KARYOPLASMIC STUDIES IN HAPLOID, ANDROGENETIC HYBRIDS OF CALIFORNIA NEWTS [1]

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The combination of a nucleus of one species acting in the cytoplasm of another is theoretically ideal for the study of the roles of the nucleus and cytoplasm in the differentiation of characters which distinguish the species. The means for achieving interspecific karyoplasmic combinations has been by heterospermic fertilization of eggs devoid of active maternal chromosomes. The preponderance of interspecific karyoplasmic hybrids in amphibians has been androgenetic haploids (Fankhauser, 1955; Moore, 1955). Although repeatedly attempted in the past, these haploids typically die prior to the appearance of recognizable species characters. Of 21 interspecific androgenetic, haploid hybrid combinations enumerated by Fankhauser (1955), none developed to a stage permitting an analysis of the relative influence of the foreign haploid nucleus or the cytoplasm on a specific character.

In a classic experiment, Hadorn (1936) overcame the difficulty of rearing haploid hybrids by grafting haploid tissue of *Triton palmatus* cytoplasm and *T. cristatus* nucleus to diploid homospermic *T. alpestris* hosts. The postmetamorphic skin of *palmatus* is characterized by projections formed by flattened epidermal cells; the skin of *cristatus* is smooth. The grafted haploid hybrid skin on metamorphosed *alpestris* hosts possessed projections typical of *palmatus,* the cytoplasmic donor in the hybrid merogon. This species character, although it appears late in development, has been considered to be "determined" in the egg cytoplasm prior to insemination, *i.e.,* the character is an expression of the genotype of the diploid oocyte from which the egg was derived. As was recognized by Hadorn, a complicating factor in this experiment is that the epidermis of *alpestris,* the diploid host, also forms skin protuberances.

Dalton (1946) produced hybrid merogons of *Taricha* (*Triturus*) *rivularis* cytoplasm and *T. torosa* nucleus. The two species differ strikingly in larval pigment patterns. Dalton transplanted haploid hybrid merogonic neural crest to diploid *torosa* hosts. The transplanted haploid hybrid tissue produced a pigment pattern essentially like that of *torosa,* the nuclear contributor. However, an early influence of the cytoplasmic donor, *rivularis,* was manifested in the rate of melanization and distribution of the pigment cells.

The circumvention of the early demise of haploid hybrid tissue by transplantation to diploid embryos has been of value, but in order to rule out the possibility of any

[1] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biological Sciences, Stanford University, Stanford, California.

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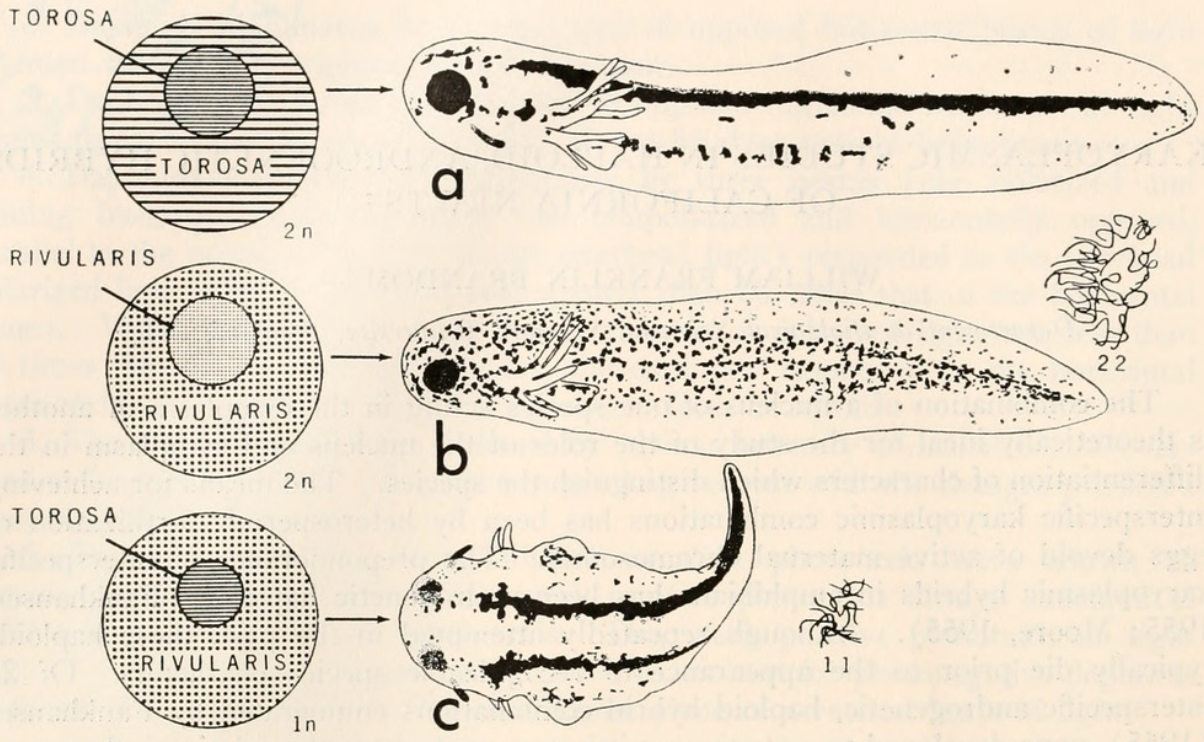


