Chromosoma (1993) 102:158–162

CHROMOSOMA

Mechanism of chromosome elimination in the hybridogenetic spermatogenesis of allotriploid males between Japanese and European water frogs

H. Ohtani

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, Higashihiroshima, Hiroshima 724, Japan

Received March 16, 1992 / in revised form July 21, 1992
Accepted July 21, 1992 by H.C. Macgregor

Abstract. Of 21 allotriploid males that possessed two genomes of *Rana nigromaculata* and one genome of *Rana lessonae* 10 produced a large number of spermatozoa in their testes. When 4 of these males were backcrossed with a female of *R. nigromaculata*, all of the resulting froglets were diploid in chromosome number and were completely *R. nigromaculata* type in appearance. These allotriploid males proved to have produced spermatozoa with one *R. nigromaculata* genome hybridogenetically. Therefore, their germ line cells were investigated for the mechanism of elimination of their *R. lessonae* chromosomes. In histological sections of testes, the great majority of spermatogonia (approximately 10^4 cells) between mitotic prometaphase and anaphase appeared normal in chromosome behavior, whereas 17 spermatogonia showed several chromosomes whose behavior deviated from the normal course during the same period. These deviant chromosomes concentrated together near the equatorial plate and remained stationary at anaphase. In metaphase chromosome preparations made from spermatogonia, 67 and 185 of the 477 chromosome spreads were diploid and triploid, respectively. The rest were aneuploid. Notably, 8 triploid spreads consisted of 26 or more normal chromosomes and 13 or fewer degenerate chromosomes. From these results it is concluded that a set of *R. lessonae* chromosomes is eliminated from some, but not all spermatogonia by becoming degenerate during the mitotic period.

Introduction

Hybridogenetic reproduction is a breeding system unique to vertebrates. It was discovered by Schultz (1961, 1966, 1969) in all-female natural hybrids of viviparous fishes in the genus *Poeciliopsis* in Mexico. Schultz showed that the hybrid females spawn only the eggs in which the paternal chromosome set has been eliminated during oogenesis. Cimino (1972) made a detailed cytological study of the mechanism of chromosome elimination in this viviparous fish. He observed that the maternal chromosomes are collected into the nucleus by an oogonial unipolar spindle, while the paternal chromosomes are left behind at the apolar end of the cell and are excluded from the oocyte nucleus.

Hybridogenetic reproduction has also been observed in *Rana esculenta*, which is distributed widely in Europe (Tunner 1973, 1974). *R. esculenta* is a species that is a hybrid of *Rana lessonae* and *Rana ridibunda* and coexists with *R. lessonae* or *R. ridibunda* (Berger 1967, 1968). In this hybrid species, however, the mechanism of hybridogenetic reproduction is more complex than it is in *Poeciliopsis* fishes. *R. esculenta* is composed of diploid and triploid bisexual hybrids. In the diploid *R. esculenta*, the females and males coexisting with *R. lessonae* produce gametes with only an *R. ridibunda* chromosome set, while the males coexisting with *R. ridibunda* produce spermatozoa with either an *R. lessonae* chromosome set or an *R. ridibunda* chromosome set (Uzzell et al. 1977). When the latter males were crossed with *R. ridibunda* females, their progeny were male *R. esculenta* and female *R. ridibunda*. The triploid *R. esculenta* consists of two *R. lessonae* and one *R. ridibunda* genome or one *R. lessonae* and two *R. ridibunda* genomes. The triploid *R. esculenta* also produces haploid gametes whose chromosome set is derived from a parental species offering two genomes to the chromosome complement (Günther et al. 1979). The mechanism of chromosome elimination in the gametogenesis of these diploid and triploid *R. esculenta* has not yet been clarified.

I was able to obtain allotriploid males with two genomes derived from tetraploid female *R. nigromaculata* and one genome derived from diploid male *R. lessonae*. Some of these were observed to produce normal haploid spermatozoa containing an *R. nigromaculata* chromosome set as a result of backcrosses of these allotriploid males and an *R. nigromaculata* female. I tried to observe the process of spermatogenesis in these males in detail, in order to clarify when and how the chromosomes derived from *R. lessonae* were eliminated. The results are described in this paper.

