Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 7463–7467, August 1994
Genetics

An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*

ELEFTHERIOS ZOUROS*†‡, AMY OBERHAUSER BALL*, CARLOS SAAVEDRA*§, AND KENNETH R. FREEMAN¶

*Department of Biology and Marine Gene Probe Laboratory, Dalhousie University, Halifax, NS Canada, B3H 4J1; †Department of Biology, University of Crete and Institute of Marine Biology of Crete, Iraklion, Crete, Greece; and ¶Halifax Fisheries Research Laboratory, Department of Fisheries and Oceans, P.O. Box 550, Halifax, NS Canada B3J 2S7

Communicated by Bruce Wallace, April 21, 1994

ABSTRACT **In animals, mitochondrial DNA (mtDNA) inheritance is predominantly maternal. In a few cases incidental transmission of paternal mtDNA was observed and estimated to account for only 10^{-4}–10^{-3} of an individual's mtDNA content. In contrast, biparental inheritance is common in mussels of the genus *Mytilus*. Here we present direct evidence that sex and mtDNA inheritance are coupled in *Mytilus*. Females inherit mtDNA only from their mother, but they transmit it to both daughters and sons. Males inherit mtDNA from both parents, but they transmit to sons only the mtDNA they inherited from their father. In pair matings, this mtDNA inheritance pattern is associated with a strong sex-ratio bias. These findings establish a newly discovered type of cytoplasmic DNA transmission. We also present evidence that the phenomenon breaks down in interspecific hybrids.**

In animals mitochondrial DNA (mtDNA) inheritance is predominantly maternal (1). In a number of organisms, repeated back-cross experiments aimed at enriching hybrid lines with paternal mtDNA have produced results consistent with the view that mtDNA was either exclusively inherited from the mother or that the father's contribution was much lower than the experiment could detect (2–4). The eventual success of this protocol in *Drosophila* (5) and mice (6) has now established the view that incidental transmission (leakage) of paternal mtDNA occurs in animals at a very low rate amounting to 10^{-4} (6) to 10^{-3} (5) of an individual's mtDNA. Additional evidence for paternal mtDNA transmission has been obtained from observations of heteroplasmy for highly diverged mtDNA molecules in *Drosophila* (7), mussels of the genus *Mytilus* (8), and the fish *Engraulis encrasicolus* (9). In honey bees, a high dose of drone mtDNA was observed in early diploid zygotes (10), but this DNA is degraded during development and is not transmitted to the next generation.

Three observations in *Mytilus* suggested that it may have an exceptional mode of mtDNA inheritance: heteroplasmy is very common in natural populations (8, 11), it is more common among males (11), and the degree of divergence between mtDNA molecules for which an animal is heteroplasmic can be as high as 20% (8). To investigate these phenomena Zouros *et al.* (12) examined offspring from pair matings of *Mytilus edulis* and *Mytilus trossulus* whose parents had different mtDNA restriction profiles. The study provided a direct observation of paternal mtDNA transmission over the span of one generation and produced a minimum estimate of paternal contribution of 1%. The phenomenon was observed in both homospecific and heterospecific crosses, in disagreement with the suggestion that paternal inheritance may be an anomaly associated with hybridization (6). These findings confirmed the claim (8) that the high incidence of mtDNA heteroplasmy is due to biparental inheritance but did not explain why heteroplasmy is more common in males or why highly diverged mtDNA molecules segregate in mussel populations. An additional observation from our first study remained unexplained: the number of progeny carrying mtDNA from both parents varied among crosses from 0 to 100%.

This study answers these questions. In mussels, the sex of an individual can be determined only if it carries a developed gonad, which was not the case in the 1-year-old progeny scored, in our first study (12). The present study was done in 3-year-old siblings, most of which carried mature gonads. The association of sex with the presence or absence of paternal mtDNA showed that in mussels mtDNA transmission follows a pattern not known to occur in any other organism for either mitochondrial or plastid DNA.

MATERIALS AND METHODS

Crosses. The crosses and the rearing of progeny have been described (12). Offspring not scored at the age of 1 year were returned to the sea for further growth. In 9 of the 16 crosses none or very few progeny survived to the age of 3 years. Details for the 7 crosses examined in this study are given in Table 1. Sex was determined by removing part of the gonad and examining it under a microscope for the detection of sperm or eggs.

