12 Osawa, T., and Jeanloz, R. W., J. org. Chem. *30* (1965) 448.
13 Chaturvedi, N. C., Khosla, M. C., and Anand, N., J. med. Chem. *9* (1966) 971.
14 Shealy, Y. F., Opliger, C. E., and Montgomery, J. A., J. Pharm. Sci. *57* (1968) 757.
15 Andreatta, R. H., and Scheraga, H. A., J. med. Chem. *14* (1971) 489.
16 Cheung, S. T., and Benoiton, N. L., Can. J. Chem. *55* (1977) 906.
17 Haq, W., Rizvi, S. Y., Kapil, A., Kundu, B., and Mathur, K. B., Indian J. Chem. *29B* (1990) 253.
18 Kundu, B., Rizvi, S. Y., Srivastava, V. P., Haq, W., and Mathur, K. B., Indian J. Chem. *29B* (1990) 409.
19 Beveridge, E., in: Chemotherapy of Leishmaniasis, vol. 1, p. 257. Eds R. J. Schnitzer and F. Hawking. Academic Press, NY 1963.
20 Pal, R., Anuradha, Guru, P. Y., and Katiyar, J. C., Med. Sci. Res. *17* (1989) 579.
21 Adinolfi, L. E., and Bonventre, P. F., Am. J. trop. Med. Hyg. *34* (1985) 270.
22 Bhatnagar, S., Guru, P. Y., Katiyar, J. C., Srivastava, R., Mukherjee, A., Akhtar, M. S., Seth, M., and Bhaduri, A. P., Indian J. med. Res. *89* (1989) 439.
23 Pal, R., Anuradha, Guru, P. Y., and Katiyar, J. C., Indian J. Med. Res. *90* (1990) 59.
24 Marshall, G. R., Intra-Sci. Chem. Rep. *5* (1971) 305.
25 Stammer, C. H., in: Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, vol. 7, p. 267. Ed. B. Weinstein. Dekker Inc., New York 1983.
26 Spatola, A. F., in: Chemistry and Biochemistry of Amino Acids, Peptides and Proteins, vol. 7, p. 267. Ed. B. Weinstein. Dekker Inc., New York 1983.
27 Freidinger, R. M., Veber, D. F., Pestoco, D. S., Brooks, J. R., Saperstein, R., Science *210* (1980) 656.
28 Rizvi, S. Y., Ph. D. Dissertation, Lucknow University (India) 1989.
29 Fessia, S. L., Kong, Y. M., Beck, P. H., and Johnson, A. G., Eur. J. Immun. *1* (1977) 508.
30 Adam, A., Devys, M., Souvannavony, V., Lefrancier, P., Choay, J., and Lederer, E., Biochem. biophys. Res. Commun. *72* (1976) 339.
31 Hawking, F., in: Experimental Chemotherapy, vol. 1, p. 129 (1963).
32 Misra, A., Katiyar, J. C., and Sen, A. B., Indian J. exp. Biol. *18* (1980) 906.
33 Katiyar, J. C., Misra, A., and Visen, P. K. S., Wiad. Parazyt. *TXXXII NHZ* (1986) 163.
34 Misra, A., Visen, P. K. S., and Katiyar, J. C., J. Helm. *55* (1981) 273.
35 Adam, A., Pettit, J. F., Lefrancier, P., and Lederer, E., Molec. cell. Biochem. *41* (1981) 27.
36 Parant, M., Parant, F., Chedid, L., Yapo, A., Petit, J. F., and Lederer, E., Int. J. Immunopharm. *1* (1979) 35.
37 Lederer, E., in: Biochemistry of Parasites, p. 206, Ed. C. M. Slutzky, Pergamon Press, Oxford 1981.

