SUPEROVULATED MATURE MICE PRODUCE MORE HETEROGENEOUS OOCYTE POPULATIONS COMPARED TO SUPEROVULATED PREPUBERAL MICE

by

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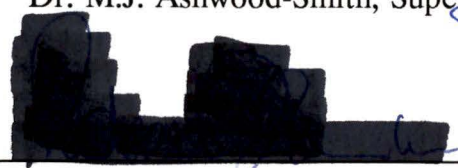
ABSTRACT

Superovulation of prepuberal and mature mice has become an established procedure. Pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) are injected to induce growth of Graafian follicles and the expulsion of secondary oocytes into the oviduct. Superovulated ova are considered normal — arrested in metaphase of meiosis II. Mouse age and hormone dose are known to affect the number of eggs ovulated, but are not considered determinants of their meiotic maturity. The current study provides evidence against this assumption, and the indiscriminate superovulation of mice to obtain metaphase II oocytes. Ovulated oocytes from individual mice were analyzed using indirect immunofluorescence of microtubules and Hoechst 33258 staining of chromosomes. Almost all of the superovulated ova from prepuberal mice were arrested in metaphase, as expected. Variation in spindle shape was the only detected heterogeneity. However, stimulated mature mice produced mixed oocyte populations 13 - 16 h post-hCG, which included non-metaphase eggs and metaphase eggs with cytoplasmic microtubule asters. The conditions of superovulation affected the type of abnormal egg observed. Putative primary oocytes were recovered after 10 IU hormone doses, and significantly more oocytes in anaphase were observed after 5 IU hormone doses, or recovery times of 15 - 16 h post-hCG. The percentage of abnormal oocytes recovered from mature mice was positively correlated with the number of eggs ovulated. Past indications of heterogeneity of superovulated mouse

oocytes were not thoroughly investigated and evidence may not have been detected due to: mouse age, hormone dose, ovulation rate, or parameter measured. Pellicer et al. (1988) proposed exogenous gonadotrophins stimulate a more heterogeneous group of oocytes compared to normal cycling, by recruitment of younger and older follicles. The current data supports this suggestion, and extends the model to include the effects of animal age. The greater heterogeneity of superovulated ova from mature mice, compared to prepuberal mice, may reflect the inherent differences in ovarian environments at the time of hormone injection.

Examiners:

Dr. M.J. Ashwood-Smith, Supervisor (Department of Biology)



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INTRODUCTION

The technique of stimulating mice with exogeneous hormones to mimic or precociously induce ovulation, known as superovulation, has been in use for over thirty years. With superovulation, egg release is precisely timed and the quantities obtained usually exceed the number normally produced. This procedure has been, and is, helpful in providing large numbers of eggs, or embryos, for study. In recent years, superovulated mouse ova have been used to investigate oocyte cryopreservation, for cold-storage of excess human oocytes recovered during *in vitro* fertilization (IVF) trials. Superovulated ova from prepuberal and mature mice are considered to be normal — the secondary oocytes arrested in metaphase of meiosis II — when collected within a few hours of ovulation.

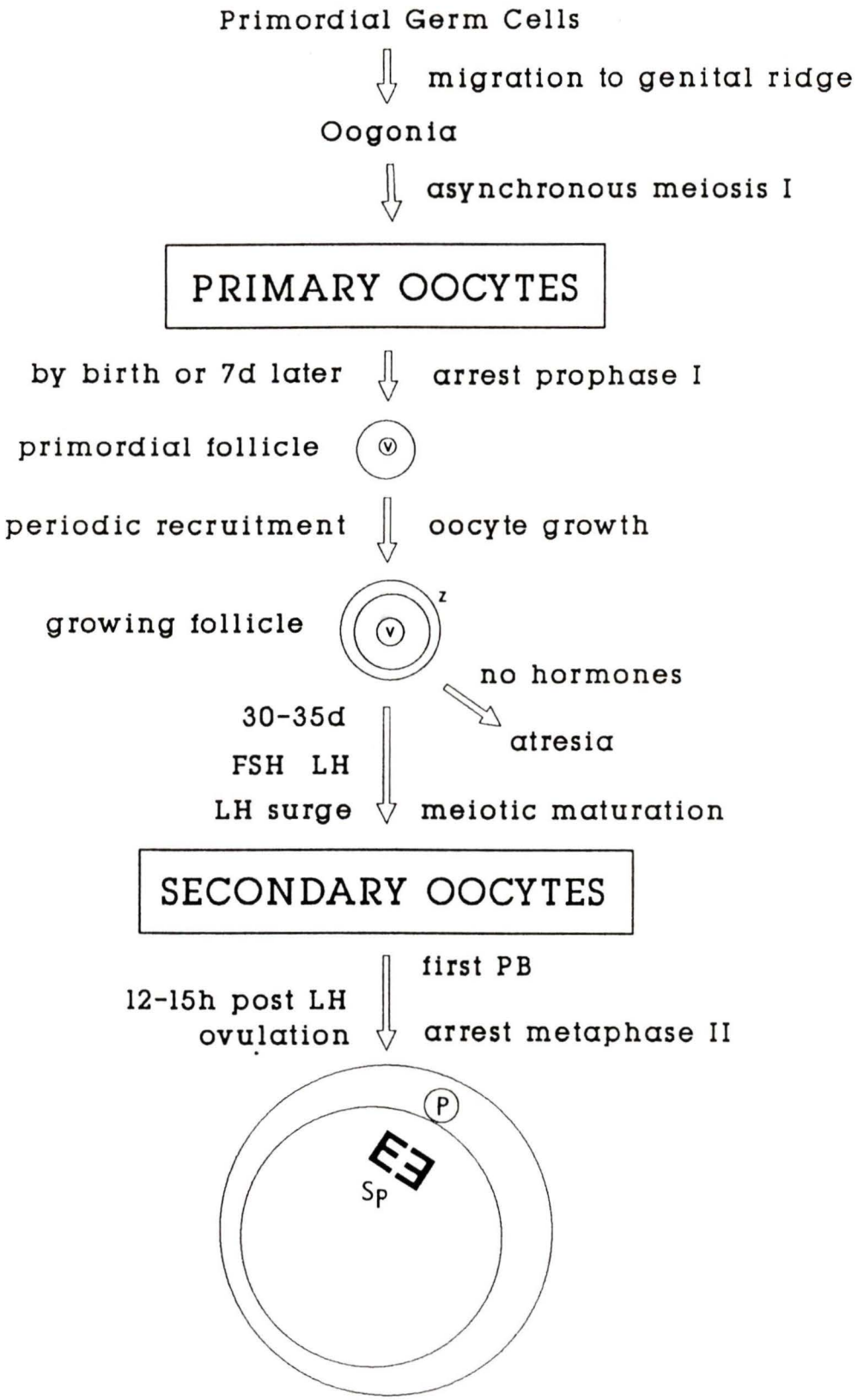
The original purpose of the thesis was to detect initial changes in the spindle of metaphase II oocytes after post-ovulatory ageing *in vivo*. Indirect immunofluorescence of microtubules and chromosomal staining were used to evaluate the eggs. However, problems in generating a control group of normal secondary oocytes from superovulated mature mice led to a re-evaluation of hormonally induced ovulation.

Oogenesis and ovulation

The secondary oocyte is the female gamete, which is fertilized by sperm during sexual reproduction (Baker, 1982). The developmental stages (oogenesis) leading to release (ovulation) of secondary oocytes in the female mouse are illustrated in Fig.1.

Primordial germ cells, originating in extraembryonic tissues, migrate to the genital ridge; once there, they are called oogonia (Byskov, 1982). The oogonia enter

Figure 1. Mouse oogenesis and ovulation. Developmental stages of germ cells (right) and associated events (left). V, germinal vesicle; Z, zona pellucida; FSH, follicle-stimulating hormone; LH, leutinizing hormone; P, polar body; Sp, spindle.


Primordial Germ Cells
migration to genital ridge
Oogonia
asynchronous meiosis I
PRIMARY OOCYTES
by birth or 7d later
arrest prophase I
primordial follicle
V
periodic recruitment
oocyte growth
growing follicle
Z
V
no hormones
atresia
30-35d
FSH LH
LH surge
meiotic maturation
SECONDARY OOCYTES
first PB
12-15h post LH
ovulation
arrest metaphase II
P
E3
SP

a period of extensive mitotic growth, but many of the cells produced die. Starting about ten days gestation, the surviving oogonia begin meiosis, asynchronously. Meiosis consists of two separate and distinct cell divisions, meiosis I and II, which upon completion produces haploid cells containing half the chromosome complement of the parent (diploid) cells (Baker, 1982). Prior to meiosis each chromosome is duplicated and meiosis I reduces the chromosome number by half. Meiosis II separates the replicate chromosome strands. The oogonia starting meiosis — the primary oocytes — only complete the first few steps of meiosis I, before they arrest in prophase; at birth, or within the following week (Peters, 1969). At this point, the nuclear membrane (germinal vesicle; GV) is still intact.

Primary oocytes are initially surrounded by a few, flattened granulosa cells; together, they constitute a primordial follicle (Brambell, 1928). Early follicle growth appears to be regulated within the ovary (Mangia and Canipari, 1977). Growth includes: an increase in oocyte size (from 20 μm to 80 μm), formation of a protective egg envelope (zona pellucida; ZP), and development of cuboidal granulosa cells in layers (Peters, 1969; Baker, 1982; Herlands and Schultz, 1984). Granulosa cells provide nutritional support for the growing egg (Herlands and Schultz, 1984).

The stimulation of primordial follicles, beginning about three days after birth (Mangia and Canipari, 1977), continues as periodic recruitment; to maintain a steady state of follicular growth (Baker, 1982). During the third and fourth week of life, small (primordial or one layer of granulosa cells), medium (thicker single granulosa cell layer to three layers), and large follicles (four or more granulosa cell layers,

sometimes containing pockets of fluid) are observed in ovarian sections (Peters, 1969).

Once a follicle has four layers of granulosa cells, gonadotrophins are required for continued growth, or the follicle becomes atretic and degenerates (Peters, 1969; Baker, 1982; Baird, 1984). The first gonadotrophin released by the anterior pituitary, follicle-stimulating hormone (FSH), promotes estrogen production (Baird, 1984). Responsive follicles develop into Graafian follicles, which contain a large, fluid-filled cavity (antrum). The second gonadotrophin released, leutinizing hormone (LH), stimulates ovarian blood flow and additional steroid synthesis (Baird, 1984). The subsequent, dramatic rise in estrogen induces maximum secretion of both gonadotophins; but, there is a much greater peak of LH (Baker, 1982). This LH surge triggers final maturation of follicles, and ovulation 12 - 15 h later (Baker, 1982). In the mouse, spontaneous ovulation begins 30 - 35 days after birth (Baker, 1982), and occurs every 4 - 5 days (Sadleir, 1972). Although many follicles begin to develop, only a portion of these eggs will be ovulated (Engle, 1927).

Primary oocytes contained within follicles undergoing antrum formation have completed their growth (Brambell, 1928; Sorensen and Wassarman, 1976), and are stimulated to resume meiosis by the LH surge (Baker, 1982). The stages of meiosis can be determined from the arrangement of chromosomes and microtubules (Donahue, 1968; Brinkley et al., 1975). Microtubules are composed of α-tubulin and β-tubulin heterodimers, which attach in a repeating sequence to create rod-like structures of varying lengths (Erickson, 1975). With the onset of cell reproduction,

the microtubule cytoskeleton is depolymerized, and new microtubules formed and assembled into a spindle (Brinkley et al., 1975). The spindle is a dynamic apparatus, which controls the movement and division of chromosomes. Cytokinesis at the end of meiosis I isolates the newly separated, homologous chromosomes into two haploid cells: a large secondary oocyte and a small polar body (PB) (Donahue, 1968). The secondary oocyte begins meiosis II, then arrests at metaphase, with an haploid number of double-stranded chromosomes aligned midway between the poles of a barrel- shaped structure (Baker, 1982). At ovulation, 12 - 15 h after the LH peak in mice, eight to twelve secondary oocytes are released (Baker, 1982; Hogan et al., 1986). The metaphase II spindle is located at the periphery of the oocyte, parallel to the egg surface (Maro et al., 1985). Fertilization (Baker, 1982) or artificial activation (Kaufman, 1973) stimulates the ovulated oocyte to complete meiosis.

The reasons for arrest of ovulated oocytes at metaphase II are unknown. The presence of a peripheral spindle has the advantage of providing polarity in the egg (Longo and Chen, 1985), which may determine successful development. Attachment of sperm to the egg membrane was found to occur, preferentially, away from the spindle (Nicosia et al., 1977).

Superovulation

In 1927, Smith and Engle injected mice with anterior pituitary tissue. The transplants stimulated the ovulation of eggs, by mature and immature mice, in numbers much higher than normal. They named this phenomenon superovulation. The ova from treated immature mice appeared normal in stained sections,

demonstrating a polar body and second maturation spindle. Based on the additional histological data of fertilized eggs from one adult mouse, they stated, "the factors involved in the production of ova are not different in the treated adult than in the precociously matured animal".

The gonadotrophins FSH and LH, from the anterior pituitary, induce final follicle growth and ovulation (Fig.1). Other hormones were found to cause similar responses. Pregnant mare's serum gonadotrophin (PMSG) demonstrates both FSH-like and LH-like properties; with FSH activity dominating (Baird, 1972; Heap and Flint, 1984). Human chorionic gonadotrophin (hCG) possesses LH-like action, stimulating ovulation from follicles primed with FSH or PMSG (Baird, 1972; Baird, 1984). hCG is extracted from the urine or serum of pregnant women (Heap and Flint, 1984; Baird, 1984).

Ova recovered from mice after injections of PMSG and hCG demonstrated successful fertilization and development (Runner and Palm, 1953; Runner and Gates, 1954; Gates, 1956); similar to eggs from spontaneous ovulations (Gates, 1956). Gross observations of oocytes detected two types of eggs: intact and surrounded by cumulus cells, or fragmented and denuded (Fowler and Edwards, 1957; Edwards and Gates, 1959). The number of fragmented ova seen, usually did not exceed the quantity of eggs normally ovulated per female (Edwards and Gates, 1959), and was thought to represent eggs from a natural cycle (Fowler and Edwards, 1957; Edwards and Gates, 1959). Edwards and Gates (1959) examined the superovulated oocytes for the presence of a polar body, and stated, "at 14 hr (post-hCG) all were ovulated and in

the metaphase of the second maturation division " (discounting degenerate eggs). Subsequent studies supported this evaluation. These included investigations of: chromosomal configurations (Johnson et al., 1975; Baumgartner and Chrisman, 1981; Kim and Schuetz, 1991), electron microscopy (Magistrini and Szollosi, 1980), and chromosomal staining with immunolabelling of spindle poles (Webb et al., 1986) or tubulin (Pickering and Johnson, 1987; Van der Elst et al., 1988).

Intact superovulated eggs were considered normal, therefore, the parameters of hormonal stimulation were examined to maximize the number of eggs recovered. Prepuberal mice, 3 weeks old, responded the best to superovulation (Gates and Runner, 1957; Zarrow and Wilson, 1961). Hormone doses of 5 to 10 IU PMS, followed 30 to 50 h later by 5 to 10 IU hCG, produced the highest number of oocytes collected 20 - 24 h post-hCG (Wilson and Zarrow, 1962). Mature mice responded to exogenous gonadotrophins irrespective of their estrus or diurnal cycle (Fowler and Edwards, 1957). Lang and Lamond (1966) found egg production was better when both injections were administered after 4:00 pm; using a twelve hour light cycle beginning at 6:00 am. However, Gates (1971) recommended the ovulatory injection be given about noon, before possible endogeneous release of LH (in response to the PMSG injection). Most descriptions of the superovulation procedure did not state the light cycle, nor the time injections were given (Edwards and Gates, 1959; Pickering and Johnson, 1987; Kim and Schuetz, 1991).

The recovery time post-hCG is critical, because post-ovulatory ageing of unfertilized oocytes leads to rapid deterioration. Ovulation occurs between 12 and

16 h after the administration of hCG (Runner and Palm, 1953). Morphological changes (spindle rotation and migration) were observed between 26 and 30 h post-hCG (Szollosi, 1971). Altered physiological responses were detected much sooner. Beginning at 16 h post-hCG, oocytes were found to be more sensitive to artificial activation (Kaufman, 1973). This lead to the classification of freshly or newly ovulated oocytes, only for eggs obtained 12 - 16 h post-hCG (Whittingham, 1977; Shaw and Trounson, 1989).

The current acceptance of an universal response in mice to superovulation is demonstrated by the use of prepuberal and mature mice (Whittingham and Siracusa, 1978; Glenister et al., 1987; Toner et al., 1991) or multiple hormone doses (Whittingham and Siracusa, 1978; Johnson et al., 1990; Vincent et al., 1990) within the same study; and, the omission of a description or reference to mouse age (Borsuk and Manka, 1988; Kubiak, 1989; Johnson et al., 1990; Vincent et al., 1990) or hormone dose (Longo and Chen, 1985; Longo, 1987). The same attitude is also reflected by claims of obtaining metaphase II oocytes from stimulated mice, without substantiation or reference to previous work (De Felici and Siracusa 1982; Longo, 1987; Kim and Schuetz, 1991). The study conducted provides evidence against the casual use of superovulation, because mouse age was found to significantly affect the quality of the eggs recovered. Metaphase ova were reliably obtained from stimulated prepuberal mice (3 wks). But, superovulated mature mice (9 - 10 wks) produced mixed oocyte populations, which included abnormal metaphase eggs and/or non-metaphase eggs, dependent upon hormone dose and recovery time.

MATERIALS and METHODS

Animals

All animals used were inbred BALB/c mice, obtained from the University of Victoria Animal Care Facility breeding colonies. A twelve hour light cycle was maintained, with lights on at 6:00 am. This system was occasionally faulty. Data from animals exposed to irregular light cycles was omitted. Prepuberal female mice were weaned at 20 or 21 days. After a minimum separation of one day, hormonal treatment was begun at 21 - 23 days. At the first injection the prepuberal animals weighed 10.5 - 13.7 g. Mature mice were 9 - 10 weeks old when exogeneous hormones were administered.

