Anatomy and Development of Racial Hybrids of *Rana pipiens*[1]

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The following experiments were directed toward discovery of the cause of the abnormalities found in embryos from crosses between races of the meadow frog, *Rana pipiens*. The existence of the abnormalities has been known for some time but no study has yet been made of the anatomy and development of the embryos.

The abnormalities themselves vary both in kind and degree. Generally speaking, they range all the way from exogastrulae to only a slight enlargement or reduction of the anterior end of a late neurula. The "direction" of the hybrid cross influences the type of abnormalities since reciprocal crosses give embryos with different syndromes.

Earlier work, discussed below, seems to indicate that the cause of the abnormalities can be linked to the fact that *Rana pipiens* is unique among its congeners in embryonic temperature requirement. Various populations of the species are adapted to different temperature conditions during early development. This possibility is discussed and a hypothesis is proposed for the evolutionary origin of this condition.

The experiments themselves consisted in raising normal and hybrid embryos in identical conditions and observing not only their external appearance but also reconstructing their internal anatomy. This was done for embryos fixed at regular intervals beginning when tissue differentiation became barely visible in stained sections and going up to stages when feeding would normally begin.

The hybrid embryos in this study, though abnormal, were considerably less abnormal than many *R. pipiens* racial hybrids reported in the literature. They were as viable as the controls (at least up to 300 hours). Their development was fairly regular so that observations could be made on a number of embryos over a long period of time. It was felt that this would be more informative than observing the more extreme cases which often die at gastrulation or soon after.

It was concluded that the abnormalities were probably caused by an excess of cells in the notochord in northern-egg hybrids and a deficiency of cells in the notochord in southern-egg hybrids. The abnormalities in both sorts of hybrids were worst at the early stages, and the embryos improved as they developed.

MATERIALS AND METHODS

Frogs from two sources were used in these experiments. For simplicity they are abbreviated by "N" for the northern race and "S" for the southern race. N was supplied by J. M. Hazen Co., Alburg, Vermont; S was supplied by Harold Williams, Dunellon, Florida. The northern frogs were collected as they went into hibernation in the fall of '59; southern frogs were collected just before the experiments began (February–March, '60).

To indicate the parentage of an embryo the abbreviations, N and S, are combined with the *maternal initial first*. Thus, N·S is a hybrid between a northern female and a southern male; S·N is the reciprocal cross. N·N and S·S are non-hybrid embryos from northern and southern races respectively.

Females were induced to ovulate by the usual pituitary injection method (Rugh, '37). Eggs were stripped from the females into large glass dishes containing sperm

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suspension. After fertilization, the eggs were flooded with 10% amphibian Ringer's solution and kept at 16°C. A complete run consisted of 4 dishes — each with one of the 4 possible parentages: N·N, N·S, S·N, S·S. For each run only one animal of each sex from each race was used. About 20 hours later the masses of eggs in each dish were cut into small clusters of less than 5 eggs each. At this time the uncleaved eggs were removed. (These unfertilized eggs always numbered less than 5% of the total.) Finally, the number of eggs was counted and the dishes returned to 16°. At about 100 hours after fertilization (about tailbud stage in N·N embryos) samples of 10 embryos were taken from each of the 4 dishes and fixed. This was repeated every 12 hours up to about 300 hours (at which time N·N embryos had opercular fold developed).

Two such experiments were used for the data in this paper — runs no. 3 and no. 5. They were identical except in the initial number of embryos per dish. In run no. 3 the numbers per dish were: N·N = 150, N·S = 191, S·N = 220, S·S = 189. In run no. 5 the numbers were set at the time of counting (20 hours after fertilization) so that there were 150 eggs per dish. The dishes were approximately 24 × 15 × 5 cm. Of course, as the embryos grew and began to move about, their numbers were being reduced by sampling so that they were never crowded. Under these conditions development was probably as normal as the heredity of each animal would allow.

Each sample was fixed in Smith's Fixative for 12 hours, then washed in distilled water for 12 hours, and preserved in 4% formalin. For sectioning, each embryo was dehydrated and embedded in paraffin in the usual manner. Sections were usually cut 10 μ thick and stained with Shumway's Polychrome (Shumway, '26). As a check on the method of reconstructing, a few embryos were bleached in 3% hydrogen peroxide, stained *in toto* with borax-carmine, and cleared in cedar oil. These were observed with a binocular dissecting microscope and compared with the reconstructions made from serial sections. In every case there was good correspondence between the reconstruction and the cleared sibling of the reconstructed embryo.

A total of over 150 embryos was sectioned and examined. The data from these include about 50 outlines and 60 reconstructions. In addition, cell counts and camera lucida drawings were made from many specimens, including a number loaned by Dr. John A. Moore that were described earlier (Moore, '46a).

Sectioned specimens were used to make graphic reconstructions of various parts of the embryos. The technique is published elsewhere in detail (Fowler, '61). The main novelty is the method of orienting sections so that an eye-piece reticle provides a coordinate system for the whole embryo. Every point in the embryo has three numbers associated with it. These represent its position in a rectilinear, three-dimensional coordinate system. From these data a drawing can be made by plotting these points as one would plot a graph of any numerical data. There is no artistic judgment required.

Most of the data are in the form of graphic reconstructions. The following plots were made for all 4 crosses (N·N, N·S, S·N, S·S) and for several different ages.

1. Side view of the embryo with the maximum extent of the following structures in the median plane: brain, neural tube, neurocoel, notochord, gut (lumen only, except for specimens less than 150 hrs.), pericardial cavity. In addition, the maximum extent of the following organs was plotted as projected to the median plane: left eye and its lens, left otic vesicle, left nasal pit, and left pronephros.
2. Top view of the embryo with the maximum extent of the following structures: brain, neural tube, neurocoel, nasal pits, otic vesicles, eyes with lenses, and pronephroi.
3. Contour-line reconstructions of the shape of the brain showing the brain in place inside the outline of the epidermis. On the same plot were put the lumen of the gut, the notochord, and the pericardial cavity.
4. Outlines of the heart in side view (the oldest stages only).
5. Outlines of about 30 embryos were made before embedding as a check

of the method of reconstruction. These were made as side and top views and served as data for figure 1.

6. Camera lucida drawings were made of various sections and parts. Figure 6 has been drawn from these data.

The data for table 1 are from 10 μ sections. Areas of the notochord cross section were calculated assuming the shape to be an ellipse. The major and minor diameters were read with an eyepiece micrometer and the areas calculated from them.

Errors and limitations

Practically all the data from these experiments are in the form of reconstructions made from fixed, embedded, and sectioned specimens. This raises the question of the accuracy of reconstructions compared with the live embryos. There is no question that the sections have shrinkage and some distortions. However, the shrinkage should be about the same for all specimens. Embryos from the same run and of the same age were always subjected to the same treatments during fixation, embedding and sectioning. The only thing that could cause a serious error in the reconstructions would be a randomly occurring distortion, such as compression due to sectioning. That this is not a serious factor is shown by the following test: Reconstructions were made by plotting the points, say, from every 5th section. After the points were plotted, the whole was done again by plotting the points intermediate to every 5th (say, 7, 12, 17 . . . etc.). This was done in a number of cases, and there never was any discrepancy between the line connecting the first series of points and that connecting the second.