0014-4754/91/050486-05$1.50 + 0.20/0

Pentaploidy in hybrid salamanders demonstrates enhanced tolerance of multiple chromosome sets

L. A. Lowcock[a] and R. W. Murphy

[a]*Department of Ichthyology and Herpetology, Royal Ontario Museum, 100 Queen's Park, Toronto, Ontario M5S 2C6 (Canada), and Ramsay Wright Zoological Laboratories, University of Toronto, Toronto, Ontario (Canada)*
Received 25 January 1990; accepted 13 November 1990

Summary. Breeding populations of unisexual hybrid salamanders (genus *Ambystoma*) are dominated by allotriploid (3n) and allotetraploid (4n) forms, however, sexually mature allopentaploids (5n) may also occur. These are the first known naturally-occurring pentaploid vertebrates, and their genesis differs from that of previously studied autopentaploid urodeles induced or observed in the laboratory. The latter always suffered severely deleterious effects in development, and could not attain sexual maturity.
Key words. Vertebrates; unisexuality; allopentaploidy; *Ambystoma.*

Cytogenetically, pentaploidy is difficult to establish in vertebrates, and unlikely to be maintained due to meiotic irregularities[1,2]. Developmental and physiological constraints in pentaploids can lead to arrested or abnormal development at any life-history stage[1,2], resulting in drastically reduced individual survival. When physical, physiological or behavioral debilities have been over-ridden in the laboratory in order to study development in pentaploid urodeles, sexual maturation is the most recognizably affected ontogenetic trait[1,3]. Thus, the presence of adult pentaploid females in breeding aggregations of hybrid salamanders is of some significance. Here we describe the discovery of these first known naturally-occurring pentaploid vertebrates, and discuss the relevance of their genesis compared to laboratory pentaploids.

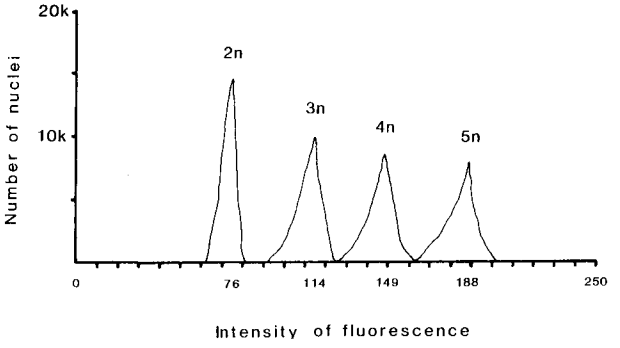
Materials and methods

During April of 1988 and 1989, we collected salamanders of the *Ambystoma laterale-jeffersonianum* complex[4] during breeding migrations to a pond in Haliburton, Ontario, Canada. In addition to *A. laterale*, a range of all-female hybrid biotypes of *A. laterale-jeffersonianum* constitute a large segment of the local breeding aggregate. In order to determine ploidy ratios, we initially separated *A. laterale* (2n) from hybrids (3n, 4n) based on morphological criteria[5]. Because ploidy classes within hybrids cannot be determined through visual inspection, ploidy analysis was carried out using flow-cytometry[6]. Individual salamanders were anesthetized in a weak solution of tricaine methanosulfate (MS 222), a few µl of blood collected from a toe-clip, and the animals released. Blood was frozen and stored following standard methods[6]. Nuclear DNA content of erythrocytes was measured[6], using propidium iodide (PI) as the nuclei stain. Samples were analyzed in an Argon-ion laser flow cytometer (EPICS V Flow Cytometer, Coulter Electronics, Hialeah, Fla). For each sample, 1000–10,000 nuclei were examined and histograms were accumulated by the MDADS computer

system (Coulter Electronics). Nuclear DNA content was determined based on comparisons to a known 2n sample. We excited PI at 488 nm using 500 mwatts of power and collected red fluorescent wavelengths above the limit established by a 595 λ interference filter.

Results

Successive ploidy levels above 2n showed characteristic increases of nuclear DNA corresponding to the addition of a full haploid set of chromosomes (fig.); no mosaics were found and we did not encounter any obvious aneuploids in the sample. The distribution of ploidy classes is detailed in table 1. Predictably, 3n and 4n hybrids predominated, but we were surprised to discover the existence of rare 5n females.