Superovulation and Egg Recovery

Pregnant mare's serum gonadotrophin (PMSG) and human chorionic gonadotrophin (hCG) were obtained from Sigma (G-4877 and CG-2). Sterile Dulbecco's phosphate-buffered saline (PBS) pH 7.4 was added to lyophilized PMSG to obtain a stock solution of 200 IU/ml. Sterile water was added to lyophilized hCG to produce a stock solution of 250 IU/ml. Sterile PBS was used to make 1/10 dilutions of both stock solutions.

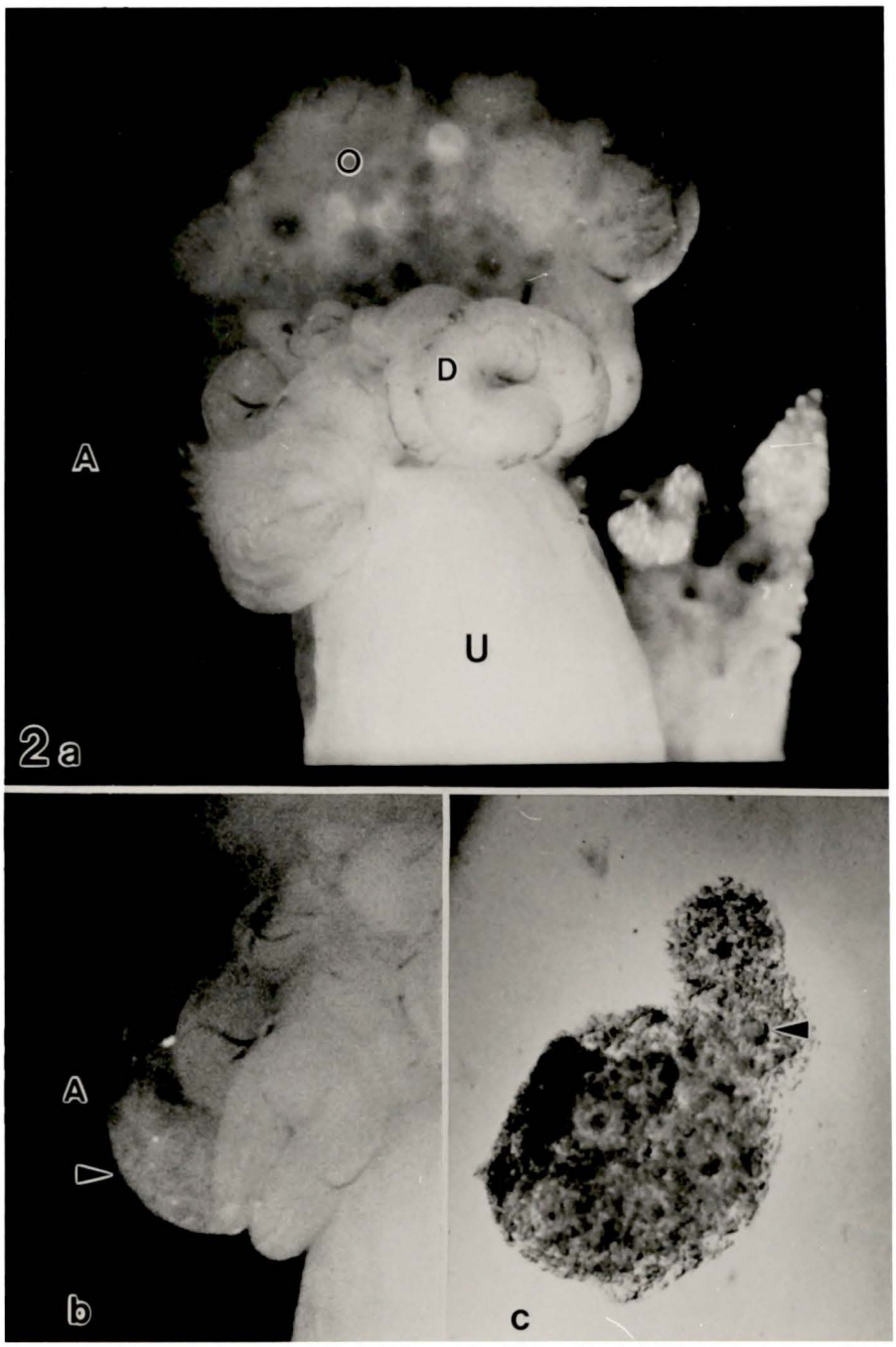
PMSG was injected into the peritoneal cavity at 8:30 pm. Forty-eight hours later, hCG was similarly administered. Mouse age (prepuberal or mature) and hormone dose (5 or 10 IU) were varied between experiments, but remained the same for the two mice used each time. Within each experiment only the oocyte recovery

time, 13 - 18 h post-hCG, was altered.

Individual aliquots of each hormone dilution for injection were kept frozen until use. It was discovered that the 5 IU hormone doses initially administered to prepuberal mice did not stimulate superovulation, as expected. These hormone dilutions had been stored the longest (6 months) prior to use, and may have lost their potency. These weak hormone doses were designated <5 IU. New 5 IU working dilutions (made immediately prior to injection) were subsequently used.

At 13 - 18 h post-hCG the mice were killed by cervical dislocation. Each oviduct (as shown in Fig. 2a), with attached ovary and upper segment of the uterine horn, was removed and placed into large drops of M2 medium (Hogan et al., 1986), in tissue culture dishes. All solutions were filter sterilized and pre-warmed to 37°C. Using a Zeiss dissecting microscope, the ovulated eggs could be clearly seen within a swollen section of the oviduct — the ampulla (arrow in Fig. 2b). With watchmaker's forceps the wall of the ampulla was torn and the eggs freed. Each oocyte (arrow in Fig. 2c) was usually surrounded by cumulus cells. Most ova were grouped within a large cluster, as shown in Fig. 2c. Sometimes individual eggs were observed. Between manipulations, the culture dishes were kept on a warming tray maintained at 35 - 37°C. When all the eggs from one animal were collected into a drop of approximately 0.5 ml M2, 1 mg hyaluronidase type II (Sigma H2126) in 1 ml PBS pH 7.4 was added to remove the cumulus cells (Pratt, 1987). Using the dissecting scope, denuded eggs were quickly removed and placed into fresh M2. Sterile, flame-tapered pasteur pipets were used for the transfer of eggs. The

Figure 2. Stimulated mouse ovary and oviduct. (a) Mouse ovary (O) with oviduct (D) and upper uterine horn (U). Swollen section of oviduct, the ampulla (A) contains the ovulated eggs. Magnification x 18. (b) Enlarged view of ampulla (A). Ovulated oocytes can be seen (arrow) through the thin ampulla wall. Magnification x 24. (c) Cluster of oocytes freed from the ampulla. Arrow points to a single egg, which is completely surrounded by a large cloud of cumulus cells. Magnification x 35.


O
D
A
U
2a
A
b
c

maximum exposure of oocytes to hyaluronidase was 5 minutes. The eggs were rinsed three times, in drops of M2 (approximately 1 ml each), and counted.

Oocyte Fixation and Immunocytochemistry

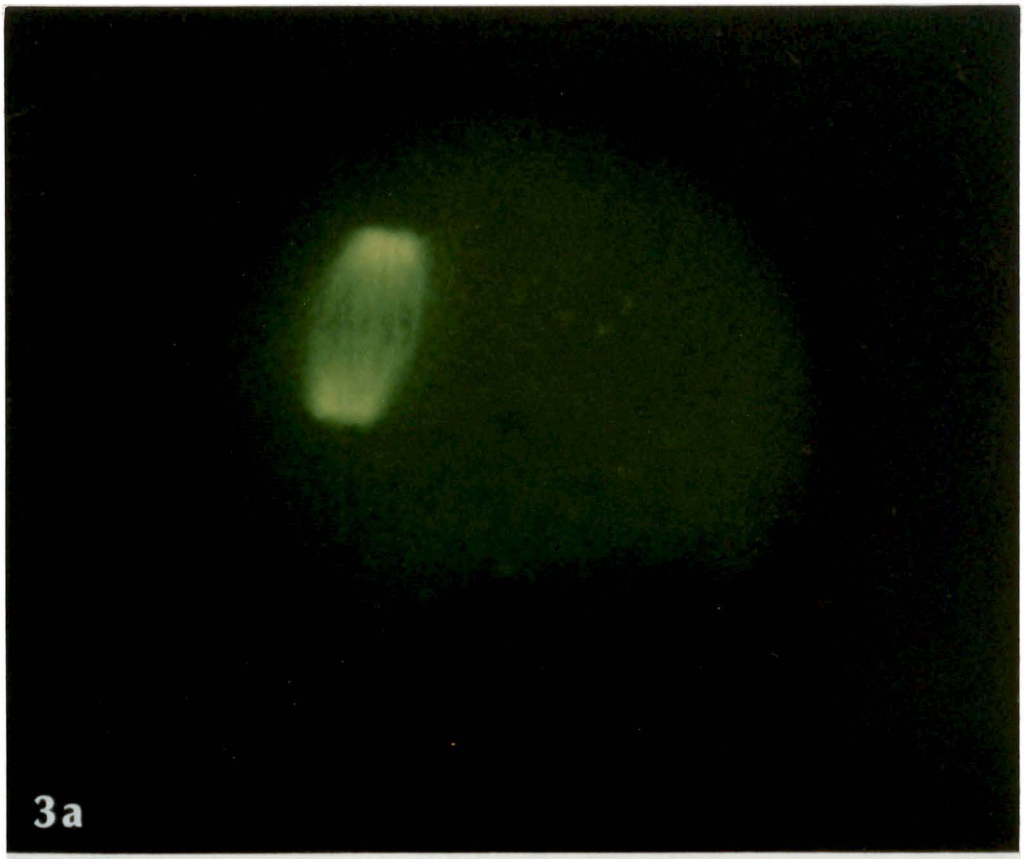
After a 15 min incubation at 36 - 37°C in M2 (to help compensate for any temperature fluctuations during egg recovery), the oocytes were rinsed once in pre-warmed PBS, then placed into a 35-mm culture dish containing pre-warmed, freshly made fixative (2% formaldehyde + 0.02% Triton X-100 in PBS pH 7.4; Pickering et al., 1988). All oocytes were fixed approximately an hour after mouse euthanasia. The eggs were kept in the fixative for 30 min, at 37°C. The ova were then removed and placed into small glass centrifuge tubes containing PBS. Eggs from each mouse, or those used for the control, were processed in a single tube. The oocytes were rinsed three times with PBS-Triton (0.5% Triton X-100 in PBS pH 7.4). For each rinse, the eggs were allowed to settle for about 10 minutes, then most of the supernatant was removed. Oocytes found in the supernatant, using the dissecting scope, were placed back into the original tube. When rinsing after fixation was complete, the eggs were placed in the fridge (up to 24 h), or processing was continued.

If oocytes were stored in the fridge overnight, the tubes were warmed to room temperature before the next step. Most of the supernatant was removed. To the remaining PBS-Triton solution (approximately 150 μl) and oocytes, 150 μl of anti-tubulin antibody (YOL 1/34; Seralab) diluted 1/300 with PBS was added. This

rat monoclonal antibody binds specifically to tyrosylated and detyrosylated alpha-tubulin (Wehland et al., 1983). To control tubes, either PBS-Triton or rat serum was added, instead of the anti-tubulin antibody. The eggs were gently mixed upon addition of the antibody and halfway through the incubation period of 30 min at 37 ° C. The eggs were then rinsed three times with the PBS-Triton solution. Next, fluorescein isothiocyanate conjugated rabbit anti-rat (ICN ImmunoBiologicals), diluted 1/60 with PBS, was added to all tubes. The oocytes were then incubated at (22 - 25 ° C), for 30 min, in the dark. The eggs were again rinsed three times. Lastly, in the same fashion, 150 μl of Hoechst DNA dye 33258 (0.05 or 0.025 mg/ml PBS) was added to all tubes. The eggs were incubated at room temperature, for 20 min., in the dark. The final rinse, after Hoechst staining, was with sterile PBS. When processing was complete, the oocytes were either stored in the fridge or mounted on slides. One to six eggs were placed into each drop of mounting medium (50% glycerol, 50% PBS, and 625 mg/ml n-propyl gallate), on glass slides. A coverslip was placed overtop and sealed with clear nail varnish.

The processed eggs were examined with an epifluorescence microscope, within 72 h. Using phase contrast and the 40x objective, oocytes were checked for the presence of a polar body and measured for egg diameter (vitellus), with a calibrated ocular scale. For those eggs having an irregular shape, the mean of the minimum and maximum diameter was used (Mangia and Epstein, 1975). Indirect immunofluorescence of microtubules is shown in Fig. 3a. Hoechst 33258 staining is demonstrated in Fig. 3b. The microtubule structures observed and the location of

Figure 3. Color micrographs showing indirect immunofluorescence of microtubules (a) and Hoechst 33258 staining (b) of a fixed mouse oocyte. (a) The slightly tapered spindle is positioned at maximum view. (b) The chromosomes form a compact row at the equatorial plate of the metaphase spindle. Magnification x 740.



3a

b

the chromosomes were noted. Spindle length (pole-to-pole) and pole width were measured for metaphase spindles aligned at maximum view; when both spindle poles could be focused in the same optical plane (Fig. 3a) and the chromosomes formed a compact row at the metaphase plate (Fig. 3b). The shape of these measured spindles was described as: barrel, slightly tapered, or abnormal. Barrel-shaped spindles have wide poles (Schatten et al., 1985), which are approximately equal in width to the spindle center (Eichenlaub-Ritter et al., 1986). Slightly tapered spindles are similar to barrel-shaped spindles, but have pole widths a bit smaller than the spindle center (Fig. 3a). Abnormal spindles vary greatly from the barrel shape (Pickering and Johnson, 1987). Tapered spindles have poles clearly smaller in width than the spindle center. Elongated spindles are quite long and narrow. Black and white photographs were taken of each oocyte, on Kodak T Max 400 film. Ektachrome 400 film (Kodak) was used for the colour photographs.

Statistics

The data from replicate experiments were combined and comparisons between groups were made with the Student t-test or chi-square analysis. Correlations were determined by the Pearson correlation co-efficient. The programme NPSTAT was used.

RESULTS

Tables 1 (mature mice) and 2 (prepuberal mice) present summaries of the quantities and descriptions of oocytes obtained from each superovulated mouse. Each paired row represents a single experiment, which consisted of two mice having the same age and hormone dose, but different recovery times post-hCG. Data from animals exposed to an irregular light cycle, during the injections, were not used. Fragmented eggs were included in the counts of ovulated oocytes, but excluded from final analysis. The omission of abnormal eggs, as well as egg loss during processing, accounts for the lower numbers of ova analyzed compared to the quantity ovulated.

Number of eggs ovulated affected by hormone dose and mouse age

At the lower hormone dose, <5 IU for prepuberal mice and 5 IU for mature mice, most of the animals ovulated 8 - 12 oocytes (Tables 1 and 2). Only two mice, both mature, produced higher quantities (19 and 22 eggs). At the higher hormone doses, 5 IU for prepuberal mice and 10 IU for mature mice, significantly more eggs per mouse were ovulated ($p < 0.01$), compared to the lower hormone doses (Fig. 4). Prepuberal mice stimulated with 5 IU hormone doses produced significantly more eggs per mouse than mature mice injected with 5 or 10 IU hormone doses ($p < 0.05$; Fig. 4).

Description of metaphase oocytes

With indirect immunofluorescence of microtubules, the spindle was clearly seen in the periphery of the eggs (Fig. 3a). The background fluorescence within

Table 1. Superovulated oocytes from mice 9 - 10 weeks old

PMSG hCG dose	Time post hCG	Number of Oocytes*							
		Ovul	Anal	PB	Diff Ast	Sp Fm	Ana	Met	Met + Ast
10 IU	13h	36	24	2	10	1	0	13	0
	16h	18	17	3	6	0	1	10	1
	15h	32	12	0	0	0	1	11	1
	17h	30	20	3	3	3	2	12	0
	13h	18	10	1	1	1	0	8	2
	18h	36	22	3	1	0	1	20	11
	13h	35	21	5	2	0	0	19	9
	18h	24	9	0	2	0	2	5	2
	13h	41	21	4	6	1	0	14	0
	18h♦	-	-	-	-	-	-	-	-
5 IU	13h	10	5	3	0	0	0	5	1
	16h	22	17	1	0	0	8	9	1
	13h	19	17	4	0	0	6	11	4
	16h	12	9	2	0	0	2	7	1
	13h	12	7	2	0	0	0	7	0
	16h	11	9	3	0	0	1	8	0

* Oocyte descriptions: Ovul (ovulated), Anal (analyzed), PB (polar body), Diff Ast (diffuse aster), Sp Fm (spindle forming), Ana (anaphase), Met (metaphase), Met + Ast (metaphase with cytoplasmic microtubule asters)

