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The effect of mitochondrial recombination on fertilization success in blue mussels

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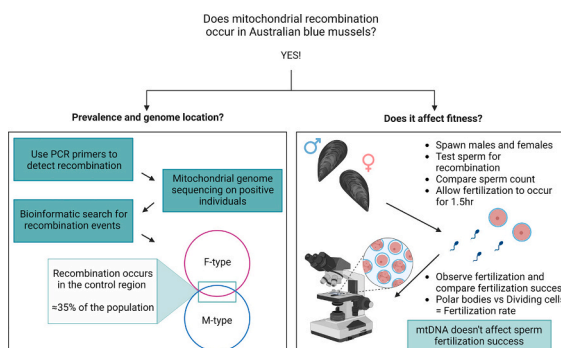
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HIGHLIGHTS

- The presence of masculinized mtDNA does not impact fertilization success in mussels.
- Two independent F-mitotypes present in Australian mussels
- F-mitotypes likely have originated from independent sources
- mtDNA recombination only detected in Southern Hemisphere lineage

GRAPHICAL ABSTRACT



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ABSTRACT

The presence of doubly uniparental inheritance (DUI) in bivalves represents a unique mode of mitochondrial transmission, whereby paternal (male-transmitted M-type) and maternal (female-transmitted F-type) haplotypes are transmitted to offspring separately. Male embryos retain both haplotypes, but the M-type is selectively removed from females. Due to the presence of heteroplasmy in males, mtDNA can recombine resulting in a 'masculinized' haplotype referred to as M^f-type. While mtDNA recombination is usually rare, it has been recorded in multiple mussel species across the Northern Hemisphere. Given that mitochondria are the powerhouse of the cell, different mtDNA haplotypes may have different selective advantages under diverse environmental conditions. This may be particularly important for sperm fitness and fertilization success. In this study we aimed to i) determine the presence, prevalence of the M^f-type in Australian blue mussels (*Mytilus* sp.) and ii) investigate the effect of M^f-mtDNA on sperm performance (a fitness correlate). We found a high prevalence of recombined mtDNA (≈35 %) located within the control region of the mitochondrial genome, which occurred

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only in specimens that contained Southern Hemisphere mtDNA. The presence of two female mitotypes were identified in the studied mussels, one likely originating from the Northern Hemisphere, and the other either representing the endemic *M. planulatus* species or introduced genotypes from the Southern Hemisphere. Despite having recombination events present in a third of the studied population, analysis of sperm performance indicated no difference in fertilization success related to mitotype.

1. Introduction

Mitochondrial DNA (mtDNA) inheritance is primarily maternal in eukaryotes (Hutchison et al., 1974), however, an alternative mode of mtDNA transmission, named doubly uniparental inheritance (DUI), has been observed in >100 bivalve species from 12 families (including freshwater mussels (Unionida) and marine mussels (Mytilida) and clams (Venerida) (Gusman et al., 2016; Stewart et al., 2020; Stewart et al., 2021)). In DUI, two distinct sex-associated mitochondrial lineages (F-type and M-type mtDNA) are transmitted, with males transmitting M-type through sperm and females transmitting F-type through their eggs. While the F-type is retained in all offspring post-fertilization, the M-type is eliminated from female progenies (Breton et al., 2007) (Fig. 1).

In species with DUI, males are known to be heteroplasmic containing both M-type and F-type mtDNA (Barr et al., 2005; Rawson, 2005; Jha et al., 2008). The F-type is more abundant in somatic tissue whereas M-type occurs in the gonads and sperm (Jha et al., 2008). Females are known to be predominantly homoplasmic for the F-type mitochondrial genome, with the M-type lineage being eliminated during embryogenesis (Rawson, 2005; Obata et al., 2006). However, there have been reports of females presenting with M-type mtDNA in eggs, gonads, and adductor muscle of *Mytilus galloprovincialis* (Hoeh et al., 1997; Sutherland et al., 1998; Obata et al., 2006; Jha et al., 2008). While one study found both M-type and F-type in all 15 mussels studied (Obata et al., 2006), other studies (Zouros et al., 1994; Wood et al., 2003) report much lower prevalence, potentially due to the low number of mitochondria containing M-type DNA (Obata et al., 2011). Dalziel and Stewart (2002)

have also shown paternal mtDNA being less expressed when present in female tissues. Both mitochondrial genomes being present and expressed in the male germline facilitates the exchange of genetic material between the genomes (i.e., recombination).

