Synchronous Oogenesis in *Engystomops pustulosus*, a Neotropic Anuran Suitable for Laboratory Studies: Localization in the Embryo of RNA Synthesized at the Lampbrush Stage

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ABSTRACT An investigation of several tropical frogs was undertaken in order to find species in which synchronous oogenesis takes place, so that the RNA synthesized at the lampbrush stage could be labelled and followed through early development. The reproductive cycle of *Engystomops pustulosus* was found to include both synchronous and asynchronous phases. Laboratory maintenance and induction of synchronous oogenesis in this organism are described. By using toads carrying out synchronous oogenesis, RNA synthesized during the lampbrush stage was shown to be retained through gastrulation. The pattern of new RNA synthesis in the embryo resembles that in other anurans. Radioautographs suggest that the distribution in the embryo of maternal RNA synthesized at the lampbrush stage is distinct from that of RNA newly synthesized by the embryo; the possible implications of these observations are discussed.

The mature ovarian oocyte contains a large store of non-ribosomal RNA's synthesized long previously during the lampbrush phase of mid-oogenesis (Crippa et al., '67; Davidson et al., '66; Davidson and Hough, '69a). Encoded in these RNA's is an enormous diversity of genetic information sufficient, in *Xenopus*, for example, to represent perhaps 5×10^4 *different* genes the size of that coding for the β-chain of hemoglobin (Davidson and Hough, '69a,b). At least a large portion of the informational RNA molecules present in the oocyte at the end of oogenesis are destined to survive the complex events of ovulation, and to be inherited by the embryo. Not until late in gastrulation does that fraction of the inherited maternal RNA sequences which has been studied begin to disappear. Some maternal RNA sequences are still present in mid-gastrulation, which is as far as available studies extend in amphibians (Crippa et al., '67; Crippa and Gross, '69). In the sea urchin some evidence exists to suggest that maternal informational RNA's persist beyond gastrulation into the pluteus stage (Whiteley et al., '66). In addition to serving as templates for the various proteins needed directly for the construction of the early embryo, it has been proposed that certain RNA's from oogenesis may perform a regulatory function in the control of early embryo gene activity (Davidson, '68; Britten and Davidson, '69). Thus the actual molecular disposition of the RNA's inherited from oogenesis by the embryo is a matter of considerable interest for the study of early development.

A persistent difficulty in investigating the fate of maternal RNA in embryogenesis has been inability to label this RNA at the time of its synthesis in the lampbrush stage oocyte and then to follow the labelled population of RNA molecules into the embryo. Gross et al. ('65) and Tyler ('67) labelled the RNA of sea urchin oocytes in situ by injecting isotope into females following an induced ovulation, and then investigated the labelled RNA's found in the unfertilized eggs presently shed by those animals, but it is likely that these studies concern only the RNA's synthesized in the terminal phases of oogenesis (Davidson, '68. In amphibians such as *Xenopus* it is not possible to label only lampbrush stage oocytes since oogenesis is asynchronous and continuous. In other words oocytes of all stages develop simultaneously, at rates apparently controlled by the hormonal state of the animal. Furthermore, the *Xenopus* female, at least under the conditions in which it is main-

tained in the laboratory, frequently does not shed *all* of its mature oocytes at any given ovulation. Thus mature oocytes are often observed in a state of atresia indicating resorption. Oogenesis is slow in *Xenopus*, as in most amphibians, requiring as long as eight months for the period between the onset of the maximum lampbrush stage and the completion of preparations for ovulation. Asynchrony of oogenesis means that the egg clutches produced by laboratory *Xenopus* females will contain eggs which have been mature for various periods of time. Hence it is impossible, irrespective of when one might label a *Xenopus* female, to be certain that label present in any given egg of a subsequent clutch was actually incorporated during the lampbrush stage of oogenesis. The project reported in this paper was undertaken in the hope that this difficulty could be circumvented if an amphibian could be found which carries out oogenesis more rapidly and in a synchronous manner. In what follows we describe a successful search for such an amphibian, its adaptation to laboratory conditions, and certain of the general characteristics of its oogenesis and early development. We also present the results of exploratory studies regarding the actual localization in the early embryo of RNA labelled during the lampbrush stage of oogenesis.

The search for an anuran amphibian with synchronous oogenesis

In the initial stages of this endeavor we were guided by the following criteria: The desirable anuran should be one in which direct evidence for synchrony of oogenesis is available; its oocytes should contain clearly recognizable lampbrush chromosomes; it should ovulate a sufficient number of eggs to permit biochemical operations to be performed on a given egg clutch; it should be adaptable to laboratory conditions, and if possible be able to carry out the complete life cycle in culture without special or sophisticated equipment being required; and it should be responsive to commercially available hormone preparations to induce ovulation, or should otherwise provide the opportunity of obtaining eggs at the desire of the investigator. Because of the great number of species available, and because of the long seasonal interruptions in oogenesis in hibernating temperate zone amphibians, it was decided to carry out a species survey in a tropical location. Thus an initial screening survey of over twenty anuran species was carried out in Panama, with the kind cooperation of the Smithsonian Tropical Research Institute located there. We are greatly indebted to this organization, which has made available to us the facilities required for our field operations in four expeditions to Panama.

In table 1 some characteristics of oogenesis in ten relatively common species representing eight of the genera we investigated are summarized. The observations of table 1 were made in June of 1965, during the breeding season for rainy season breeders. The third and fourth columns of the table provide an index of the variability of the systems of oogenesis used by these anurans. Some, such as *Eleutherodactylus*, *Colostethus*, and *Dendrobates*, mature only a very few oocytes at a time, while *Bufo marinus* and *Leptodactylus* produce enormous clutches of eggs. The ovary of *L. pentadactylus* may contain thousands of mature oocytes. Most significant for us is the suggestion in the data of column 3 that in several species oogenesis may be carried out *synchronously*, in that the ovary of these species appear to contain either mature oocytes (stage 6) and stage 4 *or* stage 5, but not both. In the case that ovulation results in the shedding of all the mature (stage 6) oocytes present, synchrony would mean that a group of oocytes traverses the lampbrush stage (stage 4) together, completes its maturation together, and is then shed. Such a group would constitute the *complete* clutch of eggs shed by the female at any one time. Synchronous oogenesis should provide the desired opportunity to label the oocytes at the lampbrush stage and later obtain a clutch of fertile eggs, all of which would bear the label in the RNA at an overall specific activity comparable to that of the RNA in the original lampbrush stage oocyte population. Table 1 shows that several of the listed species appear to have this characteristic while others do not.

TABLE 1

Some Panamanian anura investigated: oogenesis in the early rainy season

Species (typical weight female in g)	Location taken	Stages of oocyte present [1]	Character of ovary	Location and character of nucleoli at various oocyte stages	Presence and character of lampbrush chromosomes [2]
Hyla ebraccata (1.8)	Frijoles, C.Z.	1, 2, 3, 4, 5; shedding complete, leaving no st. 6	all stages of oocyte visible in each lobe of ovary. Eggs small in size	on wall of nuclear membrane until st. 3; then released to center of nucleus. Irregular in shape at all stages (figs. 1B and 1D)	barely visible
Hyla rosenbergi (27.3)	Frijoles, C.Z.	1, 2, 3, and 6 only	st. 6 eggs packed together very tightly; oocyte nuclei very small; observation of young oocytes difficult	ring nucleoli visible in st. 2 (fig. 1E)	present but thin and visualized with difficulty. By st. 6 chromatin heavily bunched (fig. 2C)
Agalychnis callidryas (9.2)	Cerro Campana R.P.	all stages present; shedding incomplete, leaving some st. 6	young oocytes very easily separated and observed	nucleolar coalescence occurs at membrane in st. 2 (fig. 1A)	large and highly extended
Colostethus inguinalis (1.7)	Cerro Campana R.P.	1, 2, 3, 4, 5, 6	disproportionately tiny ovary with few eggs	very small number of nucleoli	barely visible
Eleutherodactylus fitzingeri (9.6)	Barro Colorado C.Z.	only st. 4 and st. 6 in number	eggs are enormous in size, relatively few in number	nucleoli of the compound, lobular type	not visible

TABLE 1 (Continued)

Species (typical weight female in g)	Location taken	Stages of oocyte present [1]	Character of ovary	Location and character of nucleoli at various oocyte stages	Presence and character of lampbrush chromosomes [2]
Leptodactylus pentadactylus (310.0)	Gamboa, Colón	1, 2, 3, 4, 6 or 1, 2, 3, 5	large ovary densely packed with thousands of eggs. All stages easily prepared	nucleoli form as tiny globules on membrane at st. 1–2; become spherical, dispersed through nucleoplasm in st. 3 (fig. 1C)	large and clearly visible (fig. 2A)
Engystomops pustulosus (2.9)	Gamboa, Colón Taboga, R.P.	1, 2, 3, 4 or 1, 2, 3 and 6 or 1, 2, 3, 5 or 1, 2, 3, 4, 5, 6	small ovary bearing several hundred st. 5 or st. 6 eggs. Individual oocytes easily separated at all stages	enormous numbers of nucleoli; nucleolar generation appears to continue through st. 3	dense and bushy in appearance (fig. 2B)
Bufo marinus (535.0)	Taboga, R.P.	1, 2, 3, 4, 5 or 1, 2, 3, 4, 6	large ovary, individual oocytes easily separated at all stages	some nucleoli appear attached to chromosomes (fig. 1F)	large and clearly visible
Bufo typhonius (8.4)	Barro Colorado C.Z.	1, 2, 3, 4 and 6, 1, 2, 3, 4, 5 or 1, 2, 3, and 6; i.e., never 4, 5, and 6 in same ovary	excellent ovary for separation and observation of young oocytes	nucleoli arise at membrane in st. 1–2, are released in st. 3	large and clearly visible
Dendrobates auratus (3.0)	Taboga, R.P.	1, 2, 3, 4, 5, 6 all present	only 3–6 mature eggs per female; eggs very large in size	only a few dozen nucleoli per oocyte	very minute

[1] The staging system of Duryee ('50) is used, according to which the lampbrush chromosome begins to appear in stage 3, and reaches its maximum extent in stage 4. Vitellogenesis occurs primarily in stages 4 and 5, and by stage 6, when the oocyte attains maturity, the chromosomes have retracted and condensed. At this point they are inert in RNA synthesis.