FIGURE 1. Drawings of larval pigment patterns and balancers: a, homospermic diploid *T. torosa* (paternal nuclear contributor); b, homospermic diploid *T. rivularis* (maternal cytoplasmic contributor); c, androgenetic, haploid hybrid. Schematic karyoplasmic constitutions depicted on the left; camera lucida drawings of diploid (22) and haploid (11) chromosome complements on the right.

influence of the host tissues on the differentiation of species characters, it is still desirable to obtain whole haploid hybrid larvae. During the course of an experiment designed to study gene-dosage effects in polyploid hybrids (Brandom, 1960), two of 54 larvae from *Taricha rivularis* eggs fertilized with *T. torosa* sperm were haploids. Both developed to stages where distinctive species characters were readily visible. The parent species are advantageous for karyoplasmic studies by virtue of their distinctive larval pigment patterns and the formation of viable diploid hybrids. The larval melanophores of *rivularis* are distributed over the lateral and dorsal body surfaces (Fig. 1b), whereas those of *torosa* are confined primarily to compact dorsal bands (Fig. 1a). The diploid hybrid is intermediate to the parent species. Another character, the balancer, is always fully developed in *torosa,* but rudimentary or absent in *rivularis.*

The results of a few selected experiments bearing on the analysis of the localization of factors which direct the differentiation of species characters are summarized in Table I. The conflicting results of these experiments stress the need for further experimentation that might aid in the clarification of the karyoplasmic problem. The present report deals with this problem.

MATERIALS AND METHODS

The methods employed have been described in detail elsewhere (Brandom, 1960). Eggs of *Taricha rivularis* were fertilized by sperm of *T. torosa,* heat-shocked at 35° to 37° C. for ten minutes, and returned to room temperature. Those

TABLE I

The role of the nucleus and the cytoplasm in the determination of specific characters

Organisms	Authors	Localization of the factors for the determination of specific characters	
		Nucleus	Cytoplasm
Haploid, androgenetic hybrids			
Sea urchins	Boveri, 1889	+*	
	Hörstadius, 1936	+	
	von Ubisch, 1953	+	
Amphibians	Hadorn, 1936		+
	Dalton, 1946	+	+**
	Sambuichi, 1952	+	
	Humphrey and Fankhauser, 1957	+	
Diploid, androgenetic and nuclear-transplant hybrids			
Insects	Astaurov and Ostriakova-Varshaver, 1957	+	
Amphibians	Sambuichi, 1957	+	
	McKinnell, 1960	+	
	Gurdon, 1961	+	

