pupate, but retrogressed via molting cycles in which the postecdysial larvae were smaller than were the preecdysial forms. The first retrogressive ecdysis occurred within 10 days after isolation, and subsequent retrogressive ecdyses occurred at about 4-week intervals thereafter. After either 20 or 36 weeks of retrogression, the larvae were given food; they then regained the weight and size they had lost. Regrowth also involved two or three molting cycles. If again isolated prior to their being committed to pupation, the larvae would again retrogress. By successive cycles of retrogression and regrowth, we have produced larvae more than 2 years of age, which is in sharp contrast to the insect's normal life span of 8 weeks (from newly laid egg to death of spent adult) under our culturing conditions.

When removed from stock culture at the beginning of the sixth larval stage, the insects were programmed to molt to the pupal stage (*3*), but pupal determination was revoked by the transfer to deprived isolation; subsequent molting cycles resulted in additional but diminished larval stages. Retrogressed larvae did not retain the ability to pupate, but could regain that capability only through regrowth. The process of regrowth included not only feeding and weight gain, but also the occurence of two (sometimes three) progressive larval ecdyses. By this process the larvae attained the developmental maturity required for the programming of pupation, a developmental stage they had previously reached just prior to the initial retrogression. In this sense a real reversal of development was demonstrated, although tissue differentiation was not altered by the process of retrogression. Because these insects may be maintained in a larval state for greatly prolonged periods, it is possible to investigate the question of aging in a juvenile (larval) system, uncomplicated by adult differentiation, reproduction, and (perhaps) genetically controlled senescence.

The number of days required for regrowth was used as one measure of physiological efficiency. The hypothesis tested was that any age-related deterioration of the system might be reflected by progressively longer periods of regrowth after successive retrogressions. The experimental results tended to support this hypothesis. Repeated cycles of 20 weeks of retrogresssion followed by regrowth to the original size and weight were carried out. The required regrowth period increased from 9 days in the first to 28 days in the fourth cycle. Two cycles of 36-week retrogressions have been completed; 19 days of regrowth were required after the first retrogression, and 29 days after the second. Obviously such repeated retrogressions were accompanied by a loss in the insect's capacity for rapid recovery.

Table 1. Larval retrogression and cell ploidy in *Trogoderma glabrum*.

Retrogressions		Cell ploidy distribution			
No.	Duration (wk)	Mean (*n*)	Mode		Range (*n*)
			n	%	
		(*A*) *Epidermis*			
0	0*	2.0	2	100	
4	20	2.4	2	70	2 to 6
2	36	2.7	2	60	2 to 6
		(*B*) *Fat body*			
0	0*	3.0	2	43	2 to 6
4	20	6.8	8	40	2 to 16
2	36	8.2	8	43	4 to 22

* Control.

Histological examination of larvae after different durations of retrogression and regrowth failed to disclose pathology in any tissue except that of the fat body. It is well known that the fat body cells of many insects tend to become polyploid as the insects mature and age (*4*). The relationship, if any, of ploidy to physiological function has not been elucidated. We have found the fat body cells of retrogressed *T. glabrum* larvae to be highly polyploid (Table 1). Chromosome counts at metaphase and anaphase in spermatocytes from normal nonretrogressed larvae showed five pairs of chromosomes. Acetocarmine staining was then applied to smears of larval tissues. No mitotic figures were observed in retrogressed larvae, but typically diploid cells (such as epidermis) displayed ten hererochromatic bodies per cell, and this was taken as an approximation of the 2*n* condition. As was expected, fat body cells from normal nonretrogressed sixth instar larvae (Table 1, B, controls) showed some polyploidy, with a range from 2 to 6*n*; the mode (largest grouping) of the ploidy distribution was 2*n*, however. Larvae that had been repeatedly retrogressed showed a much greater polyploidy in the fat cells, with up to 22*n* being observed. Many instances of apparent nuclear fusion were observed, in good agreement with the observations of Wigglesworth (*4*), who studied *Rhodnius prolixus* under starvation conditions. Epidermal cells, on the other hand (Table 1, A), showed only a slight tendency toward polyploidy during retrogression, although cells with as high as 6*n* ploidy were observed.