Mitochondrial heteroplasmy and recombination have far reaching evolutionary consequences, including rescuing mtDNA from deleterious mutation meltdown (Ladoukakis and Zouros, 2017; Parakatselaki and Ladoukakis, 2021), and restoring the functionality of paternally inherited genome under certain cytonuclear incompatibility conditions (Sańko and Burzyński, 2014). In mussels, recombination often occurs in the control region of mtDNA resulting in a new 'masculinized' sequence containing an M-type control region and F-type coding sequences (Hoeh et al., 1996; Hoeh et al., 1997; Ladoukakis and Zouros, 2001; Zbawicka et al., 2014). Recombination events have also been reported in the cytochrome c oxidase subunit III region (COX3) of bivalve mitochondria (Jha et al., 2008; Ladoukakis and Zouros, 2001). This new sequence, referred to as M^f-type or 'masculinized' mtDNA, will have female protein coding genes, however, the control region will be M-type, meaning the resulting offspring that retains this mtDNA will be male (Hoeh et al., 1996).

Phylogenetic and functional studies by Stewart et al. (2009) demonstrate the replacement of the M-type mitochondrion with the recently masculinized M^f-type. These M^f-types become the new normal M-type over time which, in turn, will eventually be replaced by an even more recently masculinized M^f-type. This creates an ongoing cycle of recombination, masculinization, competition between the new M^f-type and an evolutionarily older M-type, that eventually leads to

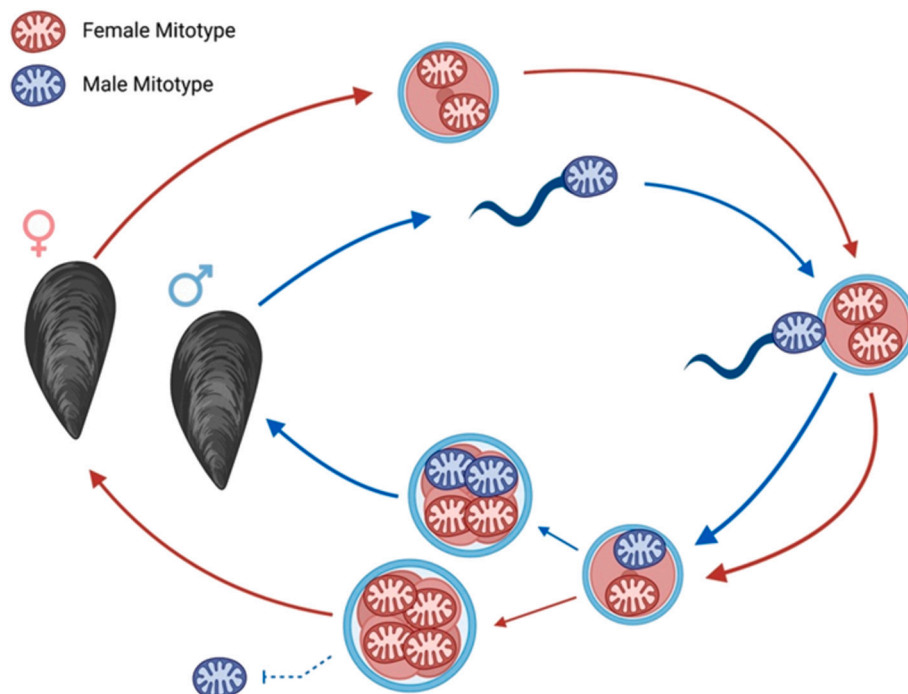


Fig. 1. Double uniparental inheritance in blue mussels.

