The Fate of Paternal Mitochondrial DNA in Developing Female Mussels, *Mytilus edulis*: Implications for the Mechanism of Doubly Uniparental Inheritance of Mitochondrial DNA

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ABSTRACT

Species of the marine mussel family Mytilidae have two types of mitochondrial DNA: one that is transmitted from the mother to both female and male offspring (the F type) and one that is transmitted from the father to sons only (the M type). By using pair matings that produce only female offspring or a mixture of female and male offspring and a pair of oligonucleotide primers that amplify part of the *COIII* gene of the M but not the F mitochondrial genome, we demonstrate that both male and female embryos receive M mtDNA through the sperm and that within 24 hr after fertilization the M mtDNA is eliminated or is drastically reduced in female embryos but maintained in male embryos. These observations are important for understanding the relationship between mtDNA transmission and sex determination in species with doubly uniparental inheritance of mitochondrial DNA.

UNLIKE most other animal species, mussels of the family Mytilidae are known to possess two types of mitochondrial DNA (mtDNA), one that is transmitted from fathers to sons exclusively (the M type) and one that is transmitted from mothers to both sons and daughters (the F type) (Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b). This system of mtDNA transmission was named doubly uniparental inheritance (DUI; Zouros *et al.* 1994a,b) to distinguish it from the standard mode of uniparental inheritance and from the rare type of biparental inheritance, where small amounts of paternal mtDNA may "leak" into the zygote's mtDNA pool (Satta *et al.* 1988; Kondo *et al.* 1990; Gyllensten *et al.* 1991; Magoulas and Zouros 1993). Presently, DUI has been demonstrated in two families of bivalves, Mytilidae (blue mussels; Geller 1994; Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b) and Unionidae (fresh water mussels; Hoeh *et al.* 1996; Liu *et al.* 1996). Because no systematic search for its presence in other taxa has been undertaken as yet, its distribution within bivalves or mollusks remains unknown.

It follows from the definition of DUI that females must be homoplasmic, males must be heteroplasmic, eggs must contain the F type, and sperm must contain the M type of mtDNA. These were indeed the basic observations that led to the discovery of the phenomenon (Fisher and Skibinski 1990; Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b). Given that this happened only recently, it is not surprising that many questions about the mechanism of operation of DUI remain unanswered. In normal heteroplasmic males, the F type is the main mtDNA in somatic tissues (M. Garrido-Ramos, personal communication), but the gonad is dominated by the M type (Stewart *et al.* 1995). Exceptional heteroplasmic (M-positive) females have been observed both in the field (Fisher and Skibinski 1990; Rawson *et al.* 1996; M. Garrido-Ramos, personal communication) and in laboratory crosses (Zouros *et al.* 1994b). Exceptional homoplasmic (M-negative) males have also been observed (Zouros *et al.* 1994a; Saavedra *et al.* 1997). It is further known that the sperm of these males contains the F type that these males inherit from their mothers (Saavedra *et al.* 1997). This is assumed to be the first step in the process of masculinization, *i.e.*, the phenomenon through which an F molecule assumes the role of an M molecule (Saavedra *et al.* 1997; Hoeh *et al.* 1977).

Cytological observations have established that the midpiece of the Mytilus sperm contains five mitochondria, which are larger in size than the mitochondria of somatic cells (Longo and Anderson 1969). The same studies have shown that these mitochondria enter the egg. In the context of studying DUI, Zouros *et al.* (1994b) and Saavedra *et al.* (1997) observed that the

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ratio of male-to-female progeny varies extensively among pair matings and that it is under the control of the female parent. Some females will produce almost exclusively daughters, and others will produce sons in ratios higher than 80%, irrespective of the male to which they are mated. This observation could provide a way to examine the developmental events that are responsible for the coupling of mtDNA inheritance and sex inheritance in species with DUI. If the sex of an egg's embryo is determined before the egg is fertilized, it is possible that eggs programmed to develop into females are also programmed, pleiotropically or by genetic linkage, to prevent the entry of the sperm's mitochondria, whereas no such prevention mechanism may exist in eggs programmed to develop into males. The observation by Longo and Anderson (1969) that sperm mitochondria enter the egg does not preclude this possibility, as these observations could have all been done with eggs destined to become males (either by coincidence or because eggs that may have not received sperm mitochondria were excluded from the data set as erratic or unfertilized). Thus, it remains an important question whether sperm mitochondria enter the egg irrespective of the sex to which it will develop. In the event this is true, the question becomes, How early in the development of a female individual is the sperm-derived mtDNA eliminated? This study was designed to answer these questions.