Materials and methods

Twenty-one allotriploid males ($3n=39$, NNL) obtained from hybridization of two tetraploid females ($4n=52$, NNNN) of *R. nigromaculata* and a diploid male ($2n=26$, LL) of *R. lessonae* were used. These males reached maturity by the breeding season of the second year after metamorphosis. At that stage, primarily in order to observe the behavior of the chromosomes in germ cells that were in mitotis and meiosis, a half testis of each of 17 of the 21 males was fixed with Nawaschin's solution for 24 h, embedded in paraffin and serial sectioned at 12 μm thickness. The sections were then stained with Heidenhain's iron hematoxylin solution. The remaining testis halves were used for fertilizing eggs to make backcrosses. Further, in order to examine the exact chromosome numbers in germ cells, preparations of mitotic and meiotic metaphase chromosomes were made by the splash technique (Schmid 1978) from all the testes of the remaining 4 males and stained with a 2% solution of Giemsa (Merck) in 0.01 M sodium phosphate buffer, pH 6.8, for 10 min.

Results

Table 1 shows the results of backcrosses of 4 allotriploid males with two *R. nigromaculata* and one *R. lessonae* genome and an *R. nigromaculata* female. In each series, the chromosome number of ten tadpoles chosen at random was examined by squashing their tail tips in acetic orcein staining solution. It was found that all of the tadpoles were diploid in chromosome number. Moreover, all of the resulting froglets were very similar in external characteristics to *R. nigromaculata* and the frogs reared grew into fertile females. From these results, it is clear that the allotriploid males had eliminated a set of *R. lessonae* chromosomes from their germ line cells.

The testes of the 17 allotriploid males (NNL nos. 1–17) were composed of three types of seminiferous tubules: those that were full of normal spermatocytes and bundles of normal spermatozoa, those that were full of spermatogonia, and those that contained no germ cells inside. In the seminiferous tubules filled with spermatogonia, there were some spermatogonia in mitotic prometaphase, metaphase and anaphase. The chromosome behavior of almost all of these spermatogonia was judged normal in each stage of mitosis. However, 3 and 14 spermatogonia at prometaphase and anaphase, respectively, showed some chromosomes whose behavior deviated from normal (Fig. 1). In each of the 3 spermatogonia at prometaphase, some of the chromosomes congregated together near the cell membrane, and the remaining chromosomes lay scattered in the cytoplasm as in a normal mitosis (Fig. 1a). The number of the latter was counted up to a maximum of 24 in each spermatogonium. Therefore, the scattering chromosomes were assumed to be equivalent to two genomes, and the congregated chromosomes equivalent to one genome. In each of the 14 spermatogonia at anaphase, some chromosomes were left behind in a body around the equatorial plate, while the rest separated toward both spindle poles as normal chromatids (Fig. 1b–d). In these spermatogonia, too, the separating and congregating chromosomes were assumed to be equivalent to two and one genome, respectively. The appearance of the chromosomes remaining around the equatorial plate differed among the 14 spermatogonia. In 3 of the 14 spermatogonia, they remained unchanged although they concentrated together in one place (Fig. 1b). In 8 others, they massed near the equatorial plate with the loss of their own shape (Fig. 1c). In the remaining 3, they separated into chromatids although remaining clustered outside the mitotic spindle (Fig. 1d).

In order to investigate the exact chromosome number of each spermatogonium, preparations of metaphase chromosomes were made from all the testes of the remaining 4 allotriploid males (NNL nos. 18–21). There were many spreads of mitotic chromosomes with those of meiotic chromosomes in the preparations obtained from 3 of the males, nos. 18–20. The spreads of meiotic chromosomes were mostly 13 tetrads and 13 dyads. Preparations from male no. 21 showed no meiotic chromosome spreads. The mitotic chromosome numbers of the spermatogonia in males 18–21 are shown in Table 2. Of the 396 cells in metaphase from males 18–20, 140 were triploid, while 66 became diploid by eliminating one chromosome set. The remaining cells had between 27 and 38 chromosomes, with the exception of a few hypodiploid, hypertriploid and tetraploid cells. Of the 81 cells from male no. 21, only 1 was diploid.