A diagram illustrating the process of double uniparental inheritance of mitochondrial DNA in blue mussels (*Mytilus* sp.). Blue indicates the movement of M-type mtDNA and red indicates F-type mtDNA. Solid arrows represent the direction of the life cycle. Dotted line signifies the elimination of M-type mtDNA from an embryo, resulting in the formation of a female mussel (Breton et al., 2018; Smith et al., 2022). Based on American Genetic Association (2023), created with Biorender.com.

replacements of the mitotypes (Stewart et al., 2009).

In *Mytilus* mussels the normal M-type and M^f-type have been found to exhibit up to 20 % divergence in nucleotide (Breton et al., 2006) and 8.7 % in amino acid sequences in coding regions of mtDNA (Everett et al., 2004). As the genetically highly diverged M^f-type mtDNA can permeate the male route of inheritance, they offer a platform for natural selection to act on and alter the adaptive value of paternally inherited mtDNA variants (Bettinazzi et al., 2020). Everett et al. (2004) proposed that amino acid differences between normal and masculinized mitotypes would affect mitochondrial energy production and concomitantly sperm performance, as seen in rainbow trout (*Oncorhynchus mykiss*) (Lahnsteiner et al., 1998). Everett et al. (2004) found no significant difference in sperm swimming performance between the mitotypes. In contrast, a similar study by Jha et al. (2008) demonstrated that *Mytilus edulis* sperm with recently masculinized mtDNA had significantly faster curvilinear and average path velocities than those with normal mtDNA. Differences in the results between these studies may have resulted from the greater sample size and sensitivity in the Jha et al. (2008) study. The authors proposed that sperm with the M^f-mitotype may have enhanced fitness, potentially due to higher fertilization success, as observed in fish (Kime et al., 2001; Burness et al., 2005). However, depending on fertilization strategy, slower sperm swimming speed, pronounced curved trajectories and higher angle change rate could represent an advantage and result in higher fertilization success, as seen in blue mussels (Liu et al., 2011; Fitzpatrick et al., 2012). In sessile, broadcast spawning marine species, longer survival time of sperm, covering larger distance and increased oscillation around the average path may benefit male reproductive success (Evans and Sherman, 2013; Crimaldi and Zimmer, 2014; Stewart et al., 2018), and such, selection may favour the normal M mitotype (Breton et al., 2007; Stewart et al., 2018). However, Levitan (2004) found that the density of mussel populations may also impact sperm fitness, with high density mussel populations containing fast, yet short lived sperm when compared to those living in low density (Levitan, 2004). In addition, apart from sperm swimming speed, other factors such as spawning densities, density and location of mussel colonies (temperature and depth), the proximity of closely related species (Levitan, 1991; Atkinson and Yund, 1996; Teo and Todd, 2018), as well as gamete level mate choice (Oliver and Evans, 2014; Sherman et al., 2015; Lymbery et al., 2017), could also dictate the evolutionary dynamics of the mitochondrial DNA polymorphisms in bivalve sperm (Bettinazzi et al., 2021).

It is therefore predictable that different mitotypes may have different selective advantages under different mate choice and fertilization strategies and diverse environmental conditions. Although Hoeh et al. (1997) and Jha et al. (2008) proposed that the M^f mitotype may eventually replace the normal M-type genome over evolutionary time, the prevalence of recently masculinized mitotypes has been reported to be relatively low: 20–25 % in *M. edulis* males in southwestern Nova Scotia (Everett et al., 2004) and 5–35 % in *M. trossulus* from the Gulf of Gdańsk (Burzyński et al., 2003), thus potentially indicating a lower fitness of this mitotype in *Mytilus*. Although, it is important to note that fitness is not absolute and is context dependent. One mitotype may be fitter under some environmental conditions while others might be beneficial under different conditions. If environments constantly change between the two conditions, then both mitotypes can be maintained in the population through balancing selection.