[2] As seen in epon sections.

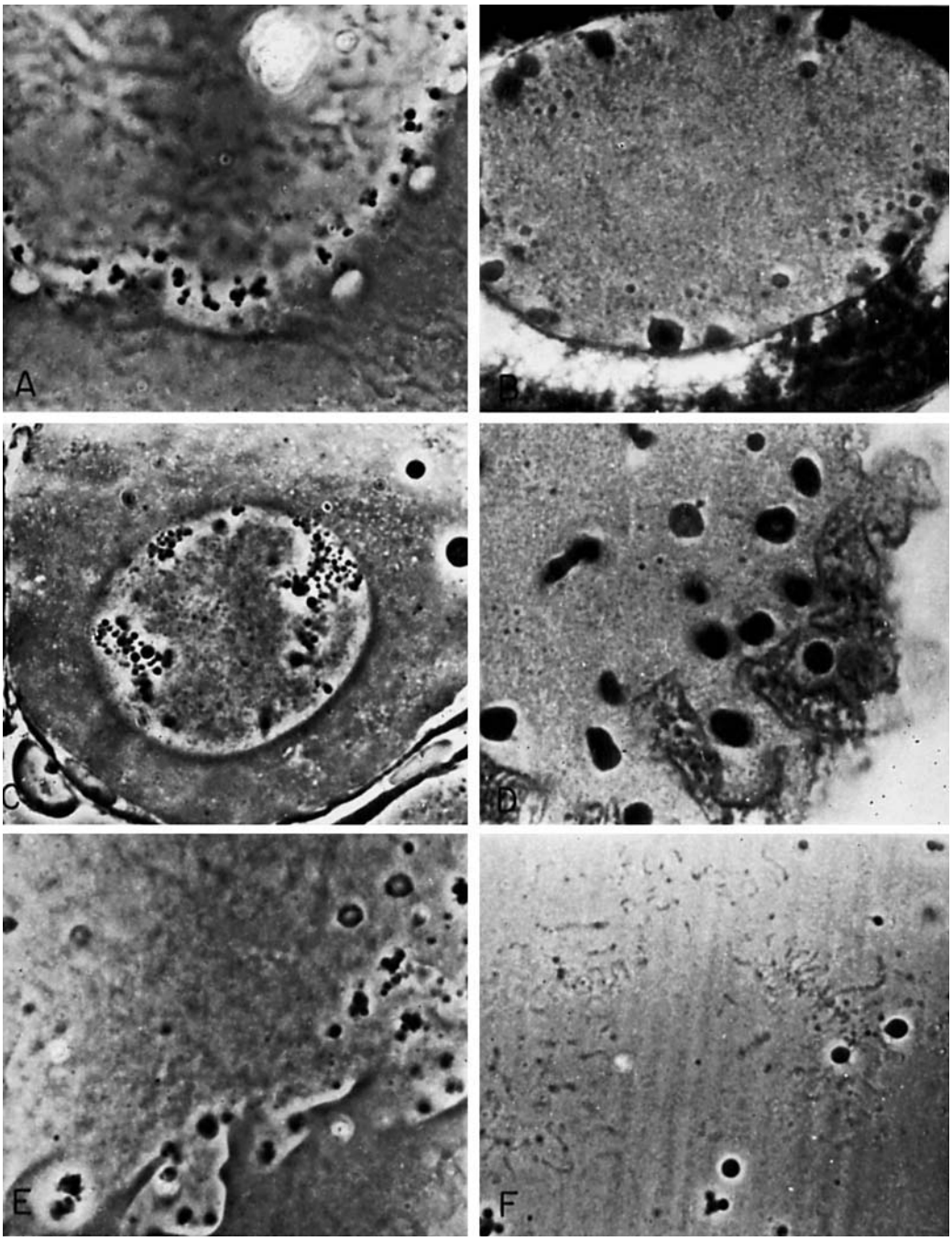

Fig. 1 Extrachromosomal nucleoli in early oocytes; sections prepared as described under figure 5. (A) Nucleoli close to the nuclear membrane in a stage 4 oocyte containing lampbrush chromosomes, in *Agalychnis callidryas*. (B) Stage 2 oocyte, *Hyla ebraccata*. (C) Stage 2 oocyte, *Leptodactylus pentadactylus*. (D) Stage 4 oocyte, *Hyla ebraccata*. (E) Stage 2 oocyte of *Hyla rosenbergi*, containing ring nucleoli. (F) Stage 4 oocyte of *Bufo marinus*, showing a pair of nucleoli attached to a lampbrush chromosome.

Figure 1 illustrates special features of the extrachromosomal nucleoli in some of the oocyte species considered in table 1 (column 6). Extrachromosomal nucleoli can be seen arising at the rim of the nucleus in early *Agalychnis* and *Hyla* oocytes (figs. 1A,B respectively), as described by many workers for various other anuran and urodele nucleoli. In stage 2 *Leptodactylus* oocytes what appear to be nascent nucleoli are present near the membrane in dense clouds composed of individual nucleoli of various sizes (fig. 1C). Large irregular nucleoli, relatively few in number, can be seen in the small stage 4 oocytes of *Hyla ebraccata* (fig. 1D) and in *Hyla rosenbergi* ring nucleoli (Lane, '67; Peacock, '65; Tyler, '67) can be observed as early as stage 3 of oogenesis (fig. 1E). In *Bufo marinus* (fig. 1F) pairs of nucleoli can be observed attached to certain lampbrush chromosome loops, perhaps representing the nucleolar organizer loci, as in *Triturus* (Callan and Lloyd, '60). These features are noted here as they may be of use to other workers, though they do not otherwise figure in the studies described below.

The various species surveyed revealed a tremendous variation in the form and size of the lampbrush chromosomes (column 7, table 1). Unfortunately genome sizes are not known for any of the organisms listed in table 1 other than *Bufo marinus*, as far as we are aware, and it is thus not possible to consider correlations between lampbrush chromosome size and DNA content per cell. The "classic" lampbrush chromosome of *Leptodactylus* is shown in figure 2A. In *Engystomops* the lampbrush chromosomes are bushy and less extended (fig. 2B). In general the pattern of events described by Duryee ('50) is adhered to, and following stage 4 the oocyte chromosomes condense as preparation for the meiotic reduction divisions which follow ovulation. The condensed chromosomes of a stage 6 oocyte of *Hyla rosenbergi* are shown in figure 2C.

As a result of the species survey a choice was made in favor of *Engystomops pustulosus*. The main factors leading to this decision were (a) the great availability of this animal, including females at breeding sites in accessible, shallow water; (b) the preliminary oocyte stage data suggesting the occurrence of synchronous oogenesis; and (c) the size of the ovary and the typical egg clutch. *Engystomops* females lay

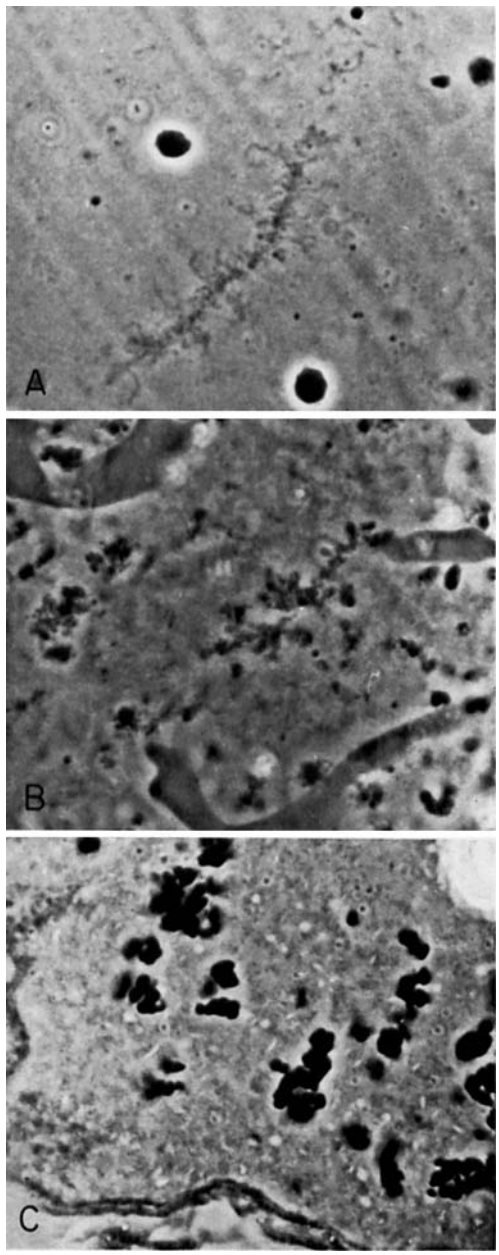

Fig. 2 Lampbrush chromosomes of some anuran species. Stage 4 oocytes (A) *Leptodactylus pentadactylus* and (B) *Engystomops pustulosus*. (C) Stage 6 oocyte, *Hyla rosenbergi*, showing condensed chromosomes.

from 100 to 500 eggs at a time, depending on their size and on prior conditions, as shown below. The choice of this species has proven to be a fortuitous one, and at the present time we can list these additional advantages: (d) the eggs develop rapidly with a high degree of synchrony, sometimes displaying no more than a 20 minute spread between the slowest and the fastest embryos throughout cleavage in a given clutch; (e) the embryos are permeable to uridine-H^3 in the medium in which they develop, after partial dejelling; (f) *Engystomops* can be carried in the laboratory through complete breeding cycles, embryogenesis, metamorphosis, and into sexual maturity; (g) mating, providing the female is ripe, can be induced at will. As becomes evident in the next two sections of this report, these features can be considered the consequence of the special adaptations which enable this organism to deal with the restricted environmental system in which it carries out its reproduction.