♦ some leakage of PMSG injection

Each row represents one experiment

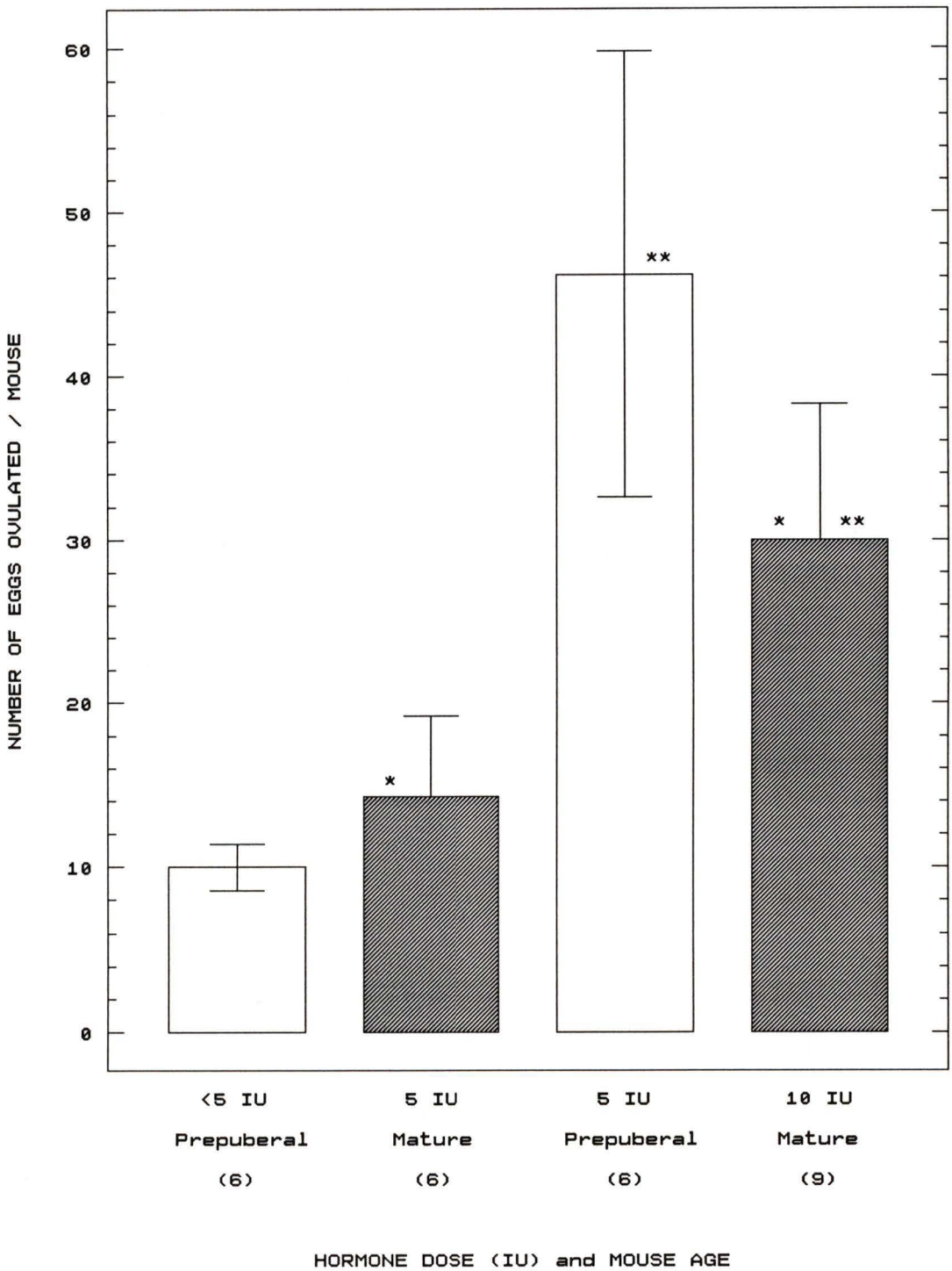
Table 2. Superovulated oocytes from mice 3 weeks old

PMSG hCG dose	Time post hCG	Number of Oocytes*							
		Ovul	Anal	PB	Diff Ast	Sp Fm	Ana	Met	Met + Ast
5 IU	13h	67	50	15	0	0	0	50	0
	15h	54	28	3	0	0	0	28	0
	13h	39	31	3	0	0	0	31	0
	15h	38	30	8	0	0	0	30	0
	13h	29	16	2	0	1	0	15	0
	15h	50	30	9	0	0	1	29	0
<5 IU	13h	10	8	3	0	0	0	8	0
	15h	11	7	4	0	0	1	6	0
	13h	9	7	2	0	0	0	7	0
	15h	8	6	2	0	0	0	6	0
	13h	10	7	1	0	0	0	7	0
	15h	12	9	3	0	0	0	9	0

* Oocyte descriptions: Ovul (ovulated), Anal (analyzed), PB (polar body), Diff Ast (diffuse aster), Sp Fm (spindle forming), Ana (anaphase), Met (metaphase), Met + Ast (metaphase with cytoplasmic microtubule asters)

Each row represents one experiment

Figure 4. The effect of hormone dose and mouse age on the number of eggs ovulated per mouse. Results are expressed as means ± S.D. Increasing hormone dose, from <5 to 5 IU for prepuberal mice and from 5 to 10 IU for mature mice significantly increased the eggs ovulated (* $p<0.01$). At the higher hormone doses, prepuberal mice produced significantly more oocytes than the mature mice (** $p<0.05$).


NUMBER OF EGGS OVULATED / MOUSE
60
50
40
30
20
10
0
*
**
*
**
<5 IU
Prepuberal
(6)
5 IU
Mature
(6)
5 IU
Prepuberal
(6)
10 IU
Mature
(9)
HORMONE DOSE (IU) and MOUSE AGE

oocytes varied between ova, but never interfered with analysis. In many oocytes discrete foci of fluorescence (1-12) could be discerned, which were distributed throughout the egg (Fig. 3a).

Different angles of the spindle were observed, in different oocytes, due to random placement of the ova on the slides. When the spindle was positioned at maximum view, both spindle poles could be focused in the same optical plane (Fig. 5a) and the chromosomes formed a compact row at the metaphase plate (Fig. 5b). When the spindle was observed at minimum view, through the poles, only one pole could be focused at a time (Fig. 5d) and the chromosomes were arranged in a circular cluster (Fig. 5e). Orientations between these two extremes were also seen. In these instances, the spindle was visible and chromosomes appeared in rows or oblong clusters. All these different arrangements, of chromosomes aligned on the equatorial plate of the spindle, were designated as metaphase.

Phase-contrast observations showed the vitellus of most metaphase oocytes was closely surrounded by the zona pellucida (ZP; Fig. 5c). A polar body (PB; Fig. 5f), whole or remnant, was not always present (Fig. 5c). Sometimes the chromosomes of the polar body were also stained with Hoechst 33258 (Fig. 5e).

Data from fourteen separate experiments, on oocytes collected 13 - 16 h post-hCG after low and high hormone doses, were pooled according to mouse age (mature or prepuberal). There was no significant difference, between the two age groups, in the percentage of eggs with a visible polar body (Fig. 6). However, the recovery of metaphase oocytes was significantly affected by mouse age ($p<0.01$).

Figure 5. Micrographs of mouse metaphase oocytes. (a) Oocyte with barrel-shaped spindle at maximum view. (b) Chromosomes are in a compact row, midway between the two spindle poles. (c) The zona pellucida (ZP) closely surrounds the egg membrane. (d) Oocyte with spindle at minimum view; only one spindle pole is visible. (e) The second metaphase chromosomes are arranged in a circular cluster (MII), between the spindle poles. (f) A polar body (PB), and the chromosomes contained within (e), are also present. (a,d) Indirect immunofluorescence of microtubules. (b,e) Hoechst 33258 staining. (c,f) Phase contrast. Bar represents 10 μm.

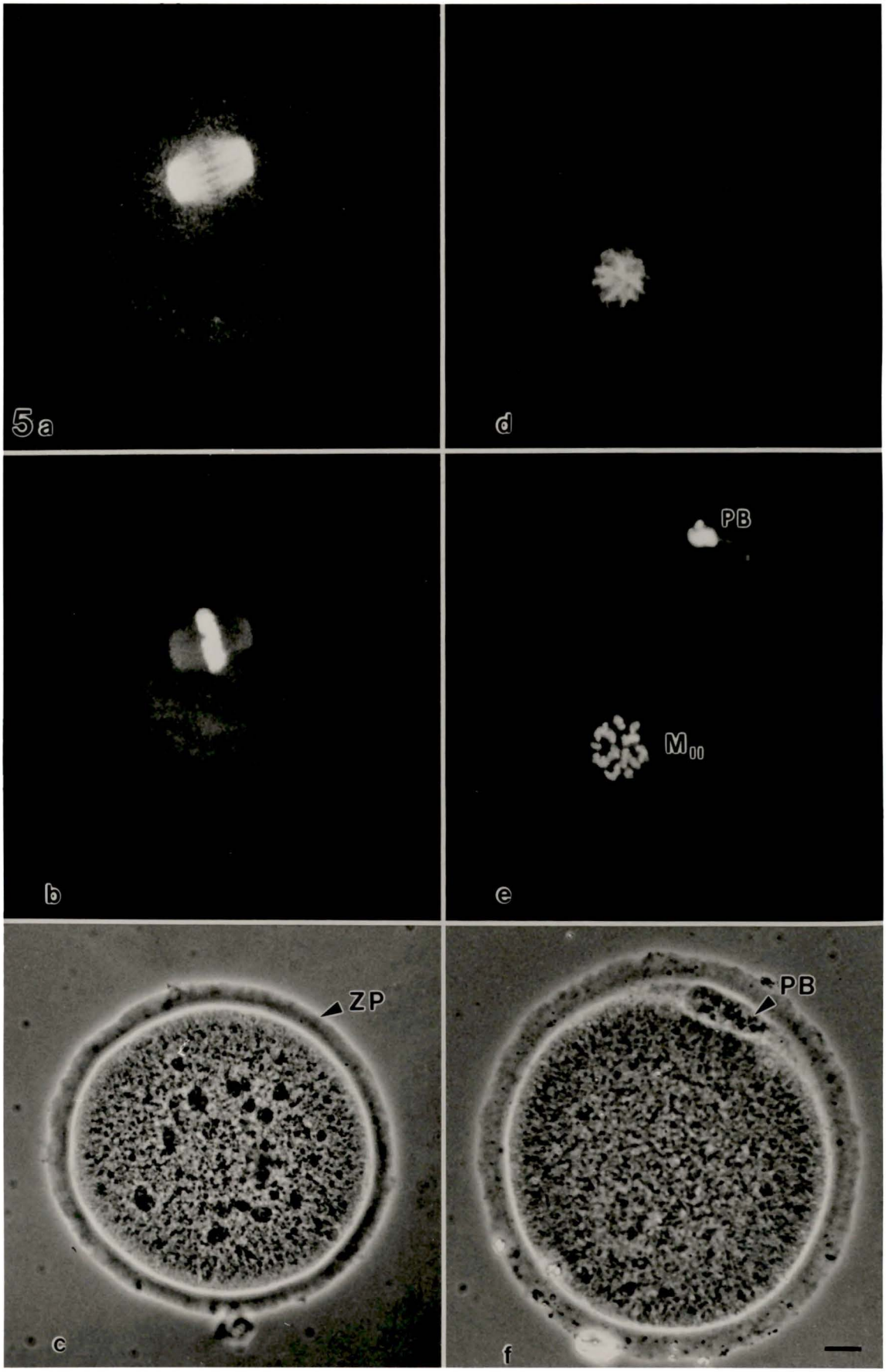
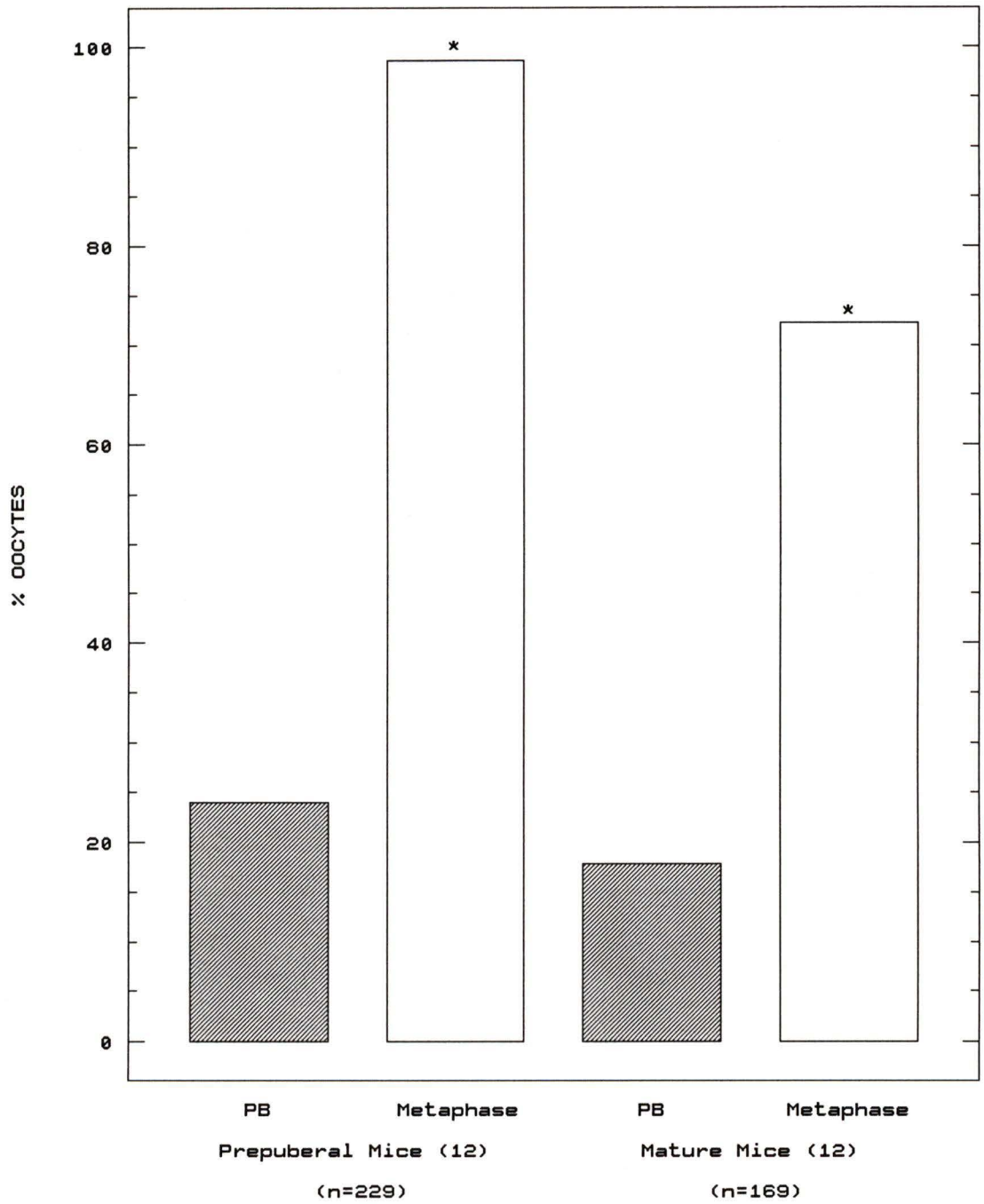

5a
d
b
PB
M_{II}
e
ZP
c
PB
f

Figure 6. Comparison of recovered oocytes from superovulated prepuberal and mature mice for possession of a polar body and meiotic stage. The percentages of oocytes (total number indicated by n) were obtained from the pooled data of fourteen experiments, which used low and high hormone doses, and recoveries of 13 - 16 h post-hCG. There was no significant difference, between the two age groups, in the total percentage of eggs with a visible polar body (PB). However, significantly more oocytes recovered from prepuberal mice contained a metaphase spindle, 98.7% in total, compared with the 72.2% from mature mice (* $p<0.01$).



LOW and HIGH HORMONE DOSES, 13 - 16 h POST-HCG

Almost all, 98.7%, of the superovulated eggs obtained from prepuberal mice contained a metaphase spindle (Fig. 6). But, only 72.2% of the oocytes recovered from stimulated mature mice were in metaphase.

Description of non-metaphase oocytes

Only superovulated mature mice produced a significant percentage of non-metaphase eggs. Oocytes which did not contain a metaphase spindle were found to exhibit one of three different microtubule configurations. About half of the non-metaphase eggs from mature mice (25/47) displayed an elaborate, diffuse aster, which filled the entire oocyte (Fig. 7a,d). The shape of the aster varied between ova; but all originated from an highly fluorescent, irregular shaped core in the center of the oocyte, with fine lines extending in all directions to the periphery. Some of these eggs also contained small cytoplasmic asters (arrow in Fig. 7d). The chromosomes were usually spread, but concentrated around the egg center (Fig. 7b). Also seen, though less frequent, was a clumping of the chromosomes (Fig. 7e). These diffuse aster oocytes always demonstrated a large perivitelline space (between the egg and the ZP), but a polar body was never detected (Fig. 7c,f).

A few oocytes from mature mice (3/47) contained microtubules, which appeared to be in the process of forming a spindle (Fig. 8a,c,e). In the center of these eggs a spindle-like structure was seen, with a large aster at each pole. Many highly fluorescent, peripheral, small asters were also observed throughout the egg; some were interconnected (Fig. 8a,c,e). Most of the chromosomes in these eggs were clumped in the center of the oocyte, sometimes with isolated chromosomes located

Figure 7. Micrographs of oocytes displaying a diffuse aster. (a,d) Some of the oocytes recovered from mature mice stimulated with 10 IU hormone doses demonstrated a large, central, diffuse microtubule aster; sometimes associated with small, peripheral asters (arrow in d). (a,b,c and d,e,f) Two are shown here. The chromosomes were usually scattered about the egg center (b), but could also appear clumped (e). All these oocytes possessed a large perivitelline space (arrow in c). CC, cumulus cell. (a,d) Indirect immunofluorescence of microtubules. (b,c) Hoechst 33258 staining. (c,f) Phase contrast. Bar represents 10 μm.

