeres. At the 8-cell stage 9.1% (4/44) of the embryos became abnormal; two embryos had excluded blastomeres, one contained unequal size blastomeres (Plate 3, Figure c) and one possessed three large white spherical bodies inside the zona pellucida. At the morula stage 2.3% (1/44) of the embryos became abnormal; one had an excluded blastomere (Plate 3, Figure e). At the blastocyst stage 22.7% (10/44) of the embryos first exhibited abnormalities; four were blastocysts with excluded material (Plate 3, Figure f), three were very small blastocysts shrunk away from the zona pellucida and three were arrested morula (Plate 3, Figure d). At the post-blastocyst stage 61.4% (27/44) of the experimental embryos became abnormal; 17 were arrested, unhatched blastocysts, 7 were unhatched blastocysts with ruptured zona pellucidas (Plate 2, Figure i). two hatched but did not attach and one attached but failed to outgrow.

In the class of 40 abnormal embryos from the A^y/a female x a/a male control crosses, 10.0% (4/40) of the embryos were judged abnormal at the 2- to 4-cell stage; two were dark, granular 4-cell embryos (Plate 2, Figure a), one 4-cell embryo had disintegrating blastomeres and one had an excluded blastomere. At the 8-cell stage 17.5% (7/40) of the embryos became abnormal; two were arrested 4-cell embryos, three were fragmented 8-cell embryos, one had large vesicles (Plate 2, Figure b) and one had disintegrating blastomeres. At the morula stage 25.0% (10/40) of the embryos became abnormal; three were arrested 8-cell

PLATE 1. NORMAL EMBRYO DEVELOPMENT

- a. Four-cell embryo from $A^y/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 56 h.p.c., showing the typical cruciform arrangement with two pairs of cells at right angles to one another. The blastomeres are nearly spherical and each is in contact with the other three. 600X.
- b. Eight-cell embryo from $A^y/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 56 h.p.c., showing spherical, distinct blastomeres. 610X.
- c. Morula with evident polar body from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 86 h.p.c., showing relatively smooth surface contour and indistinct blastomeres. 600X.
- d. Blastocyst from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 104 h.p.c., showing the inner cell mass in the lower right corner and flattened trophoblast cells surrounding the periphery of the embryo, with a large blastocoelic cavity in the center. 480X.
- e. Blastocyst hatching from the zona pellucida, from $A^y/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$, 104 h.p.c., showing the cells pushing out in the area of the ruptured zona. 372X.
- f. Outgrowth from $a/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 158 h.p.c., showing evident inner cell mass (ICM) atop flattened trophoblast cells. Trophoblast nuclei are large and the outgrowth has a fan-like appearance. 165X.

PLATE 2. ABNORMAL EMBRYO DEVELOPMENT

- a. Granular 4-cell embryo from $A^y/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 56 h.p.c., showing dark granular blastomeres of unequal size. 511X.
- b. Degenerate embryo from $A^y/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 62 h.p.c., showing dense disorganized mass with large clear vesicles on the periphery contacting the zona pellucida. 450X.
- c. Abnormal 8-cell embryo from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 56 h.p.c., showing disintegrating blastomeres (arrow) with indistinct cell boundaries. At least four blastomeres still maintain cellular integrity. 510X.
- d. Abnormal morula from $A^y/a^{\text{♀}}$ x $a/a^{\text{♂}}$ mating, 80 h.p.c., showing small granular morula with two large excluded blastomeres. Judging from their size, the blastomeres appear to have been excluded at the 8-cell stage. 500X.
- e. Abnormal blastocyst from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 104 h.p.c., showing dark excluded blastomeres (arrow), and evident bulging of trophoblast cells on the periphery. 508X.
- f. Collapsed blastocyst from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 152 h.p.c. At 104 h.p.c. the blastocyst had a small blastocoelic cavity and was shrunk away from the zona pellucida. Now there is no evident cavity and the embryo is dense and disorganized. 425X.
- g. Abnormal blastocyst from $a/a^{\text{♀}}$ x $A^y/a^{\text{♂}}$ mating, 132 h.p.c., showing blunt oblong protrusions projected in the area of the rupturized zona pellucida. The blastocoelic cavity is evident but there is no organization within the embryo. 455X.