Breeding and reproduction of Engystomops

Engystomops (or *Eupemphix*) *pustulosus* belongs to the family Leptodactylidae (Myers, '69; Oliver, '55), a group closely related to the Bufonidae, in which in fact, the genus has been included by some authorities (Breder, '46; Noble, '31). Its range extends from Central Mexico to Eastern Colombia and Venezuela. Like some Bufonidae (e.g. *Bufo*), *Engystomops* lacks both maxillary and vomerine teeth. However, it has certain skeletal characteristics, e.g. bony mesosternum, which link it to other Leptodactylidae and separate it from Bufonidae, in which the mesosternum is absent or cartilagenous (Noble, '31). This morphological alliance with the Leptodactylidae is of interest because among the *Engystomops* features of particular interest to us here is a characteristic which is widespread in the Leptodactylidae. Some genera of this family make use of a peculiar and distinctive method of egg deposition: the eggs develop within a mass of oviducal jelly which is whipped into an airfoam by the beating hind legs of the male. In *Engystomops* egg laying normally occurs at night, and the process of building the foam nest requires at least a half hour. The eggs develop suspended in the foam mass over the water in which the toads have mated. This distinguishing feature is among the significant adaptations which have permitted the species, to quote Noble ('31), "to take advantage of the limitations of their mode of life." Detailed descriptions of mating behavior and habitat of this species are available elsewhere (Breder, '46; Cope, 1864; Sexton, '66). What follows is drawn mainly from personal observations and is concerned with those aspects of reproductive behavior relevant to the laboratory culture of this species and to the studies of oogenesis and early development reported below.

Engystomops breeds mainly in *temporary* water, i.e., in the puddles appearing after heavy rain in mud ruts, in grassy and cane-bearing areas subject to partial flooding after heavy rains, in ditches or in small peripheral pools remaining near jungle streams during the rainy season. The obvious advantage in utilizing temporary water is avoidance of a variety of aquatic predators for which the small toads, their eggs, or their tadpoles might be an easy mark. Among these are the fish and reptiles which are the regular denizens of the permanent pools and ponds in the range occupied by *Engystomops*. Utilization of temporary water is intrinsically hazardous, however; many times we have observed mating pairs of *Engystomops* producing egg masses at sites which were destined to become bone dry within the following day. The temporary water chosen by *Engystomops* for egg deposition is frequently out in the open, without shade, and though the animals usually attempt to take advantage of the limited cover available — the roots of a tuft of grass, an overhanging mud bank, the shadow of a fallen leaf, etc. — there is often no protection against direct sunlight and the total desiccation which often threatens if there is a one- or two-day pause in the daily schedule of rains. Such pauses are common during the breeding season in the areas where we have collected *Engystomops*. At the swimming tadpole stage of development the embryos hatch out from

their individual jelly cases and descend through the foam mass into the underlying water, where they develop until metamorphosis. The shortest time in which we have observed metamorphosis to occur is about three weeks from hatching. Thus successful reproduction requires that water be present beneath the foam nest after hatching, although tadpoles can survive up to five days in a stranded nest (Rand, '69). Water must be accessible to the swimming tadpoles, if only an inch or two deep, for a period of several weeks thereafter. In order to utilize the relatively (though by no means completely) predator-free breeding environment offered by temporary water, several special aspects of the airfoam nest and of the nest building process are of crucial utility: (a) The outer surface of the nest tends to harden in the sun, thus providing a partially reflecting surface which decreases heat absorption. Since it is porous and foam-like in structure, however, the nest provides an enormous surface area for evaporation and probably acts to prevent overheating in this way as well. In the sun the interior of a nest which is properly located over water seems cool compared to the outside and is always moist. Thus the foam nest can provide a tolerable environment for an amphibian egg even though unshaded from the hot tropical sun. (b) Nest building tends to take place in the first half of the night, often beginning a few hours after darkness. Few new nests are initiated after one or two AM. Mating occurs after the males have attracted gravid females to potential nesting sites by calling, and this process requires some hours. The calls are complex in their informational content, according to the studies of Rand ('69) and indicate the density and level of excitement of the calling population of males. Calling behavior plays a critical role in the mating of *Engystomops* and as such represents one of the main evolutionary specializations of this species. Though beyond the scope of our immediate interest, casual observation of calling in our laboratory colony of *Engystomops* suggests that it continues in a normal fashion there. In the wild, calling begins shortly before dark or with the onset of darkness. If pair formation were to occur earlier, in daylight, the defenseless and relatively immobile mating pair would presumably be easy targets for predatory birds, which single calling males attempt to avoid by diving instantly upon perception of any moving overhead shadow. In other words, given the time required to set up and operate the vocal mate attraction system, fertilization and the beginning of development is programmed to occur about as early as possible. In this way the average embryo need be exposed to only one day of sunlight before it attains mobility itself. By dawn of the first day it is already entering gastrulation; by evening it is in the muscular twitch stage, and by the second dawn hatching is under way in optimal circumstances. Once hatched the tadpole can swim to contiguous wet areas if threatened with desiccation. The rapidity and timing of early development within the temporary protection afforded by the airfoam nest represents a critical aspect of the adaptation of *Engystomops* to temporary water breeding. (c) A further important characteristic of the breeding behavior of this species and one which is of significance in dealing with this animal in laboratory conditions, is its choice of when to breed. Mating occurs with the highest frequency one or two days after a heavy, flooding rain. It does not occur in the dry season and thus appears to be determined primarily by the availability of temporary water.

Laboratory conditions and methods for Engystomops

Engystomops can be maintained relatively easily in laboratory culture. A room equipped with a tile or other water resistant floor containing a drain, and a sink, is required, and temperature, light and humidity must be controllable. In our colony the animals live free on the floor of the room, which is covered with autoclaved earth beds about six inches deep, bounded by wooden restraining walls. In the earth we have planted *Phyllodendron*, a commonly available vine which grows profusely under our conditions. The earth beds are separated by alley ways from which one can reach any area of the earth

without difficulty. Illumination is provided by four fluorescent light banks each containing four G.E. F400W cool white 3-plus tubes suspended seven feet over the bed surface, and operated on a 12-hour light cycle, 9 AM to 9 PM. The room is kept at 80°–82°F (floor temperature) by a thermostated combination of heaters and air conditioners, and its humidity is maintained at 75–80% by a standard household humidifier (Toastmaster Automatic Humidifier). The room is 8 × 12 feet, and has housed populations ranging from 50 to over 400 individuals. Tap water runs slowly into a large tank, where it dechlorinates, and is then pumped gradually into several smaller tanks where tadpoles and metamorphosing animals are raised. This water system is also available for temporarily flooding the areas of the floor when desired, and when this is done several artificial ponds are created both in the earth and within special enclosures where given pairs of toads can be placed.

Mating can be induced simply by flooding the room. Males begin to call in the afternoon if the room is flooded by mid day, and gravid females, as in the wild, form pairs with calling males and produce eggs usually by 1 or 2 AM. Occasional deviations from this schedule are observed, such as nests still in the process of being built at 7 AM., and exceptions occur as well in the natural habitat (Rand, '69). Amplexus can sometimes be observed for one or two days preceding egg laying. If presented with an unripe female a male will frequently clasp her and remain in amplexus for such long periods despite her inability to ovulate.

Engystomops can also be induced to ovulate with commercially available chorionic gonadotropins (Parke-Davis "Antuitrin") just as can *Xenopus*. Most of the anura listed in table 2, on the other hand, are non-responsive to this hormone. The dose used with *Engystomops* is 0.05–0.10 cc of the dissolved preparation per female. If the female is gravid, ovulation can be induced by injecting the hormone under the skin of the lower back. Injection of the same dose, at least in some cases, induces males to call at abnormal hours, e.g. early morning, and stimulates attempts by one male to engage in amplexus with other males, with attendant utterance of high pitched, short calls associated with over crowding or undesirable male-male interaction.

When required for embryological studies the eggs can be removed from the air-foam nest by placing the latter over 2.5% Na thioglycolate, dissolved in 10% solution X of Barth and Barth ('59), (a simple balanced salt solution), and titrated to pH 8.6. After brief stirring at room temperature they detach themselves, fall out of the foam, and collect at the bottom of the thioglycolate solution. Exposure to this solution also removes the major portion of the individual jelly coats in which the eggs are encased. No deleterious effects on later development have been noted to result from thioglycolate treatment. The dejelled embryos can be raised in 10% solution X.