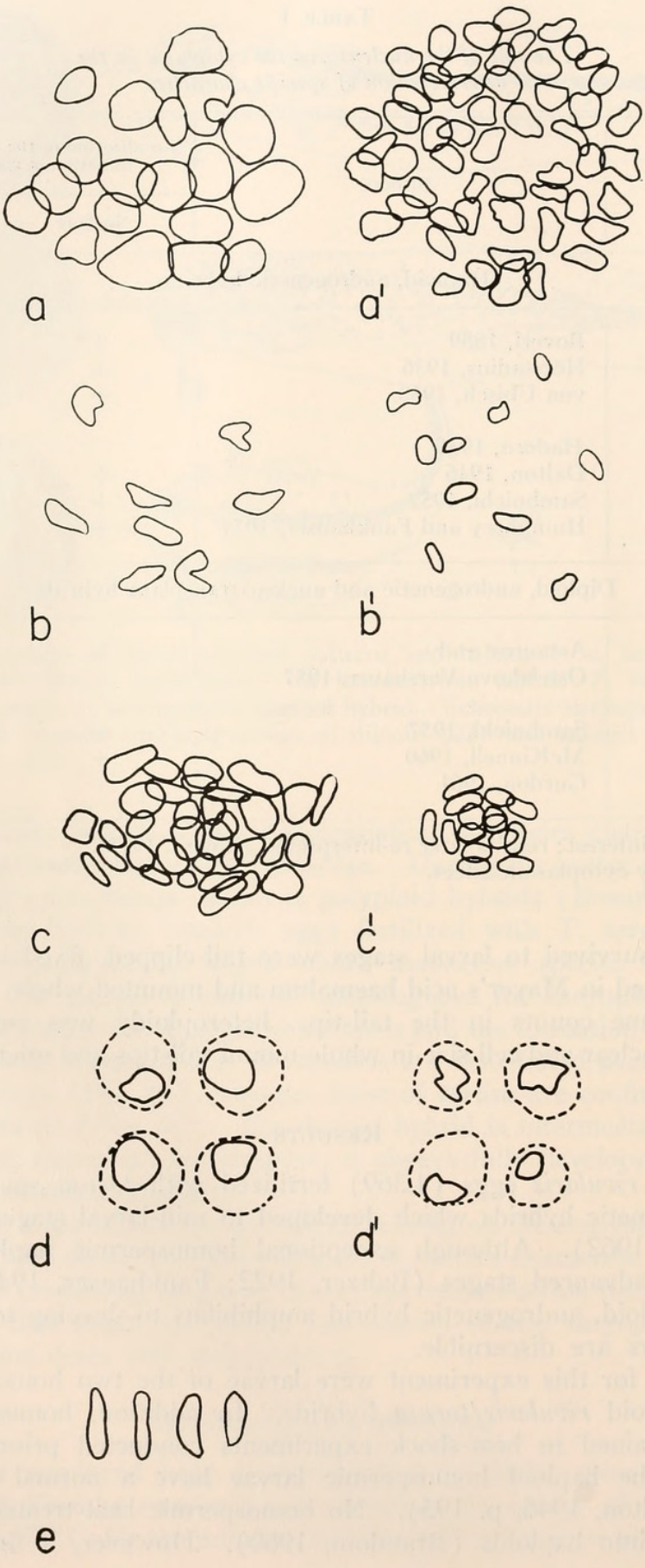
* Of historical interest; results later re-interpreted (Boveri, 1918).
** Partial, early cytoplasmic effect.

embryos which survived to larval stages were tail-clipped, fixed in Bouin's fluid, the tail-tips stained in Mayer's acid haemalum and mounted whole. In addition to direct chromosome counts in the tail-tips, heteroploidy was confirmed by the comparison of nuclear and cell size in whole-mount tail-tips and microsections.

RESULTS

Heat-treated *rivularis* eggs (4,369) fertilized with *torosa* sperm yielded two haploid, androgenetic hybrids which developed to mid-larval stages (Twitty stage 39; see Rugh, 1962). Although exceptional homospermic haploids have been reared to more advanced stages (Baltzer, 1922; Fankhauser, 1937, 1938), these are the first haploid, androgenetic hybrid amphibians to develop to a stage where species characters are discernible.

The controls for this experiment were larvae of the two homospermic diploid species and diploid *rivularis/torosa* hybrids. In addition, homospermic haploid *torosa* were obtained in heat-shock experiments conducted prior to the hybrid experiments. The haploid homospermic larvae have a normal *torosa* pigment pattern (see Dalton, 1946, p. 195). No homospermic heat-treated *rivularis* eggs have developed into haploids (Brandom, 1960). However, it may be assumed,


a
a'
b
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c
c'
d
d'
e

based on other homospermic haploid experiments, that the pigment pattern of haploid *rivularis* larvae would not be qualitatively altered.

