Laboratory development of the hybrid *Phoxinus eos* × *Phoxinus neogaeus* (Pisces: Cyprinidae)

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Summary. We chronicle the development of eggs stripped from a wild caught hybrid *Phoxinus eos* × *Phoxinus neogaeus*, that were then mixed with sperm from *Phoxinus eos*. Approximately half the eggs died in development. It is hypothesized the dead embryos are the male sex, the postulated heterogametic sex. Egg hatching time approximated that of *neogaeus* instead of *eos*. Possibly this is a maternal effect, reflecting the maternal ancestor of the hybrid. Several laboratory hybrids were karyotyped and all had triploid cells. The laboratory hybrids were also heterozygous at both diagnostic allozyme loci examined.

Key words. Cyprinid; *Phoxinus*; hybrid; polyploidy; development.

While interspecific hybridizations are not rare among the egg scattering fishes in the family Cyprinidae, the hybrids between the North American cyprinids *Phoxinus eos* and *Phoxinus neogaeus* are of particular interest. Parthenogenesis was first suspected in these hybrids by New who noted that the essentially all-female hybrids were often collected in the absence of one parental species[2]. When hybrids are found, *P. neogaeus* is usually either quite rare or absent. With respect to morphology, New found the means of several characters in hybrids were shifted towards *P. eos*. He also found individuals that had hybrid features but morphologically resembled *P. eos* more than F 1 hybrids. He postulated that the hybrids might not only be fertile, but represent a parthenogenetic species.

Support for the parthenogen hypothesis came from analyses of hybrid populations in western Nebraska. The hybrids were always heterozygous at marker loci displaying electromorph typical of F 1 hybrids yet exist in both diploid ($2N = 50$) and triploid ($3N = 75$) forms[3, 4].

A subsequent study of eastern North American hybrid populations demonstrated clonal structure in diploids, but curiously no clonal structure in triploids[5].

This unisexual complex merits investigation of its basic biological facets, not only in comparison with their progenitor species, but to other unisexual species. In this report we chronicle the development of hybrid eggs 'fertilized' with *P. eos* sperm (we are uncertain whether karyogamy occurred, see the discussion of hybridogenesis versus gynogenesis).

In May 1985 a large number of hybrid *Phoxinus* from Bone Creek and Holt Creek near Ainsworth, Nebraska were collected. These were photographed and several from each collection were preserved including a male *P. neogaeus* in good red and yellow breeding color from Holt Creek. The male *P. neogaeus* was the first of its species collected at either site despite several years of collecting.

Several dozen hybrids and male and female *P. eos* were taken back to the laboratory at the University of Nebraska at Omaha. Hybrids had been kept in tanks in the laboratory for several seasons with no resulting progeny. On May 17, 1985 several attempts were made to fertilize eggs of putative hybrids with *P. eos* sperm. The first 4 attempts did not succeed, as the eggs were not quite ripe enough and did not strip easily.

The fifth hybrid female easily released about 120 eggs. These were collected in a glass Petri dish and immediately 'fertilized' by squirting milt from a large male *eos* onto the eggs. Within 10 min it was clear that these eggs were 'fertilized' as the fertilization membrane was apparent. The animal and vegetal poles were well established in 2 h.