TABLE 2

Oocyte stage in ovaries from typical wet area females (collected in Canal Zone) and from dry area females (collected in Chitré) in early July 1966

Location	Animal	Stage of oocyte					Total number of stage 4, 5, and 6 oocytes
		3–4	% of total	5	% of total	6	
			%		%		
C.Z.	1	206	76	65	24	0	271
	2	180	77	54	23	0	234
	3	142	78	40	22	0	182
	4	399	84	78	16	0	477
Chitré	1	237	96	9	4	0	246
	2	206	92	17	8	0	223
	3	284	100	0	0	0	284

Tadpoles are easily raised in a tank of dechlorinated, slowly running tap water overlying earth. In the earth *Tubifex* worms are allowed to grow, and as an additional source of food the tadpoles are given powdered nettle leaves (S. B. Penick Co., Chicago) several times a week. After their hind legs appear they are transferred to slanted plastic tanks designated "metamorphosis tanks," and built according to the design for that purpose published by Chrapliwy ('57). Metamorphosis is typically complete within a few days, and the baby toads at this point are transferred to an aquarium where they are fed small aphids (*Acythosiphon bisun*), which are grown on bean (*Vicia faba*) seedlings. They eat this food voraciously, and after several weeks have grown to a size enabling them to take baby crickets as well as aphids. They are then transferred to an "adolescent" tank where both food sources are available. On development of recognizable secondary sexual characteristics they are considered adult and can be released to join the adult population. At this time, a minimum of 2–3 months from metamorphosis, they are capable of reproducing, and are one-half to two-thirds the average length of fully grown adults. The adult population is fed baby crickets, which are obtained commercially on a regular schedule from Selph's Cricket Ranch, in Memphis, Tenn. A supplemental source of food is baby cockroaches emerging from a large cockroach colony maintained in a covered plastic vessel with egress holes for the roaches at the bottom. In our experience the crickets alone suffice to maintain the adult toads in an apparently healthy state.

Mortality has occurred in our colony from two known causes: infection with a hookworm-like nematode which is apparently endemic in the wild population, and exposure to earth treated with insecticides. The hookworm is a parasite of the intestinal tract and was identified by Dr. George Jackson of this University as *Ozwaldocruzia*. This organism has a free-living form which requires moist earth. Therefore it can be controlled either by periodically drying the earth in and on which the toads live or simply by changing it. Valuable females in our laboratory are kept in tanks in which the earth is changed every three–four weeks.

Engystomops does not populate areas adjacent to its habitat which lack a dry season, e.g. the Bocas del Toro region of Panama or the Pacific Choco of Colombia. Our laboratory colony was maintained in "rainy season conditions" for about a year and a half with continuous breeding occurring in the population. After this, however, the females began to give infertile eggs or to fail to complete oogenesis at all, a condition we discovered to be correlated with the nematode infection, which soon became lethal. (We do not know, however, that the eventually mortal illness of these females caused by the hookworms was actually the cause of their infertility.) An essential role for the dry season in the ecology of *Engystomops* is thus suggested, *viz.* control of an endemic parasite which, as we have seen, can have disastrous effects on the *Engystomops* population. Nonetheless, it would seem that a major environmental change such as the coming of the dry season should play a more subtle and complex role in the highly adapted life cycle of *Engystomops* than this, though except for the onset of hookworm disease our laboratory populations have shown no recognizable ill effects from prolongation of wet conditions 3–4 times beyond their normal duration. The property which renders *Engystomops* valuable to us, synchronous oogenesis, however, can be viewed as a particular adaptation to the occurrence of the dry season. As we now describe, synchronous oogenesis can be induced in laboratory females by the imposition of dry conditions.

Synchronous and non-synchronous oogenesis in Engystomops

As the rainy season progresses it is advantageous for an amphibian temporary water breeder to be able to produce as *many* egg masses as possible, at the most frequent intervals the nature of the process will allow, so that the animal can maximize the possibility of depositing an egg mass in a place where the tadpoles will be able to survive. A basic limitation on egg mass size is that of space; only a certain number of yolk-filled stage 6 (mature)

oocytes can fit in the ovary at once. During the rainy season it would seem efficient for an animal such as *Engystomops* to have a supply of stage 5 oocytes ready for rapid advancement into stage 6 following ovulation, and at the same time a supply of stage 4 oocytes being prepared to enter stage 5, etc. This would result in a *non-synchronous* pattern of oogenesis in which stage 4, 5, and 6 oocytes are likely to be found in the ovary at the same time, and the interval between successive egg clutches is equal only to the time required for a clutch of stage 5 oocytes to complete vitellogenesis and become stage 6 oocytes. During the long dry season, on the other hand, no temporal pressure to complete oogenesis necessarily exists, and a different pattern of oogenesis occurs. This difference is illustrated in table 2, which records the numbers of oocytes of various stages present in recently ovulated females collected in two areas of Panama early in the rainy season. Females from the relatively wet Canal Zone region were in their mid-rainy season cycle. The second group of animals was collected at Chitré, on the border of one of the lowest rainfall areas of Panama. These animals were taken at what appeared to be the very margin of the *Engystomops* population range. Thus searches of likely breeding sites beyond Chitré failed to turn up any *Engystomops* egg nests, nor could calls be heard in this area. Immediately east of Chitré, however, a thin population of *Engystomops* was observed. The rains had begun not long before when the animals of table 2 were taken. Table 2 shows a sharp difference between the two groups: The Canal Zone females all possess a significant number of stage 5 oocytes, while the Chitré females possess hardly any stage 5 oocytes. As also evident from table 1, ovulation in *Engystomops* appears to have been complete in both groups, so that no stage 6 oocytes are left in the ovaries.

Table 2 suggests that under wet conditions stage 5 oocytes are being prepared even before the stage 6 oocytes are ovulated, while in the dry conditions which had only recently ended for the Chitré animals all the available oocytes were stored at stage 6 in preparation for the onset of breeding conditions. According to this hypothesis *synchronous oogenesis* would now ensue in the latter group of females, with a group of oocytes maturing together from the lampbrush stage (stage 4), through stage 5, stage 6, and ovulation. At any point until this next ovulation the oldest group of oocytes in the ovary would be among those which had been left in stage 3 or early stage 4 when the dry season accumulation of stage 6 oocytes had been completed. Data obtained in our laboratory colony have turned out to be consistent with this picture of events for females exposed deliberately to wet and dry conditions.

Table 3 presents oocyte counts in 21 laboratory females held under wet conditions after undergoing ovulation. By wet conditions we denote sufficient flooding in the colony room to promote calling by males and pair formation with the gravid females which are available to these males, with resulting egg laying. It can be seen that the number of stage 3 and stage 4 oocytes remains about the same during the six weeks following ovulation, while between the second and third week the number of stage 5 oocytes increases sharply. Within the next several weeks these stage 5 oocytes move rapidly into stage 6; the numbers in table 3 are such that it must be concluded that several batches of oocytes traverse stage 5 (and therefore enter stage 5) and become mature stage 6 oocytes within the period 3–6 weeks after ovulation. By six weeks these females have stockpiled large clutches of mature oocytes. In this laboratory population ovulation can actually take place anytime after 4–5 weeks following the previous ovulation, but the clutches produced are not as large as the stage 6 oocyte population in the six-week females of table 3.

In laboratory females kept in dry conditions, i.e., never exposed to ponds suitable for nesting, a pattern is observed in which a large clutch of mature oocytes accumulates, and further passage of oocytes into the maximum lampbrush stage (stage 4) from the prior stage (stage 3) is arrested. These contrasting patterns of oogenesis are summarized in table 4.

TABLE 3

Oogenesis in recently ovulated laboratory females under wet conditions

Time	Animal	st. 3	st. 4	st. 5	st. 6	Total	% st. 3	% st. 4	% st. 5	% st. 6
2nd week post ovulation	1	135	14	33	0	182	74.2	7.7	18.1	0
	2	119	50	130	0	299	39.8	16.7	43.5	0
	3	230	42	6	0	278	82.7	15.1	2.2	0
	4	95	34	15	4	148	64.2	23.0	10.1	2.7
	5	95	60	41	0	196	48.5	30.6	20.9	0
	6	87	62	10	0	159	54.7	39.0	6.3	0
	7	104	40	11	0	155	67.1	25.8	7.1	0
	8	126	80	65	0	271	46.5	29.5	24.0	0
	9	85	80	2	0	167	50.9	47.9	1.2	0
	10	100	80	54	0	234	42.7	34.2	23.1	0
	11	85	57	40	0	182	46.7	31.3	22.0	0
Av.		115	54	37	0	206	56.2	27.4	16.2	0.2
3rd week post ovulation	12	100	12	118	3	233	42.9	5.2	50.6	1.3
	13	156	82	75	0	313	49.8	26.2	24.0	0
	14	104	22	80	0	206	50.5	10.7	38.8	0
	15	95	38	35	0	168	56.6	22.6	20.8	0
	16	74	25	82	0	181	40.9	13.8	45.3	0
	17	125	33	102	0	260	48.1	12.7	39.2	0
Av.		109	45	82	0	226	48.1	15.2	36.5	0.2
6th week post ovulation (males withheld)	18	100	50	50	350	550	18.2	9.1	9.1	63.6
	19	155	95	40	520	810	19.4	11.7	4.9	64.2
	20	100 [1]	37	41	226	404	24.8	9.2	10.1	55.9
	21	100 [1]	83	64	187	434	23.0	19.1	14.8	43.1
Av.		113	66	49	321	549	21.3	12.3	9.7	56.7

[1] Estimated ± 10%.

The data available permit one to estimate the time required for each of stages 4, 5 and 6 of oogenesis. This has so far not been possible for any other amphibian species. These rates, and the nature of both wet and dry season cycles, as deduced from our observations on *laboratory females,* are presented in the model of oogenesis diagrammed in figure 3. The detailed restrictions leading to the postulation of this particular model of the oogenesis cycle are given in the legend in figure 3.