The central importance of the insect fat body in metabolism as well as a storage organ would suggest that the observed deterioration of the recuperative powers of repeatedly retrogressed larvae might logically be attributed to impaired fat body functions. A high degree of accumulative polyploidy may have been responsible for a progressive loss of metabolic efficiency, although this interpretation of our data is admittedly speculative. From the results of this study, we have concluded that some aging processes were not forestalled or prevented in the beetle larvae, even though developmental processes were repeatedly reversed and restored by retrogression and regrowth.

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Inhibition of Sperm-Egg Interaction by Specific Antibody

Abstract. *Treatment of hamster ova, in vitro, with ovary specific antibody interferes with sperm-egg interaction by inhibiting sperm attachment to the zona pellucida and subsequent zona penetration. This inhibition results from precipitation of the zona by the specific antibody.*

The possibility of utilizing immunological procedures for fertility control in mammals dates back to Metchnikoff's experiments at the turn of the century (*1*). This possibility has gained new impetus from increased knowledge in immunology and the production of specific antibodies against components of the reproductive system (*2*). Antibodies against testicular components and spermatozoa have received more attention in the last few years (*3*) as

Table 1. Sperm attachment and penetration in ova treated with antiserums.

Absorbing agent	Zona precipitation	Ova examined	No. of sperm attached per ovum		Ova penetrated		
			Range	Average	No. observed	No. penetrated	Percent penetrated
			Saline				
None	–	22	17–54	31.9			
			Normal serum				
None	–	66	17–58	38.9	58	28	48.3
			Antiserum to ovary				
Ovary	–	15	11–54	31.0	80	61	76.3
None	+	57	0–21	6.5	64	0	0
Small intestine	+	82	0–15	2.7	106	0	0

compared to investigations on the antigenic properties of tissues and hormones in the female reproductive system (*4*).

The identification and localization of antigens in the ovary (*5*, *6*) which are specific for the ova offers the possibility of utilizing complementary antibodies for inhibiting several reproductive processes including ova maturation, ovulation, sperm-egg contact, and fertilization. An ovarian specific antigen in the hamster, which is known to be associated with the zona pellucida (*6*), may be important in several of these early reproductive processes. Our results demonstrate that antibody against the zona pellucida inhibits sperm attachment and penetration through the zona.

For the production of antibody, a saline extract was prepared from ovaries excised from mature female hamsters (*Mesocricetus auratus*) and injected (intramuscularly into the subscapular or thigh regions) with Freund's complete and incomplete adjuvant into rabbits as described (*6*). Injections were given four times on successive weeks with approximately 40 mg of protein (absorbance at 280 nm) per injection. Serum obtained from blood taken from the marginal ear vein beginning on the fifth week after the initial injection was heated for 30 minutes at 56°C and tested on agar gel diffusion plates for the presence of antibodies. The antiserums to ovary produced numerous precipitin bands when tested against saline extracts of hamster ovary and somatic tissues, including liver, small intestine, lung, and kidney; normal rabbit serum was used as a control antigen.

Antiserums to ovary were rendered specific to ovary by absorption with lyophilized or fresh hamster somatic tissues. For example, antiserum absorbed with small intestine was specific to ovary since it produced a minimum of three precipitin bands when tested (as above, by agar gel double diffusion) against extract of ovary; no bands were observed against the other hamster tissues mentioned above.

Ova with their surrounding cumulus cells were obtained by injecting adult female hamsters with 25 international units of pregnant mare serum (Equinex, Ayerst) on the day when postestrus discharge was observed (*7*, *8*), and with 10 international units of HCG (human chorionic gonadotropin, Nutritional Biochemicals) between 5 and 8 p.m. on the third day after injection of pregnant mare serum. Ova in cumulus cells, recovered 12 to 15 hours after HCG injection, were then treated with either antiserums to ovary unabsorbed or absorbed with somatic tissue. Antiserums diffused through the cumulus cells in both treatments and resulted in zona precipitation. More of the precipitate formed at the zona surface as compared to that portion of the zona adjacent to the ovum. Precipitation also occurred in seminaked ova treated with antiserums (see Fig. 1a). No precipitate formed in the zonae of ova treated with normal serums or antiserums absorbed with ovary tissue.