PLATE 2 CONTINUED

- h. Small abnormal blastocyst from $a/a\text{♀}$ x $A^y/a\text{♂}$ mating, 128 h.p.c., showing excluded material on the periphery of the embryo and extending through a small rupture in the zona pellucida and outward. The blastocoelic cavity is evident. 357X.
- i. Shrunken, disorganized blastocyst (presumed lethal mutant) from $A^y/a\text{♀}$ x $A^y/a\text{♂}$, 104 h.p.c., showing a normally ruptured zona pellucida. The blastocyst was previously expanded but then collapsed and failed to hatch. 433X.

PLATE 3. ABNORMAL EMBRYO DEVELOPMENT FROM THE

 $A^y/a \times A^y/a$ EXPERIMENTAL MATING

- a. Degenerating embryo from $A^y/a \times A^y/a$ mating, 56 h.p.c., showing two granular blastomeres intact and one disintegrated blastomere. 652X.
- b. Arrested 8-cell embryo from $A^y/a \times A^y/a$ mating, 80 h.p.c., showing fragmenting blastomeres. 650X.
- c. Arrested 8-cell embryo from $A^y/a \times A^y/a$ mating, 80 h.p.c., showing blastomeres of unequal size. 640X.
- d. Arrested morula from $A^y/a \times A^y/a$ mating, 86 h.p.c., showing excluded fragmented cellular material and a clear vesicle on the periphery. 640X.
- e. Morula with excluded blastomere from $A^y/a \times A^y/a$ mating, 86 h.p.c., showing small morula with disintegrating blastomere on the periphery. 620X.
- f. Disorganized mid-blastocyst from $A^y/a \times A^y/a$ mating, 104 h.p.c., showing no evident organization of ICM and trophoblast although a blastocoelic cavity is evident (black area). Excluded material on periphery. 660X.

embryos, six were morulae with excluded blastomeres (Plate 2, Figure d) and one morula had large vesicles. At the blastocyst stage 2.5% (1/40) of the embryos became abnormal; this was an arrested morula. During post-blastocyst development 45.0% (18/40) were first judged abnormal; eight were arrested, unhatched blastocysts, two were unhatched blastocysts with ruptured zona pellucidas, three hatched but did not attach and five attached but failed to outgrow.

In the a/a female x A^y/a male control cross, 47 abnormal embryos were characterized. At the 2- to 4-cell stage 14.9% (7/47) of the embryos were first judged abnormal; one degenerating 2-cell embryo, four granular 4-cell embryos, one 4-cell with disintegrating blastomeres and one with no zona pellucida. At the 8-cell stage 23.4% (11/47) of the embryos became abnormal; six 8-cell embryos with disintegrating blastomeres (Plate 2, Figure c), two 8-cell embryos with unequal size blastomeres, one fragmenting 8-cell embryo, one with material protruding through the zona pellucida (it developed to the blastocyst stage, Plate 2, Figure h), and one arrested 4-cell embryo. At the morula stage 12.8% (6/47) of the embryos became abnormal; four morulae with excluded blastomeres, one fragmented morula, and one arrested 8-cell embryo. At the blastocyst stage 8.5% (4/47) of the embryos became abnormal; one small degenerating blastocyst, one blastocyst with excluded blastomeres (Plate 2, Figure e), one collapsed, shrunken blastocyst (Plate 2, Figure f) and one arrested morula. During post-blastocyst development 40.4% (19/47) of the embryos became abnormal; eleven were arrested, unhatched blastocysts, four were arrested blastocysts with ruptured

zona pellucidas, one hatched but did not attach and three attached but failed to outgrow.