Table 1. Results of backcrosses of four allotriploid males with a *Rana nigromaculata* female

Parents		No. of eggs	No. of normal cleavages	No. of normal neurula embryos	No. of normally hatched tadpoles	No. of metamorphosed frogs
Female	Male					
(N)NN No. 5	(N)NN No. 3	106	105 (99.1%)	103 (97.2%)	103 (97.2%)	91 (85.8%)
(N)NN No. 5	(N)NNL No. 1	139	116 (83.5%)	114 (82.0%)	109 (78.4%)	98 (70.5%)
	(N)NNL No. 5	125	119 (95.2%)	119 (95.2%)	118 (94.4%)	110 (88.0%)
	(N)NNL No. 12	124	122 (98.4%)	122 (98.4%)	120 (96.8%)	115 (92.7%)
	(N)NNL No. 15	101	78 (77.2%)	78 (77.2%)	78 (77.2%)	77 (76.2%)

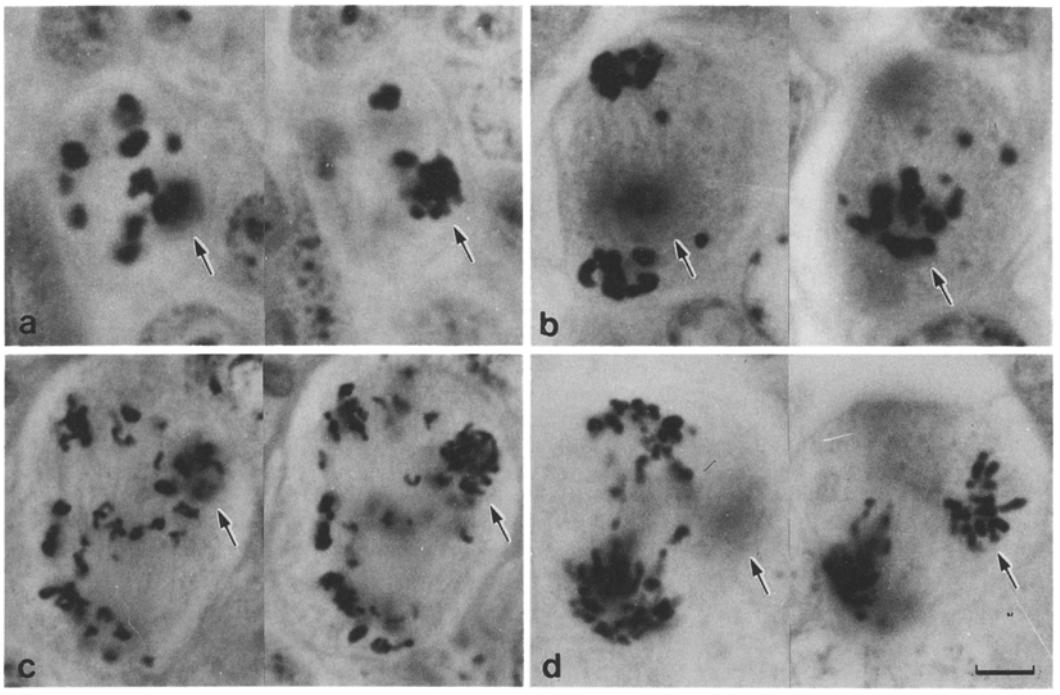

Fig. 1a–d. Chromosome behavior in four spermatogonia from allotriploid males of *Rana nigromaculata* and *Rana lessonae*. **a** Prometaphase; **b–d** anaphase. Not all the chromosomes of a spermatogonium can be seen in each photograph, because the spermatogonium was cut into two or three cross sections. The two photographs of each of the four spermatogonia were taken from one cross section by altering the focus. *Arrows* indicate degenerate chromosomes. Bar represents 10 μm

Table 2. Chromosome number of spermatogonia from four allotriploids with two *Rana nigromaculata* genomes and one *Rana lessonae* genome

Individual number	No. of spermatogonia analyzed	Number of chromosomes																										
		21	22	25	26 (2*n*)	27	28	30	31	32	33	34	35	36	37	38	39 (3*n*)	40	41	42	44	45	46	47	48	49	52 (4*n*)
18	222	1	1	1	28 (12.6%)	13	6	1	4	5	6	7	26	51	27	9	29 (13.1%)		1	4							2
19	38				20 (52.6%)	2	1		1			1	1			1	11 (28.9%)										
20	136				18 (13.2%)	3		1		2		1	2	4	3	1	100 (73.5%)	1									
21	81				1 (1.2%)		1		1	1		1		2	5	2	45 (55.6%)	7	2	1	2	1	4	3	1	1	
Total	477	1	1	1	67 (14.0%)	18	8	2	6	8	6	10	29	57	35	13	185 (38.8%)	8	3	5	2	1	4	3	1	1	2