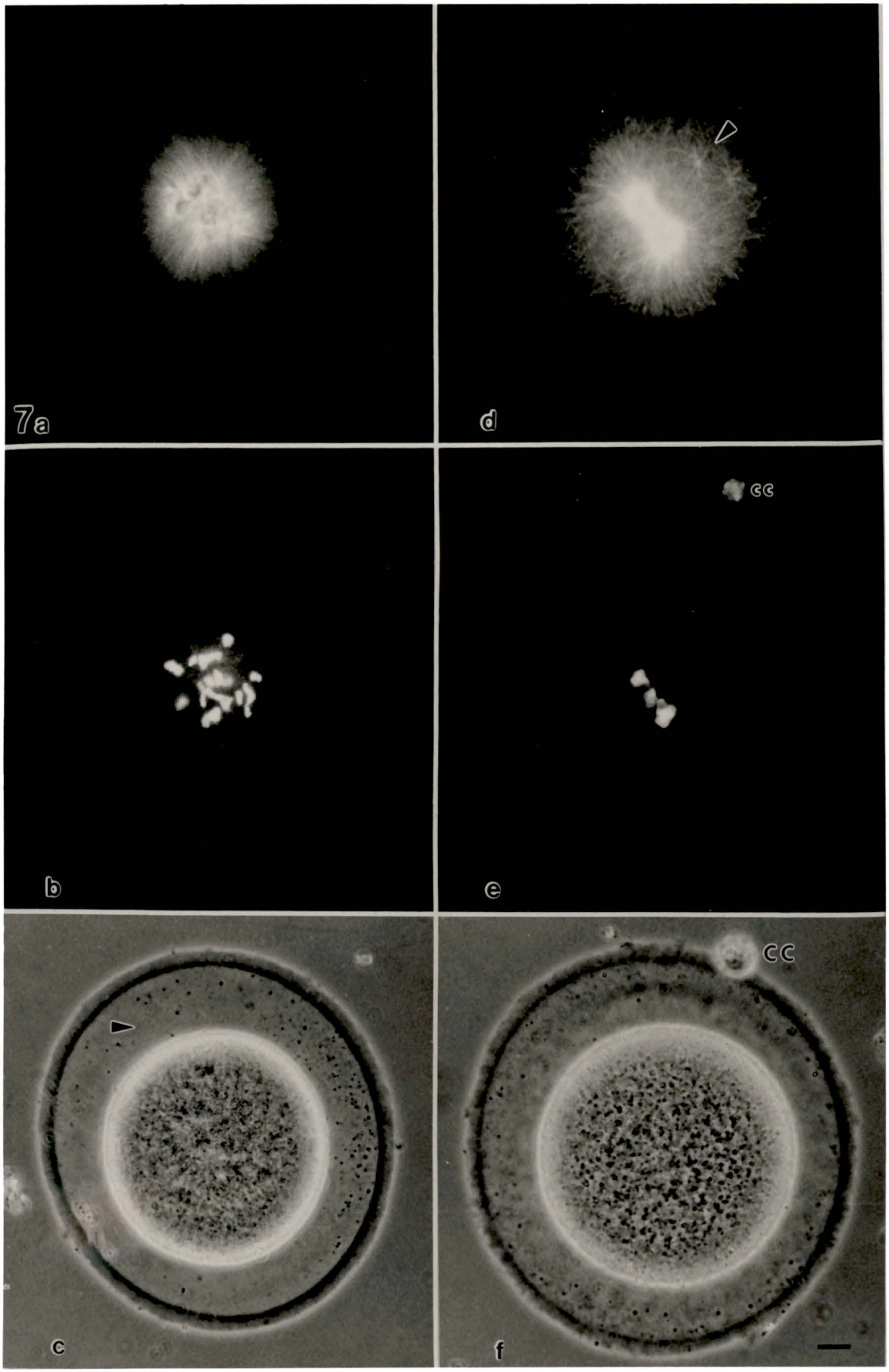
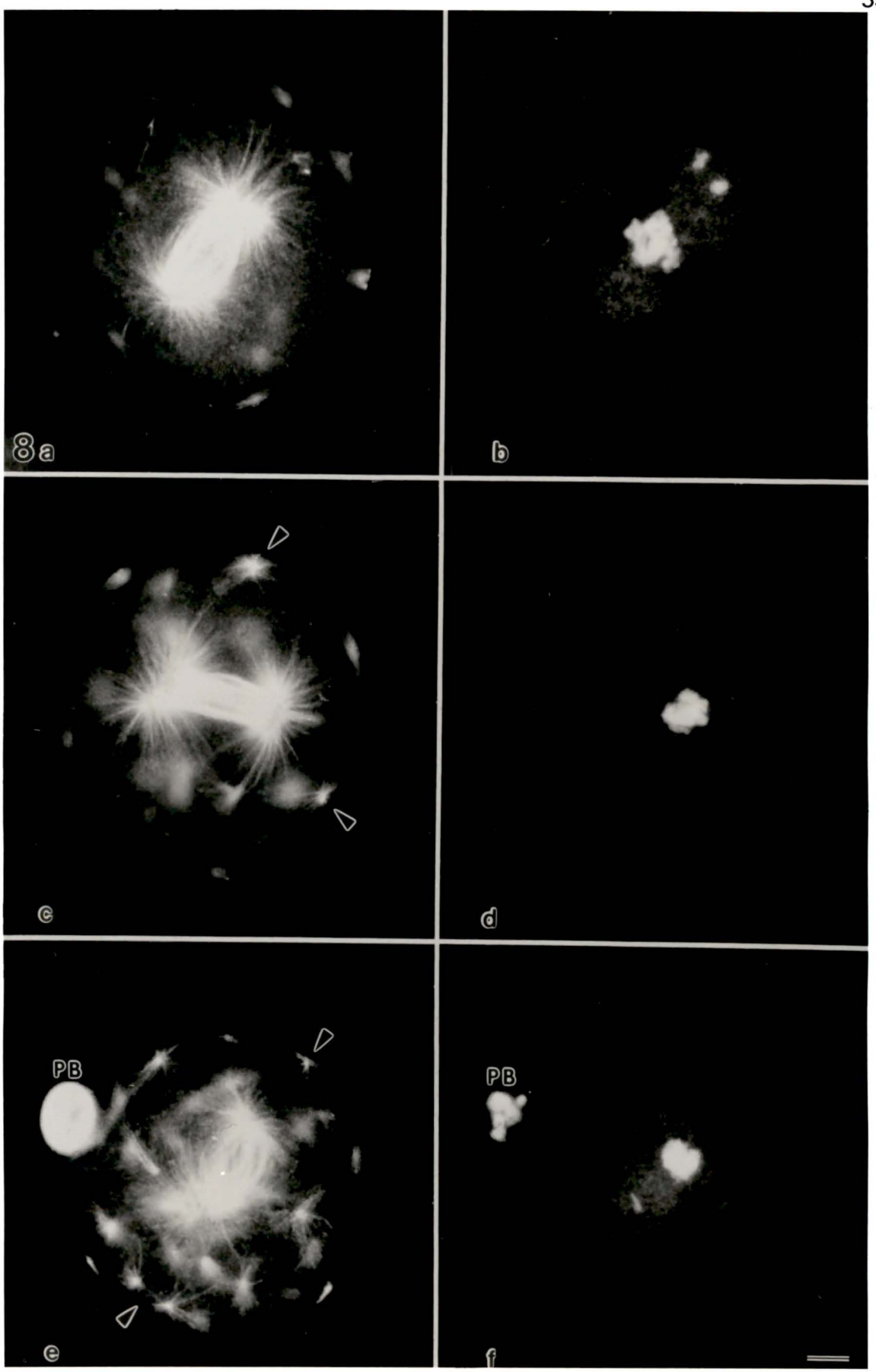

7a
d
b
e
cc
c
cc
f

Figure 8. Micrographs of oocytes displaying a spindle-forming. (a,c,e) Some of the oocytes recovered from mature mice stimulated with 10 IU hormone doses demonstrated a spindle-like structure in the egg center. (a,b; c,d; and e,f) Three are shown here. (a,c,e) Microtubules were also arranged in large polar asters and many small, but prominant, peripheral asters (arrows in c,e). (b,d,f) The chromosomes were in a central clump, sometimes with isolated chromosomes (b,f) nearby. (e) The presence of a polar body (PB) was rare. (a,c,e) Indirect immunofluorescence of microtubules. (b,d,f) Hoechst 33258 staining. Bar represents 10 μm.


8a
b
c
d
PB
e
PB
f

near one of the polar asters (Fig. 8b,d,f). The oocyte in Fig. 8e,f is not from an experiment listed in Table 1, due to irregular light exposure. This oocyte was the only egg with a forming spindle and a polar body, therefore, it was included. Similar to the diffuse aster ova, all these oocytes possessed a large perivitelline space. The mean diameter of oocytes with a diffuse aster or a spindle-forming was 79.8 ± 3.8 μm S.D. (n=28), which was significantly smaller ($p<0.01$) than the mean diameter of metaphase oocytes obtained from the same mature mice, 89.9 ± 5.8 μm (n=75) (Table 3).

The remaining non-metaphase oocytes from mature mice (19/47) contained spindles with chromosomes at each pole (Fig. 9a,b,d,e). They were designated anaphase. None of these oocytes had an obvious perivitelline space. Only 4/19 anaphase oocytes had a polar body. The diameter of these eggs ranged between 75 μm (Fig. 9c) and 92 μm (Fig. 9f). The mean diameter of the anaphase oocytes was 84.4 ± 7.0 μm (n=19) (Table 3).

Conditions of superovulation affect type of non-metaphase oocytes recovered

As previously mentioned, significantly more non-metaphase oocytes were recovered from mature mice than from prepuberal mice (Fig. 10). Within these two age groups, the hormone dose or recovery time post-hCG (including collection at 17-18 h post-hCG) did not significantly change the total percentage of non-metaphase eggs observed (Fig. 10).

The type of non-metaphase oocytes obtained from mature mice was

Table 3. Diameters of metaphase and non-metaphase eggs obtained from mature mice 13 - 16 h post-HCG (5 - 10 IU).

Metaphase (μm)	Diffuse Aster or Spindle Forming (μm)	Anaphase (μm)
83	77	83
84	77	95
104	81	85
94	82	84
95	79	92
94	84	75
98	86	89
89	78	92
87	77	77
86	77	77
91	78	83
93	90	83
90	74	85
82	77	76
89	79	86
96	74	82
79	79	99
87	77	88
92	83	73
99	83	
84	85	
95	83	
83	77	
89	77	
88	81	
87	77	
85	84	
91	78	
86		
89		
88		
92		
90		
88		
89		
79		
91		
98		
85		
79		
76		
87		
95		
86		
96		
95		
97		
104		
92		
90		
95		
87		
90		
94		
94		
94		
95		
97		
90		
92		
90		
85		
MEANS ± S.D. :		
89.9 ± 5.8 *	79.8 ± 3.8 *	84.4 ± 7.0

* $p < 0.01$

Figure 9. Micrographs of oocytes displaying an anaphase spindle. (a,b and d,e) Some of the eggs recovered from mature mice contained a spindle (Sp) with chromosomes located at the poles. (a,b,c and d,e,f) Two are shown here. Some of these oocytes had a polar body (PB). (a,d) Indirect immunofluorescence of microtubules. (b,e) Hoechst 33258 staining. (c,f) Phase contrast. Bar represents 10 μm.

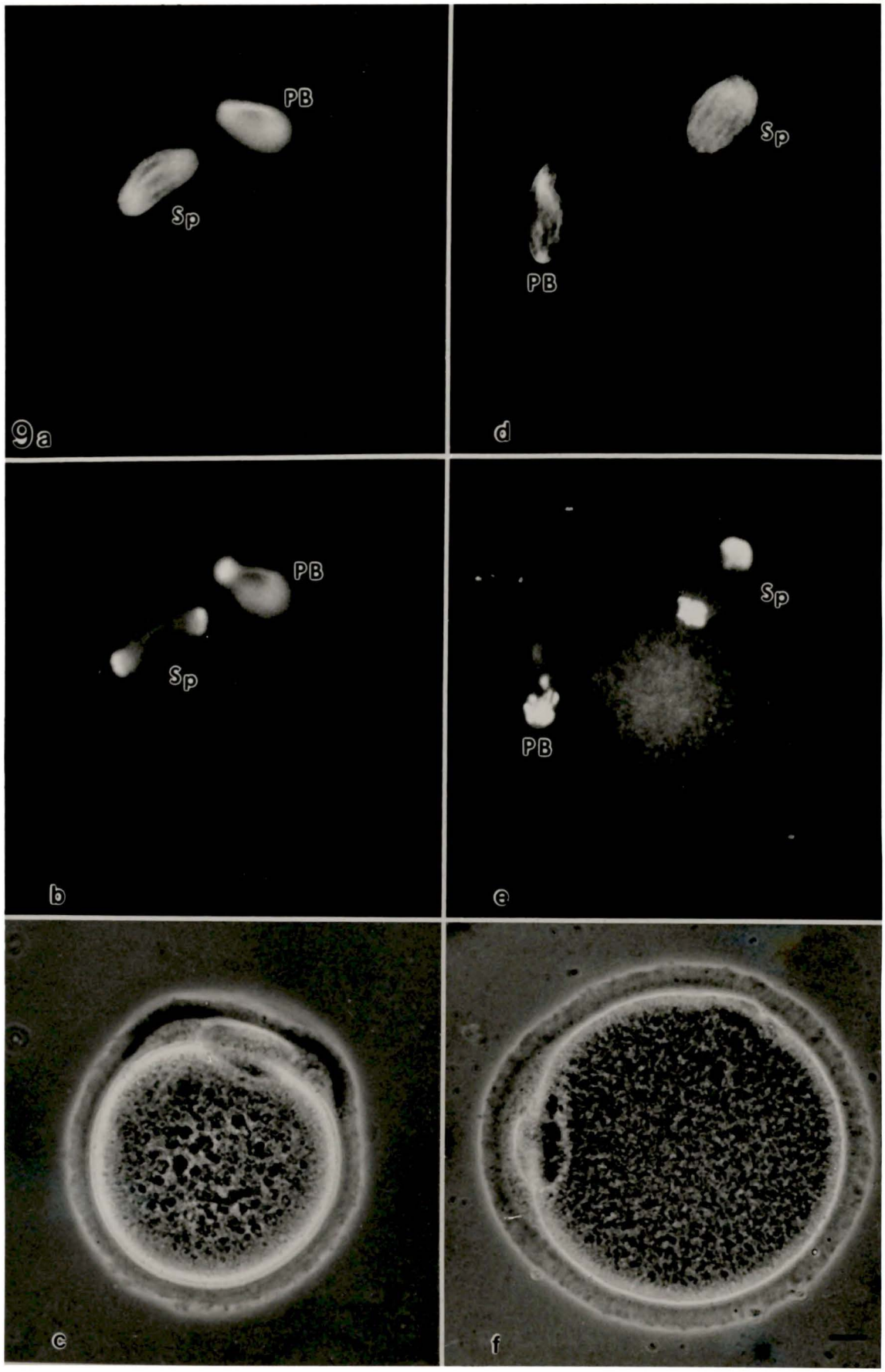
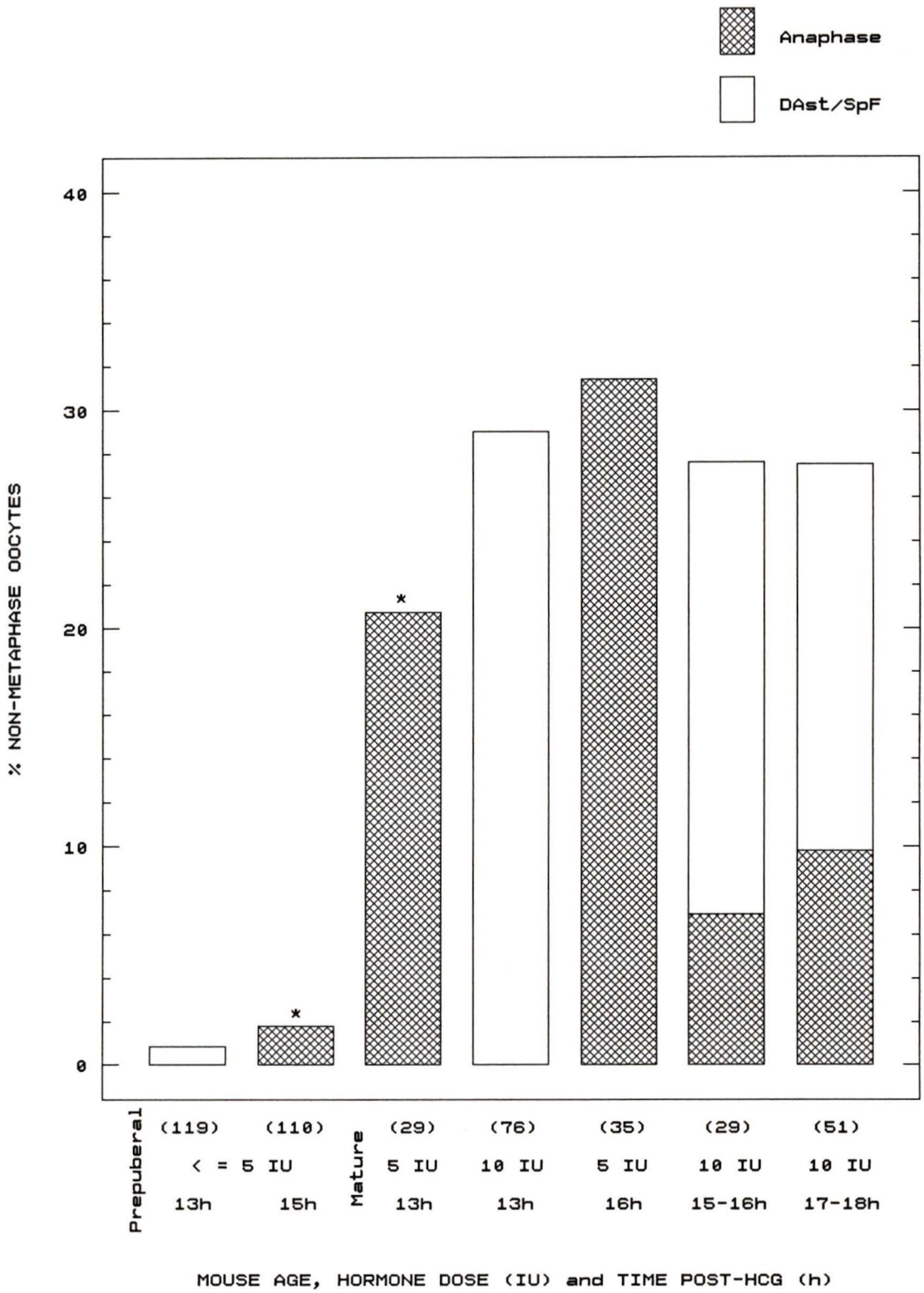

PB
Sp
9a
Sp
PB
d
PB
Sp
b
Sp
PB
e
c
f

Figure 10. The effect of mouse age, hormone dose, and recovery time post-hCG on the type of non-metaphase oocytes observed. Results are expressed as a total percentage of pooled data from 2 - 6 replicate experiments for each condition. A significantly greater percentage of non-metaphase oocytes — diffuse aster (DAst), spindle-forming (SpF), and anaphase — was detected from mature mice than from prepuberal mice (* $p<0.01$). Hormone dose and recovery time post-hCG did not significantly affect the percentage of non-metaphase eggs obtained, but did determine the type of non-metaphase egg observed. Diffuse aster and spindle-forming oocytes were only recovered after the high hormone dose (10 IU). Significantly more anaphase oocytes were recovered after the low hormone dose (5 IU) or with recoveries of 15 - 18 h post-hCG ($p<0.01$). Results from prepuberal mice were not significant, but followed a similar pattern.