Obliquity of sections could not be avoided because many embryos were curved. In all cases an effort was made to embed the embryo so that the head would be cut transversely, but this orientation was probably only accurate to within about 5 or 10°. In the graphic reconstructions, however, there was no error due to obliquity. If the sections were not truly transverse, the reconstruction was replotted by using the reconstructed top view of the same embryo to establish the axis of symmetry as a base line for replotting the side view. Measurements, on the other hand, were not corrected for obliquity. This added some error to the notochord areas (table 1). But the error introduced by having a section as oblique as 20° cannot exceed two per cent which is well within the range of repeatability of these measurements.

The great range of variability of the hybrids makes it impossible to describe anything like "the average N·S (or S·N) hybrid." Whether such a description would be very meaningful is hard to say. In any event, there is no doubt that the two classes of hybrids could be separated from normals and from each other by a number of signs. The Results below should be understood as a listing of the points of difference which were always found, rather than as a description of the two sorts of hybrids. In other words, this investigation is intended to find the developmental path followed by hybrids. The path turns out to be rather wide but it still has a general direction which is clear, and it nowhere intersects the normal path (at least for the

TABLE 1

Notochord cross section areas in square microns × 10^3. Vacuole number in parentheses. Each figure is mean of 10 non-adjacent sections at level of otic vesicles

Age (hours)	Parentage			
	N·N	N·S	S·N	S·S
Fla. × Vt.—at 16°				
117	4.5	7.6	3.4	4.3
123	5.0	12.1	4.1	5.7
141	11.8(7.7)	13.6(10.2)	5.6(4.2)	6.7(5.0)
225	13.0(6.2)	30.1(10.5)	5.7(2.9)	8.7(4.7)
309	10.9(5.5)	19.6(9.2)	9.3(4.6)	10.6(6.0)
Tex. × Wis. — at 19°				
125	14.5(7.7)	15.1(13.9)	4.1(3.2)	10.1(6.4)

time interval covered here, 100 to 300 hours at 16°).

RESULTS

Variation

The hybrid animals were very variable in almost every respect. This would have been quite dismaying except that there never was any possibility of confusing a hybrid with a normal or of confusing N·S with S·N hybrids. The older embryos could be separated by external appearance, and even as early as 100 hours the internal anatomy was markedly different. There were no consistent differences between the two runs. The normal embryos from the two runs were indistinguishable when matched in age and parentage (N·N or S·S). Because of the variability of hybrids it is difficult to make such a statement comparing the hybrids of the two runs. No consistent difference could be found, but neither could any good matching of embryos (of the same age and parentage) be made. Since the normals were alike in the two runs and the hybrids were consistently different from the normals, it seems safe to combine the data from runs no. 3 and no. 5. This has been done here. The reconstructions figured here, however, are all of animals from run no. 5. Except for this, the reconstructed embryos were chosen at random from samples at the desired age.

Of course, in considering variation it is necessary to say that not only the appearance of a particular organ at a particular time was studied, but also the changes of that organ with time. The aim of this study was not purely anatomical, but it was to observe the developmental patterns of the hybrids compared with the normals.

External appearance

There is an appreciable difference in the size of the two types of eggs. N eggs were about 1.7 mm in diameter, while S eggs were only about 1.3 mm. Thus the N eggs had over twice as much volume as the S eggs. For this reason it is necessary to compare N·S hybrids with N·N normals, and S·N hybrids with S·S normals. Added to the size difference is the difference due to hybridity. As a general rule, the hybrids were more extreme in size than the normals, although there was some overlap in this characteristic. That is, northern-egg hybrids were even larger than northern normals; southern-egg hybrids were smaller than southern normals.

In developmental rate, the hybrids seemed to keep pace with the normals (in external appearance only) except in the time of hatching. N·S embryos hatched sooner than N·N normals. S·N hybrids did not hatch at all. A number of S·N animals did get out of the egg capsules but I attribute this to damage to the capsule at the time the egg masses were cut into smaller clusters. S·N hybrids did not make any spontaneous movements which would help them to hatch. Even when touched with a hair loop or given a strong current of water from a pipette, they rarely moved. On the other hand, N·S hybrids and both kinds of normals responded to a touch or a current of water by vigorous twitching and at later stages by swimming. By 200 hours, moving a dish of embryos was enough to start them all swimming, except the S·N embryos which showed no response.

Because of their inability to hatch, the S·N hybrids were constrained in the egg well past the usual time, and this could be expected to give them the strong curvature of the body that was observed. But this was not the only cause, because those that developed outside their egg capsules (because of damage to the capsules in cutting the egg mass) were also curved in much the same way.

The external shape of the embryos at different ages can be seen in figure 1. Here the outlines have been drawn as if the body axis were straight. Any other presentation would make comparison impossible. The embryos of figure 1 were representative of their siblings except that no edematous ones are shown. The following main points were observed in the external appearance of hybrids as compared with normals:

1. The head region of the N·S hybrids was appreciably larger than that of the N·N normals.
2. The head region of the S·N hybrids was appreciably smaller than that of the S·S normals.

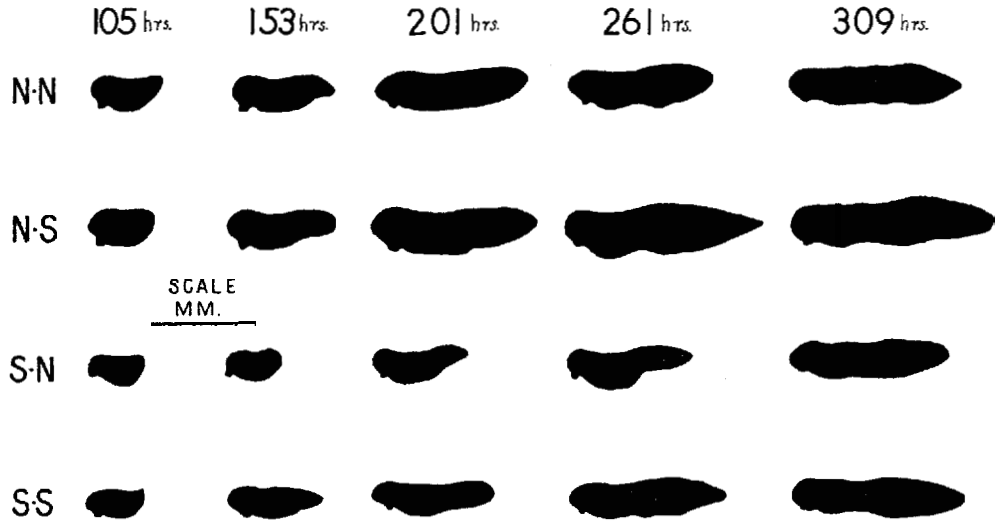

Fig. 1 Outlines of fixed embryos reconstructed with straight body axes.

3. The suckers (adhesive organs) of the N·S hybrids were overdeveloped and secreted more matter than those of the normals.
4. S·N hybrids had only a single sucker, usually median, which was much smaller than a normal (S·S) sucker.
5. The S·N hybrids suffered in varying degrees from edematous swellings in the mid-body region and were quite asymmetrical.
6. There was much more variation in the appearance of the hybrids than there was in the normals. But hybrids could never be confused with normals, especially after 150 hours (about the time of hatching for N·N normals).