The cycle portrayed in figure 3 provides an indication of the procedure required to obtain synchronous oogenesis in *Engystomops.* After ovulation wet-season females are placed in dry conditions for 12 weeks or more, and then exposed to males and breeding sites. The following period, labelled in figure 3 "initial adaptation to wet conditions" is one in which synchronous oogenesis takes place, with the next clutch of eggs developing as a group from stage 3 through the lampbrush stage and to maturity. If an isotopic RNA precursor is introduced a few days after the ovulation with which the dry phase terminates, it is incorporated mainly into lampbrush stage oocyte RNA, and the egg clutch which appears 4 to 6 weeks later bears this RNA. As is evident from figure 3, lampbrush stage oocytes forming later in the wet season do not appear together as a single group of mature eggs. Instead they are "diluted" with ooctyes which have traversed the lampbrush stage in a previous cycle. Thus the period labelled in figure 3 "initial adaptation to wet conditions" is the only period in which the labelling experiment we are interested in can be carried out.

Labelling of RNA in lampbrush stage oocytes under synchronous conditions: Persistence and inheritance of the RNA label in the embryo

Using a labelling schedule designed as the above considerations would indicate, females whose oldest oocytes were at the lampbrush stage were labelled by four injections of uridine-5-H^3. The radioactivity in the cold acid-soluble pool and in the RNA was measured in mature oocytes and

TABLE 4

Oogenesis in laboratory toads under wet and dry conditions

	Wet conditions	Dry conditions
Minimum time between successive egg clutches [1]	30–45 days	80–125 days
Stages of oocyte present in ovary during cycle	1, 2, 3, 4, 5 or 1, 2, 3, 4, 5, 6	1, 2, 3, 5, 6 or 1, 2, 3, 6 or 1, 2, 3, 4, 6
Stages of oocyte present immediately after ovulation at end of cycle	1, 2, 3, 4, 5	1, 2, 3
Size of clutch [2]	80–300 eggs	450 eggs

[1] As estimated by presenting males and water suitable for breeding.
[2] If males are not withheld as in the six week females of table 3.

at the gastrula stage in embryos derived from the labelled oocytes. Gravid females were also labelled at the *end* of oogenesis, and the distribution of radioactivity in these same stages studied. The data from this experiment appear in table 5, which shows that the labelled RNA synthesized at the lampbrush stage is inherited by the embryo and retained therein at least into gastrulation. Thus the remaining acid-soluble counts in lampbrush labelled eggs cannot account for the RNA counts found in the gastrulae since the samples labelled at the end of oogenesis show that less than 10% of the pool counts available to the embryo at the onset of embryogenesis have been incorporated by late in gastrulation. Given the pools measured in the eggs of animal B new incorporation from acid soluble precursor could account for only 10 or 11 CPM of the 160 RNA CPM present. The ratio given in table 5 (RNA-CPM/pool CPM) summarizes the sharp difference in label distribution in these two groups of animals. In table 6 data are presented regarding variation in the labelled RNA content of individual embryos deriving from oocytes labelled at the lampbrush stage. It can be seen that a certain range of labelling rates may be characteristic of each animal, but that wide differences among individual embryos exist. According to results with *Xenopus* (Davidson et al., '64) over 95% of the maternal label should be in ribosomal RNA, and the reason for the specific activity differences is not understood. Possibly they represent differences in follicle vascularization affecting the efficiency of precursor uptake into the follicle. Various other possibilities exist as well. In any case table 6 shows that reasonably high specific activities can be obtained with no particular difficulty, and by introducing a larger amount of isotope even higher specific activities could presumably be attained.

Incorporation of uridine-H³ by dejelled embryos

The use of animals labelled at the end of oogenesis as in the experiment of table 5 is often inconvenient, since the procedure is effective only if the female is gravid with completely mature eggs. If she is still two weeks away from ovulation, for example, some of the precursor present in the clutch ovulated two weeks after isotope injection will have been incorporated into immature oocytes, and some will have been lost from the oocyte in acid-soluble form before embryogenesis ever begins. In *Engystomops*, at least with the limited colony size we have employed, it is difficult to accumulate females which are certainly and completely ripe, and we have made many wrong guesses in choosing animals for late labelling experiments which in fact were not yet ready to yield eggs. It therefore became of interest to find a method of labelling the RNA of eggs during development by a means other than in vivo injection of isotope into the gravid female. A simple procedure turns out to be readily available, for, as mentioned above, the eggs of *Engystomops* are permeable to exogenous uridine once removed from the foam nest and partially dejelled with 2.5% thioglycolate. The eggs are dejelled and washed several times in clean 10% Solution X. After developing to the desired

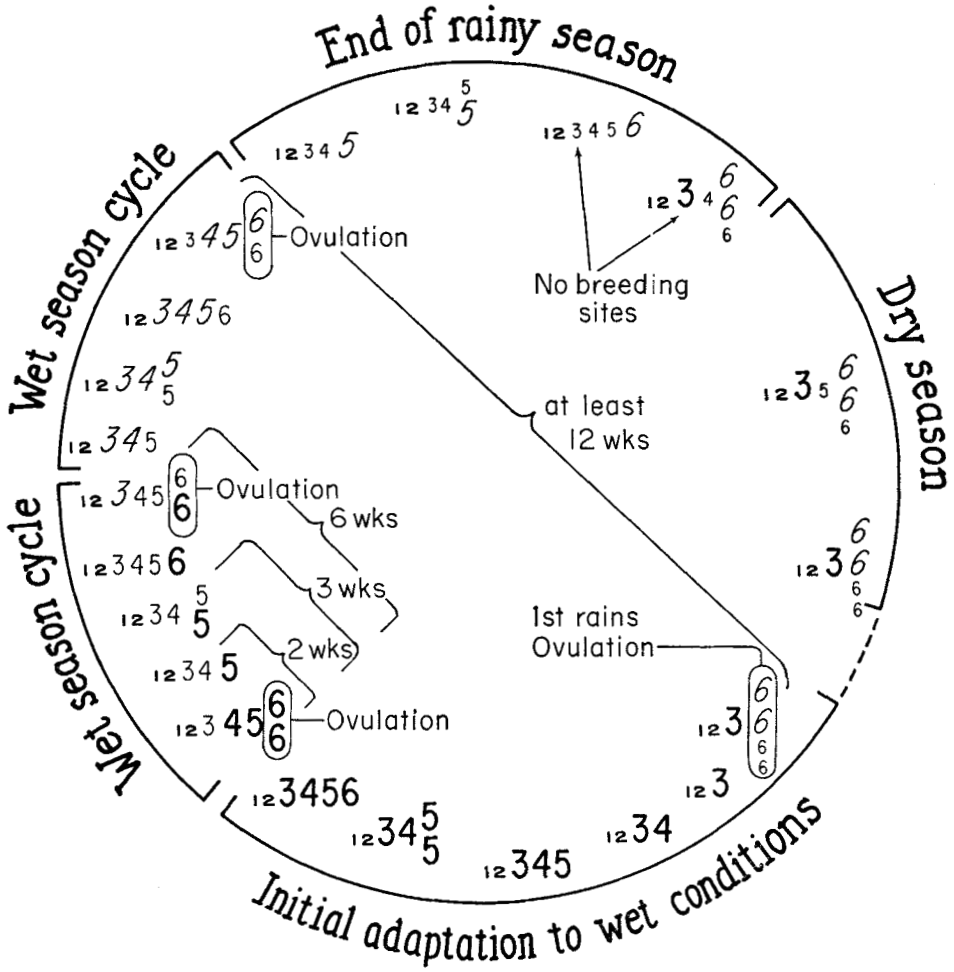

Fig. 3 The rules which any model of oogenesis in *Engystomops* must follow are: (a) new stage 3 oocytes enter only on ripening of stage 6 clutch (table 3); (b) the stage 6 oocyte population under wet conditions is at least the size of the stage 4 and stage 5 populations (table 3); (c) after ovulation in wet conditions stage 5 oocytes transiently increase in number (table 3); (d) the stages of oocyte present in wet and dry toads must conform to those listed in table 4; (e) the initial post-dry season clutch is larger than any wet season clutch; (f) there is continuous input of new oocytes from stage 3 to stage 4 during the wet season.

The model indicates that the major effect of dry season conditions is to stop the flow of eggs from the stage 3 to the stage 4 population.

The number of cycles under wet conditions is not meant to be specified. Two are shown, but the actual number for any given female will depend on the weather, the location, presumably the population, the endocrine equipment of the individual and various other factors.

The time periods in the diagram are those measured *in the laboratory* and may well be shorter in the wild, or in any case different.

stage at room temperature they are placed in a small beaker in 1 cc of uridine-5-H^3 in 10% Solution X, containing 1000 units of penicillin and 400 μg of streptomycin. Figure 4 describes uptake and incorporation of this precursor into embryos at different stages after various periods of incubation at 27°. The embryos become somewhat more permeable as development progresses, but the main change is in rate

TABLE 5

Pool radioactivity and RNA specific activity in oocytes and embryos of Engystomops labelled at the lampbrush stage and at the end of oogenesis

Time of labelling [1]	Animal	Stage of assay [2]	Pool radioactivity cpm/egg [3]	RNA radioactivity cpm/egg [4]	Counts in RNA/pool
Lampbrush stage (4 months before assay)	A	mature ovarian oocyte	74	109	1.47
	B	early gastrula	114	160	1.40
	B	late gastrula	108	157	1.46
Mature oocyte stage (4 days before assay)	C	mature ovarian oocyte	278	10	0.04
	D	early gastrula	817	85	0.10
	E	late gastrula	905	78	0.09

[1] Just-ovulated animals which had been kept under dry conditions were labelled by four injections of 50 μC uridine-5-H^3 (Sp. Ac. 15 C/mM) in the dorsal lymph sac, given at 2-day intervals.
[2] Individual determinations were made on six eggs at each stage, except for lampbrush-labelled mature oocytes, where 18 individual determinations were made, and late-labelled mature oocytes, where 12 determinations were made. Values given are the averages.
[3] Pool radioactivity was determined as total radioactivity present in the supernatants of two successive washes with cold 0.20 N perchloric acid (PCA).
[4] RNA radioactivity was determined as radioactivity insoluble after three washes with cold 0.20 N PCA which was rendered cold 0.2 N PCA-soluble by exposure of the PCA precipitate to 200 μg RNA'se in 100 λ 0.01 M Tris pH 7.6–0.10 M NaCl for four hours at 37°C.