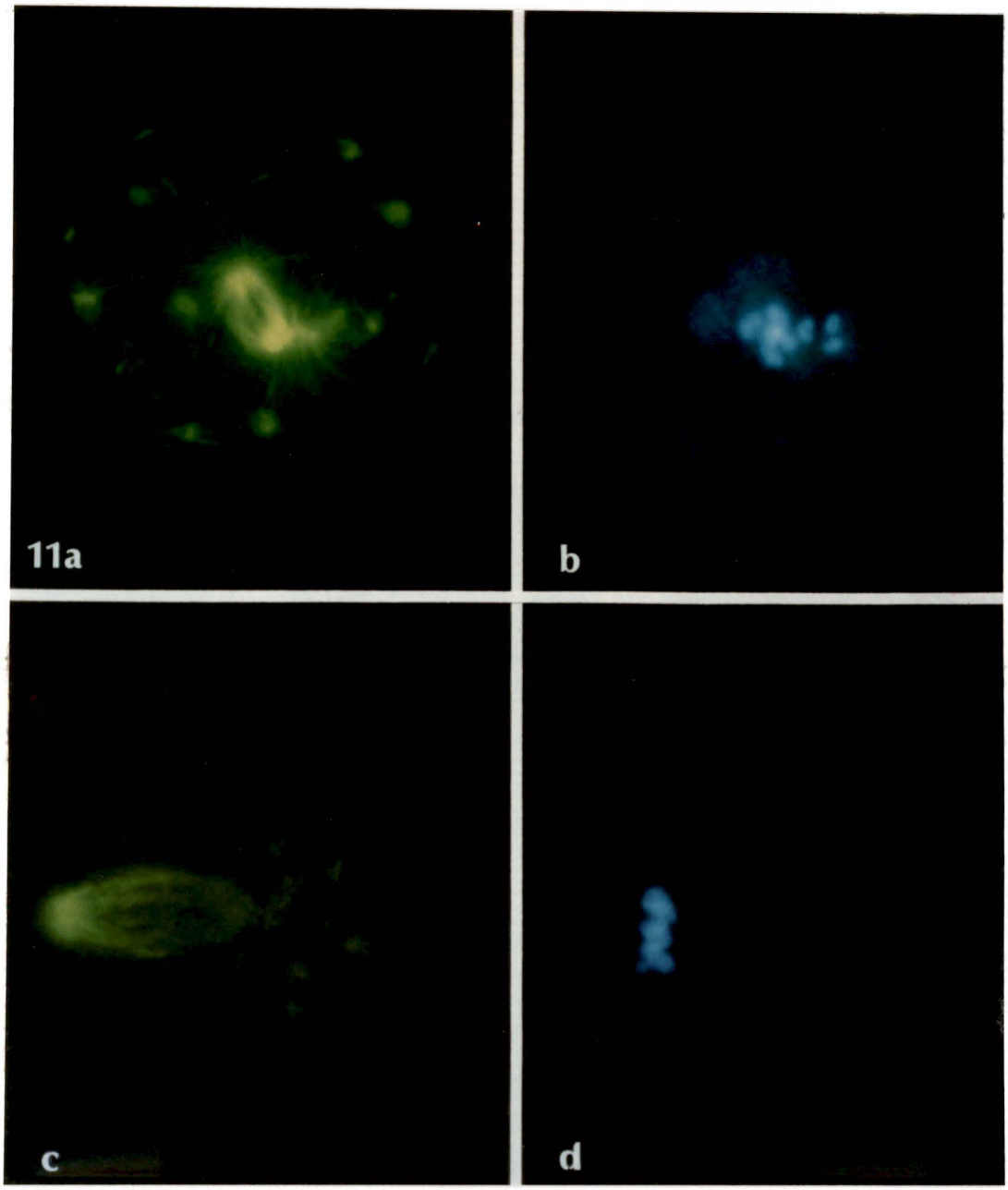

Anaphase
DAst/SpF
% NON-METAPHASE OOCYTES
40
30
20
10
0
*
*
Prepuberal
Mature
(119)
< = 5 IU
13h
(110)
15h
(29)
5 IU
13h
(76)
10 IU
13h
(35)
5 IU
16h
(29)
10 IU
15-16h
(51)
10 IU
17-18h
MOUSE AGE, HORMONE DOSE (IU) and TIME POST-HCG (h)

significantly affected by the conditions of superovulation. Ova demonstrating a diffuse aster (DAst) or spindle-forming (SpF) were only recovered from mice given 10 IU hormone doses (Fig. 10); 8/9 mice ovulated 1 - 11 such eggs (Table 1). Anaphase oocytes were obtained after both hormone treatments, but significantly more were recovered after stimulation with 5 IU ($p<0.01$). Following 5 IU hormone doses, anaphase oocytes were the only type of non-metaphase eggs detected. Mice stimulated with 10 IU of hormones demonstrated anaphase oocytes only with recoveries of 15 - 18 h post-hCG (Fig. 10). During this period at least one anaphase oocyte was observed from each mouse (Table 1). When data from both hormone doses were grouped, more anaphase oocytes were recovered 15 - 16 h post-hCG, compared with 13 h post-hCG ($p<0.01$). The presence of all three types of non-metaphase oocytes was observed only with the combination of 10 IU hormone stimulation and recovery at 15-18 h post-hCG (Fig.10).

Heterogeneity of metaphase oocytes

Some metaphase oocytes obtained from mature mice contained cytoplasmic, microtubule asters (Fig. 11c). The number and size of the asters varied, but were usually fewer in number and less intense in fluorescence compared to cytoplasmic asters associated with spindle-forming eggs (Fig. 11a). The faint cytoplasmic asters were more easily seen with the microscope, than is demonstrated in the micrographs. Cytoplasmic asters were observed in eggs with barrel-shaped spindles, as well as abnormal shaped spindles, such as the tapered metaphase spindle seen in Fig. 11c. Not all oocyte populations from individual mice contained metaphase oocytes with

Figure 11. Colour micrographs of mouse oocytes displaying cytoplasmic microtubule asters. (a,b and c,d) Two are shown. (a) A spindle-forming oocyte, with many, prominant, peripheral asters. (c) A metaphase oocyte demonstrating an elongated spindle, with chromosomes at the equatorial plate (d), and many, faint, central asters. (a,c) Indirect immunofluorescence of microtubules. (b,d) Hoechst 33258 staining. Magnification x 650.


11a
b
c
d

cytoplasmic asters (Table 1). Analysis of separate populations, containing 60% and greater of the eggs ovulated, found no relationship between the number of metaphase oocytes with cytoplasmic asters and the number of eggs ovulated (Fig. 12); but did find a positive correlation ($p < 0.05$) with the number of metaphase oocytes present (Fig. 13). Most of the metaphase oocytes with cytoplasmic asters (20/27) were found among the two populations containing the highest number of metaphase oocytes (19-20).

The oocytes obtained from prepuberal mice appeared homogeneous; 226/229 eggs were in metaphase, and none of these contained cytoplasmic asters (Table 2). The three non-metaphase oocytes found, followed patterns seen with oocytes from mature mice. The one spindle-forming egg was recovered after the high hormone dose (5 IU), and the two anaphase eggs were obtained at 15 h post-hCG. There was, however, considerable variation in spindle length and shape.

All individual oocyte populations, from prepuberal mice given 5 IU hormone doses, had a range in spindle length of 24.3 - 31.5 μm (Tables 4 and 5). The recovery time did not correlate with the mean spindle length. Oocytes obtained at 13 h post-hCG had a mean spindle length of 26.9 ± 3.1 μm (Table 4), which was not significantly different from the 27.6 ± 3.4 μm length measured from eggs recovered at 15 h post-hCG (Table 5).

Spindle shape was assessed, during measurement of spindle length, as: barrel-shaped (Fig. 5a), slightly tapered (Fig. 3a) or abnormal. Abnormal shaped spindles were either tapered, at one pole (Fig. 14a) or both poles (Fig. 14c), or

Figure 12. No correlation (Pearson correlation co-efficient) was found between the number of eggs ovulated per mature mouse and the recovery of metaphase oocytes with cytoplasmic asters. Each point on the graph represents an oocyte population from one mouse. Only mice which had analysis of 60% or greater of the ovulated population were included.

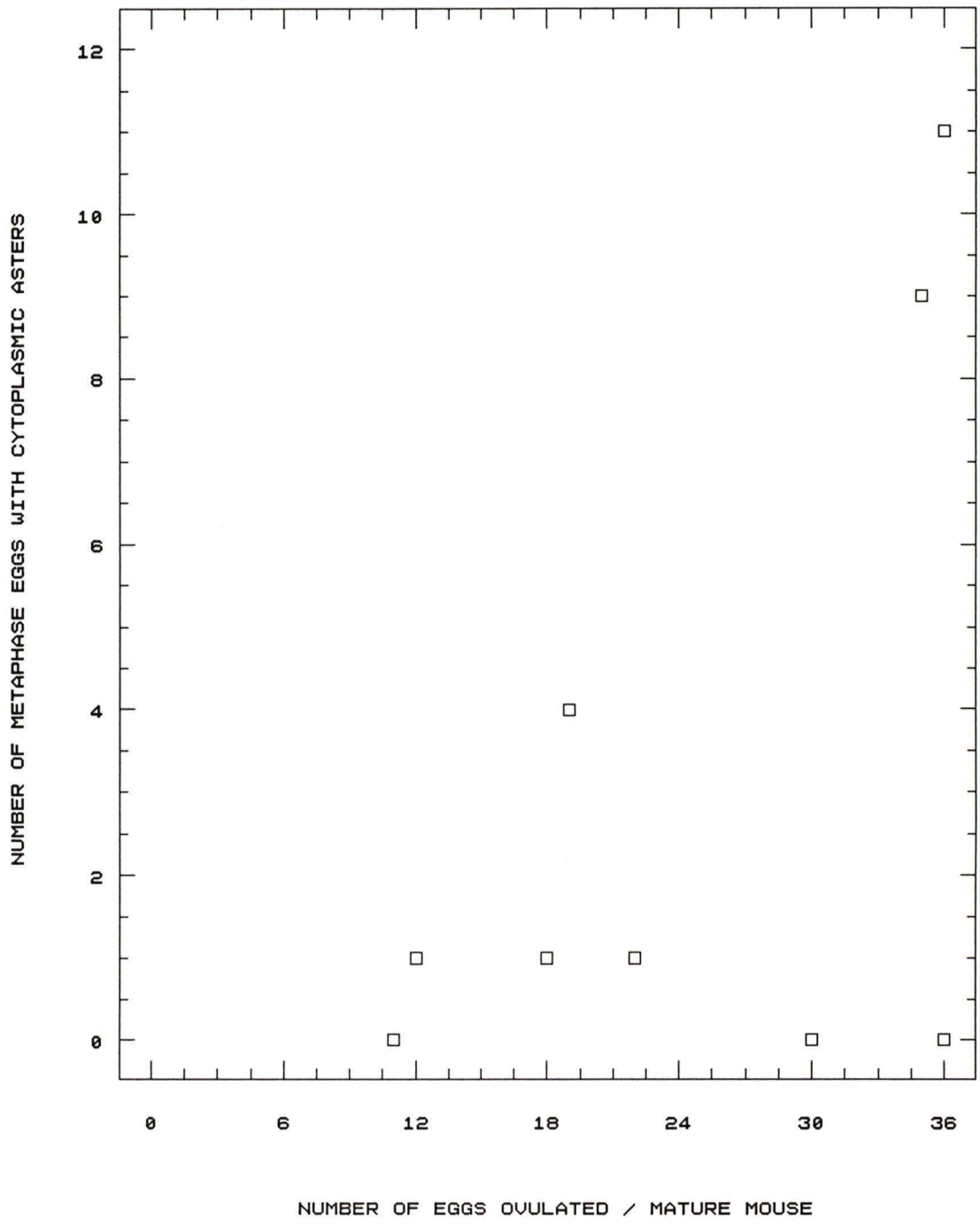

NUMBER OF METAPHASE EGGS WITH CYTOPLASMIC ASTERS
12
10
8
6
4
2
0
0
6
12
18
24
30
36
NUMBER OF EGGS OVULATED / MATURE MOUSE

Figure 13. The number of metaphase eggs present in an oocyte population, from a single mouse, was positively correlated ($p < 0.05$; Pearson correlation co-efficient) with the number of metaphase oocytes containing cytoplasmic asters. Each point on the graph represents an oocyte population from one mouse. Only mice which had analysis of 60% or greater of the ovulated population were included.

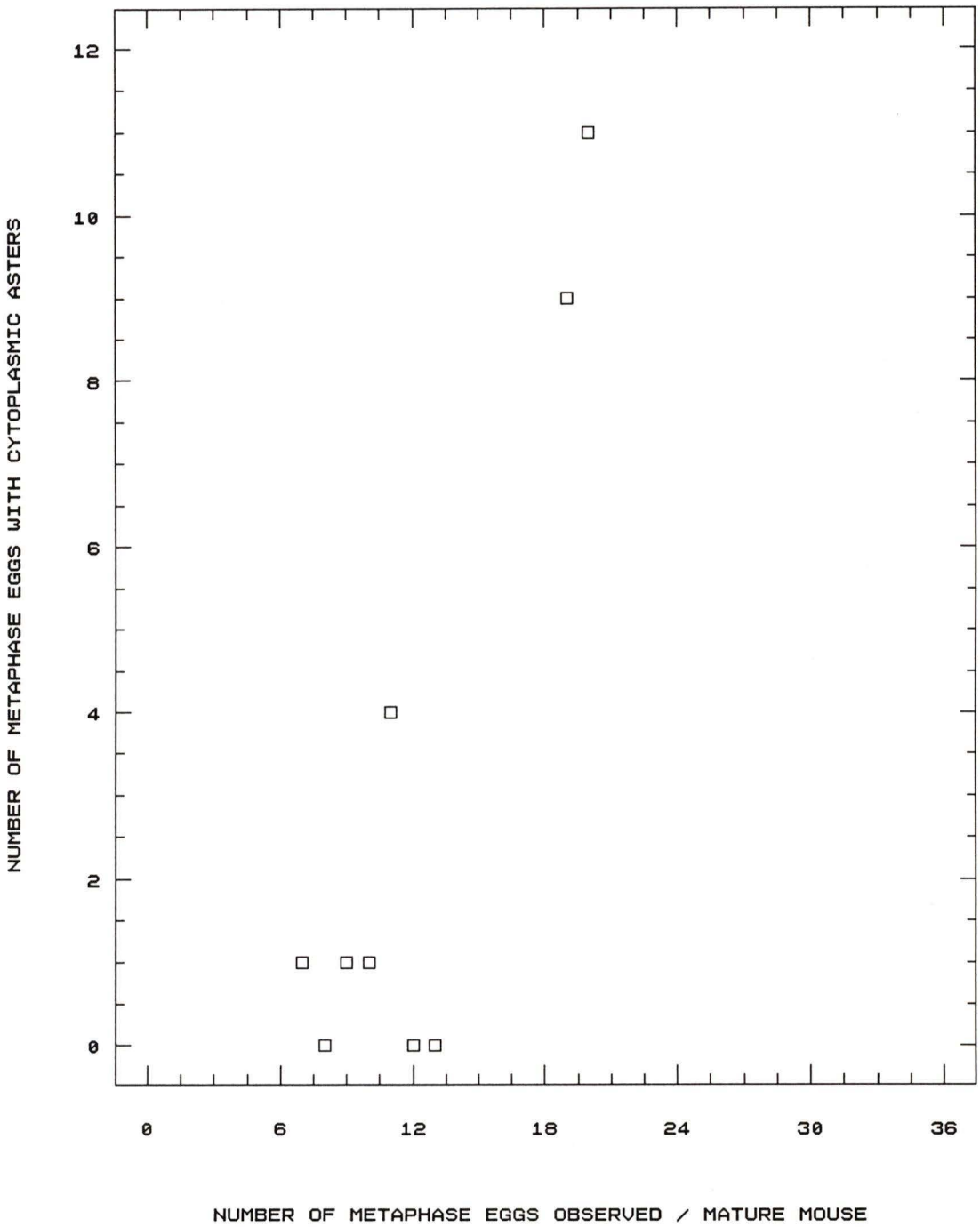

NUMBER OF METAPHASE EGGS WITH CYTOPLASMIC ASTERS
0
2
4
6
8
10
12
0
6
12
18
24
30
36
NUMBER OF METAPHASE EGGS OBSERVED / MATURE MOUSE

Table 4. Length, pole widths, and spindle shape of metaphase spindles obtained from prepuberal mice at 13 h post-HCG (5 IU).

Oocytes analyzed/ov per mouse	Spindle Shape*	SL** (μm)	PW1 (μm)	PW2 (μm)	PW1/SL + PW2/SL
18/67	sT	30.6	9.9	9.9	0.64
	sT	27.9	7.2	9.0	0.58
	B	28.8	12.6	12.6	0.88
	sT	28.8	9.0	8.1	0.59
	B	25.2	10.8	9.9	0.82
	B	24.3	9.0	9.0	0.74
	Ab	27.9	6.3	7.2	0.49
	sT	27.0	9.0	9.0	0.66
	Ab	30.6	8.1	7.2	0.51
	B	23.4	10.8	10.8	0.92
	B	25.2	10.8	12.6	0.93
	sT	31.5	9.9	11.7	0.68
	Ab	28.8	9.9	10.8	0.72
	Ab	27.9	9.0	9.0	0.64
	B	22.5	9.0	8.1	0.76
	Ab	28.8	9.0	9.0	0.62
	B	26.1	10.8	10.8	0.82
	sT	24.3	9.0	8.1	0.70
14/39	Ab	31.5	9.0	9.0	0.58
	Ab	32.4	9.9	9.0	0.59
	sT	27.0	9.9	9.9	0.74
	Ab	29.7	8.1	8.1	0.54
	B	27.0	10.8	10.8	0.80
	sT	27.0	9.9	10.8	0.77
	B	23.4	9.9	9.9	0.84
	B	23.4	9.9	10.8	0.88
	Ab	27.0	9.9	10.8	0.77
	Ab	21.6	9.0	9.0	0.84
	sT	25.2	9.0	9.9	0.75
	Ab	27.9	9.0	9.0	0.64
	Ab	21.6	8.1	8.1	0.76
	sT	21.6	9.0	8.1	0.80
4/29	sT	32.4	14.4	12.6	0.84
	sT	27.0	10.8	12.6	0.87
	sT	24.3	9.0	9.0	0.74
	Ab	28.8	8.1	8.1	0.56
mean spindle length = 26.9 $\pm$ 3.1 μm (S.D.)					