Of 39 abnormal embryos from the a/a x a/a control cross 10.3% (4/39) were first judged abnormal at the 2- to 4-cell stage; one degenerating 2-cell embryo, two granular 4-cell embryos and one fragmenting 4-cell embryo. At the 8-cell stage 23.1% (9/39) of the embryos became abnormal; four 8-cell embryos had disintegrating blastomeres, one 8-cell embryo was fragmented, one had no zona pellucida and three were arrested 4-cell embryos. At the morula stage 20.5% (8/39) of the embryos were judged abnormal; three were arrested 8-cell embryos, two were fragmenting morulae and three were very small morulae (shrunken away from the zona pellucida). At the blastocyst stage 10.3% (4/39) of the embryos became abnormal; two were shrunken blastocysts and two were arrested morulae. During post-blastocyst development 35.9% (14/39) of the embryos became abnormal; seven were unhatched, arrested blastocysts, four unhatched blastocysts had ruptured zona pellucidas, two hatched but did not attach and one attached but failed to outgrow.

In summary, the same types of abnormalities were present in all matings with no unique morphological characteristics identifying the class of A^y/A^y mutants in vitro through blastocyst development. Embryos with excluded blastomeres were observed in control crosses as well as in the experimental cross. Blastomere exclusion was observed as frequently in the yellow female x black male cross as in the experimental

cross. The greatest incidence of abnormal development in culture was hatching failure of embryos from all crosses, although hatching failure was greater ($P < 0.1$) in the experimental cross.

DISCUSSION

Observations reported here show that while embryos from control crosses (A^y/a female x a/a male, a/a female x A^y/a male and a/a female x a/a male) were generally 8-cell embryos at recovery (56 h.p.c.), embryos from the experimental cross (A^y/a female x A^y/a male) were more evenly distributed between the 4-cell and 8-cell stages indicating a lag in development at least as early as the 8-cell stage (see Figure 1). Observations of Pedersen and Spindle (1976) similarly show that effects of A^y homozygosity occur during early cleavage stages causing homozygous yellow embryos to develop more slowly than their normal littermates. Since in this study no corresponding developmental lag was observed in embryos from the yellow female x black male cross, delayed development is evidently due to a factor inherent in the yellow homozygous embryo and not due to development in the yellow female reproductive tract.

Breeding studies conducted by Cizadlo et al. (1975) on the same strain of mice used for this experiment indicate that development in the yellow uterine environment may be less favorable than in the black uterine environment, because litter size in yellow female x black male matings was significantly smaller ($P < 0.05$) than litter size in the reciprocal cross. Although the yellow uterine environment may adversely effect development, 8-cell embryos do not appear to be altered by conditions in the yellow female reproductive tract up to the time of recovery.

No differences were observed in the number of morphologically abnormal embryos at recovery between experimental and control groups indicating that the factor causing developmental lag in embryos from the yellow heterozygous cross did not grossly upset development at this time. Abnormalities observed in embryos at recovery were similar among all groups with no distinctly different morphological characteristic present in abnormal embryos from the experimental cross.

Reproductive tract flushings of experimental and control females at 56 to 60 h.p.c. showed no differences in mean number of embryos per female. Since no deficiency was observed in number of embryos recovered from $A^Y/a \times A^Y/a$ matings, it is assumed that the homozygous (A^Y/A^Y) class is present and viable at the time of recovery (56 h.p.c.).

Results of the present in vitro study show that cultured embryos from experimental and control groups are equally capable of developing normally to the blastocyst stage, despite the lag in development of some experimental embryos at recovery. Previous investigators (Kirkham, 1919; Robertson, 1942) employing histological techniques have reported embryo abnormalities due to the presence of the A^Y allele as early as the morula or blastocyst stage. In vitro studies of Pedersen (1974) have shown abnormalities in cultured embryos from $A^Y/a \times A^Y/a$ matings occurring as early as the fourth cleavage division. Subsequent cinematographic observations of Pedersen and Spindle (1976) showed delayed cleavage of presumed A^Y/A^Y embryos at the 2-cell to 4-cell stage.