While previous studies have investigated the associations between mitochondrial DNA polymorphism and sperm motility (Jha et al., 2008) and energy production (Bettinazzi et al., 2021) in Mytilidae, fertilization success could be a more direct proxy to measure the impact of different mitotypes on fitness (Zouros, 2013). In this study we, therefore, conducted experimental fertilization trials with sperm containing either normal or masculinized mtDNA. We used Australian blue mussels (*Mytilus* sp.) in which fertilization trials have previously been successfully conducted (Sherman et al., 2015; Eads et al., 2016). The Australian native *Mytilus* species is described as *Mytilus planulatus* Lamarck, 1819

(Popovic et al., 2020; Zbawicka et al., 2021), however this species complex is complicated and thus it is difficult to distinguish species (Bramwell et al., 2023), hence the species will be referred to as *Mytilus* sp. throughout this study.

2. Methodology

2.1. Sample collection, DNA extraction and recombination screening

Adult blue mussels (*Mytilus* sp.) were obtained from commercial suppliers located in Port Phillip Bay, Victoria, Australia (n = 231) and transported to Deakin University's Queenscliff Marine Research Facility for processing. A small portion of tissue containing digestive glands and gonads, approximately 1 cm × 1 cm, was biopsied and stored in 100 % ethanol. DNA was extracted using a Dneasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The quality and quantity of the extracted DNA was determined using a Nanodrop2000 (ThermoFisher Scientific, Waltham, MA).

Polymerase chain reaction (PCR) amplifications were performed using a BioRad T100TM Thermal Cycler (BioRad, Hercules, CA). The primers were amplified using the following conditions: an initial denaturing step of 94 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 60 s. The reaction was terminated with a final extension step of 72 °C for 10 min. The PCR reactions were performed in a final volume of 25 µL containing 30 ng genomic DNA, 0.2 mM dNTPs, 1 mM buffer, 2 mM MgCl₂, 0.2 µM reverse and forward primer and 0.05 U of Qiagen Taq Polymerase (Qiagen, Hilden, Germany). The PCR products were evaluated on a 3 % TAE agarose gel containing SYBR®Safe dye (ThermoFisher, Waltham, MA) using Easy-ladder I (BioLine, London, England) as reference. The samples were visualised under ultraviolet light on a BioRad Gel Doc XR+ Imaging System with the appropriate Image Lab Software (BioRad, Hercules, CA).

To conduct our experiments blind, 231 mussels were randomly selected from 10 kg of commercially purchased stock. Individuals were sexed using F-type, and M-type control region specific primers (Table 1) for which a total of 154 males and 77 females were identified. All individuals that resulted as male were further analysed using published recombined mtDNA specific primers (Venetis et al., 2007; Kyriakou et al., 2010) that do not amplify M- or F-type mitochondria (Table 1) using the same PCR and evaluation conditions as above. As the prevalence of recombination in previously published studies was below 35 % (Burzyński et al., 2003; Everett et al., 2004), we conducted a power analysis to determine the minimum number of mussels required to detect recombination in our samples. The power analysis was conducted using G*Power v3.1.9.4 (Faul et al., 2009). Results indicated the required sample size to achieve 95 % power for detecting a medium effect, with a significance criterion of $\alpha = 0.05$, was N = 88. Thus, the obtained sample size of N = 154 males was more than adequate for this study.

2.2. Mitochondrial sequencing and phylogenetic analysis

To identify recombination sites of male and female mitochondrial genomes, the complete mitogenomes were sequenced from 18 individuals (4 females and 14 males) at the Commonwealth Scientific and Industrial Research Organisation (CSIRO). DNA libraries were made for each sample using the QiaSeq™ UltraLow Input library kit (Qiagen, Hilden, Germany) using custom dual-indexed adapters. Final libraries were size-assessed on a Fragment Analyser using the high-sensitivity NGS Fragment Kit (Agilent, California, USA), quantified using a fluorescence method and then pooled equimolarly. Samples were sequenced using 150 bp paired end reads on a NovaSeq S1 flow cell (Illumina, California, USA.) at the Biomolecular Resource Facility, John Curtin School of Medical Research, Australian National University (Canberra, Australia).

Table 1

Primers used to detect recombination and to determine sex of specimens within this study.