Developmental rate and viability

Up to gastrulation the 4 groups of embryos were alike in their development. After gastrulation differences in rate appeared. Generally speaking, the N·S hybrids seemed most advanced (although sections show that cellular differentiation was no faster than normal and some organs were retarded in development—see below). This appearance was probably due to their slightly greater length (fig. 1) and their earlier hatching. N·N normals were slightly ahead of S·S normals. Finally, the S·N hybrids seemed most retarded. The degree of acceleration or retardation of hybrid development cannot be measured with precision. The hybrids were so abnormal that developmental stages were not comparable. For instance, the establishment of gill circulation was probably somewhat retarded in N·S hybrids and very definitely retarded in S·N hybrids when compared with the differentiation of, say, the brain. Thus no attempt has been made to measure any sort of overall rate of development for the 4 groups. I feel that to do so would hide more than it would reveal.

There was no difference in viability in the 4 groups of embryos—at least up to 300 hours. A few cytolizing gastrulae were removed from the hybrids in run no. 5 but no more than 5 in any dish. Run no. 3, although more crowded, had practically no mortality. Needless to say, the eggs in each dish were not selected in any way when they were counted and the numbers adjusted. It must be concluded that this particular cross, northern Vermont with central Florida, does not lead to decreased viability in the F1 generation, at least up to hatching. This differs considerably from other reported crosses. For example, Moore ('47) reports that the cross Vermont × Monterrey, Mexico resulted in F1's with mortality of different runs ranging from 100% at gastrulation to 80% dead at 16 days. It seems that viability varies widely

in those crosses that give a great deal of abnormality. My hybrids were not as abnormal as many reported in the literature. This is an advantage since my interest is not in the crosses but in the abnormalities of development.

Although the collection of samples did not continue past 300 hours, a few embryos from run no. 3 were allowed to develop past that time. These were S·S normals, and both sorts of hybrids. No regular observations were made on these animals but it was noted that some of each type survived for 50 days. Two of the S·N hybrids eventually did feed and swim but they were only two out of the 40 remaining at the end of the regular run. In general, this bears out the observation made below that the abnormalities appear early in development and some sort of compensating process seems to diminish the degree of abnormality at later stages.

Internal proportions

Figure 2 shows reconstructed median sagittal sections of the 4 classes of embryos at three different ages. These sections were reconstructed graphically from serial transverse sections. Only the anterior third is shown because there were no significant differences in the abdomen or tail regions. The outline of the left eye and lens has been indicated with dotted lines on those embryos that had eyes. The main observations visible on these drawings and confirmed in all the other embryos examined are:

1. The forebrain and midbrain regions were larger in the N·S hybrids than in the normals. The differences were most marked at the earlier stages.
2. Conversely, in the S·N hybrids the forebrain was almost absent and the rest of the brain was undersized and underdeveloped. Again the defect was most marked at the earlier ages.
3. Along with the enlarged or reduced brain there was a corresponding enlargement or reduction of the head region and enlargement or reduction of mouth and suckers.
4. Along with the enlarged brain in the N·S hybrid there seemed to be an enlarged notochord. Conversely, the S·N hybrids seemed to have a smaller notochord.

Central nervous system

The most marked difference between hybrids and normals was the shape of the brain. Figures 3, 4, and 5 show the outline of the anterior part of the embryo and its brain, notochord, and gut. The shape of the brain is indicated by contour-lines, such as are used on topographic maps. These lines represent the intersection of the brain surface with planes parallel to the median sagittal plane and located at distances from the median that are multiples of the contour interval, 39 μ. Where the lines are close together, the surface is steeply inclined to the paper; where the lines are far apart, the surface is nearly parallel to the paper.

The N·S hybrids had a larger brain than their N·N half-sibs, but it was less developed. The surface was relatively featureless, and the whole had an inflated appearance. Examination of the tissue shows that this inflated appearance was due to a thicker brain wall with more cells than that of a normal (N·N) brain. The S·N hybrids had just the opposite. Their brains were small but also underdeveloped — lacking the folds and thickenings of the normal S·S brain. The brain wall was generally thinner than the normal S·S. Furthermore, in the earlier stages (before 150 hours) there was no sharp boundary to the anterior end of the brain. The neural cells were separated along the front face of the brain, and occasional mesenchyme cells were seen between them. This defect disappeared at later stages.

I have examined a number of slides of hybrids between Wisconsin and Texas races (see Moore, '46a). These showed exactly the same abnormal histology as my hybrids did. Furthermore, those N·S hybrids (Wisconsin ♀ × Texas ♂) showed a second symptom that was present in all my N·S hybrids. There was an accumulation of cells in the ventricles of the brain beginning at the most anterior parts and continuing to the end of the hindbrain. They were especially numerous in the diencephalon. These cells were generally undifferentiated. They had large nuclei, sometimes pycnotic, with a very few show-