Detection of mtDNA. DNA extraction and detection of maternal-specific and paternal-specific restriction mtDNA profiles in offspring by Southern analysis were described (12). To increase the sensitivity of paternal mtDNA detection, we have used a PCR assay in this study. An 860-bp fragment from the cytochrome oxidase III gene was amplified by using the primers 5′-TATGTACCAGGTCCAAGTCCGTG-3′ and 5′-ATGCTCTTCTTGAATATAAGCGTACC-3′, which correspond to nucleotide positions 460–482 and 1326–1301 of segment 5 of the sequence of *M. edulis* mtDNA type FB given by Hoffmann *et al.* (13). Female parents were homoplasmic for the FB or the C mtDNA type (12). In both these types, the amplified fragment contains an *Eco*RI site that is missing from the M type for which most *M. edulis* males are heteroplasmic. For detection of paternal mtDNA, all PCR products were digested with enzymes that differentiated the father's and the mother's mtDNA types. For crosses in which the father's mtDNA contained no *Eco*RI site in the target region (crosses 3, 7, 15, and 16), the offspring's DNA was digested with *Eco*RI before amplification to allow for digestion of the maternal mtDNA and preferential amplification of paternal mtDNA. From experiments in which a constant amount of maternal DNA was mixed with decreasing amounts of paternal DNA, the detection limit of paternal

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‡To whom reprint requests should be addressed at: Department of Biology, Dalhousie University, Halifax, NS, Canada B3H 4J1.
§Present address: Departamento de Biología Fundamental, Universidad de Santiago de Compostela, Santiago de Compostela (La Coruña), Spain.

Table 1. Paternal mtDNA inheritance and sex ratio among 3-year-old offspring in pair matings of *Mytilus*

Cross	Parents	Offspring: Not sexed*	Females† +	Females† −	Males† +	Males† −	Males, %
3	EF4(FB),EM4(M/FB)	29	0	20	0	0	0
6	EF5(FB),EM5(D/FB)	1	0	9	16	0	64
7	EF7(C),EM7(M/FB)	4	0	8	32	0	80
16	EF16(FB),EM15(M/FA)	0	0	8	25	0	76
	Total	34	0	45	73	0	
2	EF3(FB),TM3(S/N)	3	0	14	0	13	48
11	EF12(FB),TM12(D/FB)	2	1	27	1	2	10
15	EF16(FB),TM15(T/O)	15	0	5	25	3	85
	Total	20	1	46	26	18	

*Sexing was not possible in several offspring because of undeveloped gonads or previous spawning. Simultaneous presence of sperm and eggs was detected in two cases.
†+ and − indicate the presence and absence of paternal mtDNA; all offspring carried maternal mtDNA.

mtDNA of this assay was estimated at 10^{-4} (1 molecule for 10,000 molecules of maternal mtDNA). In crosses in which preferential amplification was not possible, the sensitivity of the PCR assay was estimated at 10^{-3}. Offspring in which no paternal mtDNA was detected were reanalyzed either by repeating the same assay or by blotting and hybridizing the PCR product with a probe made from the PCR product from the father's DNA. Southern blotting analysis, as described (12), was used in several crosses to confirm that offspring showing the presence of paternal mtDNA by the Southern blot assay also tested positive by the PCR assay. The PCR conditions were as follows. Approximately 0.2 μg of total nucleic acids was incubated for 30 cycles of 94°C for 1 min, 54°C for 10 sec, and 72°C for 1 min. The initial denaturation period was 2 min and the final extension period was 4 min. The amplifications were done in 10 mM Tris·HCl, pH 8.3/5 mM NH_4Cl/50 mM KCl/1 mM $MgCl_2$/0.2 mM each dNTP/0.25 μM each primer/1.25 units of *Taq* DNA polymerase in 50-μl reaction mixtures. Nonradioactive Southern blot hybridization analysis was performed as described (12) except that the probe consisted of the entire *M. edulis* FB molecule (13) and two *M. trossulus* clones (12), and the stringency of the final wash was reduced to 2× SSC/0.2% SDS for 15 min at 20°C.