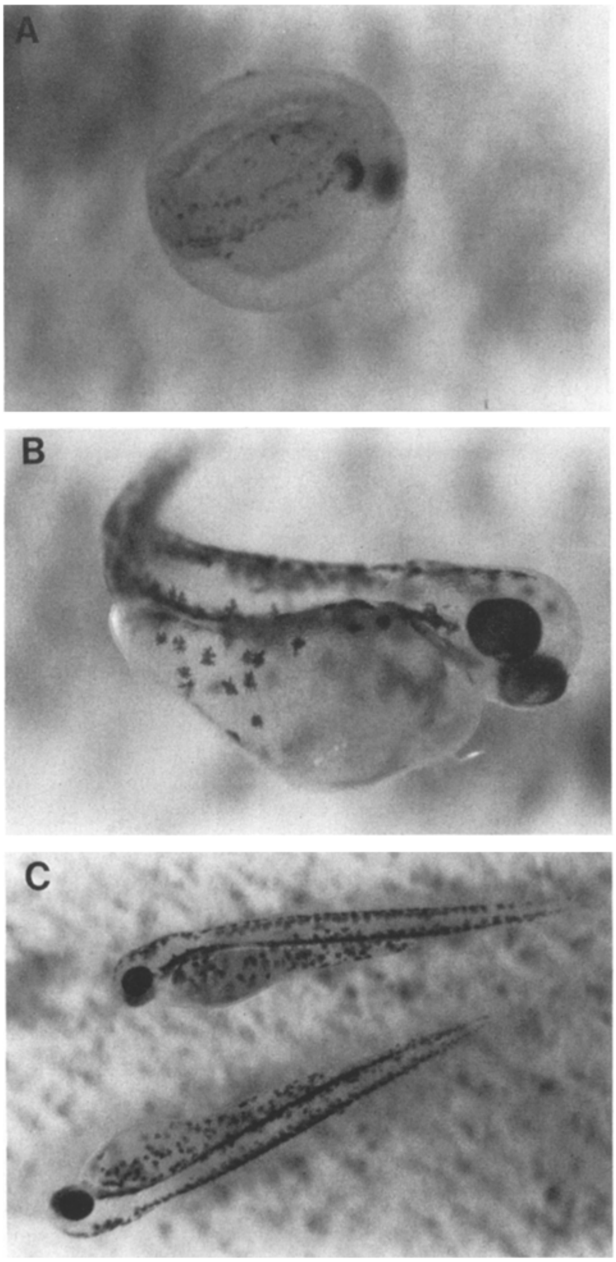
At 24 h at 21 °C, a primitive streak was obvious with about 14–15 somites. Some eggs had died; 41 were removed at this time. After 48 h eyes and otolith were apparent. At that time 17 dead embryos were removed. After 77 h at 21 °C, the first individual hatched. Hatching continued from 77 to 92 h post fertilization. The figure illustrates several hybrid larvae. We preserved 6 newly hatched progeny, some of which seemed anomalous. Size at hatching was about 5 mm.

Two weeks after hatching, about 50 fish were alive. One was noticeably different. It still had a large sac, and did not swim much, although it possessed the ability. It had a large robust body and relatively small eyes, suggestive of being more *neogaeus*-like than the rest.

Relative to the parental species, the initial hatching time of the hybrid eggs was quite rapid. Eggs of *Phoxinus neogaeus* fertilized under similar conditions required 4 days to hatch at 24 °C[6]. In his synopsis of the early life of *Phoxinus eos*, Faber cited an incubation period of 8–10 days at 21.1–26 °C for that species[7]. We hypothesize that the fact that hatching time in the hybrids more closely approximates *neogaeus* is a maternal effect. It would seem quite likely that the majority of the time *Phoxinus neogaeus* would be the maternal ancestor in the hybrids more often than *Phoxinus eos*. As proposed by Bogart, in hybridization events where one species is much rarer than the other, a rare female full of eggs would seem to have a higher chance of mating with the males of the more common species, than rare males with females of the more common species[8]. Such a hypothesis could be tested with mitochondrial DNA analysis, which is materlineally inherited.

It is interesting to speculate that perhaps the mortality of half the eggs during development might represent the death of the male embryos. Hybrid males are extremely rare although they do exist[5]. According to Haldane's rule, when hybrids of one sex are absent, rare or sterile in an interspecific cross that sex is the heterogametic sex[9]. Presuming heterogamy in *Phoxinus*, males would be the heterogametic sex, although defined sex chromosomes are not known in this genus. Perhaps in this genus there is a locus that determines sex. In their review of sex chromosomes and evolution, Jones and Barton cite evidence that in hybrids, sex chromosomes are subject to more selection than autosomes[10]. In the heterogametic sex, the sex chromosomes would have to interact with each other, often in repression, and may be very important in species differentiation. The male *Phoxinus* hybrids that do exist may be the result of rare events such as chromosomal translocations, mosaicism or developmental oddities. It would be interesting to examine their mitochondrial DNA to see if their maternal ancestor is *eos*, in contrast to the female hybrids. It may well be that reciprocal crosses in the origination of the hybrids would have different genetic consequences.