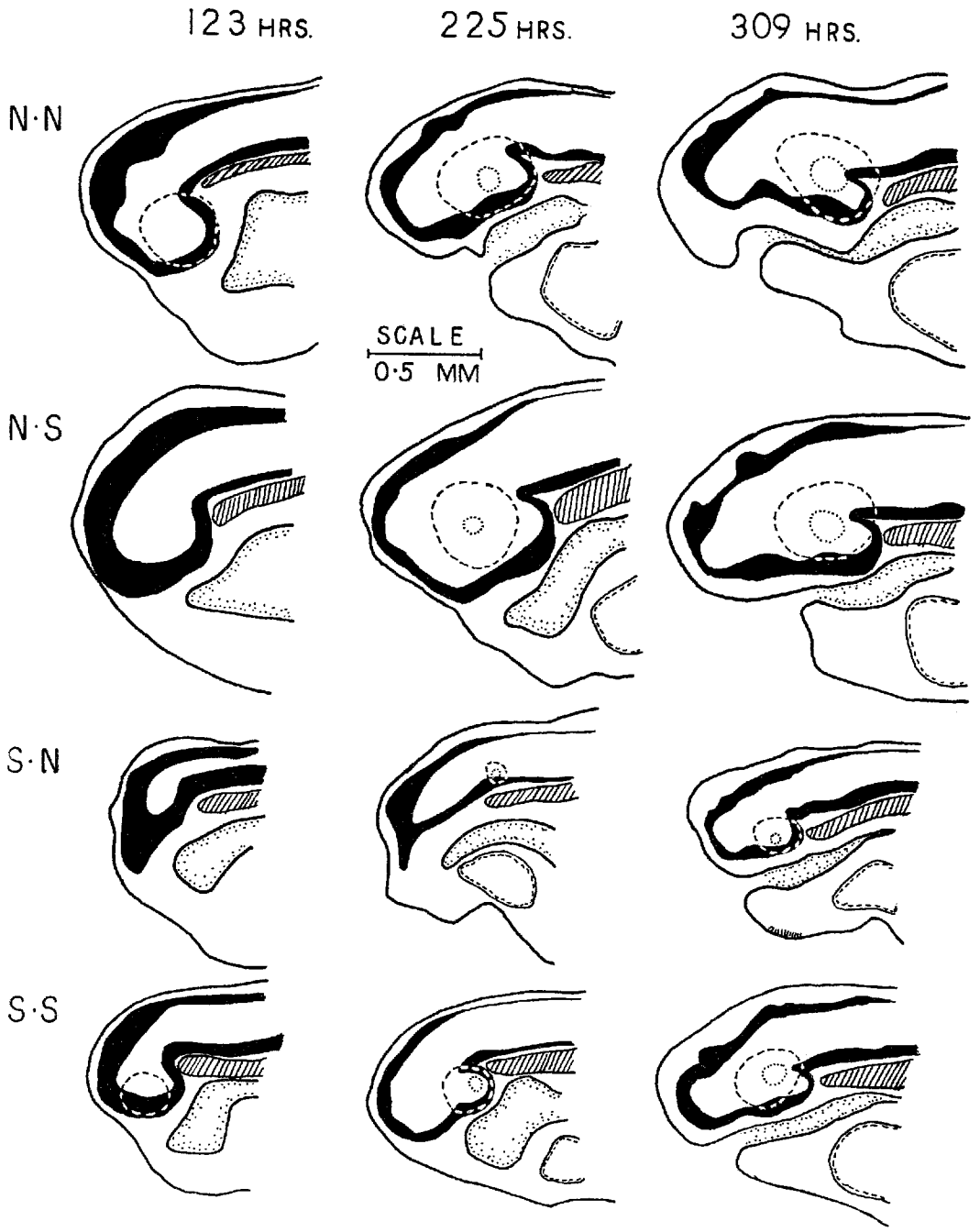

Fig. 2 Reconstructed median sagittal sections. Shown are nervous system (solid black), notochord (cross-hatched), gut (stippled outline), pericardial cavity (outline shaded with dashes), outline of eye (broken circle) and lens (dotted circle) projected to median plane. S·N—309 hr. embryo has median sucker (hatching on ventral edge).

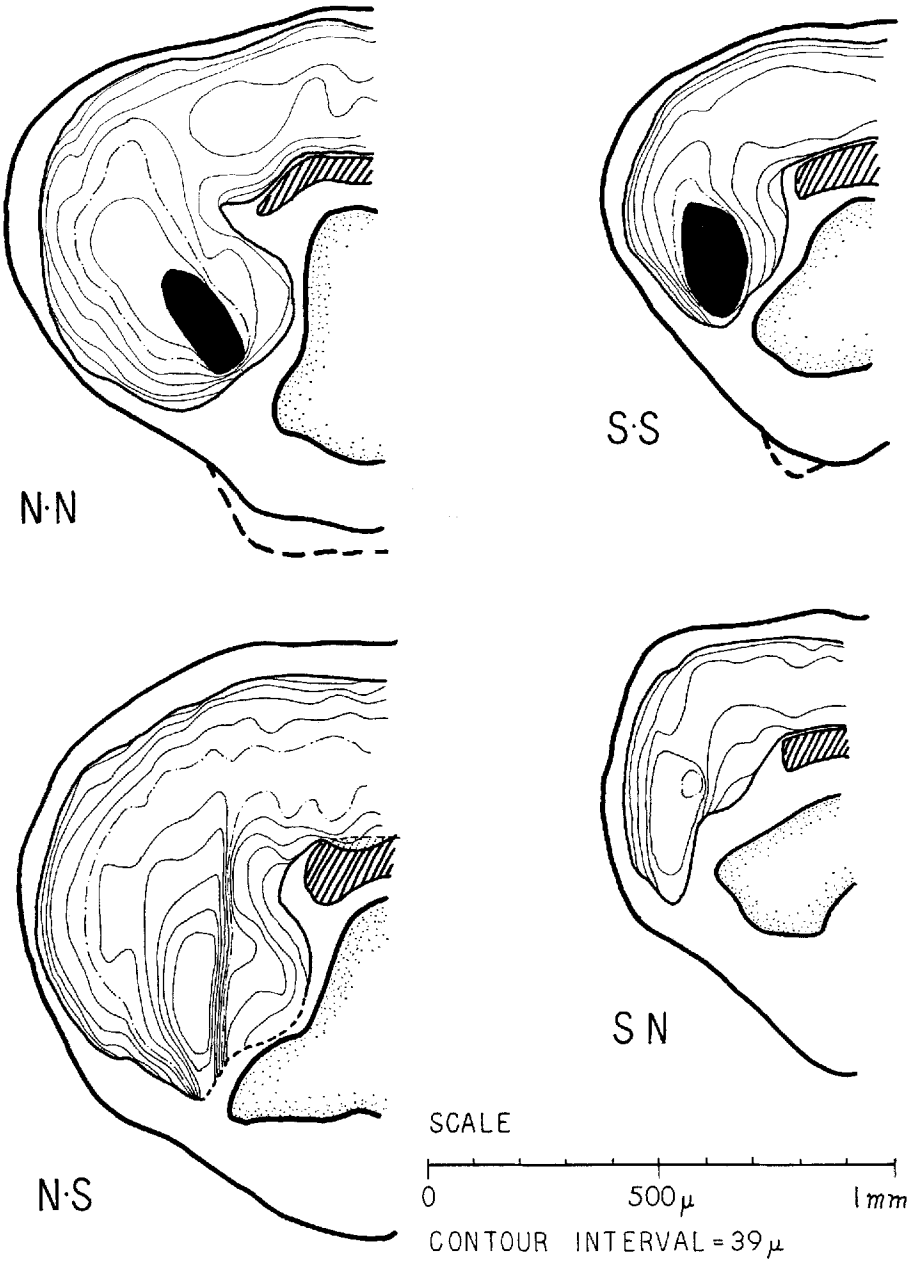

Fig. 3 One hundred and twenty-five hour embryos. Brain shape shown with contour-lines representing differences in depth of 39 μ. Cut eye stalk shown as solid black; notochord is cross-hatched; gut is stippled. Dotted lines are for outlines not in median plane such as suckers and lips around mouth. The 5th contour-line from center is always shown broken.