The two haploids did not differ noticeably from control larvae in cleavage rates. Marked developmental difficulties were first noted in yolk-plug and neurula stages. Large yolk-plugs persisted up to early tail-bud stages, and the neural folds closed irregularly. Yolk extrusion was observed through wounds in the ventral body wall of both haploid larvae. In early tail-bud larvae pronounced edema in the heart, gill, and forelimb-bud regions remained until the time when the embryos either died or were fixed. Alleviation of fluid pressure by surgical means did not materially reduce the edemic condition. The fluid imbalance and dwarf appearance of our haploids are two of the characteristics normally associated with the haploid amphibian syndrome. Microcephaly occurred in one haploid, but in the other the head was near-normal when the animal was fixed. No early localized breakdown in head mesenchyme, a difficulty previously noted in some haploid hybrids of European *Triton* (Baltzer, 1930), was found in our material.

1. *Tissue and organ architecture*

One haploid hybrid ceased development after the appearance of larval species characters but deteriorated before it could be fixed for sectioning; the other was fixed in good condition. The nuclei and cells of the haploid hybrid were smaller and more numerous than those in comparable areas in the diploid controls. This is illustrated by the outline drawings of tissues in the tailtips (Fig. 2) and micro-sections (Fig. 3). Limited nuclear pyknosis was observed in the brain but the haploid central nervous system contained mostly normal cells. The notochord was bi- and tripartite in some regions; anteriorly it was single, posteriorly it became progressively divided by thickening partitions into two and then three divisions. Duplication of the notochord was previously reported in homospermic haploid *torosa* larvae (Dalton, 1940).

The kidney tubules in the haploid were more numerous and contained larger lumina than those of the diploid control. It is not known whether there is a functional relationship between the abnormalities of the kidneys and the fluid imbalance. Rafferty (1961) concludes from homoplastic transplantation experiments (haploid to diploid and diploid to haploid kidney transplants) that factors other than the haploid kidney are likely to be involved in the fluid imbalance syndrome.

The shape of cells in the lens of the eye and the nephric duct is more cuboidal than comparable cells in the diploid control (Fig. 3). The tendency of haploids to approximate normal organ and body size in the face of decreased nuclear and cell size is partially achieved by a compensatory adjustment in cell shape and cell number (Fankhauser, 1955). As might be expected on the basis of observations of homospermic haploids, the architecture of the heterospermic haploid cells is subordinated to the achievement of near-normal organ size.

FIGURE 2. Drawings of nuclei from larval tailtips of diploid and haploid hybrids of *T. rivularis* ♀ × *T. torosa* ♂: a–e, diploid; a′–e′, haploid. Reading from top to bottom: epidermal interphase nuclei; mesenchyme cell nuclei; nuclei of lateral-line organs; epidermal glands (dotted outline) and nuclei; red blood cell nuclei (absent in haploids) × 540.

