Temperature and Sperm Incorporation in Polyploid Salamanders

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Although most animals reproduce sexually, a number of all-female groups exist. Triploid hybrid salamanders appear to maintain themselves by using a male's sperm to activate their eggs, after which the sperm nucleus is eliminated (gynogenesis). The incidence of sperm nuclear incorporation in eggs of these salamanders depends on temperature. Triploid offspring derived gynogenetically are more frequent at lower temperature, whereas tetraploid offspring derived sexually are far more frequent at higher temperatures. Temperature-dependent variability in sperm nuclear incorporation helps explain the variability in reproductive modes reported for hybrid salamanders.

THREE BASIC MECHANISMS HAVE been proposed for the perpetuation of unisexual vertebrates: (i) females reproduce by parthenogenesis (no sperm); (ii) females reproduce by gynogenesis (sperm from a sympatric male initiate development of the eggs without incorporation of the paternal genome); (iii) a hybrid state is maintained through hybridogenesis (the maturing egg eliminates an entire genome, which is retrieved in fertilization by a sympatric male's sperm). Parthenogenesis has evolved independently in several genera of lizards (*1*), gynogenesis has been found in fish (*2*), and hybridogenesis exists in fish (*3*) and frogs of the European *Rana esculenta* complex (*4*).

Mole salamanders of the genus *Ambystoma* occur in hybrid complexes throughout eastern North America and involve combinations of four species (*A. jeffersonianum*, *A. laterale*, *A. texanum*, and *A. tigrinum*). The hybrids are usually triploid females that are thought to reproduce gynogenetically by using sperm from sympatric diploid males (*5*). In some populations of hybrids, however, offspring contain alleles that were not present in the genotype of their mothers (*6*), and clutches contain offspring of mixed ploidy (*7, 8*). This observation refutes a strict gynogenetic or parthenogenetic mechanism. In order to clarify and critically test the mode of reproduction in these salamanders, we inseminated eggs from triploid hybrid females in vitro with sperm from genetically distinct males and found that temperature influenced the mode of reproduction.

We collected salamanders in the spring before breeding began, transported them to the laboratory in coolers containing snow, and held them in an incubator at 6°C. Triploid females with one complement of *A. laterale* and two complements of *A. jeffersonianum*, defined as *A. laterale*–(2)*jeffersonianum* (LJJ) (*9*), were collected near Ancaster, Ontario, as were *A. jeffersonianum* and *A. maculatum* males. *Ambystoma texanum* males were from Pelee Island, Lake Erie; *A. tigrinum* males from Illinois; and *A. laterale* males from both localities. Females were examined for the presence of sperm, and all those used in the crosses were sperm-negative (*10*). Females were induced to ovulate by hormone injections, and eggs were inseminated with sperm from the vas deferens (*11*). Hatchlings were kept in pond water and fed brine shrimp and tubifex worms. Most were raised for 3 to 4 months postmetamorphosis and fed small earthworms and crickets. Tissue samples were compared by using horizontal starch gel electrophoresis (*12*) and chromosomes were counted (*13*).

Only eggs that were inseminated began development, ruling out the possibility of parthenogenesis. The crosses involved 20 triploid LJJ females with males of all known hybrid complexes of *Ambystoma*. Out of 1288 inseminated eggs from 31 crosses, 48% cleaved and 21% developed to hatched larvae (Harrison stage 40). This high embryonic mortality is consistent with the pattern observed in hybrid salamander complexes (*6, 14*), where both field and laboratory mortality is much higher than among diploid salamander species.

In our first series of crosses, almost all of the offspring (78 out of 87) were tetraploids derived sexually (*15*) (Fig. 1). The relative proportion of electromorphs suggested that the tetraploids resulted from incorporation of haploid sperm nuclei into unreduced triploid eggs. Use of males with identifiable electromorphs, for example, LJJ female crossed with *A. texanum* male, confirmed the presence of the paternal genome. Given the low incidence of tetraploids in natural populations (*16*), the high frequency of tetraploids in our crosses was unexpected.

Hybrid *Ambystoma* breed early in the spring, as soon as most of the ice has melted in the breeding ponds. In most years, breed-

Table 1. Surviving offspring from cross combinations between triploid *A. laterale*–(2)*jeffersonianum* females and males of *A. tigrinum*, *A. laterale*, and *A. maculatum*.