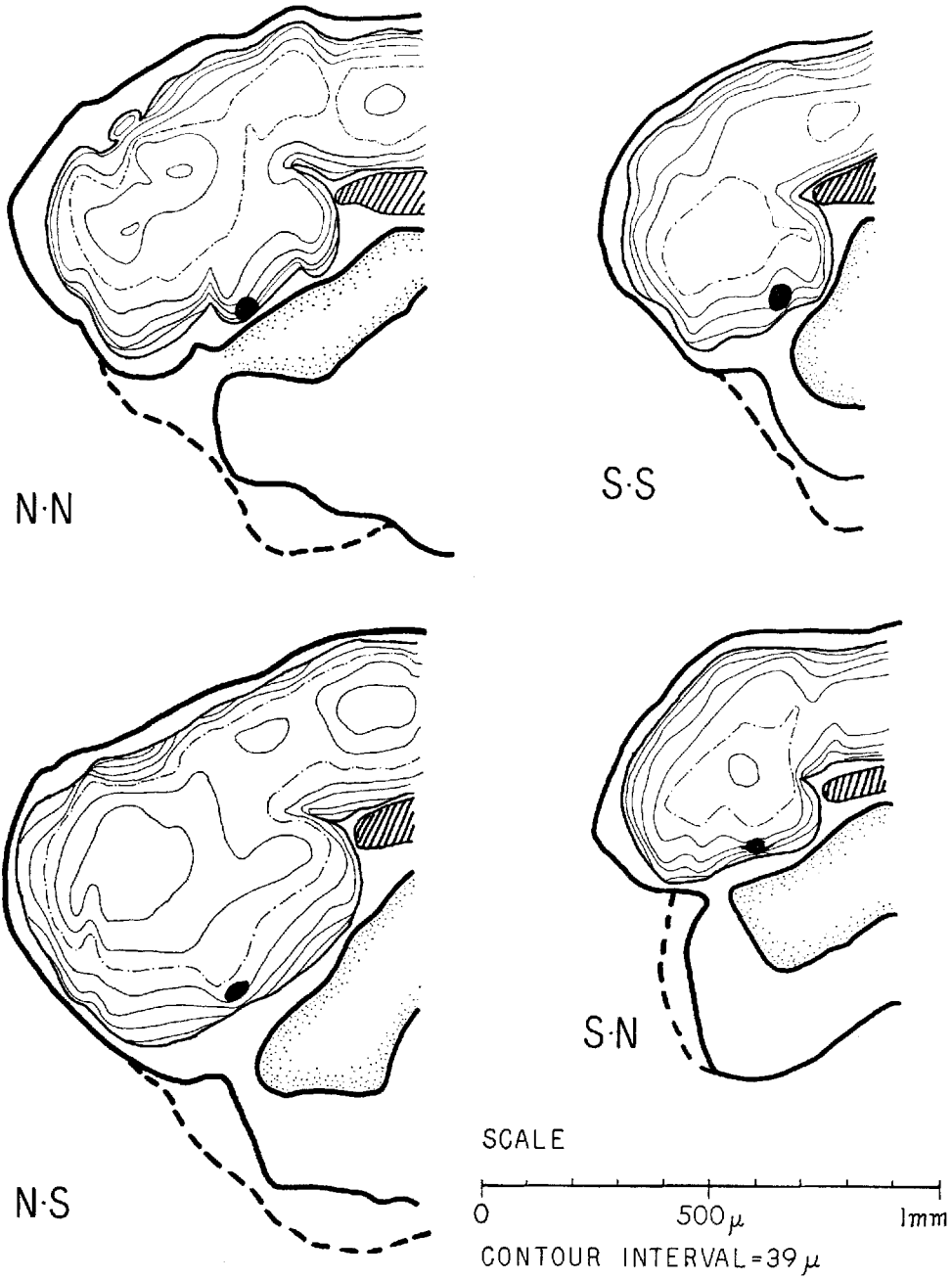

Fig. 4 Two hundred and twenty-five hour embryos. Key as in figure 3.

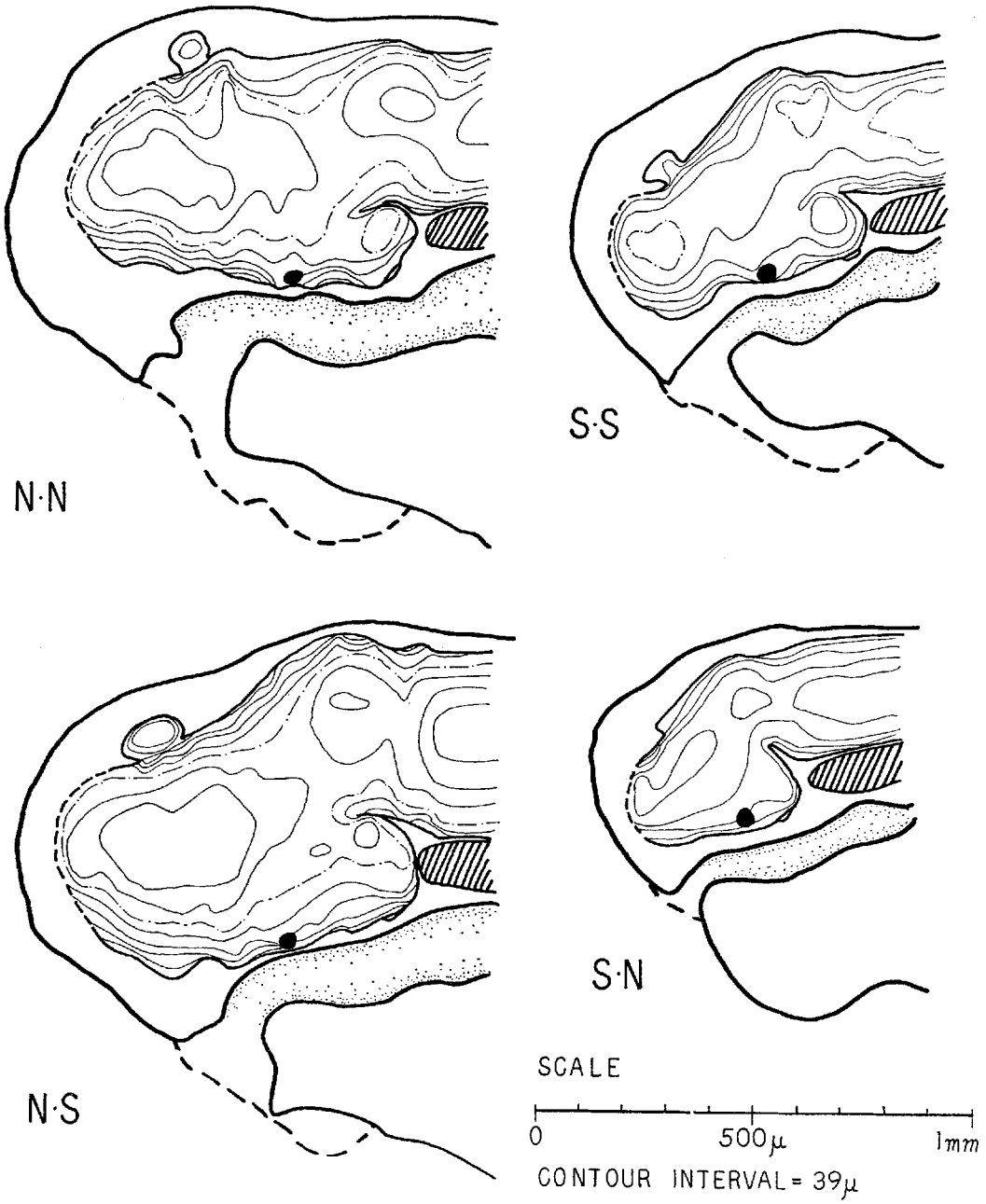

Fig. 5 Three hundred and nine hour embryos. Key as in figure 3. The dashed outline of forebrain represents that part of outline (frontal lobes) not in median plane.

ing some sort of mitotic figure. They were generally spherical unless packed together. They contained various amounts of yolk. Many contained a lot of pigment (as if they had originated at the surface of the gastrula). A search has shown that one sometimes finds one or two cells like these even in normal embryos, especially around the infundibulum. N·N and S·S embryos probably averaged about 10 or less such cells

per embryo. A few appeared to have none at all. The S·N hybrids also averaged about 5 cells of this kind per embryo. But N·S hybrids all had numbers of these cells in the thousands, and no section of the brain was without them. The significance of these cells is discussed below, but it should be mentioned here that it appears to be true that the more abnormal an N·S embryo is, the more of these neurocoel cells are found.