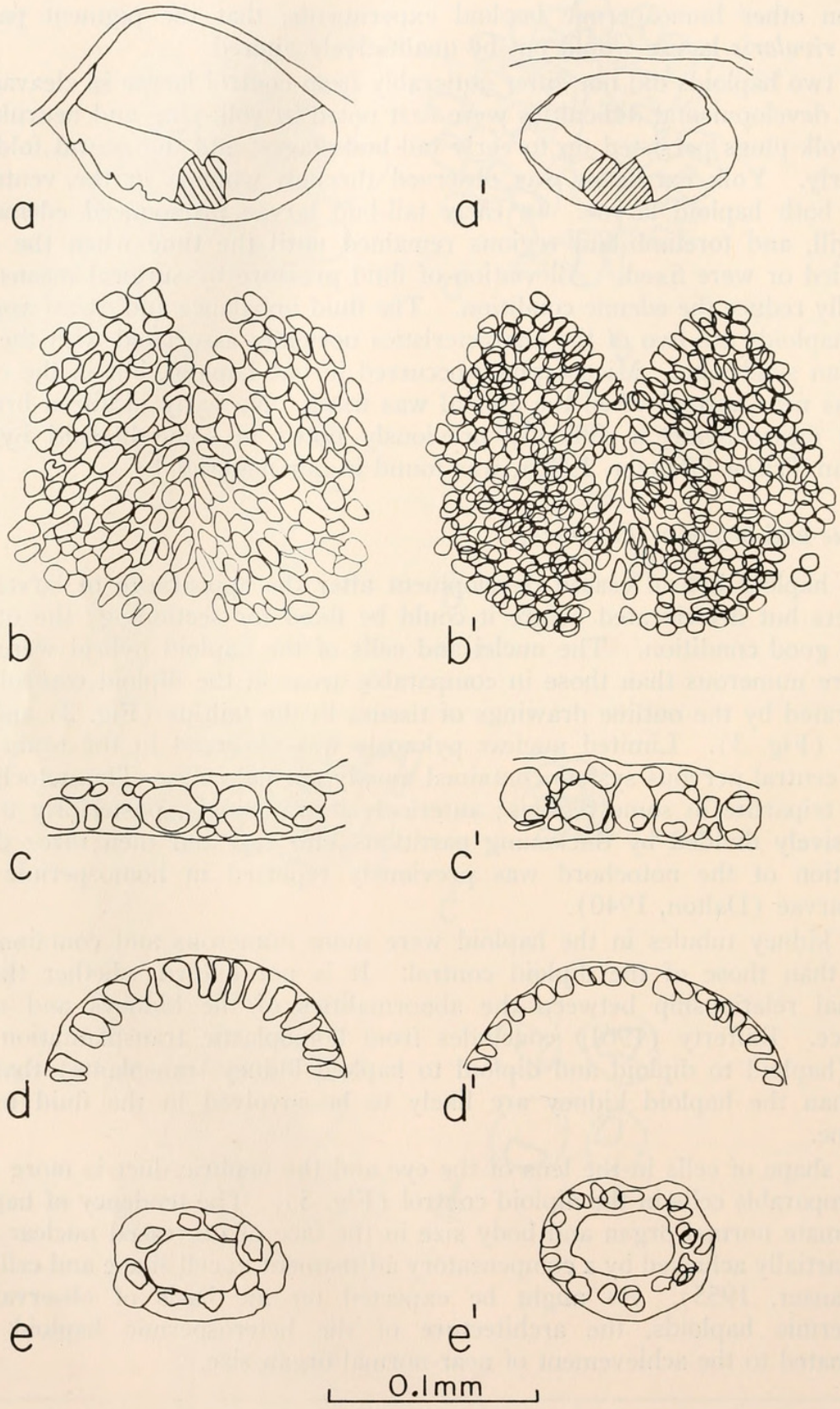

FIGURE 3. Projection-drawings of microsections of diploid (a–e) and haploid (a′–e′) hybrids of *T. rivularis* ♀ × *T. torosa* ♂: a, a′, low-power drawings of myelencephalon; b, b′, nuclei from shaded areas of a and a′; c, c′, cells and nuclei of glands of the epidermis; d, d′, nuclei of peripheral layer of the lens; e, e′, nephric ducts.

2. Balancer

As is characteristic for mountain stream-dwelling salamander larvae, the balancer is either rudimentary or absent in *rivularis,* whereas in *torosa* this organ is fully developed (Twitty, 1936). The balancer is always present in the diploid hybrid of *rivularis* ♀ × *torosa* ♂, although it may be reduced in comparison with homospermic diploid larval *torosa.* In the heterospermic haploids, the balancer was fully developed (Fig. 1). Thus, the *torosa* nucleus acting in *rivularis* cytoplasm directed the development of this organ into a strictly nuclear-donor structure.

3. Larval pigment pattern

The banded arrangement of the larval melanophores of the haploids was dominantly like that of the paternal nuclear contributor, *torosa* (Fig. 1). A few melanophores were visible on the flanks, but they were not in excess of those found in homospermic *torosa* larvae. Although a slight effect of the *rivularis* cytoplasm cannot be ruled out as a possibility, the random arrangement of the few melanophores on the yolk area can be ascribed to physical disturbances of the larval pigment pattern as a consequence of the extreme ventral and lateral body swelling. In support of the latter alternative, no strong evidence of *rivularis*-like early pigmentation was observed.

Discussion

Although the present report is concerned with nuclear-cytoplasmic haploid hybrids, several experiments involving diploid nuclei of one species acting in the cytoplasm of another bear on the problem of the differentiation of species characters.