Separate flow cytometric measurements of nuclear DNA content for 2n *A. laterale*, and 3n, 4n and 5n hybrid biotypes of *A. laterale-jeffersonianum* depicted by tracings of an overlay of single parameter histograms representing the integral of red fluorescence. Asymmetry of higher-ploidy peaks is due to machine artifact and scaling effects. Channel numbers on the X-axis correspond to the position of the peak for each histogram.

Table 1. Distribution of ploidy classes in the sample population (1988 and 1989) as determined by flow cytometry.

	N	% of total sample	
2n Male *laterale*	273	10.3	
2n Female *laterale*	377	14.3	
Total *laterale*	650	24.6	
			% of total hybrids
3n Male hybrids	3	0.1	0.15
3n Female hybrids	1711	64.7	85.70
4n Female hybrids	279	10.5	14.00
5n Female hybrids	3	0.1	0.15
Total hybrids	1996	75.4	100.00
Total sample	2646	100.0	—

Discussion

Because *A. jeffersonianum* does not occur at this site, hybrid ploidies are maintained here through activation/fertilization of reduced and unreduced hybrid eggs by male *A. laterale*. Hybrid eggs contain a single *A. jeffersonianum* (J) genome and one or more *A. laterale* (L) genomes [7–9]. Outside active zones of hybridization between these species, hybrids are mostly triploid (e.g., LLJ at our site), and occasional ploidy increases involve addition of genomes from the perpetuating bisexual species (in this case, *A. laterale*, yielding 4n LLLJ) [8]. Thus, hybrids are expected to be dominated by triploids, followed by a lower percentage of tetraploids [7, 8]. Elevation from 4n to 5n (LLLLJ) could occur in theory based on the sequence of events leading from triploidy to tetraploidy, i.e., fertilization of an unreduced hybrid ovum [7, 8, 10, 11]. However, occurrence of 5n adults was not anticipated because 1) it had not been previously revealed in hybrid complexes of *Ambystoma* [7–14]; 2) mortality of 3n and 4n eggs and larvae can be exceedingly high [10, 11, 15]; 3) fecundity decreases as ploidy increases [3]; 4) potential 4n progenitors typically represent a low percentage of hybrids [7–11, 14], and; 5) previous documentation of pentaploidy in urodeles raised under laboratory conditions (table 2) suggests little chance of occurrence or survival outside of a laboratory [1–3, 16]. Those 5n larvae studied typically had difficulty eating and processing food, water-balance problems, slow reactions, enhanced toxin sensitivity, and debilitating physical anomalies [1, 3]. As a result, growth rate was greatly reduced and metamorphosis delayed [1–3].

Autopentaploids vs allopentaploids. In the most significant work on elevated ploidy in urodeles, spontaneous and experimentally induced heteroploids of the axolotl, *Ambystoma mexicanum*, were reared to adulthood in order to study their offspring (table 2) [2, 3, 17–20]. Proportionally, far fewer pentaploids were produced from tetraploids than from diploids; as in other urodeles, the most common source of pentaploidy was the fertilization of rare, completely unreduced eggs ovulated by diploids

Table 2. Documentation of spontaneous heteroploidy in laboratory offspring of several 2n urodele species, and 3n and 4n axolotls. Listed as number of individuals and, where applicable, percentages (in parentheses).

Female parent	N	1n	2n	3n	4n	5n	6n	7n	Mosaics	Aneuploids	Refs
Notophthalmus viridescens	2448	4(0.16)	2387(97.5)	44(1.81)	1(0.04)	7(0.29)	–	–	3(0.12)	2(0.08)	1, 16
Cynops pyrrhogaster	398	1(0.25)	392(98.5)	4(1.0)	–	1(0.25)	–	–	–	–	1
Eurycea bislineata	546	2(0.37)	511(93.6)	28(5.1)	2(0.37)	–	–	–	3(0.56)	–	1
Axolotl	~ 3000	7(0.20)	most	1(0.03)	1(0.03)	2(0.07)	–	–	2(0.07)	–	1
Axolotl	~51000	few	most	few	35	71	?	?	few	?	3
3n Axolotl	445	–	8(1.8)	–	48(10.8)	3(0.65)	–	2(0.45)	7(1.6)	377(84.7)	2
3n Axolotl	> 445	?	some	?	66	few	3	?	some	most	17–20
4n Axolotl	19	–	–	2	–	–				17	17–20
4n Axolotl	many[1]	?	?	few	many	6	?	?	?	most	3

[1] Several hundred.