The thickness of the brain wall was very evident in the formation of the eye. From figures 3, 4 and 5 it can be seen that eye formation was much retarded in both hybrids. But when the eye did form, the N·S hybrid had one that was too large while the S·N hybrid had one that was too small. The large N·S eye was often rather irregular in shape. The retinal layer of cells seemed to be thicker in the N·S hybrids just as the brain wall was thicker.

Notochord

In the N·S hybrids the notochord was larger in diameter than in the N·N normals. Conversely, the S·N hybrids had smaller notochords than the S·S normals. Figure 6 shows camera lucida drawings of notochord cross sections at 225 hours. These sections were not selected in any way; they are quite typical of the sort of difference to be found at this stage. At all ages the differences were comparable. Table 1 gives the areas of notochord cross sections for 5 different ages. Each area is the mean of 10 non-adjacent sections in the region of the otic vesicles. (The reason for choosing non-adjacent sections is that a mean could be biased by having several sections in the sample represent some local aberration.) The N·S notochord became fully vacuolated considerably later than the others.

There was a difference in notochord size between N·N and S·S embryos but this was no greater than could be ascribed to the overall size difference. The hybrid notochords, however, always fell outside the range of the normals.

In table 1 the last line gives the same sort of data for Texas × Wisconsin hybrids. These were not raised at the same temperature as my embryos and should not be considered as fitting into the data in the upper

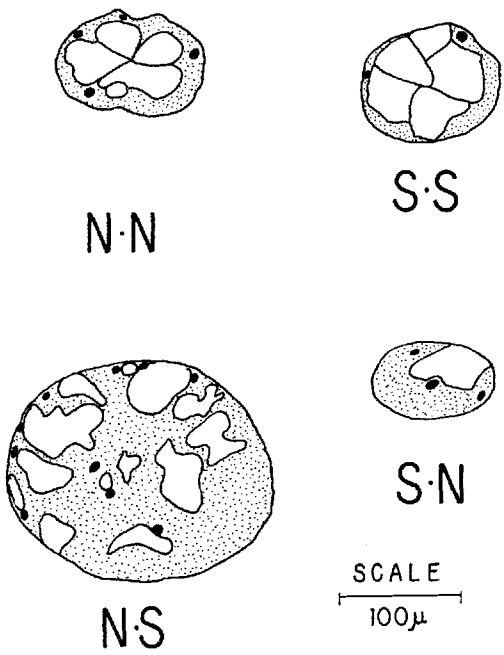

Fig. 6 Camera lucida drawings of notochord cross-sections in region of otic vesicle of 225 hour embryos. Nuclei are black; cytoplasm, stippled; vacuoles, clear.

part of the table. But reading across the line will show that, in this case too, the hybrids fall well outside the range of the normals.

Even more extreme cases of notochord enlargement can be found in Moore's material. Some had so many cells in the notochord that when they began to become vacuolated the sheath ruptured and its cross section looked not like an ellipse but like a shamrock instead. That is, it was lobed instead of circular. Many hybrids had over 30 cells in one section—compared with a normal number of 10 or less. One was found where the vacuolation caused not only the usual extension in length posteriorly but also an extension anteriorly into the third ventricle. A section through the forebrain showed a large well formed notochord in the center of the ventricle!

There seemed to be a general correlation between the size of the notochord and the size of the sub-notochordal rod, but this was not always the case. The sub-notochordal rod, especially in early stages, was often interrupted along its length and this seemed to be more common in the hybrids.

Other organs

The heart of the hybrids seemed to be the same size and shape as that of the normals. The rate of heart beat and general intensity of gill circulation did not appear to differ between normals and hybrids. The rest of the circulatory system in the hybrids was also generally normal. Of course, there were differences (such as the absence of an anterior choroid plexus in the S·N hybrids), but these differences seemed always to be associated with the differences in shape of the organs and could not be ascribed to any circulatory defects *per se*. This finding differs from those found in more extreme hybrids. For example, Vermont × southern Florida hybrids develop a heart beat very late and never have corpuscles visible in the gill circulation, and in Wisconsin × Texas hybrids only 4% develop any circulation at all (data from Moore, '46a).

No abnormalities of any organs of endodermal origin could be found. The difficulty in getting good sections of the yolk-laden cells precluded any study of the finer details of gut and liver anatomy. There was some difference in the time of mouth opening. Half the N·N embryos had open mouths at about 200 hours. But the N·S hybrids were more like their male parent in this respect and their mouths were open (50% of the cases) at the same time as the S·S embryos—about 240 hours. The perforation of the anus of hybrids was generally intermediate between the times of the normals. In all groups this occurred at about 140 hours. Gill slit perforation in N·S embryos was at the same time as N·N normals (about 210 hours). S·N embryos, on the other hand, were very abnormal around the mouth and pharynx. Mouth opening was only somewhat delayed in some embryos, and in others it never opened at all. The same irregularity was found in perforation of the gill slits.

No significant differences were found in pro- or mesonephroi or their ducts.

Changes of abnormalities with time

It is obviously impossible to make a graphic reconstruction of a sectioned embryo and then somehow let the embryo develop again. This limitation makes it difficult to make any precise statement about the course of development of abnormal organs. Since each hybrid is unique (at least among the small numbers that it is possible to reconstruct) one cannot say that a certain reconstruction of an embryo 200 hours old is exactly representative of an early stage of another embryo that was fixed when it was 300 hours old.

With this limitation in mind, I can say that there still remains a definite tendency of the abnormalities of the nervous system to decrease as the embryo developed. This is apparent from figures 2, 3, 4 and 5. This observation may represent the result of one or both of the following (hypothetical) actions: First, it is well known that amphibian embryos exhibit a strong tendency to regulate — to restore themselves to a normal condition after a departure from normal development. Such regulative processes can probably act against an abnormality that is inherited as well as against an environmentally caused one. Secondly, whatever may be the original cause of the abnormality, it could cease to act at some early stage in development so that the initial abnormality is not increased but instead is partially overcome by subsequent normal organogenesis.

Some abnormalities cannot improve. The number of loose cells in the ventricles probably does not diminish, although the size increase of the ventricles has the effect of diminishing the proportion of volume occupied by the cells. The number of notochord cells cannot decrease much except by a high mortality of these cells, and I saw no evidence that such was the case. Organs that develop in the wrong location such as the median suckers of the S·N hybrids cannot move. But in spite of these facts there remains a distinct impression that the main damage is done early in development, and later development sees the defects corrected to a certain degree.

DISCUSSION

History of the problem

The abnormality of racial hybrids of *Rana pipiens* was first described by Moore ('41) and, independently, by Porter ('41). Since that time, quite a number of different investigations have been made (see Moore, '50; Volpe, '54; Ruibal, '55). The following

is a brief summary of the conclusions as they apply to this discussion.

1. One characteristic of early development in which races differ is that of temperature adaptation during early development—both the temperature effect on the rate of development and lethal temperature limits (Moore, '49a).
2. Parents from eastern United States give abnormal hybrids if they are from populations separated by a sufficiently great distance in a North-South direction.
3. The abnormalities are variable but fall into two mutually exclusive classes. The class depends on the parentage of the hybrid as follows:
   A large-headed abnormal embryo results from the cross of a northern female with a southern male (N·S).
   A small-headed abnormal embryo results from the reciprocal of the above cross (S·N).
4. The degree of abnormality is generally greater when the parents of the hybrids are from more widely separated races. Separation is measured in a North-South direction only (East-West distance has been shown not to affect the hybrids produced).