Astaurov and Ostriakova-Varshaver (1957) reported the first adult karyoplasmic hybrids. Diploid, androgenetic hybrids of *Bombyx mandarina* and *B. mori* were obtained by temperature shock and x-ray treatment to fertilized eggs. The parent species differ in distinctive morphological characters. *Mandarina* caterpillars are of dark markings while *mori* caterpillars have different markings depending on the race. *Mandarina* moths are dark greyish-brown, while those of *mori* are white or cream-colored. In the *mandarina* cytoplasm plus *mori* nucleus combination, the species characters were all like those of *mori.* The cytoplasm did not visibly affect the species characters of the hybrid. None of the hybrids of *mori* cytoplasm plus *mandarina* nucleus developed to imagos, but four individuals were typically nuclear-like in body size, larval markings, and other characters.

Employing the nuclear transplantation technique of Briggs and King (1953), Sambuichi (1957) transplanted diploid nuclei of *Rana nigromaculata brevipoda* into enucleated eggs of *R. n. nigromaculata.* Larval character differences in these two subspecies include tadpole color, labial tooth formula, and shape of the tail. The young metamorphosed frogs differ in dorsal and ventral color pattern. The diploid hybrids are intermediate to the parents in all the characters. With one exception, the embryos, tadpoles, and young frogs of *nigromaculata* cytoplasm plus *brevipoda* diploid nucleus contained only characters of the nuclear-donor subspecies. The exceptional individual later became *brevipoda*-like.

McKinnell (1960) transplanted nuclei of kandiyohi dominant-mutant *Rana pipiens* into wild-type *Rana pipiens* egg-cytoplasm. Three of the intraspecific

karyoplasmic chimeric tadpoles underwent metamorphosis and each had pigment patterns similar to the nuclear donor, kandiyohi.

Gurdon (1961) transplanted nuclei between two subspecies of *Xenopus laevis* (*X. l. laevis* and *X. l. victorianus*). The two subspecies differ in the time of appearance of the larval body and anal melanophores and in postmetamorphic color and color patterns. The nuclear transplant larvae and frogs all showed the distinguishing characteristics of the subspecies which provided the nucleus.

Returning to the haploid experiments, Boveri (1889) first attempted combining the nucleus of one species with the cytoplasm of another by fertilizing egg fragments of *Sphaerechinus granularis* with sperm of *Parechinus microtuberaculatus*. Boveri's pioneer work on sea urchins was criticized on several counts by Morgan (1895) and Seeliger (1896) and, upon repeating his earlier experiments, he showed that it was not possible to produce viable haploid hybrid merogons that would develop beyond gastrulation (Boveri, 1918). The limited development of whole haploid, androgenetic hybrid sea urchin embryos was partially overcome by Hörstadius (1936). He surgically combined the presumptive skeletal micromeres of haploid *Paracentrotus* ♀ × *Psammechinus* ♂ hybrid with homospermic ectodermal and endodermal cells of *Paracentrotus*. In these germ-layer chimeras, the larval skeleton resembled the species which furnished the nucleus of the skeletal cells. More recently, von Ubisch (1953) obtained good merogonic hybrid plutei of *Sphaerechinus* cytoplasm plus *Psammechinus* (or *Paracentrotus*) nucleus. Skeletal characteristics and ciliated bands of the merogons all showed characters of the species which contributed the nucleus.

Finally, Humphrey and Fankhauser (1957) produced intraspecific haploid hybrids between wild, dark (DD) and recessive, white (dd) axolotls by cold-shock treatment of fertilized eggs. The embryos were predominantly white haploids, the recessive color of the males and therefore of androgenetic origin. Only one dark haploid was obtained, presumably of gynogenetic origin.

Concerning temperature shock as a means of inducing androgenesis, Böök (1945) has proposed that cold shock, if it affects the egg when it is in the second anaphase, may cause a paralysis of the spindle. According to this hypothesis, the egg chromosomes remain in the anaphase without being able to reorganize a resting nucleus. A return to normal temperature activates the sperm nucleus; the egg nucleus anaphase configuration does not have the same attraction for the sperm nucleus as does the metaphase, and the result is that the centrosome of the sperm nucleus divides, resulting in a haploid embryo with paternal chromosomes. The mode of elimination of the maternal chromosomes in the androgenetic hybrids reported herein is not known. However, since both of our haploids were androgenetic and all of Humphrey and Fankhauser's (1957) axolotls were androgenetic except one, some such mechanism may be operating in the great majority of haploids derived from temperature-shocked amphibian eggs.