Parents*		Offspring, at 6°C†			Offspring, at 15°C		
		3n			3n		
Female	Male	G	H	4n	G	H	4n
A. tigrinum males							
13451 × 13881		1	1	1	1	2	4
13459 × 13881		1	1	0	3	0	3
13461 × 13879					1	0	0
13458 × 13879		1	0	0	1	0	3
13453 × 13887					2	0	0
Total		3	2	1	8	2	10
A. laterale males							
13452 × 13880		3	0	0	2	0	2
13453 × 13885		7	0	4	3	1	1
13454 × 13885		14	3	0	0	4	10
Total		24	3	4	5	5	13
A. laterale and *A. tigrinum*							
Combined total‡		27 (73)	5 (14)	5 (14)	13 (30)	7 (16)	23 (53)
A. maculatum male							
13451 × 13487		5	0	0	2	0	0
13455 × 13487		9	0	0	2	1	0
Total		14	0	0	4	1	0

*All females were *A. laterale*–(2)*jeffersonianum*. Numbers (for example, 13451) are catalog numbers of one of the authors (J.P.B.). The specimens will be deposited in the herpetology collection of the National Museum of Canada, Ottawa. †The offspring were mostly transformed juveniles, but some larvae were also used. The triploids were determined to be the product of gynogenesis (G) or hybridogenesis (H) on the basis of the electrophoretic genotype. The total number of triploid and tetraploid offspring (99) does not equal the total number of larvae (116 at about stage 40). Some larvae were abnormal and died before chromosomes or electrophoretic information could be obtained. ‡Rounded percentages of each reproductive mode are in parentheses.

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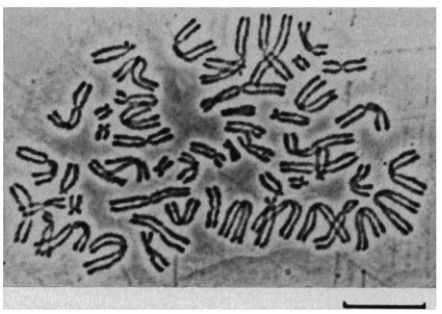

Fig. 1. Chromosomes from a tetraploid larva (4n = 56), which was an offspring from a triploid *A. laterale*–(2)*jeffersonianum* female. Scale bar, 20 μm.

ing occurs at water temperatures as low as 6°C. Our initial crosses were done in the laboratory at 15°C, higher than usual in natural ponds. We hypothesized that temperature affects the reproductive mode of the hybrids, resulting in the high proportion of tetraploid offspring. Consequently, we tested the effect of temperature on reproductive mode. Procedures for the new crosses were the same as those initially used, except that eggs were inseminated and maintained at either 6° or 15°C to at least the four-cell stage.

When sperm from *A. laterale* and *A. tigrinum* males were used at 15°C, a large number of tetraploids with paternal genomes were obtained (Table 1 and Fig. 2). When eggs were inseminated and kept at 6°C, the number of tetraploids was significantly fewer and the number of gynogens greater. Offspring were 53% (23 of 43) tetraploids at 15°C, but only 14% (5 of 37) tetraploids at 6°C (χ^2, $P < 0.005$).

Triploids arose by either gynogenesis or hybridogenesis (Table 1). The hybridogens resulted from diploid eggs formed by the elimination of an *A. jeffersonianum* genome, then incorporation of a male genome. A similar mechanism occurs in other hybridogenetic organisms (*3, 4*). It would appear that sperm nuclear incorporation is required for reproduction of reduced diploid eggs because we did not detect diploid gynogens. Tetraploids and hybridogens both represent offspring with haploid sperm nuclear incorporation. Grouping them together, sperm nuclear incorporation occurred in 70% (30 of 43) of eggs inseminated at 15°C, but only in 27% (10 of 37) inseminated at 6°C (χ^2, $P < 0.005$).