Prime name	Primer sequence 5'-3'	Target locus	Amplicon size	Optimal ann. temp.	Reference
AB15	TTGCGACCTCGATGTTGG	Complete control region	702-732 bp	55	(Burzyński et al., 2006)
AB1639 ^a	CAGGCTRTARAGCATAATCTA AAC				
16MSI-F	GTGYCCTAGAGGYTCGACGCGYCTAAG	M-type	191 bp	60	(Kyriakou et al., 2010)
muDLR	CTCTGACAAATGCTTATYAGCTG				
ssFdl1	GGTGATAGGTTGTTAAGYGTGG	F-type	187 bp	55	(Kyriakou et al., 2010)
SsFdl2	CACCGTCRCCTCTCCWCCC				
S35-vd1M-fl	GCTTGTAAMAGAARTAGTCSCTC	Recombined region (M ^f -type)	700-710 bp	55	(Venetis et al., 2007; Kyriakou et al., 2010)
QssMdl-2	CTGGTATACACGTYTACACACC				

The primers were designed to amplify the relevant genetic regions of the mussel species (all oligonucleotides were synthesised by Sigma, Kawasaki, Japan).

^a Primer AB1639 was amended from primer AB16 (Burzyński et al., 2006) to amplify *Mytilus* sp. in this study.

Bioinformatic processing was conducted using a custom pipeline written in the Snakemake language (Köster and Rahmann, 2012). Raw reads were trimmed for sequencing adaptors and quality using Trimmomatic v. 0.39 (Bolger et al., 2014). Trimmed reads were deduplicated using “Clumpify.sh” from the BBDMap package v. 38.96 (sourceforge.net/projects/bbmap/) and assembled with SPAdes v. 3.15.2 (Bankevich et al., 2012). Putative mitochondrial contigs were selected by performing BLASTn v. 2.12.0 (BLASTn, 2019) alignments against all complete mitochondrial genomes available from NCBI GenBank’s RefSeq collection and annotated using MITOS v. 2.0.8 (Bernt et al., 2013). Mitochondrial protein-coding gene annotations were cleaned by trimming spurious ends (ends of the sequence that were messy due to the sequencing process, which includes the priming site and any duplications/overhang), and phylogenetic relationships were used to manually filter out contigs arising from cross-contamination (between and within sample runs). All sequences have been deposited to GenBank under accession numbers: OR574991–OR575008.

Phylogenetic trees were created in Mega X (Jukes and Cantor, 1969; Felsenstein, 1985; Tamura and Nei, 1993; Kumar et al., 2018) using the maximum likelihood (Tamura and Nei, 1993) and neighbour joining with Kimura 2-parameter (Kimura, 1980; Saitou and Nei, 1987) methods. Phylogenetic relationships were analysed for both the full mitochondrial genome sequences, for the control region only and for the full genome sequences with the control region removed, to count for any potential “pull” effect by recombination in the control region.

Estimates of evolutionary divergence between all sequences were conducted for both the full mitochondrial genomes and for the sequences lacking the control region, by using the Kimura 2-parameter model (Kimura, 1980). This analysis involved 28 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 17,040 positions in the final dataset. To determine whether reference sequences obtained from GenBank influence the evolutionary relationship of our sequences, we generated a maximum likelihood and neighbour joining tree by only using the sequences from our samples (removed the reference sequences). Within and between group average distances in this dataset were calculated in Mega X using the Kimura 2-parameter model (Kimura, 1980). This analysis involved 17 nucleotide sequences. All ambiguous positions were removed for each sequence pair (pairwise deletion option). There was a total of 15,797 positions in the final dataset.

2.3. Detecting recombination events

To detect recombination breakpoints a total of 37 mtDNA full genome sequences were used, including the 30 specimens sequenced above and 7 reference genomes obtained from GenBank (Table 2 and 3). All sequences were aligned and manually checked for calling errors in BioEdit v7.0 (Hall, 1999). To identify possible recombination events, breakpoints were detected in the Recombination Detection Program (RDP) v4.101 (Martin and Rybicki, 2000). Parameters for

Table 2

Reference genomes obtained from Genbank and used in the recombination event analysis.