There were 8 striking, interesting triploid metaphase plates in the preparations from males 18–20 (Fig. 2). Three of these were made up of 26 chromosomes that were easy to stain and 13 that were hard to stain in Giemsa solution. Moreover, the latter chromosomes were uncondensed in shape (Fig. 2a). One metaphase plate was made up of 38 normally condensed and 1 uncondensed chromosome. Two plates were composed of 37 condensed and 2 uncondensed chromosomes (Fig. 2b). The remaining 2 plates were composed of 35 condensed and 4 uncondensed chromosomes (Fig. 2c).

All except one aneuploid metaphase plate showed no uncondensed chromosomes. The unusual plate was made up of 27 normally condensed and 1 uncondensed chromosome (Fig. 2d).

Discussion

R. esculenta is commonly known as a hybridogenetically reproducing diploid or triploid hybrid of *R. ridibunda* and *R. lessonae*. With regard to the mechanisms whereby gametes containing a chromosome set derived from ei-

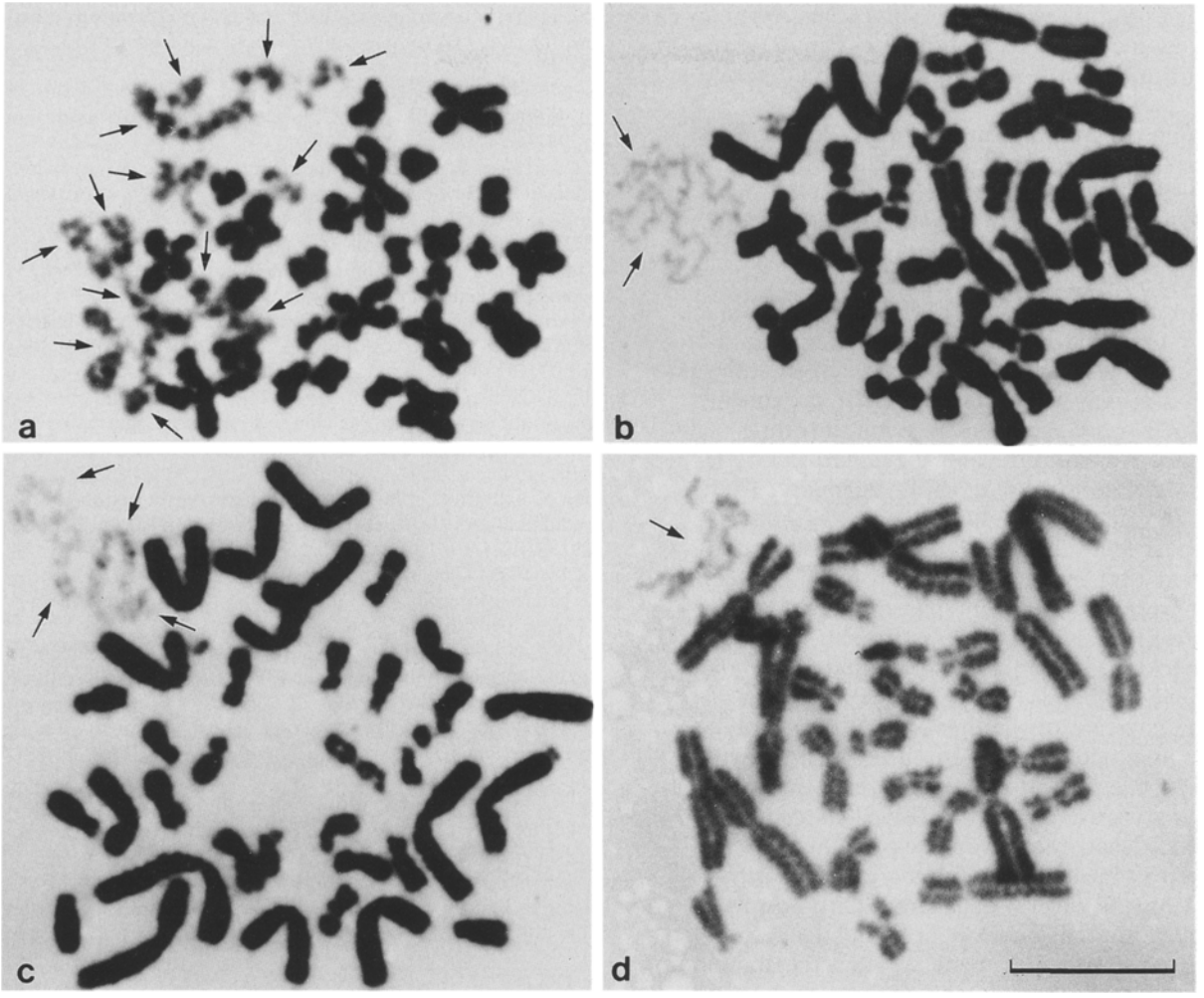

Fig. 2a–d. Metaphase plates composed of normal and degenerate chromosomes in the spermatogonia of males that were allotriploids of *Rana nigromaculata* and *Rana lessonae*. *Arrows* indicate uncondensed chromosomes. Bar represents 10 μm

ther parental species are produced, Uzzell et al. (1977) suggested that gametes develop from gametocytes in which either of the parental chromosome sets has been eliminated during meiotic division, or in which no recombination has occurred. But a study on the electrophoretic patterns of several enzymes in diploid *R. esculenta* coexisting with *R. lessonae* showed that gonadal tissues had the same patterns as *R. ridibunda*, despite the fact that somatic tissues showed hybrid patterns (Vogel and Chen 1976, 1977; Graf et al. 1977; Uzzell et al. 1980). Graf et al. (1977) insisted, on the basis of these results, that the *R. lessonae* chromosome set is eliminated prior to the onset of meiosis. Furthermore, Graf and Müller (1979) have concluded that the elimination of the *R. lessonae* chromosome set and the duplication of the remaining *R. ridibunda* chromosome set are completed before the onset of meiosis, since their primary oocytes contain 13 lampbrush bivalents, and the three kinds of enzymes of their gynogenetic progeny are identical in their electrophoretic patterns to those of *R. ridibunda*. Uzzell et al. (1980), however, maintained that the *R. lessonae* chromosome set is expected to be in an inactive state in the primary oocytes.