Allozyme Analysis. A low level of contamination of crosses detected in the previous study (12) forced the scoring of parents and progeny for a set of allozyme loci to confirm true parenthood of offspring. A set of six loci were used either because they were diagnostic for the two species involved or because they were highly polymorphic (14, 15). These included phosphomannose isomerase, esterase D, octopine dehydrogenase, phosphoglucose isomerase, phosphoglucomutase, and peptidase II. Electrophoretic techniques used were those of McDonald and Koehn (16), except that phosphomannose isomerase was run in a Tris maleate buffer (pH 7.6) and octopine dehydrogenase was run in a Tris citrate buffer (pH 7.5). The combined probability that a contaminant will be compatible with the parental genotypes at all these six loci and with the parental mitotypes is $<10^{-4}$ (12). Among a total of 122 progeny scored from seven families, 11 contaminants were detected and removed from the analysis.

RESULTS

An example of sex-specific mtDNA inheritance is shown in Fig. 1. The results are summarized in Table 1. For consistency, cross numbers and notations are the same as in Zouros *et al.* (12). Parents are identified by E (*M. edulis*) or T (*M. trossulus*), by F (female) or M (male), and by a code number. Several mtDNA *Eco*RI restriction types segregate in natural

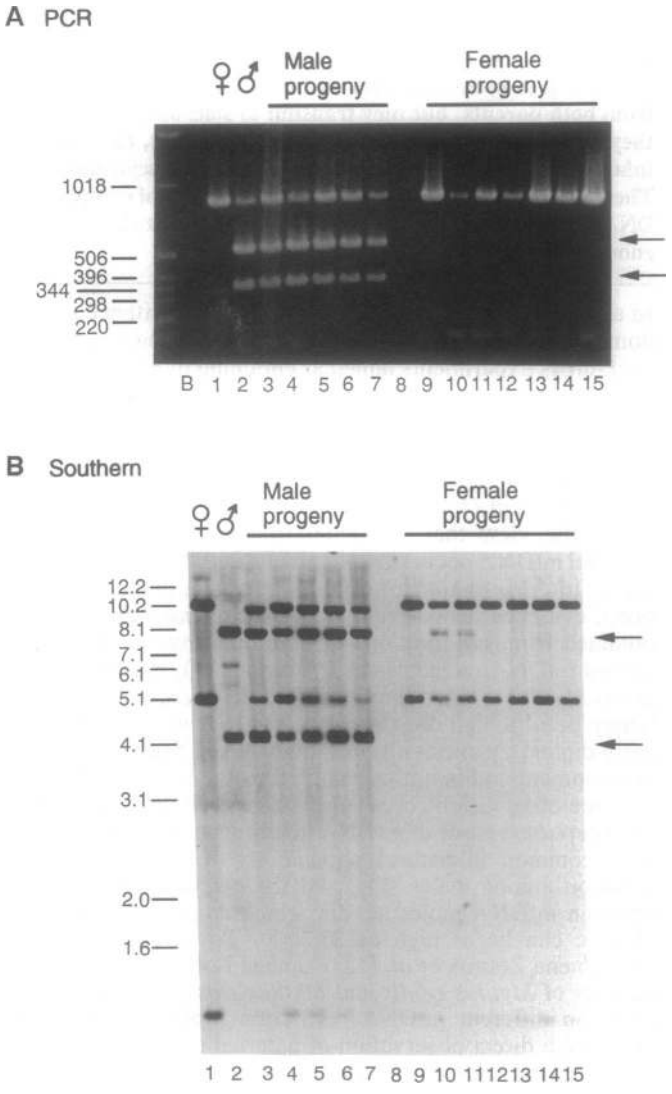

FIG. 1. Transmission of paternal mtDNA to male offspring only. The cross shown is cross 7. (*A*) PCR amplification products digested with *Bam*HI and stained with ethidium bromide. Arrows point to diagnostic paternal PCR products. Numbers in the margin give lengths in bp. Lanes: B and 8, PCR blank; 1 and 2, parents; 3–7, male offspring; 9–15, female offspring. (*B*) *Eco*RI restriction profiles of mtDNA of the parents and the same offspring as in *A*. Arrows point to diagnostic paternal bands. Faint bands migrating at 8 kb in two female offspring are products of partial digestion and do not correspond to 8-kb paternal band.

populations of *Mytilus* (8, 11, 12). Previous reports (11, 12) have noted that certain types occur almost exclusively in males and others are shared by both sexes. We found this to be true in a survey of a *M. edulis* and a *M. trossulus* population (unpublished data) that produced two distinct classes of mtDNA types: those occurring only in males (M types) and those occurring in both sexes (F types). The *Eco*RI profiles of the parents are shown in parentheses. Females were homoplasmic; males were heteroplasmic for a male and a female type (first and second profile, respectively).