MATERIALS AND METHODS

Crosses: Pair-matings (referred to here as families) were established as described in Zouros *et al.* (1992). The procedure involved spawning each male and female separately and mixing eggs and sperm to establish families. This allows using the same parent for more than one cross. We have used offspring from 17 families that were produced from nine females and 12 males (Table 1). Six of the female parents were progeny of family #3 and three of family #6, and all male parents were offspring of family #7 as listed in Zouros *et al.* (1992). Families #3 and #6 were chosen as donors of female parents because they contained the standard variant of *Mytilus edulis* F mtDNA [originally referred to as FB by Fisher and Skibinski (1990) and renamed F-ed1 by Stewart *et al.* (1995)] and because they had different sex ratios: Family #3 produced daughters only, whereas family #6 produced sons at a ratio of 65% (Zouros *et al.* 1994b). Family #7 was used as the donor of male parents because it contained the standard variant of *M. edulis* M mtDNA [originally referred to as M by Fisher and Skibinski (1990) and renamed M-ed1 by Stewart *et al.* (1995)] for which we have developed a specific PCR assay (see below).

Fourteen of the above families were established in June 1994 and three in May 1995. Progeny from the 1994 families were removed at 14 days, 3 and 6 mon after fertilization, stored at −70° and examined for presence/absence of M mtDNA. Progeny were reared to sexual maturity, approximately 2 yr of age, and sexed by examining the gonad under a microscope for presence of sperm or eggs. (Sexing is not possible in mussels that do not carry a mature gonad.) The May 1995 families were established only for the examination of early larvae (18, 24, and 48 hr after fertilization) and were terminated after sampling. Larvae were stored in 70% ethanol/seawater at 4° until examination for presence/absence of M mtDNA.

In addition to these 17 females, we examined 18-hr-old larvae from 3 pair-matings each produced by a different male and female collected from the wild. These males also contained the M-ed1 M mtDNA type.

Detection of mtDNA: All soft tissues from 3 or 6 mon-old juveniles were used for total DNA extractions using a variation of the salt extraction procedure (Miller *et al.* 1988). Total DNA from larvae was extracted as follows: Larvae were individually isolated using a drawn pasteur pipette under a phase microscope at ×40 magnification, placed in 1.5 ml distilled water, and vortexed for 30 sec to clean the larvae of cellular debris and residual sperm. This procedure was repeated five times. In the final wash, larval size was estimated using the stage micrometer, and developmental state was determined on the basis of stages described by Bayne (1976). Individual larvae were placed in 15 μl lysis solution (7.5 mm Tris-HCl, pH 8.3; 3.75 mm NH_4Cl; 3.75 mm KCl; 1.5 mm $MgCl_2$; 2 μg proteinase K), and incubated at 37° for 1.5 hr. Samples were then boiled for 10 min to inactivate proteinase K.