Pedersen (1974), Pedersen and Spindle (1976), and Calarco and Pedersen (1976) reported that A^Y/A^Y embryos could be identified as

cleavage embryos, morulae and blastocysts by the presence of excluded blastomeres. However, in the present study embryos with excluded blastomeres were common to both experimental and control groups and did not represent a distinct class in the yellow heterozygous cross. Although Pedersen (1974) used only one control mating (A^Y/a female $\times$ a/a male) and found 8.8% of embryos recovered at the morulae and early blastocyst stage abnormal, he does not list the types of abnormalities found in control embryos. It is interesting that ultrastructural studies of Calarco and Pedersen (1976) relied completely on identification of A^Y/A^Y embryos by the presence of excluded blastomeres. In fact, the works of Calarco and Pedersen (1976) and Pedersen and Spindle (1976) did not include new data from control crosses, but relied entirely on the control data from Pedersen's 1974 study. If indeed A^Y/A^Y embryos can be identified by the presence of excluded blastomeres, it was not evident in this study.

In this study embryos from the yellow heterozygous cross which failed to hatch from the zona pellucida in culture after developing to the blastocyst stage are presumed to be the lethal A^Y/A^Y mutants. Histological studies by Kirkham (1919) and Robertson (1942) reported that homozygous (A^Y/A^Y) embryos die just prior to implantation after eliciting a uterine decidual response. Later studies by Eaton and Green (1962, 1963) and Eaton (1968) suggest that A^Y/A^Y embryos are capable of implanting in the yellow uterus but die soon after. Whether A^Y/A^Y homozygotes die in vivo, because of inherent cellular malfunctions or because of improper communication with the yellow uterus is not

known. Pedersen's (1974) in vitro study reported that many presumed A^Y/A^Y mutants die in culture as a result of hatching failure. The present study supports his conclusions with the observation that 75.0% (72/96) of embryos developing normally to the blastocyst stage were able to hatch from the zona pellucida. The 25.0% (24/96) that failed to hatch may represent the class of yellow (A^Y/A^Y) mutants, although 9.3% of pooled controls were unable to hatch from the zona pellucida. Hatching failure does not explain the lethality of A^Y/A^Y embryos in vivo however, because in the uterus an estrogen-dependent zona lysis occurs freeing the embryo from the zona pellucida (Pedersen and Spindle, 1976).

Though it is generally accepted that A^Y/A^Y embryos are implantation lethals, the cause of the lethality has not yet been determined. It may be that problems inherent in the homozygous yellow embryo are responsible for the lethality. Pedersen and Spindle (1976) suggest that deficiencies accumulated by the A^Y/A^Y embryo throughout preimplantation may cause their ultimate failure at the critical time of implantation. Perhaps there is an extended phenocritical period with death of A^Y/A^Y embryos occurring at various times prior to and during implantation.

Previous researchers (Eaton and Green, 1963; Eaton, 1968; Pedersen, 1974; Pedersen and Spindle, 1976) suggest that A^Y/A^Y lethality may be due to incorrect trophoblast function. In vivo trophoblast malfunction may cause improper attachment of the embryo to the uterus. In vitro trophoblast malfunction may cause hatching failure, since

trophoblast cells must penetrate the zona pellucida freeing the embryo for attachment (Cole, 1967).

In vitro, it is necessary to denude presumed A^y/A^y embryos with pronase before outgrowth will occur (Pedersen, 1974). Even after zona removal only limited trophoblastic outgrowth occurs, and ICM cells are absent (Pedersen, 1974; Pedersen and Spindle, 1976). Gardner (1972) separated blastocysts into their component parts (pure ICM cells and pure trophoblast cells). Trophoblast segments transferred to the uteri of pseudopregnant mice implanted but did not proliferate. Gardner (1972) suggests that early implants of pure trophoblast are similar to those of homozygous lethal yellow embryos in utero. Even though presumed A^y/A^y blastocysts are capable of initial ICM and trophoblast differentiation, it is possible that continued growth and differentiation are interrupted in some way by the presence of the A^y allele.

Studies on steroidogenesis in the preimplantation rat embryo (Dickmann and Dey, 1974) suggest that the preimplantation embryo is an indispensable source of steroid hormones and that the embryo may exert a local hormonal change in the uterine endometrium which is critical for implantation. The authors suggest that most mammalian preimplantation embryos have steroidogenic capability and that a complement of steroid hormones from the ovary and the embryo is necessary for implantation to occur. If mouse embryos have the ability to synthesize steroid hormones, it is possible that A^y/A^y implantation lethals are deficient in steroid hormone synthesis and thus unable to effect

changes in the uterine endometrium necessary for implantation. Although steroidogenic capability in mouse embryos has not been tested, it may be a novel approach to the problem of homozygous (A^Y/A^Y) lethality.