Tunner and Heppich (1981) established by karyological and electrophoretic analyses that elimination and duplication of a chromosome set occur during the prolonged phase of oogonial multiplication in the diploid *R. esculenta* coexisting with *R. lessonae*. They revealed that ovaries of young *R. esculenta* 2 weeks after completion of metamorphosis were made up of diploid oogonia the enzymes of which showed the same hybrid patterns as those of their somatic cells. Older *R. esculenta*, at between 3 and 6 weeks after metamorphosis, had replaced most of the diploid oogonia with haploid, hypohaploid and hyperhaploid oogonia, the enzymes of which showed hardly any hybrid patterns. Furthermore, ovaries of 71 day old adult *R. esculenta* were filled with diploid primary oocytes that showed the same enzyme patterns as *R. ridibunda*. Nishioka and Ohtani (1984) reported that two female allotriploids consisting of two *Rana brevipoda* and one *R. lessonae* genome contain 13 lampbrush bivalents derived from *R. brevipoda* and no chromosomes derived from *R. lessonae* in their primary oocytes. This observation shows that an *R. lessonae* chromosome set was eliminated during the oogonial multiplication period. In the present study, the allotrip-

loid males consisting of two *R. nigromaculata* and one *R. lessonae* genome have been shown to eliminate an *R. lessonae* chromosome set during the spermatogonial multiplication period.

The fundamental mechanism of chromosome elimination in *R. esculenta* has not yet been elucidated. Tunner and Heppich (1981) and Heppich et al. (1982) have supposed that elimination may be a consequence of differences in centromere structure between *R. lessonae* and *R. ridibunda* chromosomes. In the present study, the 8 mitotic metaphase plates of triploid spermatogonia showed 13 or fewer degenerate chromosomes, as well as 26 or more well-shaped chromosomes. On the other hand, in testis sections, 17 spermatogonia in the mitotic period contained some chromosomes concentrating together near the cell membrane and the equatorial plate, with the rest appeared to be segregating normally. These results show that degenerate chromosomes without exception disappear from the nuclei of daughter spermatogonia. It is clear that the degenerate chromosomes seen in this study were derived from paternal *R. lessonae*, because only fertile *R. nigromaculata* offspring were produced from backcrosses of these allotriploid males and an *R. nigromaculata* female.