TABLE 6

Some RNA specific activities in individual embryos labelled in the lampbrush stage of oogenesis [1]

Stage of embryo when assayed	Animal	RNA specific activity CPM/μg	Residue CPM [2] / RNA CPM
Gastrula	A	9,208	0.075
		2,795	0.034
		4,126	0.077
		4,227	0.020
		2,981	0.041
		7,260	0.191
Gastrula	B	3,360	
		880	
		812	
		382	
		134	
		126	
		1,128	
Late blastula	C	704	
		278	
		1,095	
		507	
Unfertilized	D	2,123	
		221	
		278	
		608	

[1] Animals were labelled as in table 5 and RNA counts assayed as described there.
[2] Residue CPM refer to cold PCA-insoluble counts which were resistant to RNA'se and were rendered acid soluble by hot 0.5N PCA (70°, 20 minutes) digestion. They may represent incorporation into DNA.

of utilization of the acid-soluble pool. Figure 4 shows that during gastrulation there occurs a sharp increase in rate of synthesis of RNA from uridine relative both to total pool radioactivity and to DNA synthesis. *Engystomops* development is similar in this to other amphibian embryos. *Engystomops* presents two special advantages for studies of nucleic acid synthesis during embryogenesis: (a) the embryo can

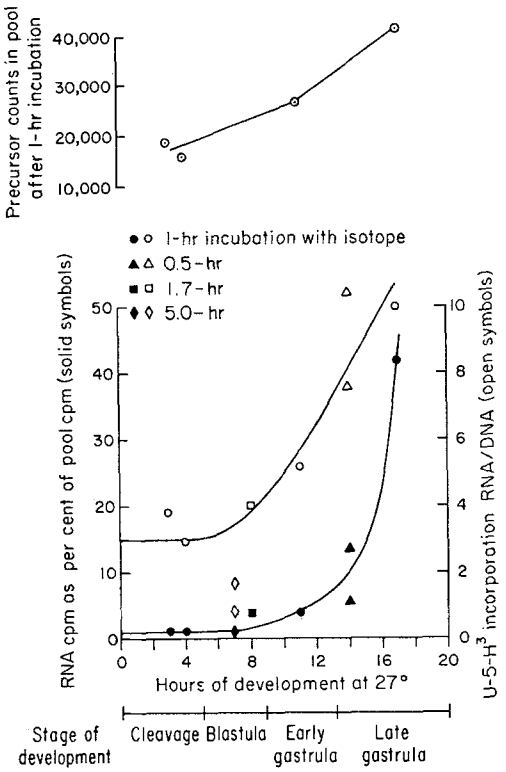

Fig. 4 Incorporation of uridine-5-H³ into dejelled *Engystomops* embryos incubated as described in text. RNA incorporation and pool radioactivity were assayed with the cold PCA-RNA'se procedure detailed in the legend to table 5. Counts incorporated in the cold acid-insoluble fraction and impervious to RNA'se treatment but solubilized by heating in 0.5 N PCA for 20 minutes at 70° were regarded as an index of incorporation into DNA, presumably by route of uridine-5-H³ conversion into deoxycytidine-5-H³.

be labelled simply by immersion in the isotope-containing solution, and (b) it is possible to avoid most of the laborious process of selecting embryos in order to obtain samples at given stages of development, since in each clutch development is almost synchronous. Sometimes as little as 20 minutes separates the slowest from the fastest embryos during cleavage. The reason for the relatively greater permeability of *Engystomops* embryos is perhaps that development in the airfoam egg nest obviates the total permeability barrier required in amphibian eggs which develop immersed in water.

Radioautographic observations on localization in the embryo of RNA labelled during oogenesis

A series of embryos was obtained from eggs which had been labelled with uridine-5-H³ at the lampbrush stage, and these were used for radioautography. Other embryos were studied in which the isotope had been injected at the end of oogenesis, immediately before ovulation (compare table 5). The embryos were fixed and embedded in epon as described in the caption to figure 5, and radioautographs such as those photographed in figure 5 made from 1 μ sections. The results, which are tabulated in tables 7 and 8, are interesting in several respects.

Most of the grains necessarily represent ribosomal RNA, since this is by far the major stable RNA component in which precursor is incorporated during the lampbrush stage of oogenesis (in *Xenopus* about 95% of label incorporated is ribosomal (Davidson et al., '64)). Therefore, most of the label would be expected to be located in the ribosome-rich oocyte cytoplasm, and later in the cytoplasm of the embryonic

Fig. 5 Radioautographs of oocytes and embryos labelled at the lampbrush stage of oogenesis or at the end of oogenesis by injection of uridine-5-H³ (20–30 μC/μM; two consecutive inoculations of 0.1 cc isotope, 1 mC/cc) into the female (see text). The eggs were fixed and held in 10% formalin, and post-fixed in 2% buffered osmium tetroxide at 4°C for 1.5 hours. They were then gradually dehydrated by sequential transfer through 50%, 70%, 80%, 95% and 100% ethanol. Precursor is removed from the eggs in the course of formalin fixation. The eggs were embedded by transfer to propylene oxide and then to 1:1 propylene oxide-epon, and the propylene oxide volatilized by incubation for one hour at 60°. After this more epon was added and the blocks were allowed to harden at 60° for 12–24 hours. The radioautographs were made from 1 μ sections using Kodak NTP-2 emulsion. (A) Labelled RNA in the cytoplasm of a stage 6 oocyte labelled 82 days earlier at the lampbrush stage. Note dense accumulation of mature yolk platelets. (B) Concentration of labelled RNA in the perinuclear area of a stage 6 oocyte from the same female as in (A). (C) Labelled RNA in one of the nuclei of a 2-cell stage embryo derived from a female labelled 108 days earlier. (D) Section of the ventral region of a gastrula from a female labelled three days prior to gastrulation. (E) Section of the equatorial region of a gastrula from a female labelled 105 days prior to ovulation. (F) Same, showing asymmetric perinuclear grain accumulations.

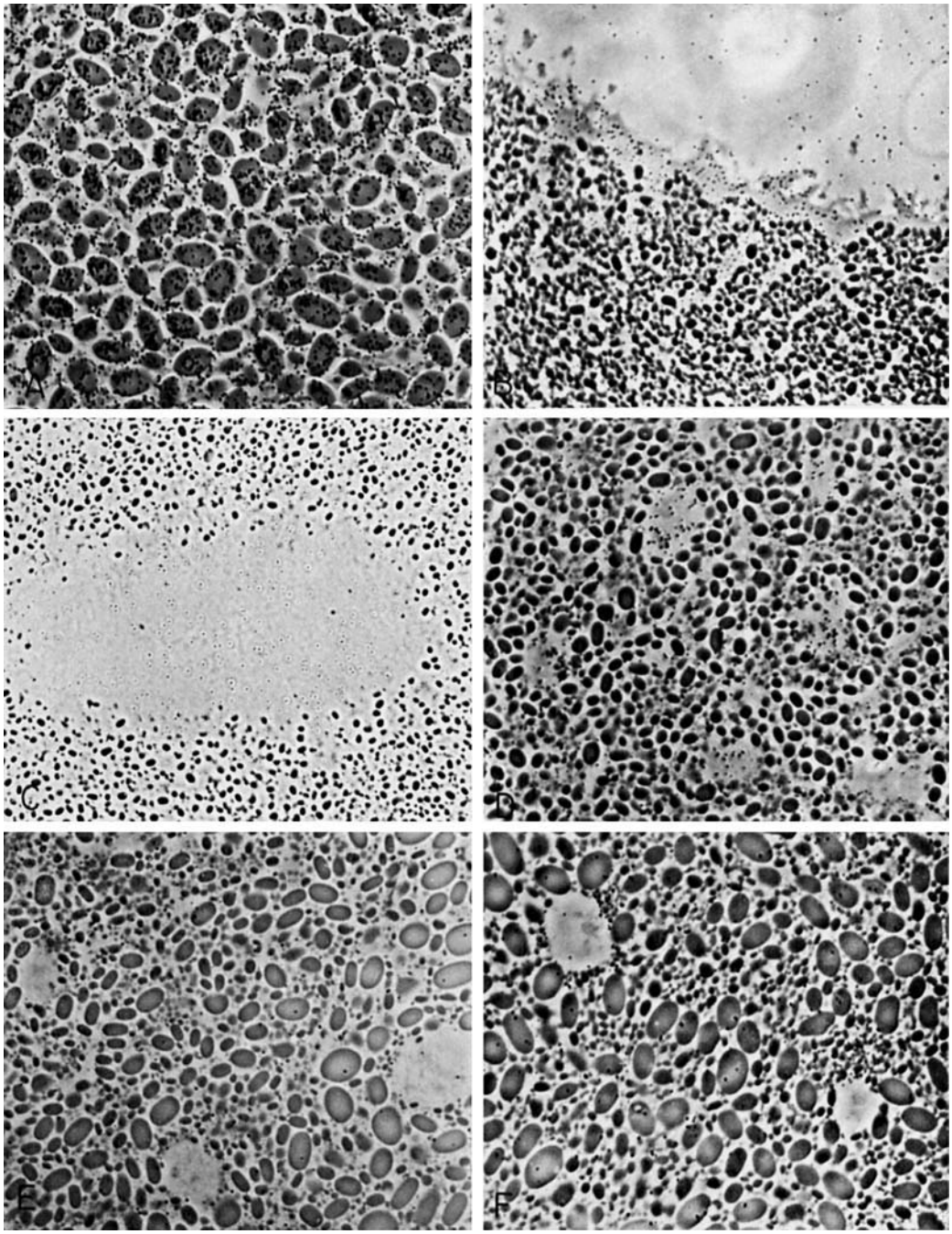

Figure 5

TABLE 7

Intracellular grain distribution in oocytes and early embryos labelled at the lampbrush stage [1]