Another hypothesis dealing with the failure of A^Y/A^Y embryos has been proposed by Eaton and Green (1963) who found that differentiation and attachment of trophoblast giant cells to the uterine endometrium did not proceed at the same rate as observed in normal embryos. The authors suggest that homozygous A^Y embryos lag behind in development and as a result may reach the uterus when the endometrium has entered a refractory period and will not respond to the embryo. Thus the embryo ready for attachment meets with a hostile environment and cannot make attachments necessary for ultimate survival. The present study and earlier study of Pedersen and Spindle (1976) support the contention of Eaton and Green (1963), because presumed A^Y/A^Y embryos show a lag in development during early cleavage.

Although the A^Y/A^Y embryo may be lethal autonomously, previous investigators (Ibsen and Steigleder, 1917; Kirkham, 1919; Robertson, 1942; Wolff and Bartke, 1966; Eaton, 1968) suggest that development of embryos within the yellow (A^Y/a) female may potentiate effects of the A^Y allele, decreasing the chances of the A^Y/A^Y embryo for survival and possibly causing death to some heterozygous (A^Y/a) embryos as well. Ibsen and Steigleder (1917) were the first investigators who attempted to control for the A^Y/a maternal background. They found a greater percentage of dead embryos (12.3%) in the yellow female x black male

(1966) suggest that the metabolic alteration responsible for the homo- control cross than in the reciprocal cross (4.5%) and suggested that the physiological difference causing yellow females to take on more fat may in some way influence the production of a greater number of dead embryos. Kirkham (1919) first suggested that viable homozygous (A^Y/A^Y) embryos might be produced in a more favorable uterine environment, i.e., that of the non-yellow female. Robertson (1942) performed ovary transplant experiments allowing A^Y/A^Y embryos to develop in a black uterine environment; he claimed that development of A^Y/A^Y embryos was prolonged. However, his data are largely qualitative and do not conclusively show enhancement of development. Wolff and Bartke (1966) concluded that the A^Y/a uterus may also be responsible for decreased survival of A^Y/a embryos as expressed by lower embryonic survival, smaller litter size and deficiencies of yellow young in the YS/ChWf strain. However, a deficiency of yellow young may have been due to cannibalism occurring immediately after birth, as pregnant females were checked only once daily, and weakened or dead offspring may have been eaten before the litter was recorded. Although Wolff and Bartke (1966) observed lower embryonic survival in A^Y/a females from the YS/ChWf strain, other strains of yellow mice (YS/Ch, YBR/He) did not show this effect. Interestingly, Wolff and Bartke (1966) reported that about 25% of implanted embryos in both a/a and A^Y/a females died before parturition. If this were true in the strain of mice used for the present study, we would have to assume that 25% of the embryos in control crosses die. This does not hold true in view of data from reproductive tract flushings and breeding data on control females. Wolff and Bartke

(1966) suggest that the metabolic alteration responsible for the homozygous (A^Y/A^Y) lethality may also decrease the viability of yellow heterozygous embryos. They state that, "Whereas A^Y/A^Y embryos are below the survival threshold, most heterozygous A^Y embryos develop successfully and the proportion that dies may depend on the specific intrauterine milieu."

Eaton (1968) thought that homozygous yellow embryos were hormonally deprived by development in the yellow uterus and thus attempted to increase the number of homozygous yellow implantations by administering exogenous ovarian steroid hormones (progesterone; progesterone and estradiol) to pregnant yellow females. However, the study was inconclusive and can be criticized because the yellow (A^Y/a) uterus has not been shown to be progesterone deficient.

In this study, embryos were recovered from the female reproductive tract at 56 to 60 h.p.c. when most were at the 8-cell stage. Thus effects of the yellow uterine environment on development were not tested, because 8-cell embryos are still in the oviduct at 50 to 70 hours after copulation (Lewis and Wright, 1935). If conditions in the yellow oviductal environment are unfavorable to developing embryos, it was not evident in the present study, because no abnormal morphological differences were observed in embryos from the yellow female control cross versus the black female control crosses.