There is the question of whether *R. lessonae* chromosomes were eliminated at once or little by little with each division. In the present study, 3 of the 140 triploid metaphase plates were composed of 26 well-shaped chromosomes and 13 degenerate chromosomes. On the other hand, there were 179 aneuploid plates together with 5 triploid plates having several degenerate chromosomes, too. However, all but 1 of these aneuploid plates showed no degenerate chromosomes. I conclude that the aneuploid spermatogonia must necessarily degenerate, together with the triploid spermatogonia that failed to eliminate *R. lessonae* chromosomes. I believe that those diploid spermatogonia that succeed in synchronous elimination of all *R. lessonae* chromosomes then multiply vigorously, filling up the space occupied by degenerate spermatogonia, and developing into haploid spermatozoa through the normal process of meiosis.

References

Berger L (1967) Embryonal and larval development of F_1 generation of green frogs different combinations. Acta Zool Cracov 12:123–160

Berger L (1968) Morphology of the F_1 generation of various crosses within *Rana esculenta* complex. Acta Zool Cracov 13:301–324

Cimino MC (1972) Egg-production, polyploidization and evolution in a diploid all-female fish of the genus *Poeciliopsis*. Evolution 26:294–306

Graf JD, Müller WP (1979) Experimental gynogenesis provides evidence of hybridogenetic reproduction in the *Rana esculenta* complex. Experientia 35:1574–1576

Graf JD, Karch F, Moreillon MC (1977) Biochemical variation in the *Rana esculenta* complex: A new hybrid form related to *Rana perezi* and *Rana ridibunda*. Experientia 33:1582–1584

Günther R, Uzzell T, Berger L (1979) Inheritance patterns in triploid *Rana "esculenta"* (Amphibia, Salientia). Mitt Zool Mus Berlin 55:35–57

Heppich S, Tunner HG, Greilhugen J (1982) Premeiotic chromosome doubling after genome elimination during spermatogenesis of the species hybrid *Rana esculenta*. Theor Appl Genet 61:101–104

Nishioka M, Ohtani H (1984) Hybridogenetic reproduction of allotriploids between Japanese and European pond frog. Zool Sci 1:291–316

Schmid M (1978) Chromosome banding in amphibia. I. Constitutive heterochromatin and nucleolus organizer regions in *Bufo* and *Hyla*. Chromosoma. 66:361–388

Schultz RJ (1961) Reproductive mechanisms of unisexual and bisexual strains of the viviparous fish *Poeciliopsis*. Evolution 15:302–325

Schultz RJ (1966) Hybridization experiments with an all-female fish of the genus *Poeciliopsis*. Biol Bull Woods Hole 130:415–429

Schultz RJ (1969) Hybridization, unisexality, and polyploidy in the teleost *Poeciliopsis* (Poeciliidae) and other vertebrates. Am Nat 103:605–619

Tunner HG (1973) Das Albumin und andere Bluteiweisse bei *Rana ridibunda* Pallas, *Rana lessonae* Camerano, *Rana esculenta* Linne und deren Hybriden. Z Zool Syst Evolut-forsch 11:219–233

Tunner HG (1974) Die klonale Struktur einer Wasserfroschpopulation. Z Zool Syst Evolut-forsch 12:309–314

Tunner HG, Heppich S (1981) Premeiotic genome exclusion during oogenesis in the common edible frog, *Rana esculenta*. Naturwissenschaften 68:207–208

Uzzell T, Gunther R, Berger L (1977) *Rana ridibunda* and *Rana esculenta*: a leaky hybridogenetic system (Amphibia, Salientia). Proc Acad Nat Sci Philadelphia 128:147–171

Uzzell T, Hotz H, Berger L (1980) Genome exclusion in gametogenesis by an interspecific *Rana* hybrid: evidence from electrophoresis of individual oocytes. J Exp Zool 214:251–259

Vogel P, Chen PS (1976) Genetic control of LDH isozymes in the *Rana esculenta* complex. Experientia 32:304–307

Vogel P, Chen PS (1977) A further study of LDH isozymes in the *Rana esculenta* complex. Experientia 33:1285–1287