Four of the seven crosses were homospecific (*M. edulis* × *M. edulis*). In three of these (crosses 6, 7, and 16) the sex ratio was biased in favor of males (in cross 6 the ratio is different from 1:1 only at the 0.16 probability level). The other cross (cross 3) contained only female offspring. All sons from these crosses contained both maternal and paternal mtDNA. In contrast, daughters contained only maternal mtDNA. For five daughters from cross 3 and for four from cross 7, the digestion of the PCR product produced faint bands corresponding to paternal mtDNA, but this observation could not be reproduced in subsequent tests. Thus, it is not clear whether these were false positives or examples of paternal mtDNA leakage.

Each of the three heterospecific crosses (*M. edulis* × *M. trossulus*) presented one or more anomalies to the pattern seen in homospecific crosses. Cross 15 was strongly male biased and no female offspring (among five) was positive for paternal mtDNA. Three of the male progeny were, however, negative for paternal mtDNA. Cross 11 was female biased. All daughters were negative for paternal DNA, except one that was unambiguously positive; also, two of the three sons did not carry paternal mtDNA (Fig. 2). Finally, cross 2 produced a pattern typical of other animal species: there was a 1:1 sex ratio and no offspring, male or female, carried paternal mtDNA.

In two of our crosses, 16 and 11, the three parental types (the mother's F type and the father's M and F types) could be readily distinguished in the offspring by Southern blot analysis. Thus, it was possible to examine in these crosses whether both types of the father's mtDNA were transmitted to sons. The same result was observed in both families: sons inherited the mother's F type and the father's M type, daughters inherited only the mother's F type, and no progeny inherited the father's F type (Fig. 2).

Cytological observations have suggested that in the blue mussel the sperm's mitochondria may enter the egg (17). The sperm's contribution to the mtDNA pool of the fertilized egg must, however, be very small compared to that of the egg (18) and should be lost in most individuals through stochastic assortment during development (19–21). The high frequency of paternal mtDNA in the sons requires that the paternal mtDNA has a replicative advantage over the maternal mtDNA. Moreover, the observation that the father transmits to sons only his paternal mtDNA requires either that the sperm contain only the male mtDNA type or that the sperm's female mtDNA type be somehow prevented from multiplying