* Subjective descriptions: B (barrel-shaped), sT (slightly tapered), Ab (abnormal; tapered or elongated)
** Measurements: SL (pole-to-pole spindle length), PW1 (one pole width), PW2 (other pole width)

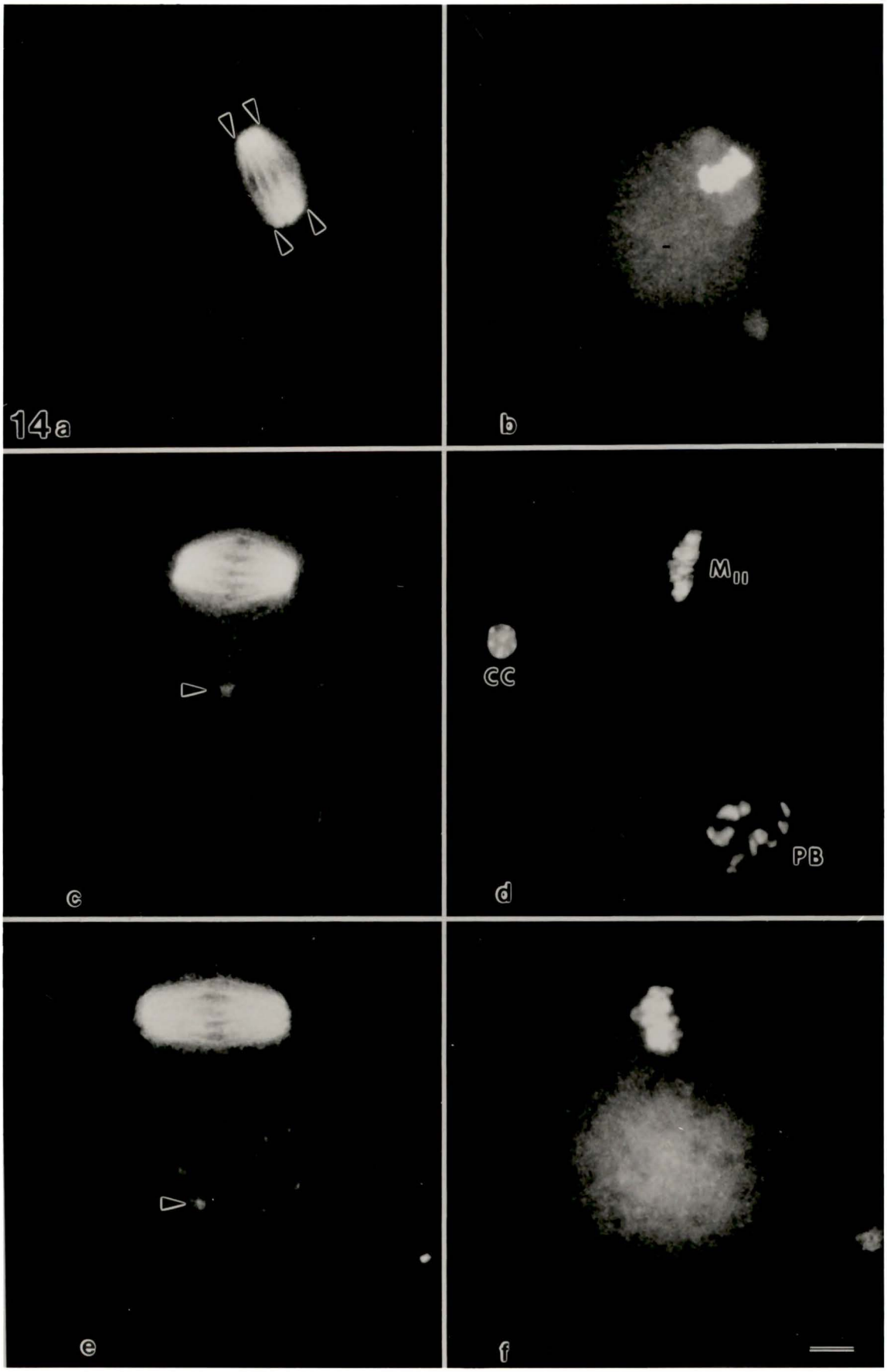
Table 5. Length, pole widths, and spindle shape of metaphase spindles obtained from prepuberal mice at 15 h post-HCG (5 IU).

Oocytes analyzed/ov per mouse	Spindle Shape*	SL** (μm)	PW1 (μm)	PW2 (μm)	PW1/SL + PW2/SL
14/54	Ab	30.6	9.0	9.0	0.58
	sT	23.4	10.8	9.0	0.85
	Ab	23.4	9.0	6.3	0.66
	Ab	30.6	7.2	7.2	0.48
	Ab	34.2	11.7	10.8	0.66
	Ab	33.3	10.8	9.9	0.62
	B	27.9	12.6	11.7	0.87
	sT	36.0	14.4	12.6	0.75
	Ab	33.3	9.0	9.0	0.54
	Ab	32.4	9.0	9.9	0.59
	Ab	28.8	9.0	8.1	0.59
	B	25.2	9.9	10.8	0.82
	B	28.8	11.7	11.7	0.82
	Ab	27.0	9.0	9.0	0.66
20/38	B	26.1	11.7	11.7	0.90
	Ab	28.8	10.8	10.8	0.76
	B	27.9	11.7	11.7	0.84
	sT	25.2	11.7	9.0	0.82
	sT	26.1	9.0	9.0	0.70
	sT	27.9	9.9	12.6	0.81
	B	27.0	10.8	10.8	0.80
	B	23.4	10.8	9.9	0.88
	sT	23.4	9.0	9.0	0.78
	sT	23.4	9.0	9.9	0.81
	B	22.5	9.9	9.9	0.88
	B	27.0	10.8	10.8	0.80
	B	23.4	9.9	9.9	0.84
	B	24.3	10.8	10.8	0.88
	sT	26.1	9.0	9.9	0.73
	sT	25.2	9.0	9.9	0.75
	sT	27.0	9.9	10.8	0.77
	Ab	31.5	11.7	10.8	0.71
	sT	22.5	8.1	9.0	0.76
	B	25.2	11.7	10.8	0.89
11/50	Ab	30.6	8.1	8.1	0.54
	sT	29.7	12.6	10.8	0.72
	sT	28.8	10.8	9.9	0.72
	Ab	23.4	9.0	8.1	0.74
	B	27.9	10.8	10.8	0.78
	Ab	28.8	14.4	10.8	0.88
	sT	27.0	11.7	11.7	0.86
	sT	28.8	12.6	10.8	0.82
	Ab	28.8	10.8	10.8	0.76
	sT	27.0	10.8	9.0	0.73
	Ab	32.4	9.0	9.9	0.59
mean spindle length = 27.6 $\pm$ 3.4 μm (S.D.)					

* Subjective descriptions: B (barrel-shaped), sT (slightly tapered), Ab (abnormal; tapered or elongated)

** Measurements: SL (pole-to-pole spindle length), PW1 (one pole width), PW2 (other pole width)

Figure 14. Micrographs of mouse oocytes with abnormal shaped metaphase spindles. (a,b; c,d; and e,f) Three are shown. (a) Spindle with one tapered pole (at the top) and one normal, wide pole. Each pair of arrows indicate the pole width. (c) Spindle with both poles tapered. (e) Elongated spindle. (c,e) Arrows indicate discrete foci of fluorescence in the egg cytoplasm. (b,d,f) All spindles have chromosomes arranged in a compact row, midway between the poles. (a,c,e) Indirect immunofluorescence of microtubules. (b,d,f) Hoechst 33258 staining. MII, metaphase II chromosomes; CC, cumulus cell; PB, polar body. Bar represents 10 μm.


14a
b
c
d
M_{II}
CC
PB
e
f

elongated (Fig. 14e). A mathematical description of spindle shape was used to evaluate the subjective classifications. The width of the spindle pole (PW), in comparison to the spindle length (SL), is an important determinant of the perceived shape. By calculating (PW/SL), a proportion value can be used to describe this relationship. Because pole widths of the same spindle can be unequal (Fig. 14a), it is necessary to calculate each pole separately. This results in the equation: PW1/SL + PW2/SL. The mean numerical values ± S.D. of spindle shape for barrel-shaped, slightly tapered, and abnormal spindles were: 0.84 ± 0.05 (n=23), 0.75 ± 0.07 (n=29), and 0.64 ± 0.10 (n=29), respectively (Tables 4 and 5; Fig. 15). These means were significantly different from each other ($p<0.01$), therefore, they represent distinct groupings.

The percentage of normally barrel shaped and abnormally shaped (tapered or elongated) spindles were analyzed, for oocyte populations obtained at 13 h or 15 h post-hCG (Fig. 16). Slightly tapered spindles were not included, because it is not known if these are normal or abnormal shapes. The range of PW1/SL + PW2/SL values used, was determined by the standard deviation associated with the numerical mean. Oocyte populations from both recovery times were heterogeneous, containing high percentages of barrel-shaped spindles (27.8 - 40.0%) and abnormal spindles (35.6 - 47.2%). There was no significant difference in the percentage of these spindle types between 13 h and 15 h post-hCG. However, differences may have gone undetected, because it was only possible to analyze 13.8% - 52.6% of each oocyte population ovulated.

Figure 15. The relationship between a numerical description of spindle shape and subjective assessment. The proportion of each pole width (PW) to spindle length (SL) was added (PW1/SL + PW2/SL) to give an objective value of spindle shape, for measured spindles collected from prepuberal mice stimulated with 5 IU hormone doses in 3 replicate experiments. The numerical means ± S.D., associated with the subjective descriptions of spindle shape, were significantly different from each other ($p < 0.01$).

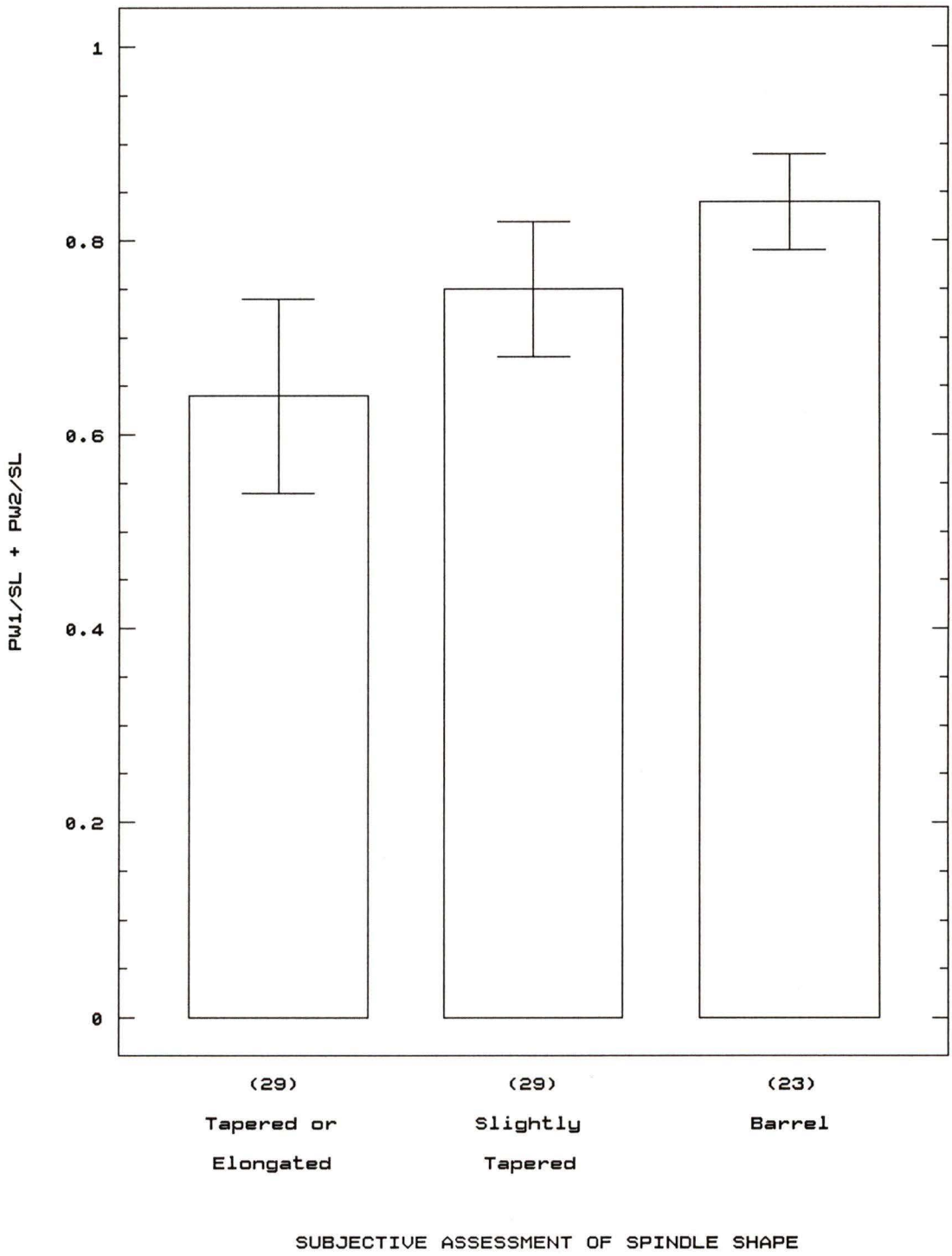
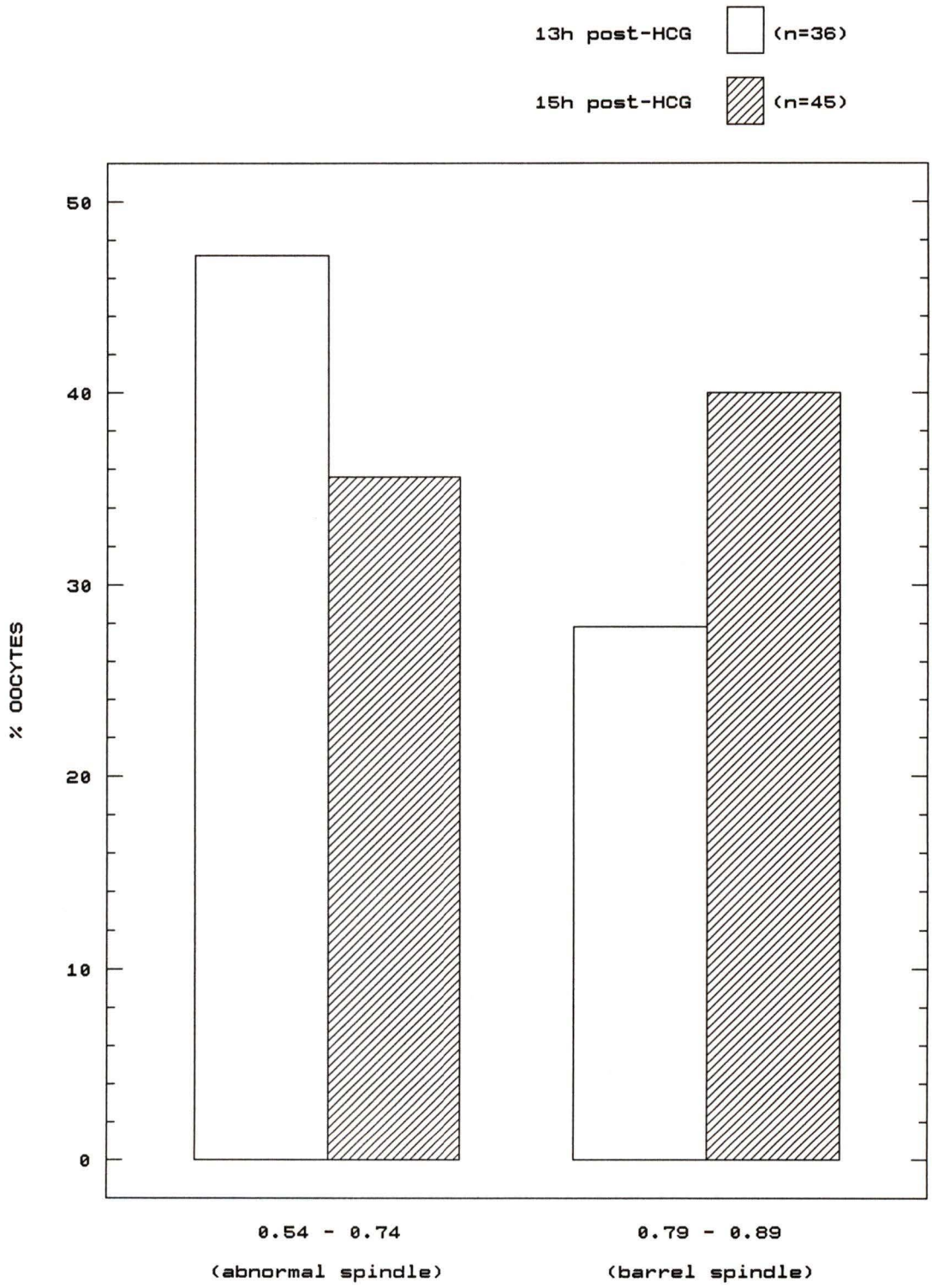

1
0.8
0.6
0.4
0.2
0
PW1/SL + PW2/SL
(29)
Tapered or
Elongated
(29)
Slightly
Tapered
(23)
Barrel
SUBJECTIVE ASSESSMENT OF SPINDLE SHAPE

Figure 16. The percentage of oocytes with barrel or abnormal shaped spindles recovered at 13 h and 15 h post-hCG from prepuberal mice stimulated with 5 IU hormone doses. The total number of spindles measured at each recovery time, collected in 3 replicate experiments, is indicated by n. An objective description of spindle length (PW1/SL + PW2/SL), which is the addition of the proportions of each pole width (PW) to spindle length (SL), was used. The range of numerical values listed, represents the mean ± S.D. associated with barrel or abnormal shaped spindles. High percentages of barrel (27.8 - 40.0%) and abnormal spindles (35.6 - 47.2%) were observed in oocytes obtained at 13 h and 15 h post-hCG. The percentage of each spindle type was not significantly affected by the recovery time.