DNA from a juvenile or the lysate of a larva was used as template for PCR amplification. For detection of F mtDNA, we used the primer pair F-ed1/FOR1 5′-TATGTACCAGGTC CAAGTCCGTG-3′ and F-ed1/REV1 5′-CACATACACTAAGC ACCACAAATG-3′REV2 described in Stewart *et al.* (1995), which amplifies a 753-bp fragment of the cytochrome c oxidase subunit III mitochondrial gene (*COIII* gene) with a much greater efficiency for the F-ed1 than the M-ed1 molecule. For detection of M mtDNA, we used the primers M-ed1/FOR1 5′-TGGAGTCGCTTTATTTATTTTATCTGA-3′ and M-ed1/REV1 5′-ATACTACAAACCACAGCCTCACTCATA-3′, which correspond to nucleotide positions 207–234 and 710–737 of the *COIII* gene. These primers were designed after comparison of the *COIII* DNA sequences of the main F and M variants of *M. edulis* and *M. trossulus* (F-ed1, M-ed1, F-tr1 and M-tr1; Stewart *et al.* 1995), so that they will amplify only from M-ed1. In trial experiments using DNA from juveniles containing both the F-ed1 and M-ed1 molecules, this pair of primers produced the expected 530-bp fragment, which upon sequencing proved to match the *COIII* gene of M-ed1, but not of F-ed1. The PCR reaction mixtures consisted of 10 mm Tris-HCl, pH 8.3; 5 mm NH_4Cl; 50 mm KCl; 2 or 3 mm $MgCl_2$ for M-ed1 or F-ed1 *COIII* specific primer pairs, respectively; 0.2 mm of each dNTP (Pharmacia, Piscataway, NJ); 0.075 μM digoxigenin-11-2′-deoxy-uridine-5′ triphosphate (Dig-11-dUTP; Boehringer Mannheim, Mannheim, Germany) for M-ed1 PCR amplifications; and 1.25 units *Taq* DNA polymerase in 25 μl reactions. Amplifications were performed in an MJ thermo-cycler (MJ Research, Watertown, MA; PTC-100™) for 40 cycles (94° for 30 sec, 59° or 63° for 90 sec for F-ed1 or M-ed1 *COIII* specific primer pairs respectively, and 72° for 30 sec). The initial denaturation period and final extension period were both 4 min.

Even though the objective of the study was to determine if a progeny carried the M mtDNA, progeny were also assayed for presence of F mtDNA. This served as an internal control against scoring a progeny as lacking the M type when in fact neither the M nor the F could be detected. Only progeny that tested positive for F were included in the data. For the detection of the F mtDNA molecule, it proved sufficient to visualize the PCR product by ethidium bromide staining of agarose gels. Because all individuals are expected to carry an F mtDNA molecule, a negative result could only be attributed to technical error. For the detection of M mtDNA, which may or may not be present in any given individual offspring, we employed two levels of testing. The first was based on staining

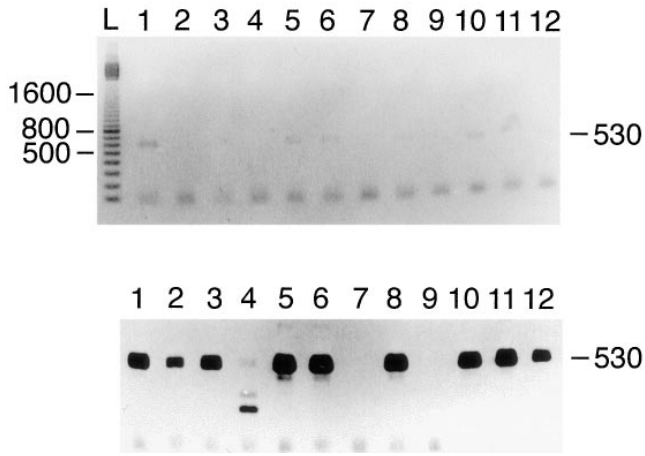

Figure 1.—Paternal mitochondrial DNA of 14-day-old larvae from family H14. Numbers in margins denote length in bp. All progeny contained the 754-bp amplification product from the *COIII* gene of the F mtDNA molecule (not shown). Top panel: stained with EtBr; the 530-bp product from the *COIII* gene of the M mtDNA molecule can be seen in lanes 1, 3, 5, 6, 8, 10, 11, and, barely, 12. Bottom panel: the same PCR product visualized with the chemiluminescence assay; the M molecule was detected in two additional lanes (2 and 4).

by ethidium bromide, as in the case of F mtDNA. The second involved the more sensitive Dig-labeling technique. For this purpose, Dig-11-dUTP was incorporated in the PCR reaction mixture, and the PCR products were transferred to nylon membranes and detected by the anti-Dig/CSPD chemiluminescence assay (Boehringer, Mannheim). To estimate the detection power gained by the second method, DNA extracted from spawned sperm (which contains only M mtDNA) was used in a dilution series. A dilution to 10^{-4} (one part of reference concentration to 10,000 parts of water) could be detected by ethidium bromide, but a dilution to 10^{-5} could not. The corresponding figures for the chemiluminescence assay were 10^{-8} and 10^{-9}, thus the sensitivity was increased by a factor of 10^3 to 10^4. The combination of the two staining methods produced three categories of progeny with regard to presence of the M genome: strong presence, when presence of M could be clearly seen with both stains (pattern I); weak presence, when presence of M could be seen only with the chemiluminescence assay (pattern II); and absence, when the M product could not be seen with either method (pattern III).

RESULTS

Fourteen-day, three-month and six-month old progeny: Typical results from testing for presence of the M mtDNA genome are shown in Figures 1–3. All three M mtDNA intensity patterns were seen in each of the three ages. The numbers for each pattern in each family are given in Table 1. The table also gives the number of female and male progeny detected at the age of 2 yr. We first asked if the distribution of the three patterns varied among ages within families. Summary statistics from the chi-square test of homogeneity are given in Table 2. A significant difference in the frequency of patterns was observed in only one family (B9, where progeny with the weak pattern were more common in the age of 6 mon than in the age of 3 mon). The heterogeneity in family X101 is not significant after the Bonferroni correction for multiple tests (Sokal and Rohlf 1995).

Given that no differences exist among ages within families, ages were pooled to provide one value for each pattern in each family. The test of homogeneity of pattern distribution among families was significant (chi square 232.094 on 26 d.f., $P < 0.0001$). Families with no male progeny tended to have no strong M mtDNA

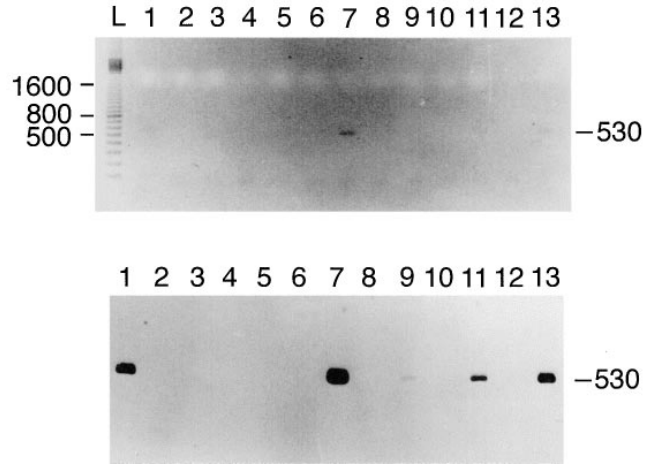

Figure 2.—Paternal mitochondrial DNA of 3-mon-old juveniles from family F7. Numbers in margins denote length in bp. All progeny contained the 754-bp amplification product from the *COIII* gene of the F mtDNA molecule (not shown). Top panel: stained with EtBr; the 530-bp product from the *COIII* gene of the M mtDNA molecule can be seen in lanes 1, 7 and 13. Bottom panel: the same PCR product visualized with the chemiluminescence assay; the M molecule was detected in two additional lanes (9 and 11).

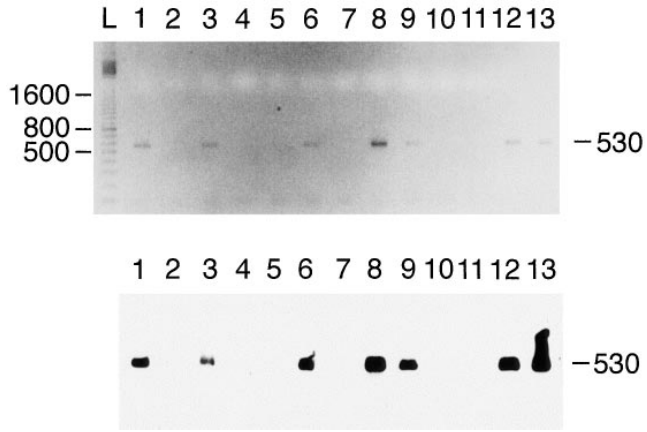

Figure 3.—Paternal mitochondrial DNA of 6-mon-day old juveniles from family F7. Numbers in margins denote length in bp. All progeny contained the 754-bp amplification product from the *COIII* gene of the F mtDNA molecule (not shown). Top panel: stained with EtBr; the 530-bp product from the *COIII* gene of the M mtDNA molecule can be seen in lanes 1, 3, 6, 8, 9, 12, and 13. Bottom panel: the same PCR product visualized with the chemiluminescence assay; and no additional M products were evident.

TABLE 1

Intensity patterns of paternal mtDNA in progeny of three different ages of *M. edulis* mussels from 14 pair-matings

Family	Age progeny examined	No. progeny examined	M mtDNA pattern I (strong)	II (weak)	III (absent)	No. progeny sexed	No. male progeny
A							
D17	14 d	38	8	0	30	40	0
Z103	14 d	30	17	1	12	50	33
X101	14 d	40	2	1	37	4	0
X101	3 m	14	0	5	9	—	—
X101	6 m	15	0	4	11	—	—
B5	3 m	17	0	8	9	37	0
B5	6 m	9	0	4	5	—	—
B6	3 m	14	0	3	11	19	0
B6	6 m	15	0	3	12	—	—
B7	3 m	24	0	1	23	45	0
B7	6 m	24	0	0	24	—	—
B9	3 m	24	0	1	23	46	0
B9	6 m	24	0	9	15	—	—
B							
H14	14 d	40	20	12	8	61	24
J15	14 d	34	25	2	7	52	39
F5	3 m	24	12	1	11	30	16
F5	6 m	24	13	0	11	—	—
F6	3 m	14	10	3	1	38	29
F6	6 m	15	13	0	2	—	—
F7	3 m	24	6	3	15	33	18
F7	6 m	23	13	3	7	—	—
F9	6 m	22	11	3	8	20	13
F9	6 m	24	9	3	12	—	—
H15	3 m	14	5	3	6	12	6
H15	6 m	15	8	0	7	—	—

In each family, the letter refers to the female parent and the number to the male parent. A, female parent is a daughter of female EF4; B, female parent is a daughter of female EF5; d, days; m, months.

TABLE 2

Tests of homogeneity of distribution of M mtDNA intensity patterns among ages within families

Family	Ages and patterns tested	d.f.	χ^2	*P*
X101	14 d, 3 m, 6 m / I, II, III	4	12.503	0.014 (ns)
X101	3 m, 6 m / II, III	1	0.277	0.599
B5	3 m, 6 m / II, III	1	0.016	0.899
B6	3 m, 6 m / II, III	1	0.009	0.924
B7	3 m, 6 m / II, III	1	1.021	0.312
B9	3 m, 6 m / II, III	1	8.084	0.004 (s)
F5	3 m, 6 m / I, II, III	2	1.040	0.594
F6	3 m, 6 m / I, II, III	2	3.695	0.158
F7	3 m, 6 m / I, II, III	2	5.469	0.065
F9	3 m, 6 m / I, II, III	2	0.915	0.633
H15	3 m, 6 m / I, II, III	2	3.739	0.154

Data from Table 1. Heterogeneity is significant in only one family (B9). The *P* value for X101 is not significant after the Bonferroni correction for multiple tests. d, days; m, months.

pattern (Table 1). To test for a correlation between sex ratio and M mtDNA intensity pattern, we regressed the family's frequency of male progeny against the frequency of progeny with presence of M mtDNA (patterns I and II) and the frequency of progeny with the strong intensity (pattern I). Figure 4 shows both regressions are positive and highly significant. The correlation is stronger when only the number of progeny with the strong intensity is used. It explains 90% of the variation among families; its slope is not statistically significant from 1, and the intercept is almost zero (Figure 4), in accord with what one would expect if the frequency of progeny with the strong M mtDNA pattern was equal to the frequency of males in the family.

Eighteen-, 24- and 48-hr old larvae: The presence of the weak M pattern in several progeny of families that produced only daughters suggests that sperm mitochondria enter the ovum irrespective of the sex to which it will develop. The nearly complete absence of the strong M pattern in these families would mean that most of the sperm's mtDNA is eliminated in female em-

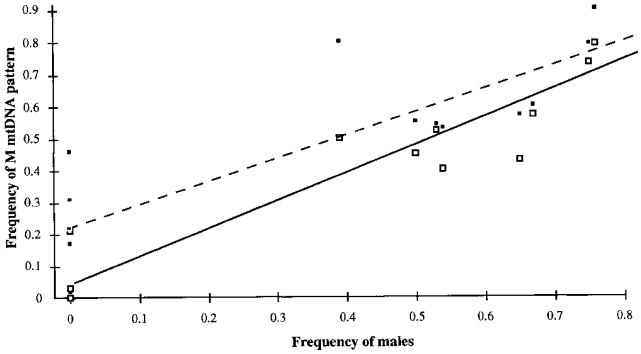

Figure 4.—The frequency of intensity patterns of M mtDNA plotted against the frequency of males in 14 pair-matings of *M. edulis*, as determined by actual sexing of 2-yr-old progeny (data compiled from Table 1). Open symbols and solid line: the frequency of strong M mtDNA (pattern I); $y = 0.041 + 0.848x$; $r = 0.951$, $P < 0.0001$ (four points have $x = 0$, $y = 0$). Filled symbols and broken line: the combined frequency of the strong and weak M mtDNA (patterns I + II); $y = 0.241 + 0.694x$; $r = 0.849$, $P < 0.0001$ (two points have $x = 0$, $y = 0.21$).

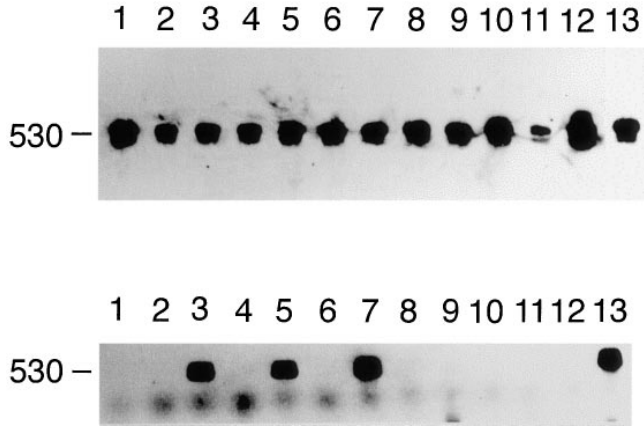

Figure 5.—Paternal mitochondrial DNA of larvae from family M210. Numbers in margins denote length in bp. The chemiluminescent assay was used in both panels. Top panel: 18-hr-old larvae; all larvae showed presence of the M product. Bottom panel: 24-hr-old larvae; the M product cannot be detected in 9 of 13 lanes.

bryos and that this event occurs at an age earlier than 14 days. To obtain further evidence for this hypothesis, we established a new set of families and examined the intensity of the M mtDNA profile at 18–48 hr after fertilization. Table 1 shows that most daughters of female EF4, which was a sonless female (Zouros *et al.* 1994b), were themselves sonless. Indeed, six of the seven daughters of EF4 produced no son, whereas all seven daughters of EF5, a female with a 1:1 sex ratio (Zouros *et al.* 1994b), produced offspring of both sexes. Therefore, to find out the earliest stage at which the M mtDNA disappears or is drastically reduced in female embryos, we used two daughters of EF4 on the assumption that they will also produce mainly or exclusively daughters. With an observed frequency of 6/7 for sonless females among daughters of EF4, the probability that at least one of the two daughters would also be sonless is 0.98 (and the probability that both would be sonless is 0.77).

In larvae of these early stages, we found no qualitative difference between the EtBr and the chemiluminescence assay. Results from two larval stages in one family are shown in Figure 5, and the results from all three families are summarized in Table 3. All larvae scored at the age of 18 hr after fertilization showed presence of M mtDNA. Indeed, the intensity of the reaction was similar to the "strong pattern" (pattern I) seen in 6 mon-, 3 mon-, or 14 day-old progeny. Larvae with no traces of M mtDNA (pattern III) were seen at the age of 24 hr and 48 hr. Table 3 also gives the results from testing the distribution of intensity patterns between different ages within families. It can be seen that there has been a marked change in the distribution of M mtDNA from 18 to 24 hr or 48 hr in all three families, in contrast to the lack of such change among the older ages of 14 days, 3 mon, or 6 mon.

The results from the three wild crosses (B1, D3, E4; Table 3) also suggest that at the age of 18 hr all larvae contain the father's mtDNA. Presence of the M molecule could not be detected in two of these larvae, but these same two larvae produced low-intensity bands for the F molecule, which suggests that the DNA extracted from these two specimens may have been of low quality. The probability that all the other 39 larvae were males is negligible given that we have so far found no family that would be completely daughterless and that the sex ratio in the progeny from a random sample of females is roughly equal to 1:1 (Saavedra *et al.* 1997).

The frequency of larvae with no M mtDNA at the age of 48 hr is not different between families L208 and L209 (chi-square 2.584, d.f. = 1, $P = 0.108$), which is consistent with the fact that these families shared the same female parent and should, therefore, have the same sex ratio. But the combined frequency of larvae with no M at the age of 48 hr from these two families is significantly higher than that at 24 hr from family M210 (chi-square 11.642, d.f. = 1, $P = 0.0006$). Viewed in terms of age only, this increase in the frequency of M-positive larvae from 24 to 48 hr is the exact opposite of the comparison of the ages of 18 and 24 hr, where the frequency of M-positive decreased from 100 to 39%. The most likely explanation for this is that the L and M females have different sex ratios. Being daughters of EF4, females L or M could either be sonless (as all their sisters except Z; Table 1) or produce progeny of both sexes (as sister Z). Preliminary results from our laboratory suggest that the sex ratio in the progeny of a female is determined by the female's nuclear genotype. A simple, as yet not tested model that assigns a homozygote genotype to a sonless female (like EF4) and a heterozygote genotype to its mate (in this case EM4) would

TABLE 3

Numbers of intensity patterns of M mtDNA in larvae of three different ages from six pair-matings of *M. edulis*

Family	Age of progeny examined	No. of progeny examined	M mtDNA pattern		*P* of homogeneity
			Presence	Absence	
L208	18 h	39	39	0	
L208	48 h	47	40	7	0.012
L209	18 h	40	40	0	
L209	48 h	26	18	8	0.0003
M210	18 h	24	24	0	
M210	24 h	18	7	11	<0.0001
B1	18 h	12	11	1	
D3	18 h	16	16	0	
E4	8 h	13	12	1	

In each family the letter refers to the female parent and the number refers to the male parent. Females L and M were daughters of female EF4. B1, D3, and E4 were crosses between animals from the wild. The probabilities (from Fisher's exact test) for homogeneity of the ages within families are also given. h, hours.

explain the segregation of the sex ratio among daughters. Unfortunately, L and M females could not be tested for the actual male-to-female progeny ratio because their broods were terminated at the larval stage.

DISCUSSION

The doubly uniparental inheritance (DUI) of mtDNA represents a remarkable exception to the nearly universal uniparental inheritance of organelle DNA. By its very nature, DUI implies a coupling of sex inheritance and mtDNA inheritance, but the mechanism for this coupling remains unknown. Saavedra *et al.* (1997) observed that in laboratory crosses the male-to-female ratio is solely determined by the mother, but whether sons will contain an M mtDNA genome is determined by the father. They found that males fall into one of two classes, the most common class being males that produce sons with the M type (DUI males) and the less common class being males that produce sons with no M type (standard maternal inheritance, SMI males). Using this information they proposed a model in which the default gender is the female in species with DUI, but presence in the early germ cells of a factor that enters the egg through the sperm's mitochondria results in masculinization of the gonad. Females that produce only or mostly daughters supply their eggs with a factor that recognizes and arrests the paternal mitochondria before they enter the germ line. This second factor is lacking or is in low amounts in eggs of females that produce mostly sons.

Our study of the fate of paternal mtDNA in developing embryos and its association with sex determination suffers from the difficulty to detect the mtDNA content of the embryo without killing it. This is compounded with our current inability to diagnose the sex of an individual before it reaches sexual maturity. In view of these difficulties, the use of families that produce either only females or produce a mixture of both sexes presents the best way to approach these questions. Our work presents evidence for a hitherto untested assumption of the above model, *i.e.*, that all eggs receive mitochondria from the sperm. We tested 144 larvae at the age of 18 hr and found that only two (1.5%) might not contain paternal mtDNA. More than 100 of these larvae were produced by females that were expected to produce exclusively or mainly daughters, so that the probability that all 18-hr-old larvae happened to be males is nearly zero.

Our study goes one step further in that it shows that the process of elimination of the paternal mtDNA in females is completed before the age of 24 hr. Fully 60% of larvae from one female (female M; Table 3) had no scorable amounts of M mtDNA at the age of 24 hr, and about 20% of larvae of the second female (female L; Table 3) produced the same result at the age of 48 hr. Moreover, we found no clear evidence of weak presence of M mtDNA among young larvae. This may suggest that the actual "decision" for retention of the paternal mtDNA (and therefore commitment to maleness), or virtual elimination of the paternal mtDNA (and therefore commitment to femaleness), takes place very early in development. If this hypothesis is correct, the "re-appearance" of the M mtDNA genome in a few older females can be explained as the result of subsequent replication, concomitant with the growth of the embryo, of a few molecules of paternal mtDNA that escaped destruction at the critical stage of sex determination. In such females, the M mtDNA will remain in the minority compared to F mtDNA, as we have observed. This interpretation is consistent with two observations. One is that the frequency of individuals with the weak pattern of M (pattern II) in our data increased from young larvae (24 hr and 48 hr of age) to old larvae (14 days) from zero to about 10% (chi-square 8.498, d.f. 1, $P = 0.004$) and from old larvae to juveniles (3- and 6-mon old) from 10% to about 15% (chi-square 4.241, d.f. 1, $P = 0.040$). There has been,

however, no increase between 3 mon and 6 mon juveniles (chi-square 0.427, d.f. 1, $P = 0.513$). The other observation is that detailed experiments with adult females have shown that many such females harbor a small amount of M mtDNA in one or another of their somatic tissues (M. Garrido-Ramos, personal communication). Exceptional females with an obviously large content of M mtDNA have been observed in natural populations (Fisher and Skibinski 1990; Rawson *et al.* 1996), but they appear to represent hybridization anomalies.

Ultrastructural studies of spermatogenesis in Mytilus (Longo and Dornfeld 1967) have shown that the ellipsoid and randomly distributed mitochondria of the early spermatid are fused to form five large spheroid mitochondria that occupy the midpiece of the mature spermatozoon and are in physical contact with the nuclear membrane. Five large mitochondria were also observed recently in the sperm of the monoplacophoran mollusc *Laevipilina antarctica* (Healy *et al.* 1995). Because each of these spheroid mitochondria is made from many original mitochondria, there is no way of knowing the percentage of paternal mtDNA in a fertilized ovum. Yet, they must represent a small minority given the large number of egg mitochondria (Billet 1979). An early study of germ-cell formation in the fresh water clam *Sphaerium striatium* (Woods 1932) made the interesting observation that a well-recognizable "mitochondrial cloud" is formed in the proximal portion of the oocyte. The cloud maintains its integrity through the first cell divisions and is finally delivered into two cells that appear at gastrulation on either side of the mesoderm and give rise to the germ line. Woods (1932) has considered this mitochondrial cloud to be a "Keimbahn," *i.e.*, a factor that is associated with the continuity of the germ line but plays no causal role in the determination of germ cells. The occurrence of DUI makes it tempting to ask whether an analogous mitochondrial cloud exists in the Mytilus egg and, if so, whether in some zygotes, those that would develop into males, the sperm's mitochondria succeed in replacing the egg's mitochondria from the cloud, thus securing their delivery into the primordial germ cells.

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