(failure of meiosis I and II). However, lab-reared 5n axolotls were malformed and non-viable, dying before the age of two years[2,3]. Degeneration of young oocytes in 5n ovaries was more extensive than in triploids and tetraploids; ovaries of older pentaploids contained few or no mature ova[3]. These studies demonstrate that the overwhelming inviability of laboratory-reared 5n larvae predicts little opportunity of survival if produced in the wild, and that chance survival through metamorphosis is unlikely to result in the attainment of sexual maturity. Finally, all previously documented cases were autopentaploid, i.e. all additional genomes were derived from the same species.

Recently, much attention has focused on unisexual hybrid complexes of vertebrates containing allopolyploid forms (bearing genomes from more than one species)[21]. Described allotriploid lineages reproduce parthenogenetically (lizards), gynogenetically (fishes, amphibians) or hybridogenetically (fishes, amphibians)[22]. Allotetraploids are known in only two groups; cobitid fishes[23] and ambystomatid salamanders[7,8,10,11,14,24,25]. In *Ambystoma*, tetraploids are symmetrical dihybrids or asymmetrical di- and trihybrids[7,8,10,11,14,24–26]. Hybrid *Ambystoma* employ either gynogenesis or a hybridogenetic-like mechanism to produce offspring of the same ploidy as the mother[7,8,10,11]. In some populations, females produce offspring of both their own and elevated ploidy; 2n hybrids give rise to diploids and triploids, while triploids produce both triploids and tetraploids[10,11] as described earlier. The known progeny of 4n hybrids are 3n and 4n[10,11]. Ploidy elevation beyond 4n in these complexes is hitherto unknown. We believe our pentaploids are derived from fertilization of unreduced 4n eggs and constitute such documentation. All available evidence supports this conclusion and precludes alternative paradigms.

First, unlike the axolotl, ploidy elevation in hybrid *Ambystoma* involves sequential acquisition of additional genomes from a syntopic bisexual 2n species[7,8,10,11], thus, as noted previously, 5n hybrids could theoretically be produced by 4n hybrids. Pentaploids were rarely produced by autotetraploid axolotls, in which egg reduction was the rule. In contrast, hybrid *Ambystoma* produce a high percentage of unreduced eggs[10,11]. Second, the percentage of adult pentaploids in our population is in keeping with the relative prevalence of tetraploidy, and an assumed concommitant production of substantial numbers of pentaploid embryos (presumably balanced by decreased viability of pentaploids in comparison to triploids and tetraploids, since those that survive all ontogenetic stages are a highly selected group). Third, autopentaploid urodeles are most frequently produced by diploids, however, those described here were clearly allopentaploids, and suitable allodiploid progenitors were not found. Finally, 4n hybrids from our site can produce unreduced 4n eggs. Test matings between LLLJ females from our site and 2n male *A. texanum* in 1989 produced high numbers of 5n larvae (LLLJT), most of which were predictably deformed.