That this is not due to a simple cline of a characteristic affecting development is shown by the crosses involving races from Central America. Although lowland Mexican (warm-adapted) races continue the clinal pattern from the United States (Moore, '47), a race from a high altitude in Costa Rica (cold-adapted) is more compatible with a race from the northern United States than are most warm-adapted, southern races (Moore, '50; Volpe, '57). Finally, Ruibal ('55) has shown that in Mexico there are races with different degrees of temperature adaptation. These give fairly normal hybrids when crossed. Such hybrids are much more normal than one would expect from experience in the United States. Those studies have recorded hybrid mortality and temperature effects on rate of development for both normals and hybrids. There is, however, little information on the type and amount of abnormality found. This is due, at least in part, to the difficulty of giving an exact description of an abnormality. It is not a measurable quantity and cannot be reported with the precision that one can give to a developmental rate, for example.

It has been suggested by previous authors that the differences in temperature adaptation between races are in some way connected with the abnormalities of their hybrids. The evidence in favor of this view is circumstantial but still impressive. Although the actual number of localities tested for both abnormality of hybrids and temperature adaptation is not very great, about 15 or so, there is no known case where races adapted for rather different temperatures give completely normal hybrids. Furthermore, the degree of abnormality seems to be roughly correlated with the amount of difference in temperature adaptation (except in Central America where the picture is still incomplete).

What data there are show that in Central America, where the "single cline" picture of hybrid abnormality breaks down, the nature of the adaptations to temperature are not the same as in North America. It was shown by Ruibal ('55) that, although the developmental rates of the high altitude races are closer to the rates of those in northern United States, the sensitivity of those rates—how much the rates are affected by a change in temperature—is very distinctly that of southern races. Volpe ('57) made a comparison between Costa Rican and Vermont races and found that, although both could be called cold-adapted, the only common factor in their reaction to developmental temperature is a similar upper lethal temperature. Obviously temperature adaptation of embryos requires adaptation of a large number of different processes within the embryos. It is not surprising that in the highlands of Central America the races have not achieved their adaptations in the same way or to the same degree as in northern United States.

It does not seem unrealistic to suppose that, whatever differences there are between warm-adapted and cold-adapted embryos, the differences will be present in the cytoplasm of the egg before fertilization. Various studies on haploids, on hybrids that block at gastrulation, and so forth

seem to hint that the nucleus and its associated organelles have little effect on development, beyond making possible an orderly pattern of cleavage, until the beginning of gastrulation. At the time of gastrulation the amount of nuclear material is several thousand times as great while the cytoplasm is essentially unchanged in quantity (this quantitative increase may be associated with the beginning of differentiation). Thus, it seems unlikely that the zygote nucleus will be the determiner of developmental rate until the beginning of gastrulation. Of course, any racial differences in egg cytoplasm would result in differences between reciprocal hybrids, although it is quite likely that many such differences could not be observed.

The hypothesis that abnormality of racial hybrids of *Rana pipiens* is associated with temperature adaptation of early development is attractive and logical. Before continuing this line of reasoning, it is best to derive as far as possible some explanation for the abnormal development of hybrids. The following paragraphs deal with the ontology of hybrids. The connection between the egg cytoplasmic factor and hybrid abnormality will be discussed later.

Hybrid development

One of the most striking characteristics of the hybrids is that they are in no way intermediate between their parents. In just about every respect where a consistent difference can be found between the parents it appeared, that the hybrids are always more extreme in this characteristic than their maternal parent. For example, northern tadpoles are larger (at a given age) than southern ones, and the N·S hybrids are even larger than the N·N normals while the S·N hybrids are even smaller than the S·S normals (fig. 1). This suggests that paternal genes do not direct the development of the hybrid in a paternal direction, but instead they seem to bring about a loss of control over the maternal traits so that the maternal traits become exaggerated.

Paternal genes, however, certainly do contribute to development. This is clearly shown by Porter's work on this problem (Porter, '41, '42). He made androgenetic (haploid) hybrids between various races. These hybrids have only the paternal genes in a normal maternal cytoplasm. Such hybrids are even more abnormal than their diploid controls. They exhibit the same abnormalities but to a much greater degree than diploid hybrids. Unfortunately, the haploidy of such animals brings about abnormalities of its own quite unconnected with heterospermy. The differences in development one might hope to see in them are masked by the "haploid syndrome." Thus, androgenetic hybrids are unsuited for the observation of developmental abnormalities due to hybridity, although, as stated below, they do suggest a hypothesis about the role of the genes in development. The effect of hybridity is then a loss of control over development. Certain tendencies, located in the cytoplasm of the egg, are not balanced by the hybrid nucleus, and the tendencies are even less balanced by the sperm nucleus alone.

The principle abnormalities and also the earliest ones detectable in the N·S hybrids are the excessive numbers of notochord and neural tube cells. In the S·N hybrids the reverse is true—there is a deficiency of notochord and neural tube cells. In the neural tube the greatest abnormality is generally at the anterior end. All other abnormalities such as those of mouth parts, circulatory system, etc., are very likely secondary effects of these two primary "errors" in early development.

It seems probable that the developmental sequence in N·S hybrids may be as follows: Some situation in the cytoplasm (which may be coupled with adaptation to cooler temperatures) results in a change in the presumptive value of blastula tissue so that more cells become notochord than normally would do so (i.e., when "balanced" by a nucleus of the N genotype). This abnormally large notochord induces an abnormally large neural plate. It is possible that the large notochord and neural plate are due to a higher rate of cell division in these regions but I saw no evidence to support this idea. No attempt was made to measure the mitotic index of those tissues. The matter can best be settled by vital staining the hybrid blastulae and tracing the movements of their presumptive regions. If the presumptive notochord areas are really larger than normal (or

thicker) then the causal sequence is probably as I have proposed above.

The oversize neural plate folds with difficulty. This accounts for the "inflated" appearance of the N·S brain at early stages. Furthermore, the folding of the neural plate forces some cells out of the inner layers which are under severe compression. These cells come to lie in the neurocoel where they continue to live for some time but do not differentiate. These are the cells found so abundantly in the ventricles of the N·S hybrids.

Probably as a result of the enlarged brain and notochord, the head is larger and the mouth and suckers are induced to form earlier and become larger than normal. In more abnormal hybrids than the ones studied here, these symptoms are even more extreme. Also they have defects in the abdomen and tail region, such as persistent blastopores, exposed endoderm in the abdomen, and so on (Moore, '46a). All of these can be accounted for by the absence of enough ectoderm to cover the embryo after so much tissue has been used to form the neural plate.

The S·N hybrids have the opposite defects. The notochord has too few cells. This induces too small a neural plate which results in a small brain, small head, and other secondary abnormalities such as irregularity of the mouth parts. There are no cells forced out of the folding neural plate so the ventricles are not filled with such cells as are found in the N·S hybrids.

The hypothesis that the neural plate abnormality is a result of the notochord abnormality can probably be tested by the classical transplantation experiments. It would be interesting to use reciprocal hybrids as host and donor pairs in such experiments. This would make possible comparisons over a much greater range of difference than if hybrids were compared only with normals.