Data from the *A. maculatum* male were considered separately (Table 1) because this species is not part of the hybrid complex and may not form viable hybrids with species of the complex (*17*). Of 19 offspring, only one expressed paternal alleles, and this larva grew slowly and died at 9 weeks. If incorporation of an *A. maculatum* genome into an LJJ egg is lethal, then survival of this cross is expected to be better at 6°C, where sperm nuclear incorporation is less likely to occur, than at 15°C. Suggestive of this pattern is the result that 44% (16 of 36) of cleaved eggs at 6°C reached stage 40 compared to 11% (5 of 46) at 15°C (χ^2, $P < 0.005$).

Our experiments demonstrate that sperm nuclear incorporation in eggs of triploid salamanders varies with temperature. Higher temperature favors incorporation (hybridogenesis or ploidy elevation), whereas lower temperature favors gynogenesis. Treatment of urodele eggs with low temperature had previously been used to produce triploid offspring from diploid females in several species, including *A. mexicanum* (*18*). Cold suppressed second polar body formation but did not inhibit sperm nuclear incorporation in these cases. Low temperature, however, prevented sperm nuclear incorporation in a few laboratory-produced frog and toad hybrids, resulting in gynogenetic development (*19*). This suggests that suppression of sperm nuclear incorporation in triploid salamanders results from a fortuitous combination of hybrid development and breeding at low temperatures.

In hybrid *Ambystoma*, temperature has ecological and evolutionary importance. At reduced temperatures, a triploid female would be expected to reproduce mainly by gynogenesis. At higher temperatures, the frequency of sperm nuclear incorporation and subsequently hybridogenesis would increase, or elevated ploidy level would increase among the unreduced eggs. Genetic variability and ploidy might be dictated by water temperature, which can vary with locality, from one year to the next, or during the breeding season in any particular year. During a given season, females breeding early may differ in reproductive mode from those breeding later. Such variation may have significant bearing on the genetic composition of hybrid populations and may account for the variability in reproductive mode in hybrid *Ambystoma*.