Conclusions. In axolotls, viability of offspring of polyploid females was low. The same range of physical and physiological defects[3,17–20] have been observed in larvae of hybrid *Ambystoma*, where embryonic mortality is as high as 100%[7,8,10,11,15]. Such defects may relate to regulatory problems connected with polyploidy, or reflect physical constraints such as large cell size. Similar physical anomalies occur in embryos of the parthenogenetic triploid hybrid lizard, *Cnemidophorus uniparens*[27], suggesting that heteroploidy, and hence chromosomal imbalance alone, is responsible for a significant proportion of developmental problems. Nevertheless, in contrast to autopentaploids, our allopentaploids had no noticeable physical anomalies, were visibly distended with eggs (many of which were extruded), ate well, and had body sizes indicating an age of several years. Apparent attainment of sexual maturity (though not necessarily viability) in these 5n individuals may be related to the effects of the hybrid genome, but requires further study. In general, the hybrid genomes of allopolyploids seem to confer a greater tolerance of higher ploidy than in autopolyploids[17–20]. Paradoxically, partial incompatibility of the different genomes appears to be responsible for the production of unreduced eggs[28,29] that often leads to the establishment of elevated levels of ploidy and/or parthenogenesis. The frequency of occurrence of allopentaploid salamanders documented here would seem to preclude ploidy elevations beyond this state. That could change if viable pentaploid genomes continue to accumulate in the population.

Acknowledgements. We thank Caleigh Garland and Alex Fishbein for assistance in collecting and processing animals, Cheryl Smith for flow cytometry, L. E. Licht and G. Klassen for comments on the manuscript and R. Elinson and J. P. Bogart for the 1989 crosses. This work was supported by grants from the Natural Sciences and Engineering Research Council of Canada to R. W. M. and the Friends of Algonquin Park to L. A. L.

1 Fankhauser, G., Q. Rev. Biol. *20* (1945) 20.
2 Fankhauser, G., and Humphrey, R. R., J. exp. Zool. *115* (1950) 207.
3 Humphrey, R. R., and Fankhauser, G., J. Morph. *98* (1956) 161.
4 Lowcock, L. A., Licht, L. E., and Bogart, J. P., Syst. Zool. *36* (1987) 328.
5 Uzzell, T. M., Copeia *1964* (1964) 257.
6 Dawley, R. M., and Goddard, K. A., Evolution *42* (1988) 649.
7 Lowcock, L. A., in: Evolution and Ecology of Unisexual Vertebrates, pp. 180–208. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
8 Bogart, J. P., in: Evolution and Ecology of Unisexual Vertebrates, pp. 170–180. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
9 Lowcock, L. A., and Bogart, J. P., Can. J. Zool. *67* (1989) 350.
10 Bogart, J. P., and Licht, L. E., Can. J. Genet. Cytol. *28* (1986) 605.
11 Bogart, J. P., Lowcock, L. A., Zeyl, C. W., and Mable, B. K., Can. J. Zool. *65* (1987) 2188.
12 Uzzell, T. M., and Goldblatt, S. M., Evolution *21* (1969) 345.
13 Bogart, J. P., Can. J. Zool. *60* (1982) 848.
14 Bogart, J. P., Licht, L. E., Oldham, K. J., and Darbyshire, S. B., Can. J. Zool. *63* (1985) 340.
15 Licht, L. E., and Bogart, J. P., Evolution *41* (1987) 918.
16 Fankhauser, G., Proc. natl Acad. Sci. USA *26* (1940) 526.
17 Fankhauser, G., and Humphrey, R. R., J. exp. Zool. *126* (1954) 33.

18 Humphrey, R. R., Am. J. Anat. *76* (1945) 33.
19 Humphrey, R. R., and Fankhauser, G., J. Morph. *79* (1946) 467.
20 Humphrey, R. R., and Fankhauser, G., J. Hered. *40* (1949) 7.
21 Dawley, R. M., and Bogart, J. P., (Eds), Evolution and Ecology of Unisexual Vertebrates. New York State Museum Bulletin 466, Albany 1989.
22 Vrijenhoek, R. C., Dawley, R. M., Cole, C. J., and Bogart, J. P., in: Evolution and Ecology of Unisexual Vertebrates, pp. 19–23. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
23 Vasil'ev, V. P., Vasileva, K. D., and Osinov, A. G., in: Evolution and Ecology of Unisexual Vertebrates, pp. 153–169. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.
24 Morris, M. A., Herpetologica *41* (1985) 267.
25 Morris, M. A., and Brandon, R. A., Copeia (1984) 324.
26 Kraus, F., Occ. Pap. Mus. Zool. Univ. Michigan *709* (1985) 1.
27 Billy, A. J., Can. J. Zool. *64* (1986) 2418.
28 Bogart, J. P., in: Polyploidy: Biological Relevance, pp. 341–378. Ed. W. H. Lewis. Plenum Press, New York 1980.
29 Dawley, R. M., in: Evolution and Ecology of Unisexual Vertebrates, pp. 1–18. Eds R. M. Dawley and J. P. Bogart. New York State Museum Bulletin 466, Albany 1989.