Stage	Av. grain densities/unit area				Grain density ratios		Total volume which is nucleus	Ratio: nuclear grains to cytoplasmic grains × 100
	in animal pole cytoplasm	in vegetal pole cytoplasm	in nucleoplasm	in perinuclear area	Animal pole cytoplasm / vegetal pole cytoplasm	nucleoplasm / animal pole cytoplasm		
							%	
Stage 5 oocyte	64.1	47.3	13.2	8.9	1.4	0.25		
Stage 6 oocyte	9.7	3.9	4.4	36.6	2.0	0.45		
	10.9	6.0	2.9	19.5	1.8	0.27		
	15.9	7.9	6.9	55.4	2.5	0.43		
2-cell	8.7	3.2	19.0		2.7	2.2	0.13	0.32
	5.7	3.2	16.7		1.8	2.9		
4-cell	2.6	2.4	8.2		1.1	3.2	0.12	0.45
	3.3	1.4	14.1		2.4	4.4		
Early cleavage (8 cell stage)	11.2	6.0	17.3		1.9	1.5	0.12	0.19
Later cleavage (32 cell stage)	5.3	2.6	14.9		2.0	2.8	1.10	3.23
	5.6	3.2	17.2		1.8	3.1		

[1] The embryos in this table were obtained from a female labelled 108 days prior to ovulation. Stage 5 oocytes were obtained from a female labelled 16 days previously, and stage 6 oocytes from a female labelled 82 days previously. The ratio of nuclear to cytoplasmic grains (last column) was calculated on the basis that the cells and the nuclei are spherical, according to the relation (ratio of grains in cell which are nuclear $= \frac{r_n^3}{r_c^3 - r_n^3} \cdot \frac{G_n/\text{unit area nucleus}}{G_c/\text{unit area cytoplasm}}$, where r denotes radius, G denotes grain number, the subscript n denotes nucleus, and the subscript c denotes cytoplasm. Each value refers to 2–5 individual fields containing 30–200 individual demarcated areas suitable for counting. The multiple values represent averages of fields counted from different slides.

TABLE 8

Grain distribution between nucleus and cytoplasm of embryos labelled at lampbrush stage of oogenesis and at end of oogenesis

Time of label (days prior to ovulation)	Stage of embryo	Cell type	Cellular area which is nucleus in section	Grains/cell nucleus in section [1]	Grains/cell cytoplasm in section [1]	Grain density in animal pole cytoplasm/ grain density in vegetal pole cytoplasm	Grain density in nucleus/ grain density in cytoplasm	Ratio of grains which are nuclear to grains which are cytoplasmic × 100 [2]
			%					
Lampbrush (108)	blastula	vegetal	4.90	4.5	59.5		1.53	1.76
				4.6	50.6	2.12	2.02	
		animal	18.5	4.0	25.0		0.80	8.33
				6.2	26.3		1.42	
Lampbrush (108)	early gastrula	endoderm	6.09	0.4	7.4		0.62	1.38
				0.4	6.2	2.46	1.19	
		ectoderm	21.2	2.4	6.2		1.67	19.8
				2.8	4.7		2.72	
Lampbrush (108)	late gastrula	ectoderm	27.1	6.7	9.7		1.89	24.0
				5.6	13.0		1.45	
		mesoderm	26.0	4.1	8.2		1.78	15.2
				3.1	12.1	5.16	0.97	
		endoderm	6.88	2.2	18.9		1.58	4.91
				4.1	13.1		4.84	
End of oogenesis (3)	early gastrula	ectoderm	21.2	12.6	4.6		10.40	100
				12.7	6.0	2.96	6.25	
		endoderm	6.09	14.1	4.0		10.2	94.1
				10.7	2.2		30.9	
End of oogenesis (3)	mid-gastrula	ectoderm	29.1	20.4	14.8		4.63	58.4
				21.0	17.5	1.84	4.97	
		endoderm	6.51	22.6	45.2		7.47	10.9
				21.3	44.6		11.45	

[1] Each value represents the average of grain counts in ten cells. Counts from two separate sections are represented for each stage and cell type listed.

[2] The assumption is made that both nucleus and whole cell are spherical, and the percent of grains which are nuclear (last column) is calculated from the relation $\frac{r_n \cdot G/\text{nucleus in section}}{r_c^3 - r_n^3 \cdot G/\text{cytoplasm in section}}$

$r_c^2 - r_n^2$

where the denotation of the symbols is as given in table 7.

blastomeres. Figure 5A shows the grains located over a cytoplasmic region of a mature, stage 6 oocyte labelled two months previously at the lampbrush stage. The fraction of the grains in the nucleus as compared to the cytoplasm of cleavage stage and later blastomeres is indicated in the last columns of tables 7 and 8, calculated on the basis that both the cells and nuclei are spherical. In early cleavage less than 0.5% of the grains are nuclear (table 7). Not until small animal pole cells appear in blastulation do the fractions of grains which are nuclear rise beyond 5%. The nuclei of these small cells and their ectodermal descendants occupy a much larger fraction of the cell than is the case in earlier appearing blastomeres (table 8, fig. 6), and from this point on the fraction of counts which are nuclear depends mainly on the cell type. Two conclusions can be drawn from the data of tables 7 and 8 regarding the fraction of labelled RNA in the nucleus: (a) In early development over 99% of the grains are in the cytoplasm, as expected for a label incorporated in mid-oogenesis and distributed mainly in ribosomal RNA; and (b) the distribution of counts in the lampbrush-labelled early gastrulae is strikingly different from that in the early gastrulae labelled at the end of oogenesis. In these embryos virtually all the label incorporated in RNA is nuclear. Table 8 shows that not until the late gastrula stage is new RNA synthesized during embryogenesis from precursor introduced just before ovulation widely distributed in the cytoplasm. The

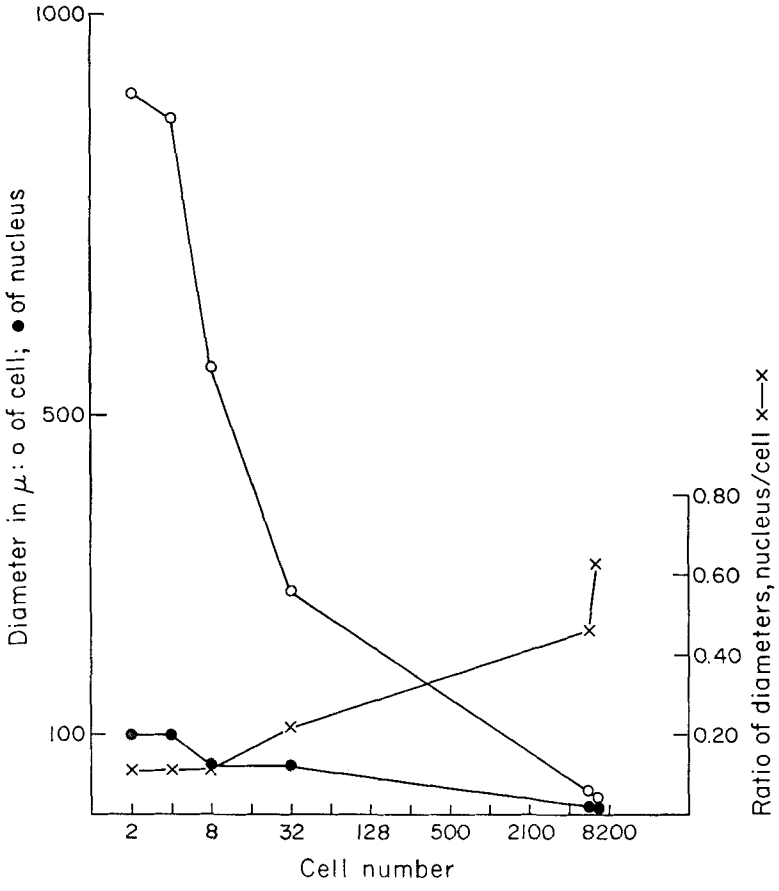

Fig. 6 Relationship between nuclear diameter and cell diameter during cleavage stages in *Engystomops*.

contrast between the two sets of early gastrulae in table 8, added to the data of table 5, makes it certain that the labelling protocol used does indeed result in embryos bearing label *mainly* in maternal RNA, rather than in RNA *newly* synthesized from either residual precursor or from precursor stemming from the breakdown of maternal RNA. This conclusion refers to the bulk of the RNA label, since table 5 shows that about 7% of the counts in a gastrula labelled at the lampbrush stage could be *incorporated from residual, acid-soluble precursor*. One is, however, primarily interested in just such a small minority of counts, those expected to represent the non-ribosomal informational RNA inherited from mid-oogenesis (Crippa et al., '67; Davidson et al., '66). Though they cannot specify the molecular species of the RNA, the radioautograph experiments summarized in figure 5 and tables 7 and 8, do in fact provide evidence relating to the localization of certain interesting minority fractions of maternal RNA. These fractions can be distinguished from the minority RNA fractions expected to be formed in the embryo as a consequence of new synthesis from residual precursor or from precursor derived by breakdown of labelled maternal RNA.