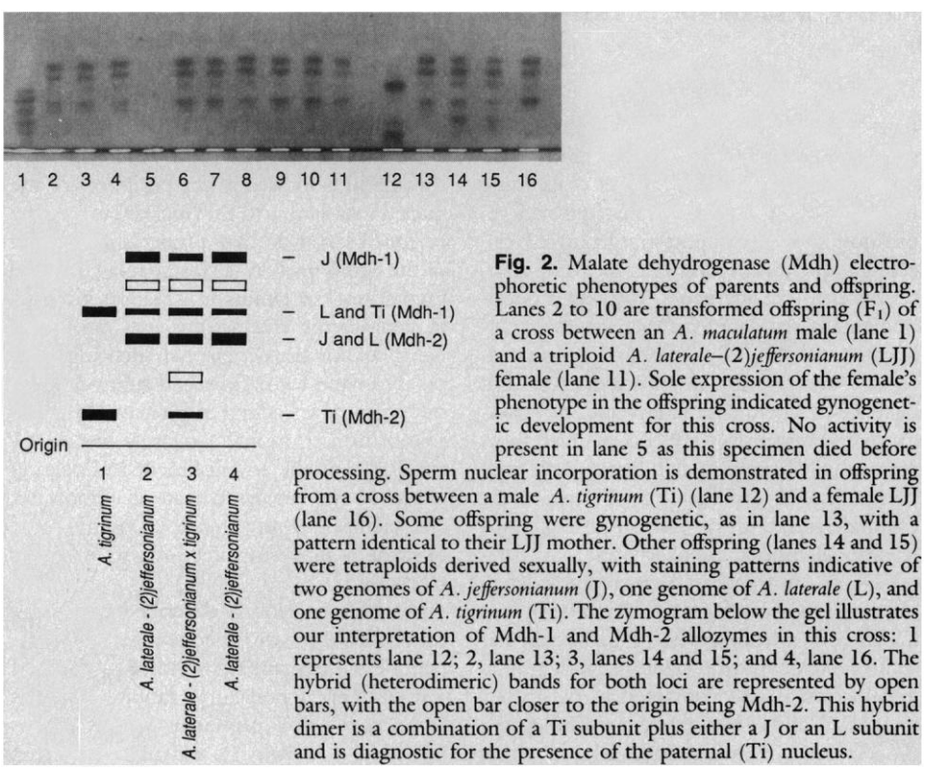

Fig. 2. Malate dehydrogenase (Mdh) electrophoretic phenotypes of parents and offspring. Lanes 2 to 10 are transformed offspring (F_1) of a cross between an *A. maculatum* male (lane 1) and a triploid *A. laterale*–(2)*jeffersonianum* (LJJ) female (lane 11). Sole expression of the female's phenotype in the offspring indicated gynogenetic development for this cross. No activity is present in lane 5 as this specimen died before processing. Sperm nuclear incorporation is demonstrated in offspring from a cross between a male *A. tigrinum* (Ti) (lane 12) and a female LJJ (lane 16). Some offspring were gynogenetic, as in lane 13, with a pattern identical to their LJJ mother. Other offspring (lanes 14 and 15) were tetraploids derived sexually, with staining patterns indicative of two genomes of *A. jeffersonianum* (J), one genome of *A. laterale* (L), and one genome of *A. tigrinum* (Ti). The zymogram below the gel illustrates our interpretation of Mdh-1 and Mdh-2 allozymes in this cross: 1 represents lane 12; 2, lane 13; 3, lanes 14 and 15; and 4, lane 16. The hybrid (heterodimeric) bands for both loci are represented by open bars, with the open bar closer to the origin being Mdh-2. This hybrid dimer is a combination of a Ti subunit plus either a J or an L subunit and is diagnostic for the presence of the paternal (Ti) nucleus.

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11. The female at 4°C was injected with 50 international

units (IU) pregnant mare's serum gonadotropin (PMSG) on the first day, transferred to 15°C and injected with 50 IU PMSG on the second day, and injected with 400 IU PMSG plus 200 IU human chorionic gonadotropin on the third day. Eggs were expressed from the females by gentle pressure on their ovisacs 30 to 36 hours after the last injection. Males were killed and the vas deferens was placed in a moist chamber. For fertilization, eggs were expressed into a petri dish. A small piece of vas deferens was cut out and used to apply sperm to the eggs. After 5 min, the eggs were flooded with 20% Steinberg's solution. As a control for true parthenogenesis, eggs from each female were expressed from the cloaca, flooded, and treated as above but not painted with sperm.

12. The heart, spleen, and a portion of skeletal muscle were dissected from the adults or juveniles and frozen with an equal volume of deionized water in 1.5-ml Eppendorf microcentrifuge tubes. The electrophoretic procedure was the same as used previously [J. P. Bogart, L. E. Licht, M. J. Oldham, S. J. Darbyshire, *Can. J. Zool.* **63**, 340 (1985)]. This study concentrated on those electromorphs that were distinctly different in the males and females. The following loci were examined: Acon-1, Adh, Ck-1, Ck-2, Got-1, Got-2, Idh-1, Idh-2, Ldh-1, Ldh-2, Mdh-1, Mdh-2, Mdh-3, Mpi, Pgi, 6Pgd, Pgm-1, Pgm-2, and Sod-1. Some loci (for example, Adh, Sod-1, and Sod-2) could not be resolved in larvae. Genomes were identified by the diagnostic electromorphs [J. P. Bogart, *NY State Mus. Bull.* **466**, 209 (1989)].
13. The females were injected with 0.5 ml of colchicine solution (1 mg/ml) 2 days before being processed for chromosomes and electrophoresis. Chromosomes from the offspring were obtained from tail tips of larvae (*7*) or gut epithelial lining of transformed individuals (*8*). Some larvae were used for initial chromosome analyses and electrophoresis. These larvae were put in 0.01% colchicine solution for 24 hours, and their tails were squashed. Larvae were frozen for later electrophoresis.
14. Undeveloped or dead eggs are common in ponds where hybrids occur. Such eggs were noted early in the century by W. H. Piersol [*Am. Nat.* **44**, 732 (1910)]. The low percentage of developing eggs that hatched in this study (27%) is consistent with other studies of hybrid female *Ambystoma* [W. Clanton, *Occas. Pap. Mus. Zool. Univ. Mich.* **290**, 1 (1934); L. E. Licht, *NY State Mus. Bull.* **466**, 170 (1989).
15. Eighty-seven offspring were analyzed from 12 crosses involving 9 LJJ females, 4 *A. jeffersonianum* males, 1 *A. laterale* male, and 1 *A. texanum* male. There were 78 tetraploids, 1 pentaploid, and 8 triploids.
16. In an intensive study of *Ambystoma* in southern Ontario, only one tetraploid was reported by D. L. Servage [thesis, University of Guelph, Guelph, Ontario (1979)]. No tetraploids were found by J. P. Bogart (*7*).
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Similarity Between the Transcriptional Silencer Binding Proteins ABF1 and RAP1