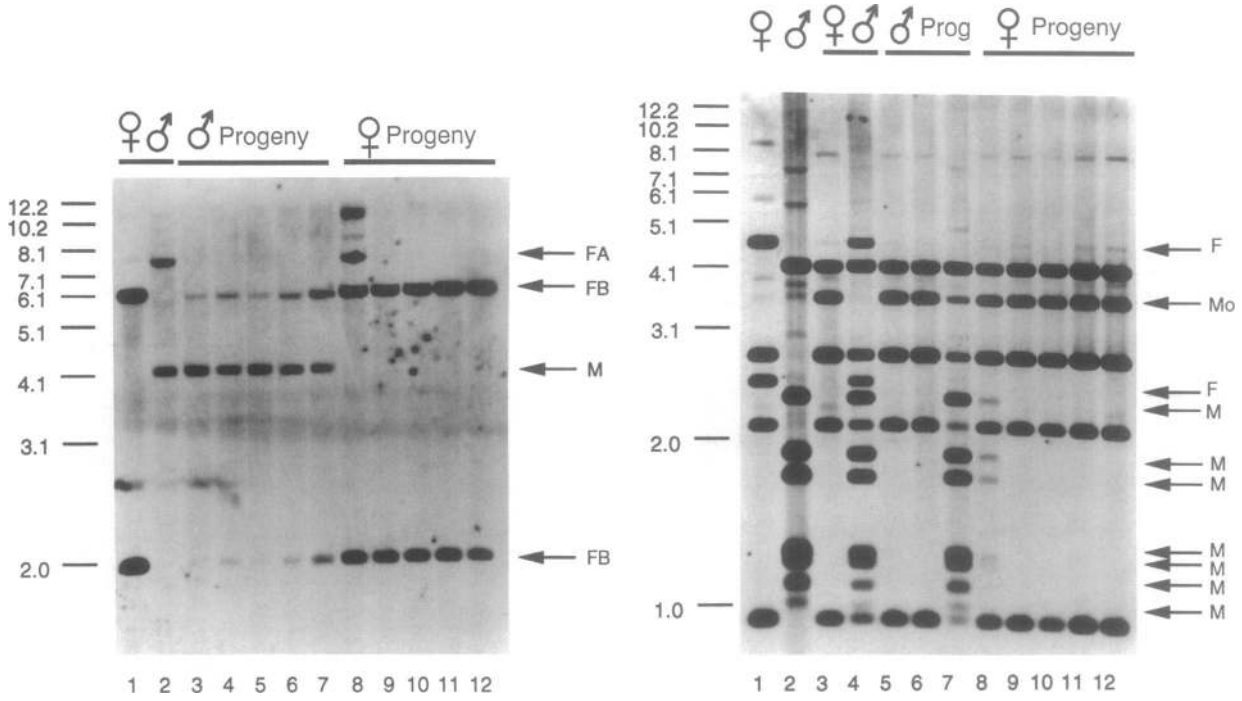

FIG. 2. MtDNA transmission in a homospecific and a heterospecific cross. (*Left*) Homospecific cross 16. DNA from the mother (lane 1), father (lane 2), five sons (lanes 3–7), and five daughters (lanes 8–12) digested with *Eco*RI and probed with a mixture of clones that hybridize to 6- and 2-kb bands of the FB profile, to the two 4-kb bands of the M profile, and to one of the two 8-kb bands of the FA profile (see refs. 11 and 12 for descriptions of these profiles). The mother had the FB profile and the father was heteroplasmic for the FA profile (a female type) and the M profile (a male type). All sons and daughters inherited the mother's mtDNA diagnostic bands (6 and 2 kb). No daughter had any of the father's bands. All sons had the diagnostic band of the father's paternal mtDNA (4 kb), but not the diagnostic band of the father's maternal mtDNA (8 kb). Bands > 7 kb in the first female offspring are due to incomplete digestion. (*Right*) Heterospecific cross 11. DNA from an unrelated female (lane 1), an unrelated male (lane 2), the mother (lane 3), the father (lane 4), three sons (lanes 5–7), and five daughters (lanes 8–12) digested with *Hin*dIII and probed with a mixture of clones that hybridized to the entire mtDNA molecules of *M. edulis* and *M. trossulus*. Each unrelated individual contained one of the two mtDNA profiles for which the father is heteroplasmic. The mother had the *Hin*dIII female type B, which shares four bands (nondiagnostic bands) with the female pattern A of the father (12). Letters on the right indicate whether a diagnostic band comes from the male (M) or female (F) mtDNA type of the father or from the mother's (Mo) mtDNA. Numbers on the left indicate approximate band size in kb. All seven offspring have the diagnostic band of the mother. No offspring carries the diagnostic bands of the father's maternal (F) type. The first two sons and the first daughter are exceptions to the rule seen in homospecific crosses: the sons inherited no mtDNA from the father; the daughter has inherited the paternal mtDNA of the father.

in the fertilized egg. Skibinski *et al.* (22) have indeed observed that sperm from heteroplasmic males contains exclusively or almost exclusively the paternal mtDNA type. Evidence for preferential increase of paternal mtDNA is presented in Fig. 3, where restriction profiles of heteroplasmic offspring at the age of 1 year are compared to profiles of their 3-year-old siblings. The intensity of bands specific to paternal mtDNA is consistently stronger in the older age class, and the inverse is true for the bands specific to maternal mtDNA.

DISCUSSION

Skibinski *et al.* (22) observed that sperm from heteroplasmic *Mytilus* males contains predominantly the male mtDNA type. Combining this with the observation that in natural populations females are usually homoplasmic and males are usually heteroplasmic for an mtDNA type not normally found in females, they reasoned that females must receive mtDNA only from their mother and that males must receive the mother's as well as the father's paternal mtDNA. Our study provides the direct evidence needed to establish this unusual pattern of mtDNA inheritance. In homospecific crosses, we failed to establish a single exception to this rule: no female carried mtDNA from the father and all sons carried the father's male mtDNA in addition to the mother's mtDNA. These findings give rise to a number of questions about how the system operates and how it evolved. Our genetic data provide some answers and point to a way for further investigation of these questions.