Precipitation of the zona by specific antibody blocks the removal of the zona by digestion with trypsin (*6*). Since a trypsin-like enzyme (acrosomal proteinase) has been postulated as being an important factor in sperm penetration through the zona during fertilization (*9*) and the zona precipitating antibody inhibits zona removal by

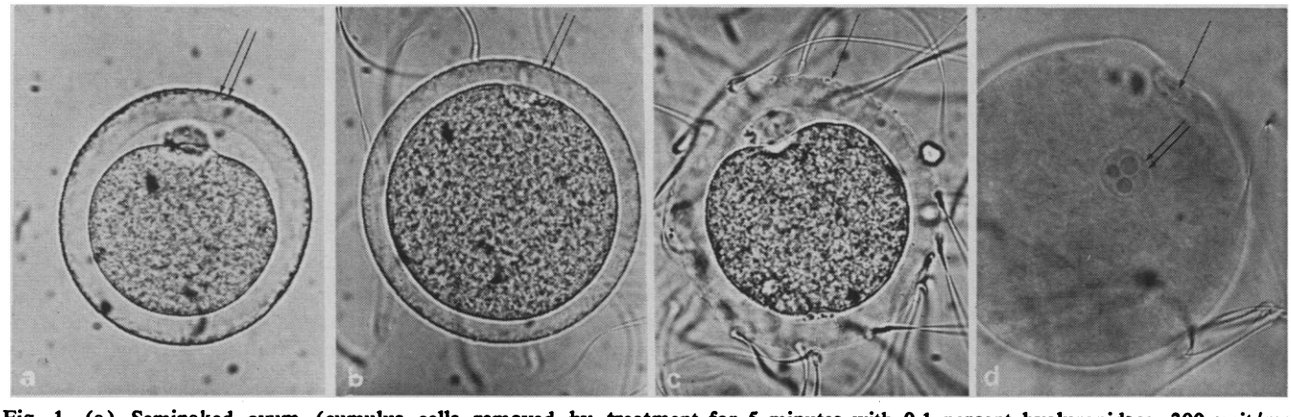

Fig. 1. (a) Seminaked ovum (cumulus cells removed by treatment for 5 minutes with 0.1 percent hyaluronidase, 300 unit/mg, in Tyrode solution showing precipitation of the zona pellucida (double arrows) by antiserum to ovary. There is a dense layer of precipitate at the periphery of the zona (× 765). (b) Ovum treated with zona precipitating antibody and then inseminated. Very few sperm can be seen attached to the densely precipitated zona (double arrows) (× 765). (c) Ovum treated with normal rabbit serum and then inseminated in vitro. Numerous sperm can be seen attached to the zona pellucida. The zona is not precipitated (single arrow) (× 765). (d) Ovum treated with normal rabbit serum prior to insemination and examined 10 hours later for sperm penetration. Fixed and stained with acetolacmoid (× 900). Note polar body formation (single arrow) and pronuclear development (double arrow).

trypsin, it is probable that the precipitation would inhibit sperm attachment to and penetration through the zona and, thereby, inhibit fertilization. Experiments were designed to test the effect of zona precipitation on attachment and penetration of the zona pellucida in vitro by sperm.