The architecture of the heterospermic haploid tissues was the same as was observed in homospermic haploids (Fankhauser, 1955). Compared to the diploid hybrid controls, the cells of the heterospermic haploids are greater in number but smaller in volume (Figs. 2 and 3). Adjustment in cell shape in single-layered tissues and organs in order to maintain near-normal organ size also agrees with prior observations on homospermic haploids (Fankhauser, 1945).

The embryological basis for specific larval pigment patterns has been extensively investigated by extirpation, transplantation, and tissue culture experiments (Twitty, 1945, 1949; Twitty and Niu, 1948, 1954). Both *in situ* and when explanted in coelomic fluid, *torosa* melanophores migrate out, become highly melanized, and then secondarily reaggregate. Under the same conditions *rivularis* melanophores neither differentiate as fully nor reaggregate as strongly as do those of *torosa*. The two species also differ in the number of larval chromatophores; *rivularis* melanophores are more numerous than *torosa*. These and other findings of Twitty and his co-workers permit us to consider the qualitative changes in the larval pigment patterns in the haploid hybrids as due to quantitative, gene-mediated changes in the pigment cells themselves.

A genetic effect of the single *torosa* genome acting in *rivularis* cytoplasm was discernible in the number of larval melanophores. Although difficult to quantitate because of the secondary banding, there were fewer melanophores in the haploid hybrids than in the diploid *rivularis/torosa* larvae. This suggests that the nuclear-donor species (*torosa*) is exercising a strong action that tends to override a typical consequence of haploidy. Ordinarily, the number of larval pigment cells is greater in homospermic haploids than in homospermic diploids (Fankhauser and Schott, 1952).

The melanophores in the two haploid hybrids were densely pigmented like those of homospermic, diploid *torosa* larvae. Hence, a diminishing effect on the melanization of the larval melanophores by the *rivularis* cytoplasm was not seen. Interpreted in the light of Twitty's findings, the higher grade of differentiation of the haploid pigment cells (visibly manifested by their highly melanized state) qualitativly affected the pigment pattern. The aggregation into dense dorsal bands in homospermic *torosa* is due to the retraction of intercellular processes and occurs only with the attainment of advanced melanophore differentiation characteristic for this species (Fig. 1a). The larval pigment pattern of the haploid, androgenetic hybrids indicates that the *torosa* nucleus was the locus of the factors which determined this larval species character (Fig. 1c).

The fully developed balancer in the heterospermic haploid (which is absent or rudimentary in *rivularis*) emphasizes the strong directive influence of the *torosa* nucleus in the progressive acquisition of this species character.

The lack of species characters of the cytoplasmic donor, *rivularis,* does not exclude the possibility that the cytoplasm produced profound but unseen effects on the propigment and balancer cells before stages when these cells were well differentiated, and subsequently assumed the larval pigment pattern and balancer characteristic for the nuclear-donor species. These results do show that the cytoplasm does not materially affect the specific characters of whole haploid *rivularis/torosa* hybrids during those stages when the visual recognition of species characters can be made.

The author is grateful to Dr. Victor C. Twitty for direction, support, and encouragement during this investigation. Thanks are also due Dr. Gerhard Fankhauser for appraising the manuscript in its incipient form.

Summary

Two species of West Coast newts differ strikingly in larval pigment patterns. *Taricha torosa* has a banded arrangement of the larval melanophores; in *T. rivularis* the larval melanophores are dispersed. *Torosa* is also characterized by a well developed balancer, whereas in *rivularis* the balancer is either absent or rudimentary.

1. Two of 54 heat-shocked, interspecific hybrids of *T. rivularis* ♀ × *T. torosa* ♂ were haploids. The two haploids are the first amphibian androgenetic, haploid hybrids to develop to stages where species characters could be observed.

2. The tissue and organ architecture of the heterospermic haploids conform to prior findings in homospermic haploids. The nuclei and cells are smaller and more numerous than in the diploid controls. A compensatory adjustment in cell shape as well as cell number was observed in single-cell layered organs.

3. The balancer was fully developed in the heterospermic haploids, thus indicating a strong directive influence of the nucleus (*torosa*) in the formation of this organ.

4. The larval pigmentation was dominantly like the nuclear-donor species in the number, degree of melanization, and pattern formation of the melanophores. No evidence was found of an influence on pigmentation by the cytoplasmic-donor species.

5. The above findings are discussed in relation to other studies on the roles of the nucleus and the cytoplasm in the differentiation of species characters.

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