Differential Segregation Patterns of Sperm Mitochondria in Embryos of the Blue Mussel (*Mytilus edulis*)

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ABSTRACT

In Mytilus, females carry predominantly maternal mitochondrial DNA (mtDNA) but males carry maternal mtDNA in their somatic tissues and paternal mtDNA in their gonads. This phenomenon, known as doubly uniparental inheritance (DUI) of mtDNA, presents a major departure from the uniparental transmission of organelle genomes. Eggs of *Mytilus edulis* from females that produce exclusively daughters and from females that produce mostly sons were fertilized with sperm stained with MitoTracker Green FM, allowing observation of sperm mitochondria in the embryo by epifluorescent and confocal microscopy. In embryos from females that produce only daughters, sperm mitochondria are randomly dispersed among blastomeres. In embryos from females that produce mostly sons, sperm mitochondria tend to aggregate and end up in one blastomere in the two- and four-cell stages. We postulate that the aggregate eventually ends up in the first germ cells, thus accounting for the presence of paternal mtDNA in the male gonad. This is the first evidence for different behaviors of sperm mitochondria in developing embryos that may explain the tight linkage between gender and inheritance of paternal mitochondrial DNA in species with DUI.

STRICTLY maternal inheritance of mitochondrial DNA (mtDNA) is the general rule in animals (Hayashi *et al.* 1978; Birky 1995), even though cases of incidental inheritance of paternal mtDNA have been reported in fruit flies (Drosophila; Kondo *et al.* 1990), mice (Gyllensten *et al.* 1991), and anchovies (Magoulas and Zouros 1993). However, in the marine mussel *Mytilus edulis*, a more complex mitochondrial transmission system was discovered (Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b). Female mussels are normally homoplasmic for one type of mtDNA, the female (F)-type, which they transmit to both daughters and sons through the egg. In contrast, males are normally heteroplasmic for two types of mtDNA, the F-type, which they inherit from their mother but do not transmit to offspring, and the male (M)-type, which they inherit from their father and transmit to their sons through the sperm (Skibinski *et al.* 1994a,b; Zouros *et al.* 1994a,b). In typical adult males, the gonad is dominated by the M-type, and the somatic tissues by the F-type (Stewart *et al.* 1995; Garrido-Ramos *et al.* 1998). There are some exceptions to this model. Further investigation has shown that occasionally the M-type is detected in adult females in various organs at very low amounts (Garrido-Ramos *et al.* 1998). Also, rarely, males whose paternal and maternal genomes both resemble the typical F genome have been observed in the field (Fisher and Skibinski 1990; Rawson *et al.* 1996; Garrido-Ramos *et al.* 1998; Quesada *et al.* 1999; Ladoukakis *et al.* 2002) and in laboratory crosses (Zouros *et al.* 1994a,b; Saavedra *et al.* 1997).

This separate transmission of M and F mitochondrial genomes gives rise to two distinct, gender-associated lineages of mtDNA, which in Mytilus may show 10–20% sequence divergence (Fisher and Skibinski 1990; Rawson and Hilbish 1995; Stewart *et al.* 1995). This system of mtDNA transmission has been termed "doubly uniparental inheritance" (DUI; Zouros *et al.* 1994a). The DUI system has subsequently been identified in the marine mussels *M. trossulus*, *M. galloprovincialis*, *M. californianus*, and *Geukensia demissa* (Mytilidae; Geller 1994; Zouros *et al.* 1994a,b; Hoeh *et al.* 1996; Beagley *et al.* 1997); in the fresh water mussels *Pyganodon grandis*, *P. fragilis*, and *Fusconaia flava* (Unionidae; Liu *et al.* 1996); and in the clam *Tapes philippinarum* (Veneridae; Passamonti and Scali 2001).

It is known from early electron microscopy studies (Longo and Dornfeld 1967) that the midpiece of Mytilus sperm at maturity normally carries five mitochondria that are much larger than egg mitochondria. As in most other animals, the sperm mitochondria penetrate the cell membrane of the ovum at fertilization. However, paternal mtDNA is a minority in the zygote at fertilization, as the egg contains many tens of thousands of F-type mitochondria (Humphreys 1962). Thus, at fertilization all embryos have a very large bias in favor of the F genome.

Clearly, in species with DUI, different mechanisms

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of mitochondrial replication and/or destruction must operate between the sexes to produce adult females in which the M genome has largely disappeared and adult males in which somatic cells are primarily or entirely F-type but the gonads are M-type. In a passive model, where sperm mtDNA has the same fate as egg mtDNA with regard to rate of replication and cell distribution in the developing embryo, the M genome would be subject to stochastic elimination or random presence in one or another tissue. This is consistent with observations made in some females using PCR techniques (Garrido-Ramos *et al.* 1998). Alternatively, active mechanisms may operate whereby M genomes are preferentially degraded in females. In males, the abundance of paternal mtDNA in gonads must be achieved through active mechanisms given that the M genome is numerically biased against at fertilization. Those mechanisms might simply involve differential replication and/or destruction of the two genomes, with the M-type being favored in the gonad and the F-type favored elsewhere. Alternatively, there may be preferential partitioning of the paternal mitochondria to the gonads during early development followed by differential replication and/or destruction.

To determine which of these mechanisms are operative, it is necessary to link *in vivo* observations of sperm mitochondria to the sex of the individual. Mussels cannot be reliably sexed histologically, even under the best culture conditions, at less than a year of age. PCR assays using sex-specific mtDNA markers may lead to false characterization of a male animal as female through amplification error, since the female state is determined by absence of amplification product using male-specific markers. Occasionally the paternal mtDNA may have an F-like sequence (Saavedra *et al.* 1997) or the specific part of the molecule used for the assay might have incorporated an F-like sequence through recombination (Ladoukakis and Zouros 2001). These difficulties may be largely overcome in Mytilus because it is possible to perform selected crosses using pedigreed females that produce progeny of one sex with very high frequency (Saavedra *et al.* 1997; Kenchington *et al.* 2002). The fate of sperm mitochondria can be followed in siblings, rather than individuals, facilitating sampling at different developmental stages.