The A^Y allele causing lethality in the homozygous condition is also responsible for the altered physiologic state of the heterozygous animal, the difference being of a quantitative nature. Although

heterozygotes (A^Y/a) are viable, they exhibit detrimental effects of the A^Y gene such as extreme obesity, reproductive inefficiency, a diabetes-like syndrome, and increased susceptibility to certain cancers (see Cizadlo, 1976 for a review).

Advances made in the understanding of either the lethal genotype (A^Y/A^Y) or the heterozygous (A^Y/a) animal may act synergistically to aid the understanding of both systems and lead to the discovery of specific cellular defects caused by A^Y allele expression. Although progress has been made in characterizing the lethal yellow mouse system in utero and in vitro, the basic deletion or biochemical alteration specified by the A^Y allele remains unknown.

CONCLUSIONS

Objectives of the studies presented in this thesis were to characterize and document the generation of the lethal yellow (A^Y/A^Y) syndrome in cultured mouse embryos. Investigations reported here have determined: (1) experimental embryos are retarded in development at 56 to 60 h.p.c. when compared to control embryos ($P < 0.01$), (2) no apparent morphological abnormality (i.e., excluded blastomeres) distinguishes the class of A^Y/A^Y mutants, (3) experimental and control embryos were equally able to successfully complete development to the blastocyst stage in culture, (4) fewer experimental embryos hatched from the zona pellucida in culture than control embryos ($P < 0.1$), (5) no decisive phenocritical period was discovered, and (6) in vitro studies can not fully explain implantation failure in utero.

In this study experimental embryos were not as developmentally advanced as control embryos at recovery; these observations confirm the developmental lag of embryos from the yellow heterozygous cross (Pedersen and Spindle, 1976). Results reported here support the conclusion of Pedersen (1974) that presumed A^Y/A^Y embryos die in culture following hatching failure; however A^Y/A^Y mutants could not be identified in culture by the exclusion of blastomeres, because embryos from control matings exhibited blastomere exclusion also. This study, in disagreement with Pedersen (1974), has shown that the exclusion of blastomeres in cultured embryos is a phenomenon common to control

embryos as well as to experimental embryos and may not aid in the exclusive identification of lethal mutants prior to hatching failure.

Although A^y/A^y embryos may lag behind in development and subsequently die in culture without becoming free of the zona pellucida, present results do not reveal a distinct phenocritical period during preimplantation development when the class of A^y/A^y embryos can be identified before death. The phenocritical period may extend throughout gestational development with the lethal homozygotes showing effects of A^y allele expression at various times.

Hatching failure of presumed A^y/A^y embryos in culture does not exemplify their developmental capability in vivo where zona lysis frees the embryo enabling attachment to occur. Thus implantation failure of A^y/A^y embryos can not be totally explained in light of in vitro phenomena.

An extensive and well controlled in vivo study of implanting embryos from yellow heterozygous parents may provide new data on implantation phenomena that will explain the lethality of A^y/A^y embryos. Histological data generated on implanting embryos could include: (1) numbers of ICM and trophoblast nuclei, (2) volume of the embryo, (3) presence or absence of the zona pellucida, (4) condition of the uterus, i.e., evidence of decidualization, (5) presence or absence of excluded blastomeres, and (6) the volume or depth of the implantation chamber. Perhaps a comprehensive study of this nature could aid in explaining the lethality. However, if the biochemical defect causing the ultimate lethality is to be defined, it may be

necessary to design specific biochemical analyses for testing physiologic systems operative in preimplantation mouse embryos, i.e., if preimplantation embryos synthesize steroid hormones necessary for successful implantation (Dickmann and Dey, 1974) perhaps staining techniques could be used to identify differential steroidogenic capabilities between A^Y/A^Y embryos and their normal littermates.

There is no reason to believe that the embryonic defect is unrelated to the heterozygous (A^Y/a) yellow syndrome. Therefore, an understanding of either system (heterozygous or homozygous) will probably aid in solving the problems of the other. The result may well be a better understanding of how seemingly diverse metabolic systems are related.