13h post-HCG
(n=36)
15h post-HCG
(n=45)
% OOCYTES
50
40
30
20
10
0
0.54 - 0.74
(abnormal spindle)
0.79 - 0.89
(barrel spindle)
NUMERICAL DESCRIPTION OF METAPHASE SPINDLE SHAPE
PW1/SL + PW2/SL

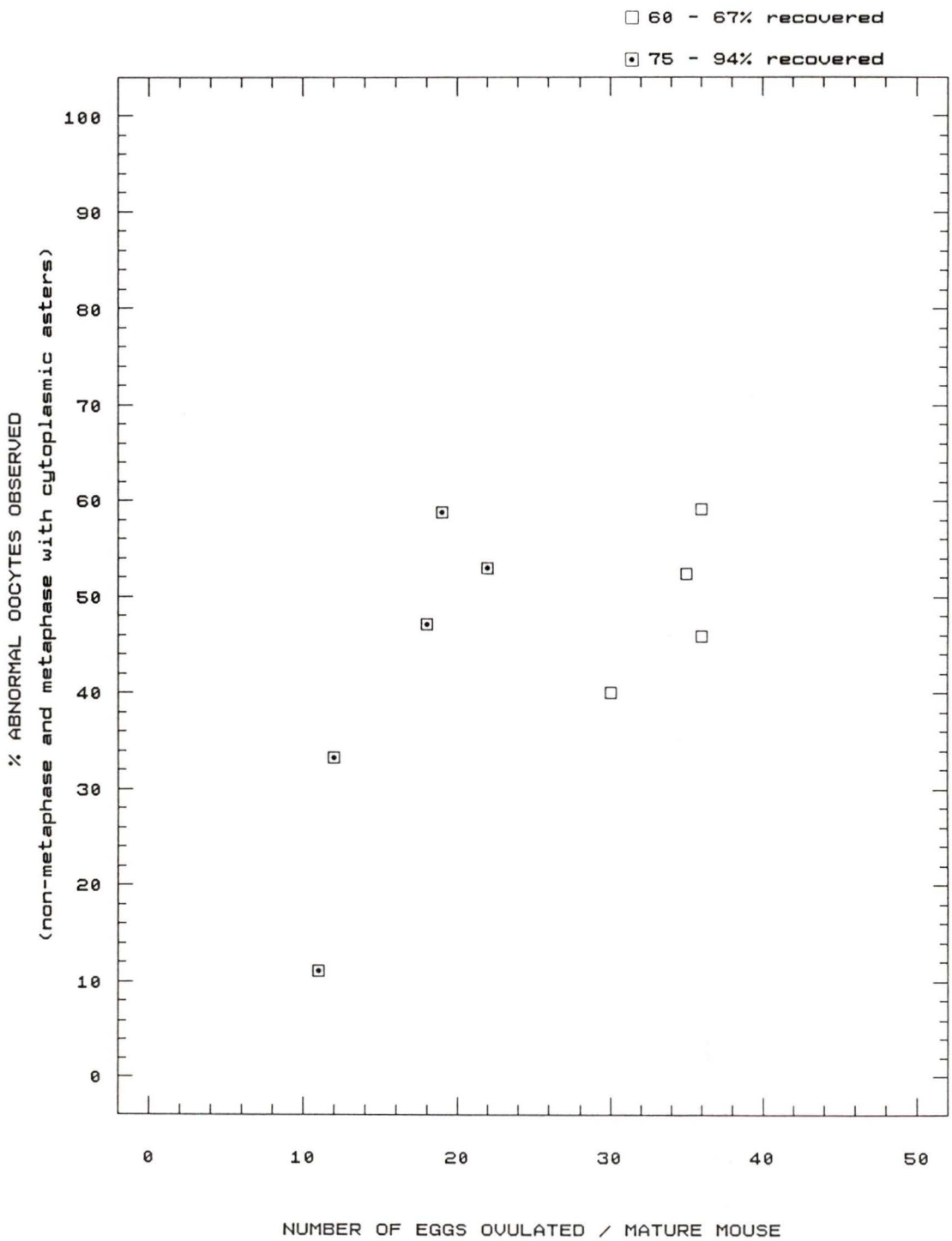
Oocytes from mature mice also demonstrated the variety of spindle shapes described. A detailed analysis was not done, because heterogeneity in these populations was more obviously expressed through the presence of non-metaphase eggs and metaphase eggs with cytoplasmic asters.

Effect of quantity of eggs ovulated on heterogeneity

The number of eggs ovulated by mature mice was positively correlated ($p<0.05$) with the percentage of abnormal oocytes observed (Fig. 17). Only populations which represented 75% or greater of eggs ovulated were included in the analysis. Fig. 17 illustrates, as the number of eggs released increased, so did the number of oocytes exhibiting some type of anomaly — either non-metaphase (diffuse aster, spindle-forming, anaphase) or metaphase with cytoplasmic microtubule asters. Thus, the degree of heterogeneity rose with increasing success of superovulation. It appears the percentage of abnormal eggs detected plateaus at 30 - 36 eggs/mouse. But, only 60 - 67% of the ovulated oocytes could be analyzed, from mature mice showing such potent stimulation. Therefore, it is not known with certainty if this is a true levelling off, or if the correlation actually continues.

An examination of any relationship between abnormal spindle shape and eggs ovulated/prepuberal mouse was not possible, because only 13.8% - 52.6% of each ovulated population could be examined.

Figure 17. The effect of eggs ovulated per mature mouse on the percentage of abnormal oocytes observed. Abnormal oocytes were non-metaphase eggs (diffuse aster, spindle-forming, and anaphase) and metaphase eggs with cytoplasmic microtubule asters. Each point on the graph represents an oocyte population from one mouse. Analysis of oocyte populations consisting of 75 - 94% of the eggs ovulated revealed a positive correlation ($p < 0.05$; Pearson correlation co-efficient) between the number of eggs released by mature mice (stimulated with 5 - 10 IU hormone doses and obtained 13 - 16 h post-hCG) and the percentage of abnormal oocytes detected in those populations. A plateau appears to occur at ovulation rates of 30 - 36 eggs/mouse; obtained from mature mice 13 - 18 h post-hCG after 10 IU hormone doses. But, for these animals, only 60 - 67% of the ovulated population could be analyzed.


□ 60 - 67% recovered
⊡ 75 - 94% recovered
% ABNORMAL OOCYTES OBSERVED
(non-metaphase and metaphase with cytoplasmic asters)
100
90
80
70
60
50
40
30
20
10
0
0
10
20
30
40
50
NUMBER OF EGGS OVULATED / MATURE MOUSE

DISCUSSION

Exogenous gonadotrophins are commonly used to induce ovulation in prepuberal and mature mice. This technique produces a large synchronous population of eggs (Gates, 1971; Kaufman, 1983), which is useful for experiments. The term synchronous was initially used to decribe the timing of egg release, but became associated with homogeneity in oocyte quality, as well (Biggers et al., 1971; Kaufman, 1983). However, the present study found the type of eggs obtained from superovulated mature mice was significantly different from those of superovulated prepuberal mice.

Mouse age affects egg quantity and quality

In agreement with previous studies (Zarrow and Wilson, 1961; Wilson and Zarrow, 1962), increasing hormone dose of PMSG and hCG increased the number of eggs ovulated per prepuberal or mature mice (Fig. 4). Also, as was found in those studies, mature mice required 10 IU hormone doses to produce high yields, and superovulated prepuberal mice produced significantly more eggs than superovulated mature mice (Fig. 4). The greater variability in egg number per mouse, at the higher hormone doses (Fig. 4), was also expected (McLaren, 1967).

The hormone doses used, 5 or 10 IU, are commonly administered to induce superovulation (Pickering and Johnson, 1987; Johnson et al., 1988; Vincent et al., 1990; Johnson et al., 1990). It is not known if the hormone doses injected for the current study were exact for each experiment, because activity can decay over time, and with mixing (Hogan et al., 1986). The experiments were conducted over a time

period of 9 months, with working solutions stored in the freezer. Extended storage was found to affect the 5 IU dose, initially given to prepuberal mice. This less potent dose was called <5 IU and the freshly made doses called 5 IU. In addition, experiments on mice of different ages, or receiving different hormone doses, were run separately. Only the recovery time post-hCG was varied for each experiment, because the original purpose of the thesis was investigation of changes with *in vivo* ageing. Therefore, the doses stated should be considered approximate.

The number of eggs ovulated was counted shortly after autopsy. The other analyses were performed after fixation and processing of the oocytes. As a result of egg loss, the analyzed oocyte populations were always smaller than the original quantities (Tables 1 and 2). Therefore, the percentages stated may differ from the true value. But, the oocytes measured represent sizable samples, and more important, indicate the extent of homogeneity present at fixation.

Healthy, ovulated eggs are arrested in metaphase II of meiosis (Baker, 1982). The presence of a polar body (PB; Fig. 5f) is an indicator of completed maturation (Donahue, 1968). However, in mice the first PB can quickly degenerate (Donahue, 1968; Austin, 1982). The current study found no significant difference in the percentage of oocytes, from superovulated prepuberal (24.0%) and superovulated mature mice (17.8%), demonstrating a polar body (Fig. 6). But, when the meiotic stage was analyzed, by indirect immunofluorescence of microtubules and Hoechst staining, significant differences were revealed. Only 72.2% of pooled superovulated oocytes from mature mice displayed a metaphase spindle (Fig. 6), compared with

98.7% from prepuberal mice.

Conditions of superovulation affect type of non-metaphase eggs observed

The 10 IU hormone dose of PMSG and hCG, given to mature mice, was associated with the recovery of diffuse aster and spindle-forming eggs (Fig. 10). There is morphological evidence these were pre-metaphase I oocytes. The scattered arrangement of the chromosomes in the egg center of the diffuse aster oocytes (Fig. 7b) is similar to the circular bivalent stage, occurring after germinal vesicle breakdown (GVBD) and prior to metaphase I (Donahue, 1968; Mattson and Albertini, 1990). Anti-tubulin immunofluorescence studies, of *in vitro* matured eggs at the circular bivalent stage, demonstrated a large fluorescent array in the middle of the oocyte (Rime et al., 1987; Mattson and Albertini, 1990), similar to the diffuse aster described in this study (Fig. 7a). The first meiotic spindle is assembled in the egg center (Van Blerkom, 1989). During early spindle formation, new tubulin polymerization at the developing spindle poles produces microtubules growing in all directions (Mitchison, 1990). This process could describe the spindle-forming structures seen (Fig. 8a,c). Eichenlaub-Ritter and Boll (1989) noted a number of microtubular asters, after GVBD *in vitro*. Cytoplasmic, microtubule asters were observed in the diffuse aster (Fig. 7d, arrow) and spindle-forming (Fig. 8a,c; arrows) oocytes examined; especially, in the latter. However, Wassarman and Fujiwara (1978) did not observe any such structures. Their lack of aster detection could be due to the higher intensity of background fluorescence, which is quite obvious in the micrographs (Wassarman and Fujiwara, 1978), or the antibody used (de Pennart et

al., 1988).

Transient cooling of oocytes can cause alterations in microtubular structures. Pickering and Johnson (1987) found incubation of unfertilized mouse eggs at room temperature (25°C) for 10 min produced significant increases in abnormally shaped spindles, plus the appearance of multiple cytoplasmic asters. In the present study, although care was taken to maintain the oocytes at 37°C, temperature fluctuations occurred. When using the dissecting scope, the eggs were exposed to room temperature. The time at room temperature was usually 2 to 5 min. The abnormalities Pickering and Johnson (1987) observed were tapered or elongated spindles, with chromosomes at the metaphase plate. The only similarity to the diffuse aster or spindle-forming oocytes described in the current study was the presence of cytoplasmic asters. Therefore, it is unlikely that the unusual microtubule assemblies and chromosome configurations were caused by the brief exposures to room temperature.

The proposed immaturity of the diffuse aster and spindle-forming oocytes is additionally supported by their distinct appearance. The mean vitellus diameter of these ova (79.8 ± 3.8 μm) was significantly smaller than the mean diameter of metaphase oocytes (89.9 ± 5.8 μm), collected from the same animals ($p<0.01$). These measurements may be larger than the true value, because the eggs were slightly compressed by the coverslip. The metaphase II oocyte of mice has a diameter of about 85 μm (Hogan et al., 1986). Also, all the diffuse aster and spindle-forming eggs displayed a large perivitelline space (Fig. 7c,f), which was never

observed in metaphase eggs (Fig. 5c,f). From other studies, micrographs of *in vitro* matured eggs did not show an obvious perivitelline space for eggs with a germinal vesicle (Wassarman and Fujiwara, 1978; Mattson and Albertini, 1990), but did demonstrate one for anaphase I and metaphase II (Wassarman and Fujiwara, 1978). The metaphase II oocytes may have shown a large gap due to *in vitro* maturation. To determine if primary oocytes are indeed ovulated, by mature mice in response to 10 IU hormone doses, the chromosome complement of the eggs needs to be examined for diploidy.

Spontaneous release of primary oocytes has been shown to occur in the mouse strains NMRI/Han (Hansmann and El-Nahass, 1979) and LT/Sv (O'Neill and Kaufman, 1987), and possibly A/He (Takagi and Sasaki, 1976). Administration of exogenous gonadotrophins to NMRI/Han (Hansmann and El-Nahass, 1979) and LT/Sv mice (Speirs and Kaufman, 1988) significantly increased the numbers of primary oocytes detected. The ovulation of primary oocytes from BALB/c mice has not been reported.

The 5 IU hormone dose of PMSG and hCG, given to mature mice, was associated with the recovery of anaphase eggs (Fig. 10). Anaphase oocytes were also obtained from mature mice given 10 IU hormone doses, but only when recoveries were 15 - 18 h post-hCG. These non-metaphase eggs contained spindles with chromosomes at opposite poles (Fig. 9a,b and d,e); similar to the late anaphase / early telophase spindles described by Eichenlaub-Ritter et al. (1988). Some of these eggs were obviously secondary oocytes, because they also possessed a polar body

(Fig. 9c,f). It is not known if the anaphase oocytes were ovulated in this condition, or developed after recovery. Activation of oocytes, to resume meiosis, has been shown to occur from handling alone (Kaufman and Surani, 1974), or a ten minute exposure to hyaluronidase (Kaufman, 1973). Incubation in hyaluronidase was kept to a maximum of 5 min for the experiments conducted in the work presented. The sensitivity of oocytes to activation has been demonstrated to increase with *in vivo* ageing (Kaufman, 1973). In the current study, significantly more anaphase eggs were recovered from mature mice at 15 - 16 h post-hCG than at 13 h post-hCG (Fig. 10). The two anaphase oocytes observed from two prepuberal mice were obtained at 15 h post-hCG (Fig. 10). Therefore, anaphase eggs may represent older oocytes.

The meiotic division, I or II, of the anaphase oocytes which did not display a polar body is unknown. None demonstrated a large perivitelline space, as seen with the putative immature oocytes previously described. Nor did egg diameter differentiate two separate classes. Two anaphase oocytes were obtained from one mouse, which had diameters of 75 μm or 92 μm, and a polar body (Fig. 9c,f). Therefore, it cannot be determined whether this group consists only of secondary oocytes, or a combination of mature and immature eggs. Analysis of the chromosome complement would be required for conclusive evidence.

Heterogeneity of metaphase oocytes

Some metaphase oocytes from superovulated mature mice also contained cytoplasmic microtubule asters (Fig. 11c,d), which were not observed in oocytes from superovulated prepuberal mice. Freshly ovulated, unfertilized metaphase II eggs do

not normally contain such asters (Maro et al., 1985; Pickering and Johnson, 1987; de Pennart et al., 1988). However, they do possess numerous, discrete foci of pericentriolar material (PCM), which can stimulate tubulin polymerization, distributed throughout the cytoplasm (Maro et al., 1985). The spots of anti-tubulin fluorescence seen (Fig. 3a; arrows in Fig. 14c,e) are probably associated with PCM foci (Eichenlaub-Ritter et al., 1986). PCM sites become active microtubule organizing centers with oocyte ageing (Schatten et al., 1985; Schatten and Schatten, 1987; Eichenlaub-Ritter et al., 1986). Cytoplasmic microtubule asters can be artificially induced by exposure to room temperature, for 10 min (Pickering and Johnson, 1987). Brief temperature fluctuations did occur, as previously discussed. However, oocytes from prepuberal mice were collected in the same manner, and cytoplasmic asters were not observed. Therefore, temperature in itself is unlikely the sole cause of this phenomenon. The results may reflect differential reactions to cold stress, due to variable egg quality; possibly as a result of differential oocyte age within each population. It is not known if the asters seen were present at ovulation, or produced after recovery.

Metaphase oocytes from both age groups demonstrated variation in spindle shape. This was the only significant heterogeneity detected in oocytes produced by superovulated prepuberal mice. Eichenlaub-Ritter et al. (1986), using indirect immunofluorescence, observed that spindles from mature spontaneously ovulating mice were barrel-shaped or slightly tapered, with a mean pole-to-pole spindle length of 26.4 ± 0.3 μm. The current study measured a much larger variation in spindle

length. For eggs recovered at 13 h or 15 h post-hCG, the means were 26.9 ± 3.1 μm and 27.6 ± 3.4 μm, respectively. The wide range in spindle length was associated with vastly different spindle shapes.