Ova in cumulus cells were expressed from oviducts of superovulated hamsters (*8*) into culture dishes (35 by 10 mm, Falcon Plastics) filled with mineral oil. The ova in cumulus cells were maintained in separate culture dishes and treated for 20 minutes before insemination with 0.1 ml of one of the following: normal rabbit serum, physiological saline, Tyrode solution (Difco), unabsorbed antiserum to ovary, antiserum to ovary absorbed with ovary, or antiserum to ovary absorbed with small intestine. Each cumulus cell mass containing ova was then washed several times with 0.15 ml of Tyrode solution to remove any unreacted serum components and cellular debris. The ova were then inseminated with a sperm suspension prepared from the epididymides of mature male hamsters. To obtain sperm we placed the epididymis under mineral oil and made a small incision in the caudal portion. Sperm were removed from beneath the mineral oil with a fine pipette and the concentration was adjusted by dilution in Tyrode solution. The concentration ranged from 12×10^6 to 57×10^6 sperms per milliliter (average 30.3×10^6) but was constant for each experiment.

For insemination, 0.05 ml of sperm was added to the ova under mineral oil, and the mixture (sperm plus ova in 0.15 ml of Tyrode) was incubated in culture dishes at 37°C for 5 to 6 hours (*10*). At intervals during the incubation period the ova were examined with bright-field and phase-contrast optics for any morphological changes. All sperm-egg mixtures were observed to exhibit motile sperm after 6 hours of incubation, cumulus cells dispersed from ova approximately 1 to 2 hours after insemination, and numerous sperm-egg collisions at the zona surfaces. The frequency of sperm-egg collision in all groups resulted in the ova being gently shaken by the vigorously swimming sperm. At the conclusion of the incubation period, the ova were placed on a microscope slide, under a cover slip supported by four Vaseline spots, and examined. The number of sperm attached to the zona were counted by making a through focus series on each ovum. Very few spermatozoa were observed attached to the zona of ova treated with the zona precipitating antibody (see Fig. 1b and Table 1). The few sperm that were attached to the precipitated zonae usually made contact with only a small portion of the convex region of the sperm head and could be easily removed by washing the ova with saline. Conversely, numerous sperm were attached to zonae in ova treated with either normal serums or antiserums absorbed with ovaries (Fig. 1c and Table 1). Sperm in these control treatments were firmly attached to the zonae, as evidenced by their greater degree of sperm surface to zona contact, depth of penetration into the zona, and inability of sperm to be removed by a saline wash of the inseminated ova. These results indicate that the inhibition of sperm attachment was of immunological origin, since the same antiserums that precipitated the zona also blocked sperm attachment; and, absorption of antiserums with ovary neutralized the inhibitory effect of the antibody.

Since precipitation inhibited sperm attachment to the zona, it follows that such treatment might also prevent passage of the sperm through the zona. Thus, additional experiments were performed to determine whether zona precipitation would prevent sperm penetration through the zona and thereby inhibit fertilization in vitro. Ova treated according to the method described above were examined for indications of sperm penetration thorugh the zona pellucida 6 to 8 hours after insemination (Table 1). Zonae in none of the ova treated with zona precipitating antibody were penetrated compared to 48.3 and 76.3 percent penetration in control ova treated with normal serums or with ovary-absorbed antiserums. Control treatments did not inhibit viability of inseminated ova, in culture, since changes in the nucleus (pronuclear development) occurred (Fig. 1d). Thus, sperm do not attach to or pass through zonae that have been precipitated by antibody (Table 1). Our data are the first to demonstrate inhibition of sperm-egg interaction in a mammalian system with the use of an antibody specific to an ovarian component.

The precise role of the zona pellucida in early stages of fertilization has not been established (*11*). It is obviously important during the initial contact between the egg and sperm since the spermatozoan must penetrate this noncellular coat to reach the vitellus of the ovum. Thus any alteration in the physical or chemical properties of the zona that interferes with attachment or penetration of the sperm could be an effective means of contraception. Inhibition of gamete fusion in vivo, resulting from precipitation of the zona by specific antibody, would be an attractive means of fertility control for several reasons including the fact that such a mechanism would presumably be free of endocrine and other side effects since the zona (antigen) has no known counterpart in body tissues other than the ovary. Also, inhibition of sperm-egg interaction by the zona precipitating antibody may not interfere with sperm attachment and penetration in subsequent ovulations, should conception be desired.

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