Here we use a combination of genetic and cytological evidence made on selected crosses of pedigreed females, known to produce different proportions of male and female offspring, to obtain strong evidence for differential mitochondrial segregation patterns between the sexes. In female embryos the sperm mitochondria disperse randomly among the cells. However, in male embryos the sperm mitochondria aggregate in the large cell (CD) at the two-cell stage and remain aggregated through subsequent cell divisions. These results are discussed in terms of the DUI model.

MATERIALS AND METHODS

Mussel pair matings: Animals were collected from a mussel farm in Country Harbour, Nova Scotia, Canada. Because *M. trossulus*, a sibling species, is known to occur in the region, we confirmed species identity by restriction fragment analyses of the internal transcribed spacer (ITS) region of the nuclear ribosomal RNA genes (Heath *et al.* 1995). All male mussels and their male progeny carried the Med-1 haplotype of the paternally transmitted genome, which is the typical M-type in *M. edulis* from the Atlantic coast of Canada (Stewart *et al.* 1995).

The methods used for *in vitro* spawning and subsequent mixing of eggs with sperm were as described in Kenchington *et al.* (2002), as were the methods for rearing the offspring. Seven females were used, four from pedigreed lines known to produce almost exclusively daughters (>97%), and three from lines that produce mostly sons (>75%). The seven were individually crossed with one of four males—the use of multiple females with the same male allowing some examination of the roles of the two parental sexes in determining the fate of the sperm mitochondria. The parents used for each cross and the resulting offspring are here termed a "family" (Table 1).

Fluorescence labeling: MitoTracker Green FM (MitoFM; Molecular Probes, Eugene, OR) is a mitochondria-specific vital dye. It is essentially nonfluorescent in an aqueous solution and becomes fluorescent when its chloromethyl moieties form covalent bonds with protein thiols in the mitochondrion (Haugland 1996). The intensity of MitoFM fluorescence in living cells depends on the potential of the mitochondrial membrane (Cummins *et al.* 1997). Loss of mitochondrial membrane potential correlates with the destruction of the mitochondrion (Kaneda *et al.* 1995; Sutovsky *et al.* 2000).

MitoFM was diluted to a concentration of 1 mM in dimethyl sulfoxide (Sigma Chemical, St. Louis) and added to the sperm suspension to a final concentration of 200 nM. The sperm were incubated in the dye for 20 min at 18° and then washed thoroughly on a Millipore filter and suspended in seawater. Labeled sperm were added to eggs at a very low concentration of ~100 sperm/ml for 10 min. The resulting zygotes were washed three times on a 20-μm filter to remove sperm that did not participate in fertilization but adhered to the surface of the eggs.

To observe the relative position of the mitochondria with regard to the nucleus, sperm nuclei were counterstained with propidium iodide (Molecular Probes), at a final concentration of 3 μg/ml for 10 min.

To examine the possibility that staining of the sperm mitochondria might have an effect on the distribution of paternal mtDNA among progeny, we conducted a subsidiary experiment in which eggs from two females were separately fertilized with stained and unstained sperm from the same male. One of the females (X102E) was known from a previous mating to produce only daughters and the other to produce predominately sons (99wF1).

Sex determination: Some progeny of all families were raised to sexual maturity and the sex ratio was determined for each family through microscopic examination of the gonads.

Visualization of labeled mitochondria in developing mussel embryos: An undetermined number of embryos from each family were extracted from the pool of offspring and mounted in seawater on glass slides under coverslips, sealed, and placed in the dark at 18° for 20 min. Epifluorescent microscopy was then used to determine the position of sperm mitochondria in individual embryos. At first we used a polyvar epifluorescent microscope (Reichert-Jung, Wien, Austria) with a 200-W mer-

cury arc lamp and an ORCA 100/C4742-95 Hamamatsu digital camera (Hamamatsu Photonics KK, Japan) operated by SimplePCI software (Compix, Mars, PA). A combination of a 450- to 495-nm bandpass exciter filter and a 520- to 560-nm bandpass barrier filter was employed. Individual embryos were examined by focusing down from one side of the embryo to the other, with several images being acquired at different focal planes. We subsequently used a Nikon (Japan) E800 epifluorescence microscope equipped with a 450- to 490-nm bandpass excitation and a 520-nm longpass emission filter block set. A series of optical sections were made by focusing down through the embryos at ~2- to 7-μm intervals. Images were captured with a Nikon DXM 1200 high-resolution color digital camera.

Epifluorescent microscopy was used to capture data on embryos at the early stages of development. After the embryos reached the four- or eight-cell stage, detection of the sperm mitochondria became more difficult. For these and later cell stages (trochophore and D stage) that acquire a background autofluorescence, and for capturing images of the sperm alone, confocal microscopy was used. However, the increased time associated with collecting images with this method prohibited its use for extracting data for analytical purposes.

For confocal microscopy, MitoFM-stained sperm and trochophore larvae were excited with 488-nm laser light, and the emission was collected with a 515- to 540-nm bandpass filter. Older D stage larvae were excited with 558-nm laser light and imaged through a 575- to 640-nm bandpass filter. This was done to distinguish the MitoFM signal from the autofluorescence background. The two images of the same larva were overlain such that orange coloration showed the autofluorescence and green coloration denoted stained sperm mitochondria within the embryo. Images of double-labeled sperm alone were obtained following the same procedure as with D stage larvae.

The number of fluorescent mitochondria and their distribution among the cells were recorded for individual embryos at the two-, four-, or eight-cell stages. Observations of later developmental stages, through to the trochophore stage, were also made from each family with varying degrees of success.