0014-4754/91/050490-04$1.50 + 0.20/0

Effects of retinoic acid on embryonic development of mice in culture

T. Watanabe[a] and R. M. Pratt[b] †

[a] *Department of Hygiene and Preventive Medicine, Yamagata University School of Medicine, Yamagata 990-23 (Japan), and* [b] *Laboratory of Reproductive and Developmental Toxicology, NIEHS, NIH, Research Triangle Park (North Carolina 27709, USA)*
Received 19 June 1990; accepted 28 September 1990

Summary. The effects of all-trans-retinoic acid (RA) (tretinoin) on the craniofacial development of mouse embryos were examined using whole embryo culture. In day 8 embryos cultured for 48 h, embryonic growth was inhibited concentration-dependently by all-trans-RA treatment. Most of the treated embryos exhibited hypoplasia of the primary palatal processes and a reduction in the development of the first visceral arches. In day 10 embryos cultured for 48 h, although embryonic growth was not inhibited at any concentrations of all-trans-RA, median cleft lip (93%), hypoplasia of the primary palatal processes (37%) and limb reduction deformities (48%) occurred commonly. Furthermore, RA treatment greatly reduced the size of the secondary palatal processes. The incorporation of ^{3}H-thymidine in the treated maxillary processes was decreased to 65% of the control value at 1.0×10^{-7} M all-trans-RA. These findings indicate that all-trans-RA is teratogenic in mouse whole embryo culture.
Key words. All-trans-retinoic acid; whole embryo culture; mouse embryos; craniofacial development; mesenchymal cells; palatal processes.

Teratogenic and embryolethal effects of retinoic acids (RA) have been observed in humans and experimental animals. In humans, there have been severe congenital malformations in newborns born to women who took 13-cis-RA [1,2]. The major malformations resulting from the embryonic exposure were cleft palate, micrognathia, and external ear and central nervous system abnormalities. In experimental animals in vivo and in vitro, developmental defects have been produced in various tissues including neural folds, the heart and limbs [3–5]. These are similar to those observed in human newborns.

In a series of our experiments, using whole embryo culture, 13-cis- and 4-oxo-13-cis-RA caused highly specific defects in mouse embryos. Day 8 embryos showed a marked reduction in the size of the visceral arches. This may be caused by 13-cis-RA inhibiting the migration of cranial neural crest cells [6,7]. A high percentage of day 10 embryos were affected with median cleft lip and severe limb reduction. In these embryos, the mesenchymal cells beneath the epithelium of the nasal and maxillary processes contained pyknotic nuclei (dead cells), and there was a dramatical reduction in the number of the mesenchymal cells and in ^{3}H-thymidine incorporation in the secondary palatal processes [8]. In culture, the treated palatal mesenchymal cells took a long time to proliferate [9]. We suggested that the cis-isomers of RA affect proliferation of cranial neural crest cells as well as oronasal mesenchymal cells. This appears to be related to the production of craniofacial malformations in rodents and humans.

Different hypotheses concerning the cellular mechanism by which retinoids produce craniofacial malformations in mammalian embryos have been proposed. Some of the malformations are considered to be due to an inhibition of the migration or proliferation of cranial neural crest cells and to programmed cell death of epithelium in facial processes [7,10,11]. Recent findings indicate that the inhibition of 13-cis-RA on palatal mesenchymal cell growth may contribute to facial clefting in mouse embryos [9,12,13]. It has also been demonstrated that the teratogenic effects of RA are attributable to excessive cell death [14,15].

In the present study, to determine the cellular mechanism of the development of craniofacial malformations in