Accession number	Genome	Species	Reference
DQ198231.2	F-type	<i>M. trossulus</i>	(Zbawicka et al., 2007)
FJ890849.1	F-type	<i>M. galloprovincialis</i>	(Burzyński and Śmietanka, 2009)
NC_006161.1	F-type	<i>M. edulis</i>	(Boore et al., 2004)
FJ890850.1	M-type	<i>M. galloprovincialis</i>	(Burzyński and Śmietanka, 2009)
AY363687.2	M-type	<i>M. galloprovincialis</i>	(Cao et al., 2004)
DQ198225.2	M-type	<i>M. trossulus</i>	(Zbawicka et al., 2007)
Kp100301	F-type	<i>M. platensis</i>	(Gaitán-Espitia et al., 2016)
KT966847.1	F-type	<i>M. chilensis</i>	(Śmietanka and Burzyński, 2017)
EF434638.2	M ^f -type	<i>M. galloprovincialis</i>	(Filipowicz et al., 2008b)
KM192124.1	M ^f -type	<i>M. trossulus</i>	(Zbawicka et al., 2014)

Table 3

Full mitochondrial genome and control region sequences of the Australian blue mussel (*Mytilus* sp.) obtained within this study. Table includes sample identification (ID), sex, mitotype, tissue type, total sequence length, control region (CR) length and the accession number. Lengths of the sequences are in nucleotide base pairs (bp). Samples containing <250 bp of the control region, and a complete genome size <8,000 bp were not included into the phylogenetic analysis. However, ML15_W was retained in the analysis due to the M-type differentiation of the coding regions.

ID	Sex	Mitotype	Tissue	Total length (bp)	CR length (bp)	Accession number
FL04_G	Male	F-type	Mantle	16,710	1123	
FL10_P	Male	F-type	Mantle	16,756	1160	
FH2_W	Male	F-type	Mantle	16,741	1158	
FH22_P	Female	F-type	Mantle	16,743	1158	
FH23_W	Male	F-type	Mantle	16,711	1122	
FLG_W	Male	F-type	Mantle	16,773	1189	
FL12_P	Male	F-type	Mantle	16,714	1123	
MH18_P	Male	F-type	Mantle	16,775	1189	
J4T	Female	F-type	Mantle	16,778	1195	
FH16_G	Female	F-type	Mantle	16,643	1048	
FH24-P	Female	F-type	Mantle	15,988	618	
FH26_W	Male	F-type	Mantle	16,742	1158	
ML15_W	Male	M-type	Mantle	14,710	16	
MH16-P	Male	M ^f -type	Mantle	9229	618	
MH10_W	Male	M ^f -type	Mantle	12,377	597	
ML29_P	Male	M ^f -type	Mantle	15,912	617	
ML30_P	Male	M ^f -type	Mantle	8928	618	
J52T	Male	M ^f -type	Mantle	16,038	616	

recombination detection followed the protocol outlined in Zbawicka et al. (2014). Only recombination events detected with $p < 0.05$ Bonferroni correction for multiple tests by at least two of the following methods were considered reliable: 3SEQ (Boni et al., 2007), Chimaera

(Posada and Crandall, 2001), Geneconv (Padidam et al., 1999), Bootscan (Martin et al., 2005), Maxchi (Smith, 1992), SiScan (Gibbs et al., 2000). Recombination events were further confirmed or excluded via manual visualisation of the sequence alignment.

2.4. Fertilization trials

Adult broodstock were collected from commercial suppliers located in Port Phillip Bay (Victoria, Australia) and transported to the Queenscliff Marine Research Facility for spawning. Adults were cleaned and transferred to 13–15 °C aerated, flowing seawater baths where they remained for 24 h to allow for stress stabilization. Mussels were then placed in a 10 L seawater bath (20 individuals/bath) at 22 °C for 20 min, then relocated to 15 °C for a further 20 min. This process was repeated until spawning was observed (approximately 1 h). Once gamete release was seen in a single individual, all mussels were removed and placed in isolated 250 mL containers of filtered seawater. Gamete release was allowed to continue for a further 30 min before sample collection. