TRIPLOIDY (AND HAPLOIDY) IN THE NEWT, *TRITURUS VIRIDESCENS*, INDUCED BY REFRIGERATION OF FERTILIZED EGGS*

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POLYPLOIDY in the animal kingdom has been found to be a rare occurrence. A few polyploid races are known which are all parthenogenetic. VANDEL (1926, 1937) has reported the existence of a parthenogenetic triploid race of isopods in *Trichoniscus elisabethae*. ARTOM (1925) found a parthenogenetic tetraploid race in the brine shrimp, *Artemia salina*. And according to GROSS (1932) and BARIGOZZI (1934) an octoploid race of *Artemia* also exists. SEILER (1927, 1936) has described parthenogenetic tetraploid races in two species of moths, *Solenobia lichenella* and *S. triquetrella*. Recently SUOMALAINEN (1940) reported an interesting discovery of polyploidy in weevils of the family Curculionidae, in which parthenogenetic triploid and tetraploid species have been found.

Occasional polyploid individuals appear in normally diploid species. Triploids and tetraploids have been found in *Drosophila* (BRIDGES 1921, 1925; L. V. MORGAN 1925). Triploids have also been discovered among preserved embryos of frogs, *Rana esculenta* (HERTWIG and HERTWIG 1920; DALCQ 1930), and of salamanders, *Triton palmatus* (FANKHAUSER 1934), and *Triturus viridescens* (FANKHAUSER and KAYLOR 1935). PARMENTER (1933) found one parthenogenetic triploid larva in *Rana pipiens* which developed from an egg that had been pricked. KAWAMURA (1939a) has obtained in a similar way parthenogenetic triploid, tetraploid, and even hexaploid larvae in the Japanese frog, *Rana nigromaculata*. Triploid individuals have been identified among living larvae of the newt, *Triturus viridescens* (FANKHAUSER (1938a), and both triploid and tetraploid larvae in a living population of the two-lined salamander, *Eurycea bislineata* (FANKHAUSER 1939b).

Experimental means of inducing polyploidy have been used extensively on plants. The methods employed have been effective both in bringing about chromosome multiplication in somatic tissue and in causing the formation of gametes with the unreduced number of chromosomes. SAX (1937) has reviewed the various agents causing the production of polyploid cells.

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One of the most successful experimental methods for inducing polyploidy has been the application of extreme temperatures. The effect of low temperature on *Datura* and other plants was described by BELLING in 1925. He found that low temperature applied during meiosis resulted in non-reduction and consequent doubling of the chromosomes in the gametes. Diploid gametes produced in this way combined with normal haploid gametes to give triploid zygotes. High temperatures have been found to cause somatic doubling. RANDOLPH (1932), working with maize, has used this treatment on root tips of seedlings, on the zygote, and on the pro-embryo. SAX (1936) and DERMEN (1938) have found that alternating cold and heat treatments provide another effective means of producing diploid and tetraploid gametes.

Exposures of eggs to low and to high temperatures have been very useful in inducing polyploidy in animals. GROSS (1932) has obtained tetraploid and octoploid individuals in *Artemia salina*, developing from eggs of diploid and tetraploid females, respectively, which had been exposed to a temperature of 4°C while the eggs were in the oviduct. This treatment caused the eggs to retain the polar body which would normally be given off during the single maturation division. Tetraploid silkworms have been produced by high temperature treatment of the eggs immediately after oviposition (HASIMOTO 1933).

Chromosome duplication arising by low temperature treatment has been obtained in amphibian eggs by ROSTAND (1934, 1936, 1938). ROSTAND studied the effect of refrigeration on hybridization between species of frogs and toads. Certain of the hybrid combinations which normally do not survive late embryonic stages do, under the influence of refrigeration immediately after fertilization, develop into swimming larvae. These larvae develop from eggs in which the sperm chromosomes are eliminated from participation in development. They are gynogenetic. Most of the tadpoles arising as a result of the refrigeration are gynogenetic haploids; few are diploid. ROSTAND believes that the cold treatment determines not only gynogenetic development, but also causes a duplication of maternal chromosomes, giving rise to diploid eggs. His work suggested the use of cold treatment for inducing triploidy in *Triturus viridescens*. Experiments carried out in the late fall of 1938 were at once successful. The results have been reported in a preliminary communication (FANKHAUSER and GRIFFITHS 1939). These experiments have since been repeated and confirmed (GRIFFITHS 1940).

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MATERIALS

The eggs of the common American newt, *Triturus viridescens*, were used in these experiments. Adult females were collected in October, 1938, and again in November, 1939, from a pond near Hellertown, Pennsylvania. The animals were brought into the laboratory and were kept in the refrigerator at about 10°C, a temperature which will prolong the normal activity of the ovaries of captive animals.

The normal breeding season of the newt is in the spring, during April and May. Eggs are obtainable during the fall and winter months by means of pituitary stimulation. In November, 1938, and again in December, 1939, and January, 1940, large females were implanted subcutaneously with anterior pituitary lobes from females of *Rana pipiens*, according to the technique developed by KAYLOR (1937). A total of twelve females were used in these experiments. Each female was implanted twice, underneath the skin of the lower jaw, with a single gland, allowing an interval of two days between two implantations. The animals began to lay eggs on Elodea leaves usually on the first or second day following the second implantation and deposited from 30 to more than 100 eggs in the course of eight to nine days. Both freshly collected specimens and animals kept in the laboratory for almost two months before implanting gave these results. Eggs from animals kept in captivity for more than two months were not used, since they are usually less viable than eggs from freshly collected animals.

METHODS

Newt eggs are deposited singly and are fertilized as they pass through the cloaca by spermatozoa stored in the spermatheca connecting with the cloaca. The female requires only about three minutes for the deposition of each egg, and it is therefore easy to obtain the eggs within a very short time after insemination. At this time the egg is in the metaphase of the second maturation division.

Eggs were collected immediately after laying and were placed in small dishes at the desired low temperature. After the treatment which lasted two to twenty-four hours, they were allowed to develop at room temperature (20° to 23°C). In the preliminary work in November, 1938, an ordinary electric refrigerator was used. Last fall further experiments were undertaken to determine more precisely the factors involved in the induction of triploidy, by a more careful control of the temperature, of the stage when the eggs were exposed, and of the duration of the exposure. In these experiments a constant temperature water bath was employed and so equipped that covered stender dishes could be completely immersed in water at the desired temperature. All control eggs were left at room temperature.

When the larvae developing from treated and control eggs reached the stage of three finger buds (three to four weeks), just before they began to

feed, the posterior one-third of their tails was cut off and fixed in Bouin's solution, after the method described by FANKHAUSER (1938a). These tail-tips were then stained with Harris' acid haemalum. Mitotic figures can usually be found in the epidermis of the tailfin. Chromosome counts in metaphase plates were made for each larva.

After tail-tip amputation, the larvae were allowed to continue development. All the experimental and control animals from the same female were kept under the same conditions of temperature, food, volume of water per animal, and of water change. Each young larva was put into an individual watch-glass after tail-clipping and was fed small *Daphnia* until the hind limb buds appeared (seven weeks). At this time each larva was transferred to a larger finger bowl and fed *Enchytraeus* worms from then on.

Most of the animals were fixed at some time during metamorphosis, since they are difficult to raise beyond this stage. All animals were fixed in Michaelis' fluid, and a number of them were sectioned at 15μ . The sections were stained with the nuclear stain, Harris' acid haemalum, and counterstained with Orange G. Drawings of the nuclei were made with the camera lucida, using a $10\times$ ocular and the $43\times$ objective.

RESULTS

General results

A total of 245 eggs, from a dozen different females, were refrigerated in the course of these experiments. This number represents four groups of eggs, each group being subjected to treatment at a different temperature and for different periods of time. Summaries of the results in each series are presented in table 1 and table 2.

TABLE 1
Summary of development of refrigeration and control eggs.

	SERIES A		SERIES B		SERIES C		SERIES D	
	REF.	CON.	REF.	CON.	REF.	CON.	REF.	CON.
Total	114	146	78	67	39	33	14	10
Died before end of gastrulation	64	10	32	11	21	—	7	—
Died in early embryonic stages	15	19	3	—	2	—	1	—
Normal larvae	24	117	41	56	15	33	5	10
Slightly abnormal larvae	1	—	2	—	1	—	1	—
Dwarfed, more or less edematous larvae	10	—	—	—	—	—	—	—

One hundred out of 245 refrigerated eggs were successfully reared to swimming larval stages. The remaining 145 eggs died in greatest numbers during cleavage, which in many cases was very irregular, and during the critical period of gastrulation. A few underwent irregular neurulation and

died in abnormal embryonic stages. This mortality represents 59.1 percent of the total number of treated eggs. This rate is much higher than in untreated control eggs where the rate is only 11.7 percent of the total. The mortality varied greatly between groups of treated eggs obtained from

TABLE 2
Summary of the effects of variation in the treatment.

SERIES	TEMPERATURE	STAGE AT WHICH TREATED	DURATION OF TREATMENT	NUMBER OF LARVAE		
				3N	2N	N
A	0.5° to 3.0°C	Immediately after fertilization	16 to 26 hours	24	1	10
B	1.55° to 1.6°C	Immediately after fertilization	2 hours	—	2	—
		Immediately after fertilization	5, 10, 12, 14, 15, 20, 24 hours	34	—	—
		$\frac{1}{2}$ hour after fertilization	20 hours	—	1	—
		1 hour after fertilization	20 hours	—	3	—
		$1\frac{1}{2}$ hours after fertilization	20 hours	—	3	—
		Immediately after fertilization	6, 10, 20 hours	16	—	—
C	0.45° to 0.5°C	Immediately after fertilization	20 hours	6	—	—
D	0°C.	Immediately after fertilization	20 hours	80	10	10
Total						

different females. It ranged from 25 to 100 percent. In the groups of control eggs from different females, the mortality varied from 0 to 33.3 percent. No correlation could be found between egg mortality and the length of time the animals had been in captivity before egg-laying was induced.

The results of chromosome counts in the tail-tips of larvae developing from treated eggs and control eggs are summarized in table 3.

TABLE 3
Results of chromosome counts in the tail-tips of larvae.

	FROM REFRIGERATED EGGS	FROM CONTROL EGGS
Total	100	216
Triploid	80	1
Diploid	10	215
Haploid	10	—

Identification of triploids

The stained tail-tip is excellent material for establishing the triploid condition of the larvae, both by the number of chromosomes in the mitotic figures and by the size of the nuclei in various tissues (FANKHAUSER 1938a).

Direct counts of the chromosomes in the epidermal cells of the tailfins of the young larvae were made. The first indications of the presence of more than the diploid number of 22 chromosomes were the larger size of the mitotic figures and the crowding of the chromosomes (fig. A-D).

Polar views of metaphase plates were preferably selected for counts. In two cases, the triploid number of 33 chromosomes was accurately counted. In 41 tail-tips, where the metaphase figures were not too crowded for a fairly accurate count, counts of 30 or more chromosomes were made. In 32 tail-tips, only 27 or more chromosomes could be counted, and in six tail-tips only 25 or 26 chromosomes could be distinguished. In these last 38 cases, the chromosomes were too close together and often too overlapping for more accurate counts. It was evident, however, that there were actually more chromosomes present than the number that could be clearly distinguished.

The size of the nuclei and cells was larger on the average in the triploid than in the diploid tail-tips, though there is considerable variation in the size of tail-tip nuclei. This fact is strikingly demonstrated in the camera lucida drawings of epidermal nuclei (fig. 1). Such nuclei were drawn in a similar manner on graph paper and their surface areas measured by counting the number of squares within nuclear outlines. Measurements of the surface areas of 30 epidermal nuclei in a triploid and in a control tail-tip indicate the ratio to be about 1:1.5. Since epidermal nuclei are flat discs, this ratio can be considered approximately the one for nuclear volumes. The size of the nuclei is therefore about proportional to the number of chromosomes.

The nuclei in the lateral line sense organs also show this difference in size between triploid and control nuclei. The number of nuclei in each triploid sense organ is reduced, however, so that the organ as a whole is about the same size as the diploid sense organ (fig. 2). Erythrocytes found in a few capillaries have nuclei which are also correspondingly larger in the triploid than in control tail-tips (fig. 3).

Evidence of triploidy may again be obtained later from pieces of skin shed by the larvae at metamorphosis. Such pieces were fixed and stained in the same way as tail-tips. The epidermal nuclei in the triploid skin are larger and show a wider spacing than in diploid skin (fig. 4). Since the nuclei in moulted skin are degenerating, they were in many cases in a poor state of preservation and could not be relied upon to give any conclusive indication of their actual original size.

A final evidence of triploidy, which has been of use in some cases as a method for tentatively identifying triploids among living larvae, is the size of the melanophores. Individual pigment cells, especially on the head, are larger, fewer, and more widely spaced in the triploid than in the control during early larval stages (fig. M). The melanophores of some of the triploids did not seem to be consistently larger than in controls because of differences in degree of expansion, although in all triploids they were fewer in number and much more widely spaced.

FIGURES 1 to 4.—Camera lucida drawings of nuclei in the tail-tips, from whole mounts of the tail-tips, stained with Harris' acid haemalum. Nuclei from triploids are shown on the left; those from controls are on the right. 365 $\times$. FIGURE 1.—Epidermal nuclei. FIGURE 2.—Lateral line sense organs. FIGURE 3.—Nuclei of erythrocytes. FIGURE 4.—Nuclei of moultskin.

Development of triploid and control larvae

The development of 81 triploid larvae and 166 diploid control larvae has been observed from the time of tail-tip amputation (table 4). The great majority of the triploid larvae showed perfectly normal development as did all controls, until the time of death. About one-half of the triploids and controls that died between the time of tail-clipping and metamorphosis were killed by an attack of mould. The other half of the animals died from

unknown causes. Five triploid larvae were slightly abnormal. Only one of these was able to reach metamorphosis. This animal possessed dwarfed gills on the left side, and it had no digits on the left fore-limb until it was four weeks old. As this animal grew larger, its body developed a lateral curvature so that the left side was slightly concave. It was found to be partly haploid.

TABLE 4
Fate of triploid and control larvae.

	TRIPLOID	CONTROL
Normal larvae		
Fixed during late fore-limb stages	8	4
Died during late fore-limb stages	12	24
Fixed during hind-limb stages	3	16
Died during hind-limb stages	4	19
Died just before moulting	1	5
Reached metamorphosis	48*	98
Abnormal larvae		
Fixed during late fore-limb stages	4	—
Reached metamorphosis (partly haploid)	1	—
Total	81	166

* Includes 1 natural triploid.

The triploids showed no consistent differences from control animals in their rate of development. There was some variation within particular groups, but a similar variation was noted also in the controls. In the first group (A) of 25 triploids at the time of tail-clipping, 15 triploids were slightly behind controls of the same age; the other ten triploids were at the same stage of development. In the course of the next few weeks, three of the triploids had caught up with the controls, 13 were now slightly ahead, and eight were still somewhat behind the controls. The advance made by many of the triploid larvae can probably be accounted for by the fact that the limited supply of small *Daphnia* available at the time when the larvae began to feed was preferably given to the triploids. In the group of 54 tri-

EXPLANATION OF PLATE I

FIGURES A to L.—Photomicrographs of mitotic figures in the epidermis of tail-tips, from whole mounts of tail-tips, stained with Harris' acid haemalum. 348×.—A, Triploid prophase. B, Triploid metaphase. C, Triploid anaphase. D, Triploid telophase. E, Diploid prophase. F, Diploid metaphase. G, Diploid anaphase. H, Diploid telophase. I, Haploid prophase. J, Haploid metaphase. K, Haploid anaphase. L, Haploid anaphase.

FIGURE M.—Photograph of a triploid (left) and a diploid control (right) larva, four weeks old. Note size and distribution of melanophores on the triploid. 6.9×.

FIGURE N.—Photograph of a triploid (left) and a diploid control (right) larva, nine weeks old. 3×.

ploids observed in recent experiments, all the triploids were approximately at the same stage of development as the control group, when identified by the tail-tip method. At the time when these animals were transferred to larger bowls, during the early hind-limb stages, only six were slightly ahead of the controls, four were slightly behind, and 36 others were at the same stage of development.

There were never any appreciable differences in size between triploid larvae and controls at the same stage of development (fig. N-P). The slight deviations in developmental rate in some cases were reflected in small differences in size between triploids and controls of the same age, however (fig. M).

The triploid larvae which successfully reached metamorphosis began moulting at about the same time as most of the controls. In series A, this occurred between the ages of 11 weeks and 15 weeks. One triploid, the slightly abnormal animal with a spinal curvature, began to moult at 14 weeks, while the majority of its controls had already moulted at 11 to 11½ weeks. Another triploid, the slowest developing of the entire first group of triploids, began moulting at the end of the 14th week, but most of its controls had moulted at 12 weeks. In the group of larvae raised this year, metamorphosis was not reached until the 15th week. Here also the triploids and controls underwent almost simultaneous metamorphosis.

Microscopical anatomy of triploid larvae

Cell size and organ size

Serial sections of 15 triploid and 13 diploid control metamorphosing animals, between the ages of 12½ and 16 weeks, were studied. Camera lucida drawings reveal that the nuclei and cells in the various organs and tissues are distinctly larger in the triploids than in the controls (fig. 5-12). In many cases the nuclei are irregular in shape and show considerable individual variation in size, but on the average the nuclei in triploid organs

EXPLANATION OF PLATE 2

FIGURE O.—Photograph of a triploid (left) and a diploid control (right) larva, 11 weeks old. The same larvae as shown in Figure N. 2.8×

FIGURE P.—Photograph of a metamorphosing triploid (left) and a diploid control (right) animal, 14½ weeks old. 2.8×

FIGURES Q to S.—Photomicrographs of transverse sections through ovaries. 220×

FIGURE Q.—Left ovary of a triploid animal, 15 weeks old. Note small size of ovary, undifferentiated germ cells with typical lobed nuclei, oogonia, and young oocytes. Larger oocytes are absent.

FIGURE R.—Right ovary of a triploid animal, 15½ weeks old. This triploid ovary possesses some large growing oocytes, an unusual occurrence for triploid ovaries at this stage of development.

FIGURE S.—Left ovary of a diploid control animal, 15 weeks old. Note size of ovary, and the large growing oocytes.

FIGURES 5 to 8.—Camera lucida drawings of nuclei in the various tissues and organs of a triploid and a control animal, $13\frac{1}{2}$ weeks old. Nuclei from triploid are shown on the left; those from control are on the right. $365\times$. FIGURE 5.—Nuclei from spinal cord. FIGURE 6.—Nuclei from notochord. FIGURE 7.—Nuclei from spinal ganglion. FIGURE 8.—Nuclei from cartilage of the pelvis.

are larger than nuclei in diploid organs. Despite this increase in nuclear and cell size, however, the organs as a whole in the triploids are not larger than corresponding diploid organs in controls, but are actually about the same size. This is accounted for by the fact that triploid organs consist of fewer cells than diploid organs. These observations are in full agreement with those of FANKHAUSER (1939a, 1941) on two natural and two presumably cold-induced triploids.

FIGURES 9 to 12.—Camera lucida drawings of nuclei in the various tissues and organs of a triploid and a control animal, 13½ weeks old. Nuclei from triploid are shown on the left; those from control are on the right. 365X. FIGURE 9.—Nuclei from kidney. FIGURE 10.—Nuclei from lingual glands. FIGURE 11.—Nuclei from liver. FIGURE 12.—Nuclei from intestine.

The only abnormal triploid animal which reached metamorphosis has been found to be not entirely triploid throughout. At metamorphosis its body was bent laterally, producing a concave left side. The tail-tip showed the presence of a number of small, haploid epidermal nuclei, and sections of the entire animal revealed that the animal is predominantly haploid on the left side.

Sex differentiation in triploids

FANKHAUSER (1938b, 1940) has recently described the normal process of sex differentiation in *Triturus viridescens* and also sex differentiation in four triploid animals which he obtained. At the time of metamorphosis, the gonads of normal diploid animals are already differentiated into ovaries or testes. The ovaries of the females are characterized by a central ovarian cavity surrounded by large growing oocytes. At the periphery of the ovaries are nests of darkly-stained oogonia and young oocytes distinguishable by their round nuclei with typical prophase structure. Occasional undifferentiated germ cells with lobed nuclei may be found in all the ovaries. The testes of the males are small and solid, and they show a uniform distribution of spermatogonia, the nuclei of which are deeply-lobed. In the group of four triploids, one was a male with normal testes, and three were females with rudimentary ovaries.

Of the 13 metamorphosing control animals sectioned and studied by the writer, five were females and eight were males. In the group of 15 metamorphosing triploid individuals investigated, the gonads of all 15 were definitely differentiated in a female direction. The ovaries were smaller than normal control ovaries and appeared to be rudimentary. The central cavity in each was very narrow, and the body of the ovary consisted mainly of oogonia and young oocytes, in addition to many undifferentiated germ cells (fig. Q). Oocytes in early growth stages were lacking in all triploid ovaries excepting those of one animal. Both ovaries in this individual contained a number of growing oocytes (fig. R).

Haploid larvae

Ten larvae developing in the first group of cold-treated eggs were conspicuously smaller and less advanced in development than controls, at the time of tail-clipping. The mitotic figures in the tail-tips were much smaller and obviously contained fewer elements than corresponding control figures (fig. I-L). Chromosome counts in metaphase figures in the tail-tips of nine of the larvae gave the haploid number of 11 chromosomes. The nuclei in the epidermal layers and in the lateral line sense organs were considerably smaller than in controls (FANKHAUSER and GRIFFITHS 1939, figs. 1, 2). Only three of the tail-tips were completely haploid, however. The other six tail-tips contained sectors with larger, presumably diploid, nuclei.

All these individuals developed from eggs of one female. The bodies of all except one were somewhat edematous. The melanophores on the head, particularly, were very small and much more numerous than in controls (FANKHAUSER and GRIFFITHS 1939, figs. 3, 4). All haploids had gill, limb, and jaw abnormalities. They were inactive, and all except two took no

food. Three of these larvae died within a short time after tail-clipping; six of them were fixed during late fore-limb stages; only one survived further development. This exceptional animal was the most normal-appearing of the group. It fed well and lived for 16 weeks, though it did not become very large and did not begin to metamorphose during this time. As this animal grew older, it developed a lateral body curvature, the left side becoming concave. The left gills remained stunted, and the body cavity became swollen with fluid.

Seven of these larvae were sectioned. Two of them, fixed at the age of five weeks, were haploid throughout. The other five larvae were mixtures of haploid and diploid cells. All of them were haploid, or predominantly haploid, at least on one side of the body. Larger diploid nuclei could be found in all the organs. The oldest larva (16 weeks) had a greater proportion of diploid cells than had any of the others. The left side of this animal seemed to be almost completely haploid, while the right side was mainly diploid. The gonads were very small and resembled ovaries in that they possessed a central cavity. They were closely applied to the under surfaces of the mesonephroi, and they had a great proliferation of sex-cord material from the mesonephroi. There were a few undifferentiated germ cells in each ovary. The small size of the nuclei in the interstitial tissue of both ovaries indicates that they were probably completely haploid.

DISCUSSION

Natural triploidy

The occurrence of occasional triploid frog and salamander embryos and larvae, reported by several investigators, suggests that natural triploidy is not rare in the Amphibia, at least not among salamanders. FANKHAUSER (1934) discovered two triploid embryos of *Triton palmatus* in a group of 14 and later reported two triploid embryos of *Triturus viridescens* in a group of 11 embryos (FANKHAUSER and KAYLOR 1935). More recently FANKHAUSER (1939b) found 13 triploids in 134 living larvae of the salamander, *Eurycea bistineata*. Previously, he (1938a) reported the discovery of four triploids in a lot of 100 living larvae of *Triturus viridescens*, two of which were naturally-occurring. Two others developed from eggs which had been placed in a refrigerator for some hours after laying.

During the past three years, a total of 489 living larvae, raised from normal, untreated newt eggs from 25 different females, have been tail-clipped and examined for chromosome numbers by the present investigator. Only two of these, both from different mothers, were triploid. FANKHAUSER (1938a) had previously found two natural triploids in 100 living larvae. Recently, one naturally-occurring triploid was observed in a group of 60 *viridescens* larvae (CROTTA, unpublished data). These series together would

indicate the frequency of natural triploidy in this species to be about 0.7 percent.

Origin of triploidy

Triploid frog and salamander embryos may arise by the fertilization of diploid eggs. HERTWIG and HERTWIG (1920) found that their triploid embryos of *Rana esculenta* developed from eggs laid by one female. These triploid frog embryos undoubtedly arose by fertilization of diploid eggs, since eggs from the same female gave rise to gynogenetic diploid embryos after insemination with the sperm of *Bufo viridis*. BATAILLON and TCHOU-SU (1929) have described in *Bufo calamita* one egg in which the reduction division was apparently omitted. Diploid oocytes may arise from tetraploid ovarian tissues by normal maturation. One other likely possibility, however, is that diploid oocytes might undergo a normal maturation but remain diploid because of failure of extrusion of chromosomes into a second polar body.

Abnormal temperatures applied during maturation of the gametes may suppress meiotic divisions in both animals and plants. The chromosomes can divide, but their separation during anaphase is prevented. This may be explained by disturbances in the spindle mechanism resulting from changes in the viscosity of the cytoplasm brought about by changes in temperature (SAX 1937; DERMEN 1938).

It has been remarked that ROSTAND (1934, 1936, 1938) obtained chromosome duplication in frog and toad eggs by exposing them to freezing for several hours immediately after insemination with sperm from males of a different genus. The eggs develop without participation of the male chromatin and may give rise to normal diploid gynogenetic tadpoles. ROSTAND suggested that refrigeration caused a duplication of the maternal chromosomes which led to the production of diploid eggs. Since the amphibian egg at insemination is in the second maturation division, cold treatment at this time must suppress this division. It is very likely, then, that the chromosome duplication is actually due to a retention within the egg of the haploid set of chromosomes normally going into the second polar body.

This hypothesis of the origin of triploid newts was offered in a recent communication (FANKHAUSER and GRIFFITHS 1939) and has been supported by new evidence in recent experiments (GRIFFITHS 1940). It has been found that triploid newt larvae develop only from eggs that are refrigerated immediately after laying. Eggs on which cold treatment is delayed until as little as one-half hour after laying always develop into diploid larvae. Since the second maturation division is normally completed within one hour after deposition of the egg, refrigeration applied immediately can inhibit this division from going to completion. By this treatment

the egg retains the second polar body chromosomes and therefore, becomes diploid. Union of a diploid female pronucleus with a haploid sperm nucleus already present would give rise to triploid zygotes and eventually to triploid larvae. Direct confirmation of this scheme could be provided only by detailed cytological studies of cold-treated eggs, which have not been undertaken so far.

It was hoped that variations in the cold treatment would give some information on the conditions which led to the production of the ten haploid larvae in the first series (A) of experiments (table 3). Unfortunately, no other haploids have again been produced. Since all the haploids developed from eggs of the same female, it is quite probable that conditions peculiar to these particular eggs were accountable for haploid development. This could not have been characteristic of all eggs from this female, however, since other refrigerated eggs developed into triploid larvae, and the control eggs gave diploid larvae without exception. Further modifications of the treatment may give some evidence for the origin of haploid larvae.

Effects of triploidy

General effects

Triploid newt embryos which have undergone successful cleavage and gastrulation usually show perfectly normal development through later stages. In the entire group of triploids, discovered at the age of three to four weeks (tail-clipping), 60 percent were reared to metamorphosis. In the controls the corresponding figure was 59 percent. After the first moult the larvae usually refused to take any food, and they could not be induced to feed again. This factor, in addition to the severe metamorphic changes within the animals, made it difficult to raise the larvae through metamorphosis. This was found equally in triploid and control groups. The majority of animals were, therefore, fixed during metamorphosis, when they seemed no longer able to survive.

Triploidy in newts does not seem to have the same physiological and morphological consequences that have been described for many triploid plants and some triploid animals. In plants, triploidy is often associated with a slow growth rate. The growth rate of triploid newt larvae, however, parallels very closely the rate of diploid controls. The deviations within the triploid group are no greater than among control animals.

The nuclei and cells in the various tissues and organs of triploid larvae are larger than diploid nuclei and cells, just as in the case of triploid plants as exemplified by triploid mutants of *Oenothera* (STOMPS 1912; LUTZ 1912). In many triploid plants and in some triploid animals, the cell number in the organs remains more or less the same as in diploids, and the triploids are as a whole, therefore, larger. Triploid evening primroses are charac-

teristically large plants, and they have been called the "semigigas" mutants. Among the animals, VANDEL (1927) reports that triploid females of a parthenogenetic race of isopods (*Trichoniscus provisorius*) are definitely larger than diploid females of the same species. ARTOM (1925, 1928) has discovered a similar phenomenon in tetraploid *Artemia*, and SEILER (1927) in tetraploid *Solenobia*.

Unlike these forms, however, triploid newt larvae do not show any signs of gigantism. Likewise, triploid and even tetraploid larvae of *Eurycea bislineata*, are not significantly larger than diploid individuals (FANKHAUSER 1939b). This fact indicates that some regulatory factor must come into play in the triploid and tetraploid individuals, to offset the increase in cell size. This regulation is realized by a decrease in the number of cells in the various organs. The final result is that triploid and tetraploid organs, and consequently the whole animal, remain close to the normal size of diploid individuals (*cf.* FANKHAUSER 1941). Among plants, also, there are tetraploid forms which do not show any evidence of gigantism, even though the size of the cells is larger than in diploid forms—namely, *Euphorbia granulata* (HAGERUP 1932).

On Sex

Triploidy affects one of the sexes in animals in which sex is determined by a distribution of sex chromosomes. The disturbances are reflected in the gonads of the heterogametic sex, while in the homogametic sex there is no effect. In *Drosophila*, where the female is homogametic, triploids are either normal females or intersexes (BRIDGES 1922). In moths, however, where the female is heterogametic, triploids are normal males or intersexes (GOLDSCHMIDT and PARISER 1923).

The mechanism of sex determination in the Amphibia has not yet been conclusively shown, although breeding experiments with frogs and toads have indirectly contributed some information as to what it may be. The experiments of CREW (1921) and of WITSCHI (1923, 1929) with hermaphroditic frogs indicates that the male sex is the heterogametic one in frogs. The breeding experiments with sex-reversed male toads have not given consistent results. HARMS (1926) has evidence which suggests male heterogamety. PONSE (1931), however, carrying out similar experiments, obtains results which indicate male homogamety in toads.

On the basis of the hypothesis of CREW and WITSCHI for frogs, parthenogenetically developed frogs should become females. KAWAMURA (1939b) has found this to be the case in *Rana nigromaculata* and *R. japonica*. Most of his parthenogenetic diploid frogs were females, although he did find one male and several other animals with transforming gonads.

Studies on the gonads of triploid newt larvae reveal that the male has

typical testes, but that the female has small, poorly-developed ovaries (FANKHAUSER 1938b, 1940). BÖÖK (1940) has recently reported a triploid testis in a group of testes taken from adult *Triton taeniatus* males. This testis was of the same size and appearance as diploid testes from normal males, and sections through it reveal no indications of intersexuality.

In the group of 15 triploid animals investigated by the author, all 15 animals were females possessing rudimentary ovaries. No triploid males appeared in this lot of experimental triploids. The effect on the ovaries appears to be the same as that described by FANKHAUSER (1940) in natural triploids, with the exception of one animal, the ovaries of which, although smaller than in the control, contained a few large, growing oocytes (fig. R). The higher degree of development of the ovaries of this animal may indicate that triploidy affects the rate of growth and differentiation of the ovaries rather than the fundamental character of their structure. The observations confirm FANKHAUSER'S conclusion that in newts, triploidy has a pronounced effect on the female sex, probably comparable to that observed in triploid moths. This may mean that the female sex is heterogametic in newts and other salamanders, in contrast to frogs where male heterogamety is more probable. The absence of males among the fifteen experimental triploids examined so far is also important in this connection and presents an interesting problem which will be discussed later when the condition of the gonads of all experimental triploids will be known.

SUMMARY

1. A total of 245 eggs of the newt, *Triturus viridescens*, obtained by pituitary stimulation of 12 adult females, were exposed to low temperatures between 0° and 3°C.

2. One hundred of the treated eggs were successfully raised to swimming larval stages. The tail-tips of these 100 larvae and 216 controls were amputated, fixed, and stained, and examined for chromosome numbers. After tail amputation, the larvae were allowed to continue development.

3. Eighty of the 100 larvae developing from refrigerated eggs were triploid; ten were diploid; ten were haploid. All controls were diploid except one which was a naturally-occurring triploid.

4. Triploid larvae developed only from eggs refrigerated for five hours or more immediately after laying. Eggs to which application of cold treatment was delayed until one-half hour after laying all gave rise to diploid larvae. These results support the hypothesis that cold treatment produces triploidy by inhibiting the second maturation division, since this division is completed within one hour after deposition of the egg.

5. The frequency of natural triploidy in newts, based on an examination

of the tail-tips from 649 larvae developing from normal untreated eggs, is about 0.7 percent.

6. Seventy-five triploid larvae showed perfectly normal development during larval stages. Five triploid larvae were slightly abnormal.

7. There were no appreciable differences in developmental rate between triploid and control larvae, and such deviations that did occur were very slight.

8. None of the triploid larvae showed any signs of gigantism. All the triploids were identical or almost identical in size with controls of the same age.

9. Nuclei and cells in the epidermis, lateral line sense organs, and the erythrocytes in triploid tail-tips are on the average larger than in diploid control tail-tips. Serial sections of metamorphosing triploid and control animals reveal that the nuclei and cells in the various organs are larger in the triploids than in controls but that the number of cells is reduced. Such a mechanism of regulation accounts for the fact that triploid larvae are no larger than diploid controls.

10. Fifteen of the metamorphosing triploid larvae were investigated microscopically. All were females with rudimentary ovaries.

11. Ten larvae from refrigerated eggs were dwarfed, more or less edematous larvae. Tail-tips showed that they were haploid. Sections of six five-week-old animals reveal that only two were completely haploid, while four others were predominantly haploid. One haploid lived for 16 weeks. Sections through it show that the left side of the animal was almost completely haploid, while the right side was diploid.

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