In many embryos the observed number of sperm mitochondria was less than five. This can be attributed to several factors, such as the actual number of mitochondria in the parental sperm, the position of the mitochondrion in the cell interfering with visualization, the density of staining, and organelle death. All microscopic observations were made without prior knowledge of the family codes to avoid bias in the results.

Statistical analyses: Chi-square tests were used to evaluate the nonrandomness of (1) the distribution of sperm mitochondria among the cells of the embryo within families at the two- and four-cell stages according to the number of sperm mitochondria observed, (2) the occurrence of aggregated and dispersed sperm mitochondrial distribution patterns among embryos within families irrespective of the number of sperm mitochondria observed, (3) the specific location of sperm mitochondria at the two-cell stage (small AB cell or large CD cell) within the distribution pattern (aggregated, replicate equals embryo; dispersed, replicate equals sperm mitochondrion) within families, (4) the occurrence of aggregated and dispersed sperm mitochondrial distribution patterns among families sired by the same male and among families producing only daughters and those producing mostly sons, and (5) the specific location of sperm mitochondria at the two-cell stage (AB or CD cell) among families sired by the same male and among families producing only daughters and those producing mostly sons.

For the first of these tests, the number of possible scenarios of the distribution of sperm mitochondria within families varied according to the number of mitochondria observed and the number of cells. Embryos with one observed mitochondrion were excluded from the data, leaving observations of two, three, four, or five sperm mitochondria per embryo. In a two-cell embryo in which three sperm mitochondria were observed there are two possible scenarios: 3:0 (all three mitochondria in one cell) and 2:1 (two mitochondria in one cell and one mitochondrion in the other cell). Symmetrical scenarios (*e.g.*, 3:0 and 0:3) were pooled together. In most of these tests the expected numbers were small and so a Monte Carlo approach to estimating the probabilities was followed (*cf.* Manly 1997). The set of the first 10,000 integers was divided into subsets of sizes proportional to the probabilities of the scenarios of the tested distribution. (In the "two cell, three mitochondria" case 2500 randomly chosen numbers specified the set of the 3:0 scenario and the remaining 7500 specified the set of the 2:1 scenario.) A random-number generating function was employed to draw from the entire set of 10,000 as many numbers as the embryos examined. The numbers drawn from each subset were compared to the expected proportions to generate a chi-square value. This process was repeated 1000 times. The chi-square values generated in this way were compared to those from the observed numbers. The proportion of the 1000 chi-square values that were equal to or larger than the ones produced from the real observation specified the probability of the null hypothesis. A significance level of $\alpha = 0.05$ was used throughout; however, by using this level of α we accept a number of type I errors, with three falsely significant results expected across the 62 tests performed.

At the two-cell stage developmentally defined cells could be distinguished, and we tested whether one or the other of these cells received more or fewer sperm mitochondria than expected by chance alone, by assuming that a mitochondrion had an equal chance to land in one or the other cell.

Because the aggregation of all mitochondria in one cell is the main feature that differentiates the aggregated from the dispersed pattern, we tested whether embryos with all mitochondria in one cell are more than expected by chance. For this we produced the composite "aggregated" class by summing all embryos where the two, three, four, or five sperm mitochondria were observed in one cell. All other scenarios were lumped together to form the complementary "dispersed" class.

RESULTS

Sperm of *M. edulis* stained with MitoFM showed a ring of five large mitochondria in the midpiece (Figure 1). This is consistent with previous transmission electron microscopy studies (Longo and Dornfeld 1967). The enlarged size facilitated the tracing of mitochondria in the fertilized ovum.

In all families, sperm penetrated the egg at various points in relation to the location of the first meiotic spindle, as observed by Longo and Anderson (1969). Following fertilization, the five sperm mitochondria separated from each other and dispersed into the egg cytoplasm (Figure 2). At this time the zygote extruded the first and second polar bodies. Subsequently, the zygote formed a protrusion, referred to as the polar lobe, with decreased cytoplasmic density. The position of the polar

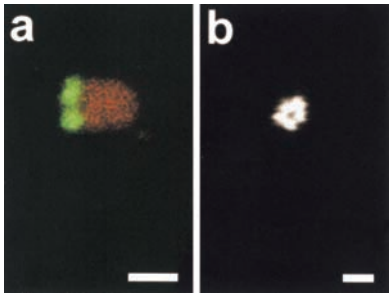

FIGURE 1.—Sperm of *M. edulis*. (a) MitoTracker Green FM-labeled mitochondria (green) and propidium iodide labeled nucleus (red), lateral view. (b) MitoTracker Green FM-labeled mitochondria, showing five mitochondrial bundles, posterior view. Confocal microscope was used. Bars, 2 μm.

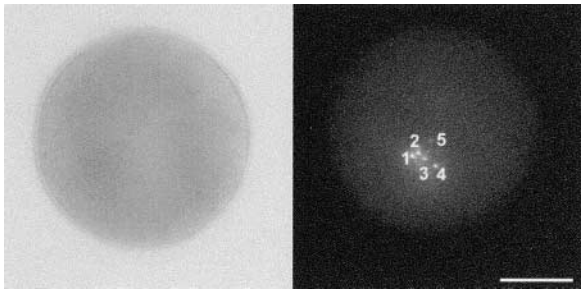

FIGURE 2.—Zygote of *M. edulis*. Bright field was followed by fluorescent field. Sperm mitochondria were labeled with MitoTracker Green FM. Numbers indicate individual sperm mitochondria. Epifluorescent microscope was used. Bar, 20 μm.

lobe identified the vegetal pole of the embryo. Concomitantly, a vertical "cleavage furrow" developed, dividing the embryo into two blastomeres. The polar lobe connected to one of the blastomeres and flowed back into it when the first cleavage was completed. As a result, a two-cell embryo with cells of unequal size was formed.

Following the system of nomenclature developed by CONKLIN (1897), the larger cell was designated CD, and the other, AB. At the second cleavage, the CD blastomere formed a polar lobe again in the same fashion as in the first division. As a result, a 4-cell embryo was produced with three equally sized blastomeres, A, B, and C, and one larger D blastomere. At the third cleavage, the D blastomere gave rise to the 1d-micromere and the 1D-macromere. At the fourth cleavage, the 1D divided into the 2d-micromere and the 2D-macromere. Similarly, 3d-3D and 4d-4D were obtained at the fifth and the sixth cleavages as the embryo developed into 64 cells. It is generally assumed that the germ cells and the gonad originated from 4d (VERDONK and VAN DEN BIGGELAAR 1983).

The sperm mitochondria retained the vital stain through to the late D stage, indicating that the organelles were still alive. During this phase of larval development, replication or fusion of the sperm mitochondria was never observed. Rare observations of more than five mitochondria were made but these were present at fertilization, of equal large size, and attributed to polyspermy, which is common in bivalves.

The number of embryos examined and the total number of sperm mitochondria observed in each family at the two-cell and four-cell stages are given in Tables 1 and 2, respectively. At the two-cell stage the number of embryos in each family varied from 31 to 44 and the average number of sperm mitochondria per embryo varied from 2.97 to 4, with a mean across families of 3.27 (SD = 0.36). For the four-cell stage the range was 3–3.92 (mean across families 3.47, SD = 0.46), and for the eight-cell stage, 3.08–3.9 (mean across families 3.41, SD = 0.30; not shown in the tables). Thus, the observed number of sperm mitochondria per embryo did not vary among families (or among cell stages).

As early as polar lobe formation in the first division, sperm mitochondria appear to follow two distinct patterns of behavior, which we have named dispersed and aggregated:

The dispersed pattern: In some embryos the sperm mitochondria appeared to disperse randomly in the egg before cell division (Figure 3A) and segregate also at random into different cells at the various developmental stages (Figure 3, B–D). This pattern was observed as late as the early trochophore stage (Figure 3E), but could not be identified at the D stage. This may be because dispersion makes the detection of the mitochondrial dye at late developmental stages more difficult.

The aggregated pattern: In other embryos, all five sperm mitochondria moved into the polar lobe while it was developing. It appeared that these mitochondria were pushed back later by the cytoplasm of the polar lobe as it fused with the CD blastomere, along either the cleavage furrow that divided the AB and the CD blastomeres (Figure 4A) or the margin of the CD cell (Figure 4B). This process appeared to occur very quickly and was observed repeatedly. Consequently, all of the sperm mitochondria were sequestered in the CD blastomere at the two-cell stage (Figure 4, C and D). The five sperm mitochondria were also found to aggregate in D and 1D cells at the four-cell (Figure 4E) and the eight-cell (Figure 4F) stages, respectively. When all sperm mitochondria aggregated in one cell, they were usually located close to the cell membrane. As with the dispersed pattern, the aggregated pattern was observed to persist as late as the trochophore stage (Figure 4G) and even in larvae of the D stage (Figure 4H), almost 72 hr postfertilization.

No effect of sperm mitochondria labeling on sex ratio: All of the progeny of a female known from a previous mating to produce only daughters (X102E) were fe-

TABLE 1

Distribution of sperm mitochondria in two-cell embryos of *M. edulis*

No. of mitochondria	Distribution pattern	*P* of distribution pattern	Family 1: X102B98a, wm2 (30, 0)		Family 2: X102E, wm23 (198, 0)		Family 3: X102H, wm24 (116, 0)		Family 4: WF20, wm26 (30, 0)		Family 5: 98A, wm24 (30, 142)		Family 6: WF21, wm26 (6, 15)		Family 7: 98B, wm26 (9, 39)	
			No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*
2	2:0	0.5	2(2.5)		5(4.5)		6(6.5)		4(4)		9(5.5)		10(6)		10(7)	
	1:1	0.5	3(2.5)	0.377	4(4.5)	0.494	7(6.5)	0.601	4(4)	0.719	2(5.5)	0.014	2(6)	0.008	4(7)	0.047
3	3:0	0.25	0(0.75)		6(3.75)		3(2.5)		4(2.75)		10(2.5)		9(2.25)		6(2.25)	
	2:1	0.75	3(2.25)	0.156	9(11.25)	0.142	7(7.5)	0.491	7(8.25)	0.313	0(7.5)	0.001	0(6.75)	0.001	3(6.75)	0.003
4	4:0	0.125	4(1.5)		2(1.75)		4(2)		5(1.38)		3(1)		1(0.875)		4(0.875)	
	3:1	0.500	6(6)		7(7)		6(8)		2(5.5)		2(4)		4(3.5)		0(3.5)	
	2:2	0.375	2(4.5)	0.032	5(5.25)	0.951	6(6)	0.277	4(4.13)	0.001	3(3)	0.052	2(2.625)	0.776	3(2.625)	0.001
5	5:0	0.0625	2(0.8125)		2(0.375)		0(0.05)		1(0.25)		2(0.313)		2(0.188)		2(0.1875)	
	4:1	0.3125	6(4.0625)		2(1.875)		0(0.625)		1(1.25)		2(1.563)		0(0.938)		1(0.9375)	
	3:2	0.6250	5(8.125)	0.107	2(3.75)	0.020	2(1.25)	0.220	2(2.5)	0.240	1(3.125)	0.006	1(1.875)	0.003	0(1.875)	0.001

For any number of sperm mitochondria (two to five) the possible distribution patterns and their probabilities to occur by chance are given. For each family, the code for each parent (female, male), the number of daughters and sons (in parentheses), the observed number of embryos by total number of mitochondria observed by distribution pattern (expected number in parentheses), and the probability that the observed numbers are not different from the expected determined from Monte Carlo simulations are listed.

TABLE 2

Distribution of sperm mitochondria in four-cell embryos of *M. edulis*

No. of mitochondria	Distribution pattern	*P* of distribution pattern	Family 2		Family 3		Family 5	
			No. of embryos	*P*	No. of embryos	*P*	No. of embryos	*P*
2	2:0	0.25	1(1)		2(1.25)		2(0.75)	
	1:1	0.75	3(3)	0.588	3(3.75)	0.318	1(2.25)	0.012
3	3:0	0.0625	1(0.25)		1(0.5)		3(0.3125)	
	2:1	0.5625	0(2.25)		4(4.5)		2(2.8125)	
	1:1:1	0.3750	3(1.5)	0.039	3(3)	0.744	0(1.875)	0.001
4	4:0	0.015625	1(0.109)		0(0.047)		2(0.109)	
	3:1	0.187500	2(1.3125)		1(0.5625)		2(1.3125)	
	2:2	0.140625	1(0.984)		1(0.422)		0(0.98)	
	2:1:1	0.562500	1(3.9374)		1(1.6875)		3(3.937)	
	1:1:1:1	0.093750	2(0.6562)	0.028	0(0.281)	0.601	0(0.656)	0.005
5	5:0	0.003906	0(0.0117)		0(0.0039)		2(0.035)	
	4:1	0.058594	0(0.1758)		0(0.0586)		3(0.527)	
	3:2	0.117188	0(0.3516)		0(0.117)		1(1.055)	
	3:1:1	0.234375	1(0.7031)		1(0.234)		2(3.164)	
	2:2:1	0.234375	1(1.055)		0(0.362)		0(2.109)	
	2:1:1:1	0.351563	1(0.7031)	0.879	0(0.234)	0.173	1(2.109)	0.001

See Table 1 for column explanations.

male, and all of her progeny arising from unstained ($N = 28$) and stained sperm ($N = 79$) were female. Progeny of a female known to produce mostly sons (99wF1) produced 65% male offspring when eggs were fertilized by unstained sperm ($N = 29$) and 61% male offspring when eggs were fertilized with stained sperm ($N = 28$). These results provide evidence for our assumption that labeling the mitochondria with the fluorescent tag did not bias the sex ratio ($\chi^2 = 0.1412$, $P = 0.707$).

The distribution of the two patterns in families and correspondence between mitochondrial distribution pattern and gender: The distribution of sperm mitochondria was studied in two- and four-cell embryos from the seven families. Table 1 lists all possible distribution patterns in two-cell embryos for each observed number of mitochondria (two to five). Families 1, 2, 3, and 4 produced only daughters and families 5, 6, and 7 produced mostly sons (Table 1). Table 2 presents the equivalent information for four-cell embryos from families 2, 3, and 5. No observations beyond the two-cell stage were made in families 1 and 4, and for families 6 and 7 the numbers of examined four-cell embryos were small and are not given in the table. There were marked differences between the families. In families that produce no sons, the observed distribution of mitochondria among the cells rarely deviated significantly from expectation. There were three exceptions in the 16 comparisons in Table 1 and two in 8 in Table 2. The distribution of sperm mitochondria among the cells within these families is random. In contrast, the observed distribution of sperm mitochondria in the families producing mostly sons deviated strongly from expectations in 10 of 12 comparisons in Table 1 and in all 4 comparisons in Table 2.

In two-cell embryos we could consistently record if an observed sperm mitochondrion was in the AB or the CD cell. However, unambiguous cell identification was not always possible at the four-cell stage. Chi-square tests of the frequency of occurrence of the aggregated and dispersed patterns within families were significant in two of the four daughter-producing families (families 2 and 4) and for all families producing predominantly sons (Table 3A). In the four daughter-producing families, an aggregated pattern is equally likely to be found in the AB (small) or CD (large) cell (Table 3B). Among embryos with an aggregated distribution of sperm mitochondria, from families producing mostly sons, the aggregation was more commonly found in the CD cell (Table 3B). For the dispersed pattern, in all families, there is no tendency for the sperm mitochondria to be delivered preferentially to the CD cell, and location of the sperm aggregation is random (Table 3B).

Noninvolvement of the male parent: Of the four males used in our families (Table 1), one (wm24) was used to produce families 3 and 5 and another (wm26) to produce families 4, 6, and 7. Families with the same male parent but different female parents were heteroge-

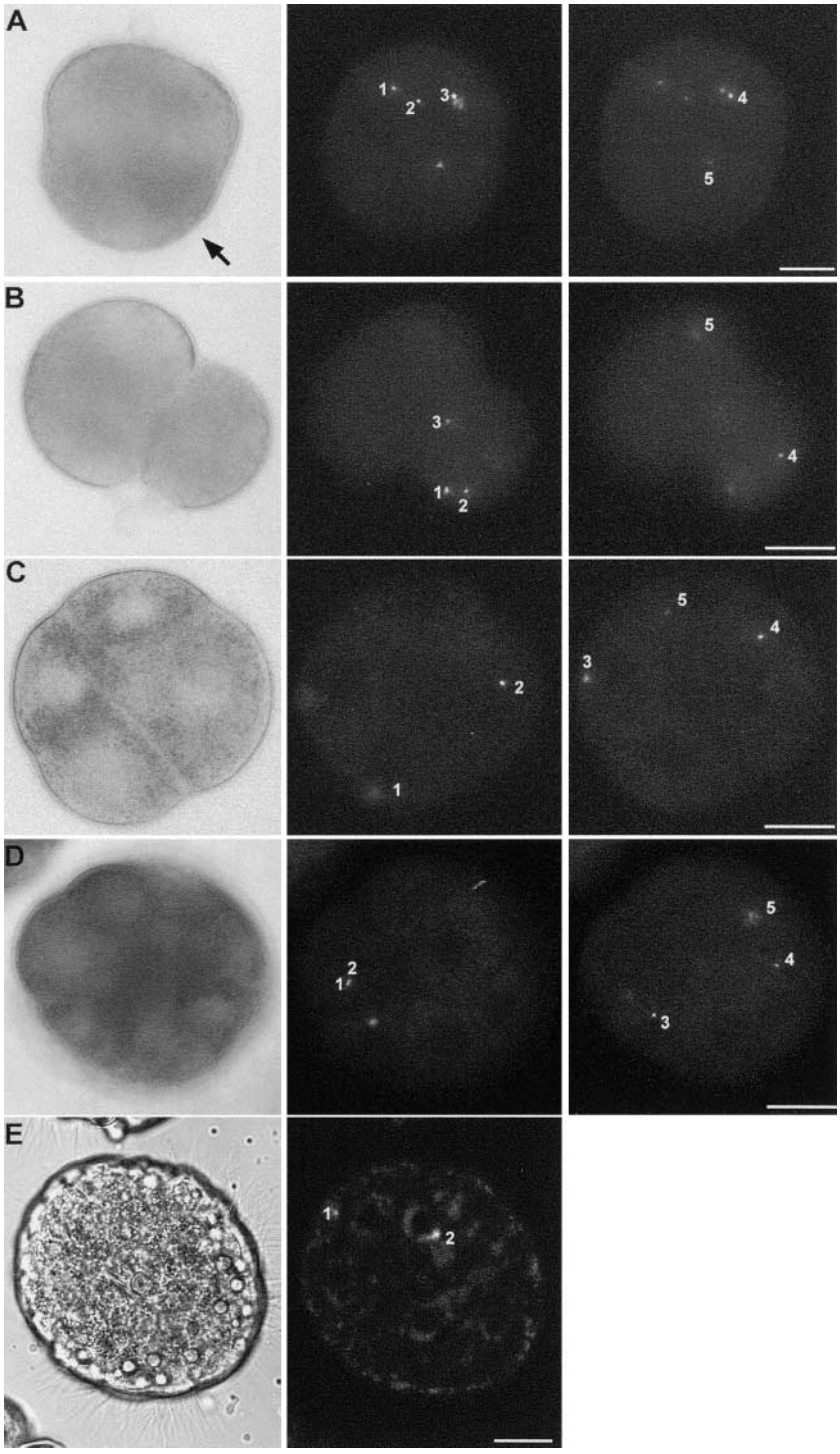

FIGURE 3.—Dispersed pattern of sperm mitochondria at various cell stages in embryos of *M. edulis*. Bright field was followed by fluorescent field for each embryo. Fluorescent images were taken at different focal planes. Numbers indicate individual sperm mitochondria stained by MitoTracker Green FM. (A) One-cell zygote with polar lobe (arrow). (B) Two-cell embryo. (C) Four-cell embryo. (D) Eight-cell embryo. (E) Trochophore larva. A–D, epifluorescent microscope was used; E, confocal microscope was used. Bars, 20 μm.

neous for two characters, the frequency of the aggregated pattern in two-cell embryos and the distribution of mitochondria between AB and CD cells (Table 4). In contrast, the grouping of the seven families according to whether the female parent produces only daughters or mostly sons, regardless of the male parent, results in two homogeneous classes for both characters of sperm mitochondrial distribution. This test provides further evidence that the fate of sperm mitochondria in the fertilized egg is under the control of the female parent.

DISCUSSION

Our observations of the distribution of sperm mitochondria in the early developmental stages of mussel embryos allow several inferences to be made about the

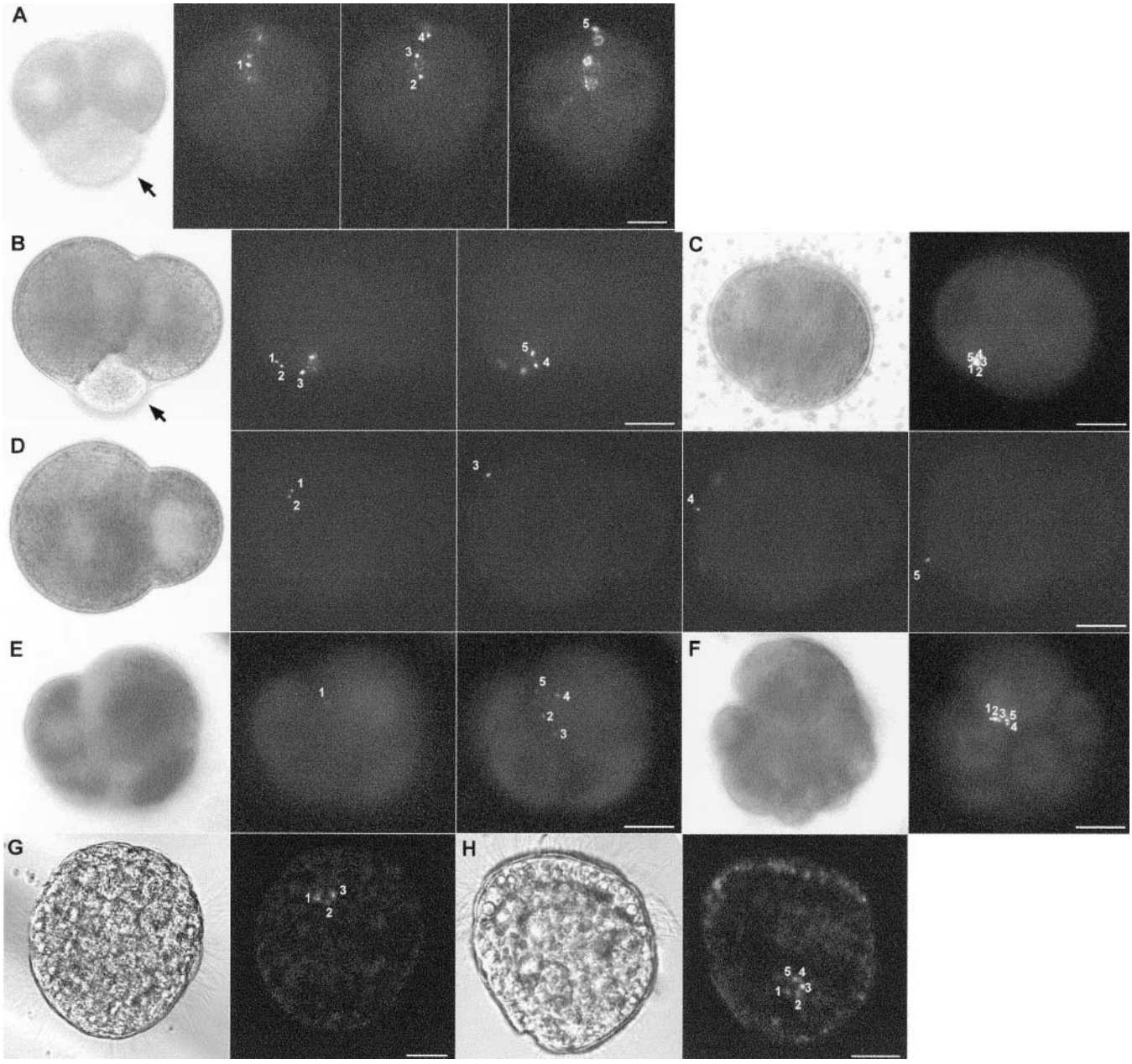

FIGURE 4.—Aggregate pattern of sperm mitochondria at various cell stages in embryos of *M. edulis*. Bright field was followed by fluorescent field for each embryo. Fluorescent images were taken at different focal planes. Numbers indicate individual sperm mitochondria stained by MitoTracker Green FM. (A) Late one-cell zygote with polar lobe (arrow). Sperm mitochondria are along the cleavage furrow. (B) Late one-cell embryo with polar lobe (arrow). Sperm mitochondria are along the line between the polar lobe and the large cell. (C and D) Two-cell embryo. (E) Four-cell embryo. (F) Eight-cell embryo. (G) Trochophore larva. (H) D-stage larva. A–F, epifluorescent microscope was used; G and H, confocal microscope was used. Bars, 20 μm.

fate of these mitochondria and, by extension, about the fate of paternal mtDNA. The first conclusion is that sperm mitochondria destruction in *M. edulis* does not occur during the early developmental stages. A mechanism of this kind involving ubiquitin is known to exist in mice and bovines, where it appears to operate almost immediately after the entrance of the sperm into the ovum (KANEDA *et al.* 1995; SUTOVSKY *et al.* 1996, 1999). In all of our families we could see the persistence of sperm mitochondria to the stage at which the embryo may consist of 128 cells. After this, our data were insufficient to make claims on the fate of sperm mitochondria; however, at least in some embryos, they were observed to persist through to the D stage.

To explain the abundance of paternal mtDNA in the male gonad, early publications on the phenomenon of DUI (SKIBINSKI *et al.* 1994b; ZOUROS *et al.* 1994b) speculated that the paternal mtDNA had an early repli-

TABLE 3

Distribution of the aggregated and dispersed sperm mitochondrial patterns from two- and four-cell embryos within families of *M. edulis* (A) and cell localization of sperm mitochondria in two-cell embryos within pattern within family (B)

	Family						
Distribution pattern	1	2	3	4	5	6	7
A.							
Aggregated	8(5.562)	18(11.746)	16(12.851)	14(8.375)	33(10.520)	22(9.313)	22(10.313)
Dispersed	25(27.438)	44(50.254)	42(45.149)	20(25.625)	25(47.480)	9(21.687)	11(22.687)
Probability:	0.257	0.043	0.319	0.025	0	0	0
B.							
	No. of embryos with sperm mitochondria from aggregated pattern						
In AB cell	2	6	4	6	3	5	1
In CD cell	6	9	9	8	21	17	21
Probability:	0.157	0.439	0.166	0.539	0.000	0.011	0.000
	No. of mitochondria from dispersed pattern						
In AB	47	50	49	30	19	12	15
In CD	55	53	44	38	20	21	19
Probability:	0.428	0.768	0.604	0.332	0.872	0.117	0.493

cation advantage over the maternal mtDNA in male-destined embryos. This would increase the amount of paternal mtDNA relative to maternal mtDNA in the mtDNA pool of the early zygote and, thus, increase the chance that it would be included in the mtDNA pool of the first primordial cells. Our observations render this hypothesis unlikely on two grounds. First, an early replication of sperm mitochondria in male- but not female-destined embryos would not be compatible with our observation that there is no variation among female- and male-biased families in the number of sperm mitochondria that can be seen at early stages. Second, our observation that sperm mitochondria form an aggregate suggests another way through which these mitochondria may find their way to the male primordial cells, other than through mere chance boosted by early replication.

Our observations suggest a mechanism that would account for the difference between the genders in the mitochondrial type of the gonad and provide a description of the very first steps of that mechanism. Our hypothesis is that in early-stage female embryos, sperm mitochondria behave like egg mitochondria. They are assorted at random among the early cells and start replicating at the same time and rate as maternal mitochondria, sometime after the D stage of larval development. In sharp contrast, sperm mitochondria follow a very regimented path in male embryos. They aggregate together just before the first cell division and are sequestered in the large CD cell. This aggregation into the same cell appears to continue for several successive cell divisions. We could not identify, in terms of developmental destiny, the chain of cells through which the aggregate passes, nor do we have data as to when and where the aggregate is dissolved. The fact that germ cells descend from the CD rather than from the AB cell is compatible with the hypothesis that the aggregate follows a path that leads to primordial germ cells. However, our observations are not of a nature to provide direct confirmation of this hypothesis.

The existence of a mechanism through which sperm mitochondria are delivered to the first germ cells would explain why the paternal genome is found almost exclusively in the male gonad, but cannot explain why the gonad contains only paternal mtDNA. One way this could happen is if sperm mitochondria are the only mitochondria that enter the first germ cells. If, however, both egg and sperm mitochondria enter the germ cells, then we would require either that the former are actively eliminated from the germ line or that during gonad development the paternal mtDNA multiplies at a much faster rate than maternal mtDNA. There is indirect evidence in support of the latter hypothesis. One well-established feature of DUI is that occasionally a maternally transmitted mitochondrial genome may invade the paternal transmission route. The phenomenon, known as "masculinization" (Saavedra *et al.* 1997) of the mitochondrial genome, amounts to producing new paternal genomes out of the pool of maternal genomes. It has been observed in laboratory crosses of mussels (Zouros *et al.* 1994a,b; Saavedra *et al.* 1997) and in natural populations (Fisher and Skibinski 1990; Rawson *et al.* 1996; Quesada *et al.* 1999; Ladoukakis *et al.* 2002). The occasional replacement of paternal by maternal mtDNA suggests that maternal mitochondria can also enter the first germ cells.

Whereas the stochastic behavior of sperm mitochon-

TABLE 4

Probability of homogeneity of families grouped according to common sire (A) and sex bias of progeny (B) for two characters: frequency of sperm mitochondrial pattern (aggregated *vs.* dispersed) and number of mitochondria in the specific cells of two-cell embryos (AB *vs.* CD)

	Aggregated	Dispersed	$\chi^2(P)$	AB	CD	$\chi^2(P)$
A.						
wm24						
F3	13	28	11.241	61	69	12.507
F5	24	10	(0.001)	27	82	(0.001)
wm26						
F4	14	20		48	65	
F6	22	9	7.111	26	68	13.715
F7	22	11	(0.029)	19	79	(0.001)
B.						
Female bias						
F1	8	25		56	76	
F2	15	29		69	80	
F3	13	28	2.23	61	69	0.918
F4	14	20	(0.526)	48	65	(0.821)
Male bias						
F5	24	10		27	82	
F6	22	9	0.175	26	68	1.872
F7	22	11	(0.916)	19	79	(0.392)

dria in female embryos requires no further explanation, the aggregation of these mitochondria and the delivery of this aggregate to specific cells in male embryos require the existence of a specific mechanism. At present we can offer no explanation for how this mechanism might operate. Our study is not, however, the first to report a nonrandom distribution of mitochondria after cell division. In yeast (*Saccharomyces cerevisiae*), mitochondria are transmitted from the mother cell to the bud daughter cell through a directed linear movement facilitated by actin (Simon *et al.* 1997). A similar process may be involved in the movement of paternal mitochondria within male Mytilus embryos.

The existence of a mechanism that operates in male mussel embryos, but not in female embryos, is the central piece of a model that was put forward to explain the two main observations of DUI in mussels: the presence of paternal mtDNA in the male but not female gonads and the strong female-dependent sex bias. The model (Saavedra *et al.* 1997; Kenchington *et al.* 2002) predicts the presence of a factor that is expressed in females, perhaps only during oogenesis. It is encoded by a nuclear locus that segregates for two alleles, the active allele Z and the inactive allele z. Females of genotype zz produce eggs that lack the factor Z. In these eggs the sperm mitochondria would have the default fate, which according to our observations is a random distribution in the developing embryo. Thus zz females produce almost exclusively daughters. Females of genotype ZZ supply their eggs with factor Z. This factor initiates a battery of events, the end result of which is the delivery of sperm mitochondria into germ cells. The ZZ females produce mostly sons. The fact that we have rarely seen females that produce exclusively sons suggests that there is always a small probability that eggs with the Z factor will fall back onto the stochastic mode of sperm mitochondria segregation. Females with the Zz genotype produce eggs whose supply with factor Z falls sometimes above and sometimes below the threshold required for the directed mode of sperm mitochondrial segregation. These are the females that produce intermediate sex ratios. The model implies that the development of a mussel egg into a female or male individual is determined at the moment that the mother produces it.

This study takes this model one important step forward by indicating that the mechanism through which the mitochondrial factor is delivered to the germ cells operates through the aggregation of sperm mitochondria. Further evidence for the model comes from the presence of paternal mtDNA in somatic tissues. As stated, most females (~70%) contain no paternal mtDNA, but when they do it is found in low amounts and in specific tissues that vary from individual to individual (Garrido-Ramos *et al.* 1998). This is expected from the stochastic distribution during development of a tiny minority of mitochondria, including their accidental loss. Garrido-Ramos *et al.* (1998) reported that in males, paternal mtDNA is often found in the adductor muscle but less so in the mantle and in the foot; however, their results may have been influenced by contamination. The adductor muscle originates from the mesoderm, as do the

germ cells, whereas the mantle and the foot originate from the ectoderm (Van Den Biggelaar *et al.* 1994). Thus the Garrido-Ramos *et al.* (1998) findings are what would be expected if a sperm mitochondrion breaks away from the aggregate and is trapped in an adjacent cell.

In conclusion, we have identified two distinct gender-associated patterns of distribution of sperm mitochondria in developing embryos of the mussel *M. edulis*. These results are significant because they provide the first demonstration of a sex-specific behavior of sperm mitochondria and because they open an important path toward the understanding of the cellular and molecular mechanisms that underlie the phenomenon of doubly uniparental inheritance of mtDNA.

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