High developmental mortality is seen in the hybrid embryos. Such mortality has also been noted in unisexual salamanders in the genus *Ambystoma* where only a few percent of the eggs give rise to juveniles[11]. This type of endogenous selection certainly would be an important component in the estimation of fitness in the cyprinid hybrids. Given that in the hybrid populations of Nebraska, 20% of the *Phoxinus* are *eos* and almost all the rest are hybrids, further studies of the population biology of the hybrids are certainly warranted.



Several stages of laboratory hybrid development. *A* Hybrid *Phoxinus* embryo just prior to hatching at about 75 h. *B* Anomalous larva 2 days post-hatching. Note unusually large yolk sac. *C* Larvae 5 days after fertilization, 2 days post-hatching at 21 °C.

The two parental species *P. eos* and *P. neogaeus* are not particularly closely related, having fixed allelic differences at a number of allozyme loci[12]. Zymograms of three diagnostic loci, two muscle enzymes, phosphoglucomutase and malate dehydrogenase and a liver enzyme, superoxide dismutase have indicated that wild caught hybrids have patterns expected of F 1 hybrids[3].

Three of the laboratory hybrids were electrophoresed for the two muscle enzymes, phosphoglucomutase and malate dehydrogenase, as a test for whether recombination had occurred. Evidence for this would be had if a laboratory hybrid was homozygous for either parental allele at one or both loci. However all three were heterozygous at both loci with zymogram patterns identical to those of wild caught hybrids. These results support both the hybrid nature of the offspring and that recombination did not occur between the diagnostic loci, as would be typical in independent assortment.

Wild caught hybrids are known to exist in diploid and triploid forms; a chromosome spread from a triploid has been published elsewhere[4]. The chromosomes in North American *Phoxinus* are quite small, with no obvious gross morphological differences[13].

All three laboratory hybrids possessed triploid cells. Because the parents of the hybrids were immediately preserved after the crosses were performed, cytological and allozymic analyses of the parents were not possible.

In our discussion we use parthenogenesis in the broad sense to mean all modes of reproduction in which one or more genomes are inherited clonally as defined by Moore[14]. In his review Moore notes that all parthenogenetic reptilians are female and reproduce by spontaneous parthenogenesis. This is thelytoky, where egg nuclei have the same chromosome as somatic cells and fertilization. In contrast, Moore states that all parthenogenetic amphibians and fishes require at least the physical stimulus of sperm to induce embryogenesis. Two separate sperm-dependent mechanisms exist: hybridogenesis and gynogenesis. In hybridogenesis a reduction division occurs with the same genome, typically the maternal one, segregating intact into haploid gametes. The diploid state is restored by fertilization with sperm. In the meiosis of the resulting offspring only the maternal genome is once again segregated intact into gametes, and is thus perpetuated. Gynogenesis produces eggs with chromosome numbers identical to somatic cells. Sperm merely initiates cleavage and the male chromatin is not expressed in the progeny.

From our data hybridogenesis and gynogenesis as modes of hybrid reproduction cannot be distinguished.

The hybrids apparently are sperm-dependent however, in that progeny never resulted from females isolated without male *P. eos*. The fact that the laboratory hybrids were heterozygous at both loci supports the hypothesis that typical sexual recombination has not taken place. The morphological variability seen in the laboratory hybrids suggest either developmental instability or perhaps a mixture of hybridogenetic and gynogenetic events among the hybrids. This latter is consistent with the clonal structure found in eastern diploid hybrids which must be gynogens, while triploids lack clonal structure and consequently are probably the result of hybridogenetic events[5].

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