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The yeast ARS binding factor 1 (ABF1)—where ARS is an autonomously replicating sequence—and repressor/activator protein 1 (RAP1) have been implicated in DNA replication, transcriptional activation, and transcriptional silencing. The *ABF1* gene was cloned and sequenced and shown to be essential for viability. The predicted amino acid sequence contains a novel sequence motif related to the zinc finger, and the ABF1 protein requires zinc and unmodified cysteine residues for sequence-specific DNA binding. Interestingly, ABF1 is extensively related to its counterpart, RAP1, and both proteins share a region of similarity with SAN1, a suppressor of certain *SIR4* mutations, suggesting that this region may be involved in mediating SIR function at the silent mating type loci.

A CRITICAL FEATURE IN THE LIFE CYCLE of the yeast *Saccharomyces cerevisiae* is that two loci, *HML* and *HMR*, which contain mating type information and the cis elements required for expression, must be maintained in a transcriptionally silent state (*1*). Silencing involves the binding sites for two sequence-specific DNA binding proteins, ABF1 and RAP1, located within regulatory regions (silencers) flanking these silent loci (*2–4*). Four genes, *SIR1* to *SIR4*, are also required for silencing (*5*) and appear to act, at least in part, through ABF1 and RAP1 (*6*). ABF1 and RAP1 are multifunctional proteins implicated not only in transcriptional repression, but also in transcriptional activation (both proteins), telomere function (RAP1), and initiation of DNA replication (ABF1) (*2, 3, 7, 8*).

Eukaryotic cells are capable of regulating the amount of transcription of a given gene over eight to nine orders of magnitude. Of this range, five to six orders of magnitude have been attributed to transcriptional repression (*9*). An important group of cis-acting sequences involved in repressing transcription of specific genes, often in a developmentally regulated manner, has been delineated (*7, 10, 11*). These sequences can repress transcription in an orientation- and distance-independent manner and, by analogy to transcriptional enhancers, have been termed "silencers" (*7*). Understanding how these silencers function may be important for understanding the full breadth of transcriptional control in eukaryotes.

Cell type in the yeast *S. cerevisiae* is determined by the expression of either one of two alleles, α or **a** from the *MAT* locus near the centromere of chromosome III. Mating type information is also contained at two other loci, *HML* and *HMR*, which are found near the telomeres of chromosome III and which serve as stores of α and **a** mating type information, respectively, during mating type switching. *HML* and *HMR* are actively maintained in an inert state by four short sequences flanking each silent locus (*12*). Each of these sequences can function as an autonomously replicating sequence (ARS), implicating the initiation of DNA replication in silencing. It is within these sequences that ABF1 or RAP1, or both, binds and appears to act in silencing with the products of the four *SIR* genes.

Like general transcriptional repression (*9*), the repressed state of *HML* and *HMR* is stably propagated during DNA replication and, in fact, requires passage through S phase for its establishment (*13*). Furthermore, silencing involves the generation of a chromatin structure reminiscent of heterochromatin (*14*). Since most cellular heterochromatin is located at the periphery of the nucleus, subjacent to the nuclear lamina, the recent observation that a functional domain of the SIR4 protein is closely related to the human nuclear lamins in a region involved in lamin-lamin interactions may provide a link between transcriptional silencing, heterochromatin formation, and subnuclear localization of cis-acting sequences (*15*). The role of transcriptional activators like ABF1 and RAP1 in the establishment and maintenance of the repressed state is important, and analysis of the genes encoding these proteins is likely to offer insights into this mechanism.

We have previously described the purification of ABF1 to homogeneity as a polypeptide with an apparent molecular mass of 135 kD (*3*). A polyclonal rabbit antiserum to purified ABF1 protein was affinity-purified (Fig. 1A, lane 1) and used to screen

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