A mathematical description of spindle shape (PW1/SL + PW2/SL) demonstrated significant differences between barrel (Fig. 5a) spindles (0.84 ± 0.05), slightly tapered (Fig. 3a) spindles (0.75 ± 0.07), and tapered (Fig. 14c) or elongated (Fig. 14e) spindles (0.64 ± 0.10). The recovery time (13 h or 15 h post-hCG) did not significantly change the distribution of barrel and abnormal spindle shapes, obtained from prepuberal mice given 5 IU hormone doses (Fig. 16). However, differences may have gone undetected, as a result of incomplete analysis. Only a small percentage of spindles/population could be measured (13.8% - 52.6%), due to egg loss and spindle orientation.

Barrel-shaped spindles were measured in 27.8% - 40.0% of the oocytes, collected 13 - 15 h post-hCG from prepuberal mice given 5 IU hormone doses (Fig. 16). Pickering and Johnson (1987) reported 89% barrel spindles, recovered 12.5 h post-hCG from prepuberal mice, with indirect anti-tubulin immunofluorescence. However, their measurements of spindle shape was based on subjective classification only, and could be falsely high. If the percentage of slightly tapered spindles is included in the current assessment, the percentage of barrel-shape spindles rises to 52.8% - 64.4%. Some loss of barrel shape, in the current study, may have resulted from temperature fluctuations. Pickering and Johnson (1987) found barrel-shaped spindles decreased from 89% to 37%, after cooling at room temperature for 10 min.

As mentioned before, manipulations on the dissecting scope resulted in room temperature exposures of 2 to 5 min. Still, these brief temperature changes may have contributed to the lower percentage of barrel shape spindles observed between this study and Pickering and Johnson (1987), which used a heated stage. It is not known if the heterogeneity in spindle shape was present at ovulation, or occurred after recovery.

Effect of number of eggs ovulated on heterogeneity

Analysis of oocyte populations, consisting of 75% or greater of the eggs ovulated, revealed the percentage of abnormal oocytes detected (non-metaphase and metaphase + cytoplasmic asters) increased as more eggs were ovulated from mature mice (Fig. 17). The positive correlation was found for mice releasing 11 - 22 eggs. When populations of 60% - 67% recovery were incorporated, the increase in percentage of abnormal oocytes appeared to plateau (Fig. 17). But, it is not known if this is an accurate pattern, because of incomplete data. The highest ovulation rates (24-41 eggs/mouse) were excluded, because only low proportions of these populations were measured (37.5% - 66.7%). This is much lower than the values obtained from prepuberal mice, which had recoveries of 75% or greater for 3/6 mice with high ovulation rates. The discrepancy in recovery rates may be due to the presence of fragmented eggs. Marston and Chang (1964) found superovulated mature mice produced more fragmented eggs than superovulated prepuberal mice. In the current study, the fragmented eggs were not counted. It is also possible that an additional class of abnormal eggs was released by mature mice — a group which

appeared normal, but was unable to withstand the experimental procedure and disintegrated.

The observed number of metaphase oocytes with cytoplasmic asters was not correlated with the number of eggs ovulated from mature mice (Fig. 12), as expected. The number of metaphase oocytes present was a better predictor; when more than the usual 8-12 metaphase eggs were recovered from mature mice, the extra metaphase eggs displayed cytoplasmic asters (Fig. 13). The lack of complete analysis of ovulated populations, due to varying recoveries, resulted in this paradox.

The recovery of putative primary oocytes (diffuse aster and spindle-forming) was only observed after the 10 IU hormone dose (Fig. 10), which also produced a high ovulation rate (Fig. 4). This association, between egg number and the release of meiosis I eggs, has been demonstrated for immature rats given low (4 IU) or high doses (20 IU) of PMSG (Yun et al., 1989). In mouse strains which naturally ovulate immature ova, high egg production is also present (Bartels et al., 1984). However, Beerman et al. (1986) found no such correlation. But, the large variability in the ages of the females used (8-16 wks) may have confounded their results. Speirs and Kaufman (1990) demonstrated that with increasing maternal age the frequency of primary oocyte ovulation decreased.

Past evidence of heterogeneity and homogeneity

There has been evidence of oocyte heterogeneity, but this issue was not thoroughly investigated. The original paper on superovulation of mature mice (often cited) showed significantly lower success with pregnancy, compared to naturally

cycling mice (Fowler and Edwards, 1957). Overcrowding of embryos or hormonal imbalance were suggested causes, rather than suspecting egg quality (Kaufman, 1983).

Differences between oocytes, prior to ovulation, have been demonstrated. Eggs obtained from the antral follicles of unstimulated mice vary in size, 70 - 100 μm in vitellus diameter (Mangia and Epstein, 1975; Mangia and Canipari, 1977). Maturation of germinal vesicle eggs induced by *in vitro* culturing, from PMSG primed follicles (Hashimoto and Kishimoto, 1988) or naturally cycling mice (Eichenlaub-Ritter et al., 1988), was asynchronous. Edwards and Gates (1959) found the *in vivo* progression of meiotic maturation within a single superovulated mouse was also staggered. Phase-contrast analysis of oocytes revealed a range of meiotic stages, from metaphase I to first PB, shortly before ovulation. Once ovulation had begun, ovarian oocytes from anaphase I and later stages were observed. The ovulated eggs possessed polar bodies and were classified as metaphase II.

Physical differences among ovulated eggs has been observed. If the zona pellucida is removed from superovulated mouse oocytes, a bulge in the egg surface, over the spindle, becomes apparent (Johnson et al., 1975; Phillips and Shalgi, 1980; Longo and Chen, 1985). This nipple-like area is relatively smooth, compared to the many microvilli covering the rest of the egg, and can vary in shape between oocytes (Johnson et al., 1975; Phillips and Shalgi, 1980). Phillips and Shalgi (1980) suggested the differences they found may result from natural variation and/or artifacts produced from specimen processing for electron microscopy. Rodman (1971a) noted variabilities, within and between mice, in the type of cumulus masses surrounding the

superovulated eggs. In addition to compact (fresh) cumulus, dispersed masses were observed. Oocytes with aged cumulus showed a significantly higher percentage of premature chromatid disjunction.

Heterogeneity is evident in the physiological responses of ovulated eggs. Asynchrony occurs with natural fertilization (Gates, 1965) and ageing (Longo, 1980). Exposure to stress, such as hyaluronidase induced activation (Kaufman, 1973) or cold temperatures (Pickering and Johnson, 1987) uncovers additional differences among superovulated eggs. The loss or appearance of a measured characteristic or function was not homogeneous, but only occurred in portions of the populations tested. The percentage of eggs affected depended on the nature of the stress imposed. Activation of oocytes obtained 15.15 h post-hCG significantly increased from 3.1% to 44.6%, after pre-incubation in calcium-free medium (Whittingham and Siracusa, 1978). Shaw and Trounson (1989) found activation rates varied significantly, with different reagents.

Based on the results of the presented work, heterogeneity in meiotic stage of superovulated eggs could have gone undetected in the past due to: mouse age, hormone dose, or ovulation rate. Many studies used superovulated prepuberal (3 - 5 wks) mice (Gates, 1971; Sathananthan et al., 1988; Shaw and Trounson, 1989; Badenas et al., 1989), especially in experiments which directly assessed meiotic stage through immunolabelling of tubulin and chromosomal staining (Maro et al., 1985; Pickering and Johnson, 1987). Metaphase oocytes were easily recovered from stimulated prepuberal mice in the current and past studies. The use of low hormone

doses (1 - 5 IU) to superovulate mature mice was, and is a common practice (Fowler and Edwards, 1957; Magistrini and Szollosi, 1980; Van der Elst et al., 1988; Nakagata, 1989; Kim and Schuetz, 1991). In these experiments, any activated eggs found could be attributed to the physical stress of the procedures. The recovery of metaphase eggs is expected with a low ovulation rate. No abnormal oocytes were detected from mature mice given 7.5 IU hormone doses (Ducibella et al., 1988). These mice produced 10 - 15 oocytes per female.

The parameter measured may have falsely given the appearance of homogeneity in meiotic stage, among superovulated oocytes from mature mice (6 wks and older) given high hormone doses (7.5 - 10 IU). Examination of fertilization rate (Wolf and Hamada, 1976; Carroll et al., 1989) can be misleading. The gross morphology of sperm penetration and pronuclei formation of primary oocytes is similar to normal fertilization of secondary oocytes (O'Neill and Kaufman, 1987). Abnormal development, such as triploid embryos (3n chromosomes), can result from the fertilization of primary oocytes, or secondary oocytes which have retained the second polar body (O'Neill and Kaufman, 1987). The chromosome configurations of these two events can be differentiated (O'Neill and Kaufman, 1987), but precise analysis of all embryos is usually not possible (Glenister et al., 1987). The long *in vitro* incubation periods (3 - 7 h post-ovulation) used to obtain scores for fertilization and development (Glenister et al., 1987) or artificial activation (Whittingham and Siracusa, 1978), may have allowed primary oocytes to complete maturation. GV eggs, maturing *in vitro*, take approximately 8 h to progress from pro-metaphase I to

metaphase II (Donahue, 1968). Oocytes released at the prometaphase I stage may develop at a slightly faster rate. Some measurements required the pooling of oocytes to obtain a single data point. Endo et al. (1986) used groups of 100 oocytes for values of protein phosphorylation. In this instance, any differences between eggs would not be noticed.

Results can initially appear homogeneous, but reveal heterogeneity with additional analysis. Edgar et al. (1987) superovulated mature mice with varying doses of PMSG (5, 10, or 15 IU), followed by 5 IU hCG. The oocytes were recovered 13 h post-hCG and fertilized *in vitro*. The higher doses of PMSG, 10 and 15 IU, gave similar rates of development to the 2-cell stage, when compared to the use of 5 IU. However, examination of more advanced development (blastocyst stage) uncovered a significant decrease when 10 or 15 IU hormone doses were used. The presence of a polar body is used to classify eggs as metaphase II (Edwards and Gates, 1959). But, the current study found the observation of a polar body was not a reliable indicator of meiotic arrest. Six anaphase oocytes (Fig. 9) and one spindle-forming egg (Fig. 8e,f) demonstrated polar bodies.

Hormonally induced heterogeneity

Exogeneous hormonal stimulation seems to extend the range of oocyte variation, normally occurring. Longo (1981) presented more direct evidence. Spontaneously ovulated and superovulated (5 IU) eggs from mature mice had different responses to alpha-chymotrypsin digestion. The zona pellucida was removed from 90 ± 6% of naturally ovulated eggs, but only 76 ± 7% of super-

ovulated eggs had a similar reaction.

Pellicer et al. (1988) proposed a more heterogeneous group of follicles were developing to pre-ovulatory stages under conditions of superovulation, compared to spontaneous cycling, to explain the observed asynchrony in follicles of hormonally stimulated humans. Pellicer et al. (1988) suggested exogenous gonadotrophins may re-recruit follicles beginning to undergo atresia, and surpass the threshold FSH requirements needed to stimulate younger follicles.

There is support for the Pellicer et al. (1988) model. Graafian follicles, of PMSG-primed prepuberal and spontaneously cycling mice, have been stimulated to ovulate after a period of overripening; after the early stages of atresia have begun (Laing et al., 1984). The ovulation of primary oocytes is rare for most mammals (except dog and fox) during natural cycling, but is more likely after hormone treatment (review - Austin, 1969). Immature rats injected with a 4 IU PMSG dose produced 1.8% primary oocytes; but, following a 20 IU PMSG dose, 51.7% meiosis I eggs were recovered (Yun et al., 1989). In mice, PMSG doses of 10 IU were found to significantly increase triploid and fragmented embryos; compared to the use of 1.5 IU PMSG (Sato and Marrs, 1984). The fertilization of primary oocytes may have contributed to the presence of triploid embryos. Maudlin and Frazer (1977) also observed increased polyploidy (in mice) after 10 IU PMSG stimulation. However, they determined the extra chromosomes resulted from the presence of more than one sperm.

Studies with PMSG-primed sheep have demonstrated premature maturation

of ovarian oocytes (Moor et al., 1985). The authors proposed the longer half-life of PMSG (compared to FSH), plus the added LH-like activity of PMSG, contributed to this overstimulation. During a natural cycle, the ovaries are exposed to fluctuating, rather than constant levels of LH and FSH (Baker, 1982). In immature rats, the ratios of follicular steroids demonstrated significantly less variation over time after 20 IU PMSG, compared to controls given 4 IU PMSG (Yun et al., 1989).

The present work showed heterogeneity of oocytes after superovulation was more strongly expressed in mature mice, than in prepuberal mice. Oocytes from stimulated prepuberal mice varied only in spindle length and shape. Oocytes from stimulated mature mice also displayed significant numbers of non-metaphase oocytes and metaphase eggs with cytoplasmic asters. These results might reflect the different ovarian envivironments present at the time of treatment.

The dynamics of follicle recruitment changes with age (Pedersen, 1969; Peters, 1969). The first wave of follicle growth produces an unusually high number of large follicles, at 21 days (Pedersen, 1969). Inbred BALB/c mice demonstrated the maximum number of large follicles, about 150 per mouse, at three weeks (Gates, 1971). These follicles normally degenerate, due to lack of needed hormones in the prepuberal mouse. With increasing age, continued follicle recruitment leads to depletion in the total number of oocytes present in the ovaries (Baker, 1982). At 35 days, when sexual maturity begins, only 25 large follicles were observed in one ovary, compared with 51 large follicles detected at 21 days (Pedersen, 1969; Peters, 1969). Prepuberal mice (3 weeks old) possess a larger and more homogeneous group of

follicles ready for stimulation by gonadotrophic hormones, compared to older mice. Mature mice normally ovulate only 8 - 12 oocytes (Hogan et al., 1986). Therefore, to increase egg production in the mature mouse, follicles at different stages may need to be recruited.

The current data support this suggestion. Ovulated eggs collected from stimulated mature mice ranged from putative primary oocytes (diffuse aster, spindle-forming) to eggs exhibiting properties associated with ageing (cytoplasmic asters or activation). It seems, in mice, the 5 IU hormone dose recruited older follicles, as well as, the next group ready for final maturation. Recruitment of older follicles was not consistent, and may reflect the stage in the estrus cycle the injections were given. The 10 IU hormone dose appeared to stimulate younger and older follicles; but seemed more inhibitory to older ones compared to the 5 IU dose. The randomness of the estrus cycle in the mice used may have falsely created this impression. Rodman (1971b) also observed a similar age range in ovulated ova 15 - 18 h following high hormone doses. Eggs with cumulus masses varying from intact to loose, as well as primary oocytes (chromosome analysis), were detected after mice (age not stated) were given 10 IU follicle-stimulating hormone (Equinex) and 20 IU luteotrophic hormone (APL). To confirm the proposed effects of mouse age and hormone dose, the follicles of prepuberal and mature mice need to be examined before and after treatment with different hormone doses (at known stages of the estrus cycle). The chromosome complement of ovarian oocytes from stimulated follicles, and ovulated eggs, would also need to be determined. The endogeneous

release of LH may have contributed to the heterogeneity observed (Gates, 1971). To control for this possibility, the ovulatory injection should be given about noon, or the light cycle adjusted accordingly (Gates, 1971).

Implications

Past investigations may have unknowingly used oocyte populations with different degrees of heterogeneity, from superovulated mice. For example, experiments on cryopreservation of mouse oocytes have given mixed results. Comparison between studies is difficult, due to different protocols. But, the source of the eggs should also be considered.

Kono et al. (1991) demonstrated *in vitro* fertilization and development of mouse oocytes after vitrification (ice-free cryopreservation), at about twice the rate of previous studies using frozen-thawed oocytes. The less successful studies superovulated mature mice (or mixed ages) with 5 - 7.5 IU of PMSG (Whittingham, 1977; Glenister et al. 1987; Carroll, et al. 1989). Kono et al. (1991) also used mature mice (5IU), but incorporated a post-recovery selection procedure — only collecting oocytes with a PB — not used by the comparative papers cited. Thus, Kono et al. (1991) may have tested a more homogeneous oocyte population. Significant variation in results from the same treatment groups of replicate experiments (Glenister et al. 1987), including the control group (Whittingham, 1977), was noted by the authors of the freeze/thaw experiments. Heterogeneity in oocyte age may have affected this variability. Immature oocytes appear to be more sensitive to cooling than mature eggs (Whittingham, 1977), and aged oocytes have been shown to be less resilient to

stress (Kaufman, 1978). Parkening and Chang (1977) found mouse maturity affected the recovery and fertilization of frozen-thawed superovulated eggs. However, their results demonstrated oocytes from mature mice had higher success rates, than ova from immature mice. The reason for this apparent contradiction to the current findings is unknown.

The work on cryopreservation of mouse oocytes is used to help establish procedures for cold storage of excess human eggs, recovered during *in vitro* fertilization (IVF) procedures (Chen, 1988; Siebzehnrubl, 1989). Optimum conditions for freezing and thawing metaphase II oocytes still need to be determined. According to the current study, only superovulated eggs from prepuberal mice should be used in cryopreservation experiments.

CONCLUSIONS

Based on the work presented, the indiscriminate superovulation of mice to obtain metaphase II oocytes needs to be thoroughly examined. The accepted protocol, using 5 - 10 IU PMSG and hCG with recovery 13 - 16 h post-hCG, produced significantly different responses in mice of different ages. Indirect immunofluorescence of microtubules and chromosomal staining with Hoechst 33258 demonstrated eggs recovered from superovulated prepuberal mice were homogeneous for metaphase, but eggs from superovulated mature mice were heterogeneous. The conditions of superovulation determined the type of heterogeneity observed in mature mice. Putative primary oocytes were released after administration of 10 IU hormone doses. Anaphase eggs were recovered more frequently after 5 IU hormones doses, or recovery times of 15 - 16 h post-hCG. The percentage of egg abnormalites (non-metaphase oocytes and metaphase eggs with cytoplasmic microtuble asters) was positively correlated with the number of oocytes released by superovulated mature mice; except at the highest ovulation rates, which had poor recoveries. Different ovarian responses to exogeneous hormones by prepuberal and mature mice is the suggested cause. Given these findings, when mature mice are hormonally stimulated, substantiation should accompany the claim of using freshly ovulated eggs.

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Title of Thesis: Superovulated Mature Mice Produce More Heterogeneous Oocyte Populations Compared To Superovulated Prepuberal Mice

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MARY-ANNE HAMMER

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