Finally, developmental disturbances brought about by the expression of a lethal gene can be compared to normal developmental processes for the purpose of understanding factors involved in the control and integration of successful development. Inferences drawn from the study of an embryo that is lethal at implantation may provide a better understanding of this critical reproductive process. In return, a greater understanding of events surrounding implantation could be of value in areas of reproductive efficiency and mammalian fertility control.

LITERATURE CITED

- Calarco, P. G., and R. A. Pedersen. 1976. Ultrastructural observations of lethal yellow (A^Y/A^Y) mouse embryos. *J. Embryol. exp. Morph.* 35:73.
- Castle, W. E., and C. C. Little. 1910. On a modified Mendelian ratio among yellow mice. *Science* 32:868.
- Cizadlo, G. R. 1976. Physiologic and embryologic studies of the yellow (A^Y/a) mouse (Dissertation). Brookings: South Dakota State University.
- Cizadlo, G. R., S. K. Hoffman, and N. H. Granholm. 1975. Mating characteristics and embryonic losses in the yellow (A^Y/a) mouse. *Proc. S. D. Acad. Sci.* 54:157.
- Cole, R. J. 1967. Cinemicrographic observations on the trophoblast and zona pellucida of the mouse blastocyst. *J. Embryol. exp. Morph.* 17:481.
- Cuenot, L. 1905. Les races pures et leurs combinaisons chez les souris. *Arch. Zool. Exper. et Gener.* 4^e Serie, T. III:123.
- Dickmann, Z., and S. K. Dey. 1974. Steroidogenesis in the preimplantation rat embryo and its possible influence on morula-blastocyst transformation and implantation. *J. Reprod. Fert.* 37:91.
- Eaton, G. J. 1968. Stimulation of trophoblastic giant cell differentiation in the homozygous yellow mouse embryo. *Genetica* 39:371.
- Eaton, G. J., and M. M. Green. 1962. Implantation and lethality of the yellow mouse. *Genetica* 33:106.

- Eaton, G. J., and M. M. Green. 1963. Giant cell differentiation and lethality of homozygous yellow mouse embryos. *Genetica* 34:155.
- Gardner, R. L. 1972. An investigation of inner cell mass and trophoblast tissues following their isolation from the mouse blastocyst. *J. Embryol. exp. Morph.* 28:279.
- Ibsen, H. L., and E. Steigleder. 1917. Evidence for the death in utero of the homozygous yellow mouse. *Am. Nat.* 51:740.
- Kirkham, W. B. 1917. Embryology of the yellow mouse. *Anat. Rec.* 11:480. (Abstr.).
- Kirkham, W. B. 1919. The fate of homozygous yellow mice. *J. Exp. Zool.* 28:125.
- Lewis, W. H., and E. S. Wright. 1935. On the early development of the mouse egg. In *Contributions to Embryology*. Carnegie Institute, Washington. 25:115.
- Little, C. C. 1919. A note on the fate of individuals homozygous for certain color factors in mice. *Am. Nat.* 53:185.
- Pedersen, R. A. 1974. Development of lethal yellow (A^Y/A^Y) mouse embryos in vitro. *J. Exp. Zool.* 188:307.
- Pedersen, R. A., and A. I. Spindle. 1976. Genetic effects of mammalian development during and after implantation. In Elliott, I. and M. O'Connor (eds.) *Embryogenesis in Mammals*. Ciba Foundation Symposium, New York.
- Rafferty, K. A., Jr. 1970. Methods in experimental embryology of the mouse. John Hopkins Press, Baltimore.

Robertson, G. G. 1942. An analysis of the development of homozygous yellow mouse embryos. J. Exp. Zool. 132:51.

Wolff, G. L. 1967. Practical mating systems and record-keeping in a breeding colony. In The UFAW Handbook on the Care and Management of Laboratory Animals. The Williams and Wilkins Co., Baltimore.

Wolff, G. L., and A. Bartke. 1966. Decreased survival of embryos in yellow (A^y/a) female mice. J. Heredity 57:14.