Cytoplasmic differences

The usual explanation offered for a case where reciprocal hybrids are very different is that there is some "cytoplasmic factor" which is inherited only through the cytoplasm, hence from only one parent. For reasons given above it seems most reasonable to postulate that egg cytoplasms are different in different races of *Rana pipiens*. If such is the case, then the cytoplasmic factor is not quite like any that have so far been found in other animals. For one thing, it varies in intensity—each race has its own amount. It is hard to see how this case can be compared with such self-reproducing cytoplasmic factors as "kappa" in *Paramecium* (Sonneborn, '43) or "sex-ratio" in *Drosophila* (Malagalowkin and Poulson, '57). A closer parallel would be the sort of situation where the maternal genotype affected the egg cytoplasm and this was made apparent in the offspring. A well known case is the coiling of the shell in the snail *Limnea* (Boycott and Diver, '23). These "maternal determinations" are generally found as all-or-none characteristics and they are not ordinarily associated with a particular race of a species.

There are two studies, however, that have shown cytoplasmic inheritance associated with various races of a species. One is the work of Goldschmidt on the intersexuality in racial hybrids of the gypsy-moth, *Lymantria* (reviewed in Goldschmidt, '31). Some races transmit a "strong" factor and others transmit a "weak" factor through the cytoplasm. The factor modifies the development of the moth towards one sex. If the factor is strong enough, this can result in an intersex when the sex-chromosomes and the factor are in opposition. This has some similarity to the kind of inheritance studied here. The mode of action, however, must be quite different for in *Lymantria* the factor affects an adult trait (and is thus only apparent after metamorphosis) while in *Rana pipiens* the factor is most effective at the beginning of cellular differentiation.

The second case of racially variable maternal determinations is the situation in the mosquito, *Culex pipiens*. Laven and Kitzmiller have demonstrated a rather complex distribution of cytoplasmic factors in various populations (reviewed in Laven, '59), and they have postulated what seems to be a unique mechanism of speciation (Kitzmiller and Laven, '59). They have suggested that an alteration in the egg cytoplasm appears in one step—they call it a "mutation"—that has the effect of making male offspring sterile but only in crosses with non-mutant females. Such a

mutation would tend to spread in a population if not opposed by selection. It would eventually isolate that population reproductively from other, non-mutant populations. This case, unlike *Lymantria,* has a maternal determination which is most active in early development—in fact, it stops development at fertilization. But there is no variation in the strength of the factor and the distribution of the several factors found appears to be the result of chance, not selection.

It is interesting to speculate on the possible evolutionary origin of the cytoplasmic differences in *Rana pipiens* and see if this can further explain why a hybrid nucleus exhibits the loss of control over development. At one time, no doubt, *Rana pipiens* was a species with a uniform temperature adaptation for the embryos of all populations. This is apparently the present situation in all the other species of *Rana* studied in eastern North America (Moore, '49b). Natural selection managed to alter development in some populations in such a way that their temperature limits and developmental rates were better adapted to warmer regions. This allowed a southward spread of the species and favored a greater diversity of geographic races. Very likely the process still continues today.

Along with the origin of this ability to alter early development in such a subtle but advantageous way must have gone two changes in the gene pool of each such evolved population. The first, and most obvious change was a change in the genes that control the cytoplasm of the egg. The second change is less obvious but equally important. Those genes which affect early development—for example the gastrular map or the extent of invagination at gastrulation—must have been altered so that the result of their action on the newly evolved cytoplasm of the eggs was not different from what it had been on the earlier, unadapted cytoplasm. Only then would normal development of each embryo be assured, in spite of the change in egg cytoplasm.

In hybrids of the N·S type, as Porter ('42) suggested, the N and S genes are in opposition and are not able to modify the cytoplasm toward normality. The S genes would have an action more or less in opposition to the N genes because S genes are "correct" only for the S type of cytoplasm. The zygote nucleus would thus have little effect on some developmental processes, which is what is observed. An S·N hybrid, of course, would also have the same ineffective genotype, but the cytoplasm would be of the opposite type and, hence, abnormalities of development would be opposite in direction to that of the N·S hybrid.

I know of no test of this hypothesis, but one sort of experiment has thrown some light on the matter. Eggs of the various races of *R. pipiens* can be fertilized with sperm of a different species. If this other species is capable of giving intermediate hybrids with *R. pipiens,* then it would be most instructive to see the effect of this compatible foreign sperm. Presumably this sperm would not be adapted for any particular *pipiens* egg cytoplasm and would be neutral in balancing the cytoplasmic effects. This experiment has been performed. Moore ('46b) has made crosses between various races of *R. pipiens* and the closely related *R. palustris.* The hybrids developed normally and metamorphosed into frogs that appeared to be intermediate between the parents. Moore's conclusion includes this statement about *R. pipiens,* "every strain of this species tested can be successfully hybridized with *Rana palustris.*" But he does note that the cross of a *palustris* female with the *pipiens* male from Texas gave embryos with a "slight but definite enlargement" at the anterior end. The reciprocal cross gave embryos whose anterior ends were "slightly reduced in size."

Symbolizing the *palustris* by "P" we can say that P·S embryos are slightly large-headed and S·P embryos are slightly small-headed. The nucleus of the P·S hybrid should be genetically like that of the S·P hybrid. By the hypothesis above, the P genotype is neutral but the S genotype acts to oppose the action of S cytoplasm. It has been demonstrated that S cytoplasm tends to give small anterior ends; thus, S genes should give a tendency to large anterior ends.

What has apparently happened in this experiment is that the S cytoplasm in the S·P hybrid is not completely balanced by

one "dose" of the S genes so such a hybrid has the slight anterior reduction. The P·S hybrid has a neutral cytoplasm so the single dose of S genes gives a slight anterior enlargement. It should be stated, however, that the effect of the genes probably is not strictly additive. Hybrids between *pipiens* from Texas and a race of *pipiens* adapted for about the same temperature as *palustris* would most likely be more abnormal than any of these *R. pipiens* × *R. palustris* hybrids.

Of course the mere association between temperature adaptations and hybrid abnormalities is not proof of these hypotheses. And even the *R. pipiens* × *R. palustris* experiments can be interpreted other ways. But perhaps an investigation of the hybrids by methods used in this study would reveal whether the same developmental sequence is present. If so, then the hypothesis above would be strengthened considerably, although strict proof would still be lacking.

SUMMARY

1. Hybrid and normal embryos of *Rana pipiens* from Vermont and Florida were raised under identical conditions and 12-hour samples were taken from tailbud stage up to late larvae. These were examined externally, sectioned and stained, and graphic reconstructions made for a number of them.

2. Northern-egg hybrids had an excessive number of cells in notochord and central nervous system. Furthermore, there were many loose cells in the ventricles of the brain.

3. Southern-egg hybrids had a deficiency of notochord and neural cells.

4. These initial abnormalities probably were the cause of the other defects involving the shape of the head, development of the mouth parts, etc.

5. It is suggested that the primary abnormality was an excess of notochord cells in the northern-egg hybrids and a deficiency of notochord cells in the southern-egg hybrids. These abnormal notochords then induced neural plates which were too large and too small respectively.

6. Theories about the location and origin of the factor causing these abnormalities are proposed and discussed.

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