The coupling of sex inheritance with mtDNA inheritance implies a network of interactions between nuclear and mtDNA. The observation that the coupling breaks down in interspecific crosses either partly (crosses 11 and 15) or fully (cross 2) provides independent evidence for these interactions. It is clear from these crosses that the mtDNA is not involved in sex determination. Nothing is known about sex determination in *Mytilus* except that it is dioecious (23) (even though hermaphroditism must occur in low frequency; see Table 1) and that in natural populations the sex ratio is not noticeably different from 1:1 (refs. 11 and 23; unpublished data). This work reports on sex ratio in controlled crosses in this species. The extreme variation of sex ratio among crosses may provide a clue to explaining the association between paternal mtDNA and maleness. In most families, the ratio was highly biased, with the proportion of males varying from 0 to 85%. Two of the crosses (crosses 15 and 16) shared the same female parent (EF 16) and had very similar sex ratios (P = 0.35 from test for homogeneity). Two other crosses (crosses 3 and 4) shared the same male (EM4). Cross 3 was found to be sonless in this study and none of the 88 progeny scored in the previous study (12) carried paternal mtDNA and can be assumed, on the basis of this report, to be females. Cross 4 was among the ones lost, but 12 of 28 progeny tested positive in the previous study and can be assumed to be males. A hypothesis that is compatible with the results and may serve as a working model for further studies is that sex ratio is controlled by the mother's genotype, which also controls, pleiotropically, the fate of the sperm's mtDNA. Eggs determined to become females also become able to prevent the entrance of sperm mitochondria, destroy them after entrance, or suppress the replicative advantage of the male mtDNA. Alternatively, this advantage