Figure 5B shows the rim of the nucleus of a stage 6 oocyte several months after uridine-H^3 was administered at the lampbrush stage. Between the nucleus and the yolk-filled cytoplasm is a peripheral, yolk-free area in which labelled RNA appears to be concentrated. Since there is hardly any new RNA synthesis in stage 6 oocytes (table 5), the grains in figure 5B represent RNA synthesized previously, at the lampbrush stage. The grain density in the perinuclear region of the stage 6 oocyte is higher than in any other area of the oocyte; it exceeds that of the nucleoplasm proper by a factor of 6–8, as shown in table 7. Interestingly, the perinuclear RNA concentration is not established until stage 6, and the stage 5 oocyte nucleus proper has a greater grain density than the perinuclear region. Thus the accumulation and storage of a certain small fraction of previously synthesized RNA around the nucleus is a localization phenomenon associated with the final maturation of the oocyte. The actual nature of this RNA is of course unknown.

Figure 5C shows a section through one of the nuclei in a two-cell embryo. The perinuclear area has disappeared (it does not reform after germinal vesicle breakdown). What is of interest here is the concentration of grains in the nucleoplasm. There are, according to table 7, over twice as many nuclear grains per unit area as in the animal pole cytoplasm. This is in contrast to the case before fertilization, where the grain density is 2–3 times higher in the animal pole cytoplasm than in the nucleoplasm. Furthermore, table 7 shows that the nuclear grain concentration persists, though it does not particularly increase, through cleavage. Could these grains simply represent *new* RNA synthesis from residual precursor or degraded maternal RNA? Certain arguments militate against this possibility: tables 7 and 8 show that the ratio of nuclear to cytoplasmic grain densities remains essentially constant, around two, all the way into gastrulation, irrespective of widely different nuclear sizes (fig. 6). This is definitely not the case for animals labelled at the end of oogenesis, in which the pattern of label distribution mimics that which would occur in the case of new synthesis from residual precursor or degraded maternal RNA. Here the nuclear grain densities climb sharply at gastrulation, paralleling the increase in total synthesis (fig. 4); virtually all the counts in new RNA in these embryos are nuclear (table 8). In the maternally labelled embryos about the same relative concentration of labelled RNA is present in the two-cell nucleus as in the nuclei of gastrular cells. While the grains in the latter could indeed be due to new synthesis, that in the two-cell embryo nucleus evidently cannot be explained in this way. In maternally labelled eggs re-utilization of degraded maternal RNA or utilization of residual precursor could account for about 7% of the labelled RNA in the gastrula (table 5). However the amount of total RNA synthesis in the gastrula is more than 10 × the amount in early cleavage, according to figure 4. Thus on this basis as well the grains in the early cleavage stage nu-

clei of maternally labelled embryos can simply not be due to new RNA synthesis. Evidence for RNA synthesis in amphibian blastomere nuclei as early as the two-cell stage is lacking in any case. Another possibility to be considered is that the nuclear grains found in embryos after lampbrush stage labelling represent incorporation into DNA from labelled deoxyribonucleotides in the precursor pool. The acid soluble pool in embryos labelled in this way is sufficient to account for only about 7% of the counts incorporated by gastrulation (see above) in RNA, and according to figure 4 only 0.1–0.2 of this could represent DNA synthesis. This amount of DNA label would have to be divided amongst the 10,000 or so cells in the early gastrula. Thus the proportion of counts which are nuclear in cleavage stage embryos is several orders of magnitude too large to be accounted for as DNA synthesis. We conclude, pending further information, that the early cleavage stage nuclei contain a fraction of RNA inherited from the lampbrush stage. As development proceeds it is possible that some maternal RNA moves from cytoplasm to nucleus since the nuclear to cytoplasmic volume ratio increases, while the nuclear concentration of maternal RNA label remains about the same.

In figures 5D, E and F radioautographs of gastrular cells from embryos labelled at the lampbrush stage and at the end of oogenesis are compared. Cells of the latter embryos (fig. 5D) show the expected concentration of nuclear grains, including grains over a nucleolus visible in the section. The section from the maternally labelled embryo in figure 5E also shows nucleoli but in this case they are *not labelled.* Since gastrular nucleoli are strongly active in RNA synthesis this contrast suggests that even by this comparatively late stage there is little significant contribution to the labelling pattern from *new* incorporation in maternally labelled embryos. An additional interesting feature is illustrated in figure 5F, *viz.* a striking, asymmetric, concentration of grains just outside the nuclear membrane. This phenomenon is widespread in maternally labelled gastrulae, and it is never observed where new RNA synthesis is being studied. We conclude that at gastrulation there occurs a previously unknown localization phenomenon in which certain maternal RNA's are accumulated in direct apposition to the blastomere nuclei. Though less easily distinguished from the pattern of grains characterizing *de novo* synthesis, an additional aspect of the perinuclear grain concentration in maternally labelled gastrulae is a tendency for grains to be preferentially distributed in a ring around the nuclear membrane as well as asymmetrically concentrated in a mass at one side.

DISCUSSION

In this paper we have attempted to provide the necessary foundation for continuing studies on the distribution and fate in embryogenesis of lampbrush stage oocyte RNA's. We have found the neotropic toad *Engystomops pustulosus* to be particularly suited for such studies. Adaptation to the conditions of its environmental niche has produced a fortuitous cycle of events in oogenesis, one which permits the investigator to label RNA at the lampbrush stage of oogenesis, and to recover a few months later a clutch of eggs bearing this labelled RNA at high specific activity. In no other creature has this so far been accomplished, and it should now be possible to capitalize on the enormous experimental advantage which should derive from this stage-specific labelling procedure in investigating the fate and function of maternal RNA in early development.

Among the observations we have reported in this study several stand out as particularly interesting. The cycle of oogenesis portrayed in figure 3 provides a splendid example of adaptation at the level of a cellular process, oogenesis, to that particular environmental niche which *Engystomops* occupies. Thus oogenesis is asynchronous in the rainy season, thereby providing a statistical defense against the hazardous nature of the temporary water method of breeding. Asynchrony of oogenesis yields the greatest possible number of egg clutches while the rains last. In the dry season, on the other hand, the process is such that the largest possible egg clutch will be deposited with the first rains. Undoubtedly this cycle is a hormonally regulated func-

tion. Thus we see here a continuum of regulation levels extending, via the hormones secreted by the animal, directly from regulation of mating behavior in response to environmental conditions to regulation of the molecular events occurring within the nuclei of the oocytes.

The pattern of oogenesis *Engystomops* has evolved includes the fortuitous synchronous phase on which we have focussed in this exploratory study. Other peculiar adaptations of this anuran to temporary water breeding, have also turned out to provide advantages for the investigator. Thus the rapid, nearly synchronous development of the embryos makes staging of embryos far easier to accomplish than with *Xenopus*, for example. Similarly, the natural response of the animal to the appearance of potential breeding sites renders it possible to induce mating in a laboratory population merely by flooding the colony floor. The unusual permeability of the early embryos may fall in this class of characteristics as well, since, as pointed out above, this permeability may be an indirect result of the most elegant of the adaptations of *Engystomops* to temporary water breeding, suspension of the embryos in the airfoam egg nest. A further advantage probably deriving indirectly from the airfoam egg nest is the lack of pigment in the eggs. Unlike most amphibian eggs *Engystomops* eggs are completely white, as is the airfoam nest in which they are embedded. The paucity of pigment granules renders radioautograph grain counting considerably easier than in pigmented anuran eggs.

The total synthesis and radioautograph experiments show that the distribution in the embryo of maternal RNA synthesized at the lampbrush stage is clearly distinct from that of RNA synthesized anew by the embryo. Particularly intriguing is the apparent presence of the three minority fractions of the maternal RNA discussed above; the perinuclear accumulation of previously synthesized RNA at the very end of oogenesis; the apparent localization of maternal RNA within the early blastomere nuclei; and the sharp perinuclear accumulations of maternal RNA much later, at gastrulation. We know nothing of the molecular species of these RNA fractions, and therefore it is as yet possible only to speculate on the significance of this distribution pattern. Most of the maternal RNA is cytoplasmic, just as expected for maternal ribosomal RNA. If the perinuclear and intranuclear RNA of the stage 6 oocyte includes informational RNA's as well, it would perhaps explain the observation of several workers that the germinal vesicle contains substances later required in order for embryonic organization to occur. Thus Briggs and Justus ('68) have shown that the microinjection of germinal vesicle contents into the eggs of axolotl mutants unable to gastrulate cures them of this defect. Similarly Smith ('66) has demonstrated by microinjection into irradiated eggs that germ cell determination in the early amphibian egg requires substances stored before ovulation in the germinal vesicle. Theoretical reasons exist, and have been detailed elsewhere (Britten and Davidson, '69; Davidson, '68), for considering that the patterns of embryo genome function may be determined by regulative RNA's synthesized in oogenesis and inherited by the embryo. The finding of a small fraction of maternal RNA in the embryo blastomere nuclei is therefore perhaps the single most interesting aspect of this study. The nature of this RNA and of its interaction with the components of the blastomere nuclei constitute an experimental target of general significance, and it would seem that evolution has rendered *Engystomops* uniquely suited to the pursuit of that objective.

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