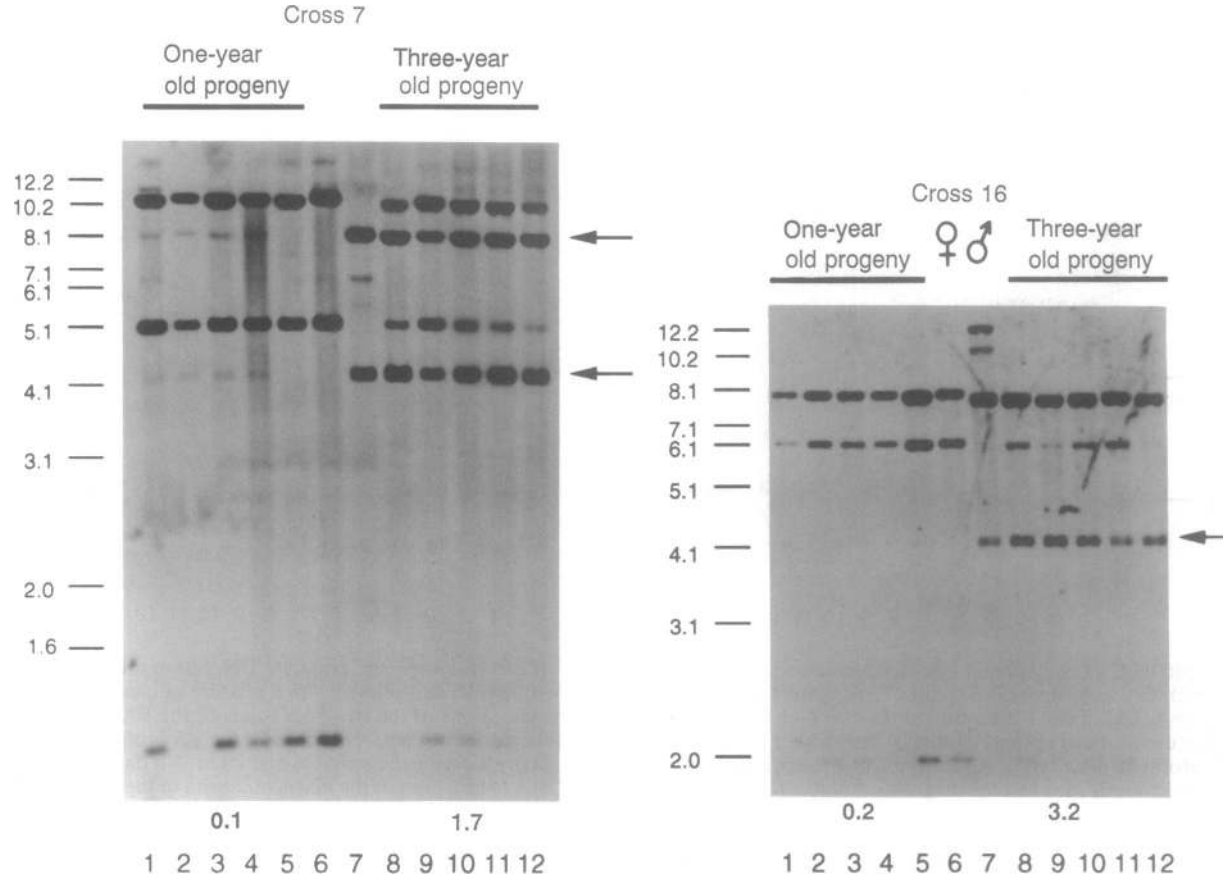

FIG. 3. Increase of the paternal/maternal mtDNA ratio with age in heteroplasmic offspring. Arrows point to diagnostic paternal mtDNA bands, and numbers on the left are lengths in kb. Two exposures of each blot were scanned with an HP Scan Jet II scanner and the peak areas corresponding to each band were integrated by scan analysis. Number below each age class gives the mean ratio of the intensity of the 8-kb paternal mtDNA band to the intensity of the 5-kb maternal mtDNA band (cross 7) or the mean ratio of the 4-kb paternal mtDNA band to the 6-kb maternal mtDNA band (cross 16). Bands > 10 kb are due to partial digestion.

may be present only in eggs determined to become males. The assumption of pleiotropy may be replaced by one of two genetic factors in strong linkage disequilibrium, with one factor affecting the sex and the other the fate of the sperm's mtDNA. A gene cluster affecting mating-type determination and uniparental inheritance of chloroplast DNA has been recently identified in *Chlamydomonas* (24), and a theoretical argument of why extreme sex ratios affected by nuclear genes may eventually become maternally controlled was presented by Haig (25).

The fact that females receive mtDNA only from their mother and that males transmit to their sons only the mtDNA type they inherited from their father explains the high degree of divergence between male and female mtDNA types (8, 11, 12). It also provides empirical evidence for the theory that uniparental inheritance of organelle DNA evolved because it provided a mechanism for the containment of selfish cytoplasmic DNA mutations within the lineage through which they arose (26–29). In *Mytilus*, selfish mutations arising in a male's maternal mtDNA will not be transmitted to offspring, and mutations arising in a male's paternal mtDNA will spread only through descending male lineages. Similarly, selfish mutations arising in a female's mtDNA will spread only through descending female lineages. If deleterious, these mutations will be removed from the population through lineage extinction.

All the peculiarities of the mussel mtDNA noted in previous reports (8, 11, 12) can now be understood as manifestations of the type of mtDNA transmission described here. This includes the presence of males with only the female mtDNA type and of females with both the female and male type in a zone of hybridization between *M. edulis* and *Mytilus galloprovincialis* (11). Such atypical cases are expected from the breakdown of the phenomenon in interspecific hybrids. An exceptional case of paternally inherited mtDNA was described in the plant *Sequoia sempervirens* (30). The "doubly uniparental" (31) mtDNA transmission in mussels appears to present an even more complex and intriguing phenomenon, both from the standpoint of the nuclear/cytoplasmic mechanism involved and of the population contingencies under which it evolved.

We acknowledge the input of Drs. M. Ball, R. Hoeh, R. Hoekstra, L. Hurst, E. Kenchington, G. Kotoulas, A. Magoulas, D. Skibinski, D. Stewart, and R. Trivers at various stages of this work. This research was supported by a Natural Sciences and Engineering Research Council of Canada grant to E.Z. C.S. was supported by a postdoctoral fellowship from the Consellería de Educación, Xunta de Galicia (Spain).

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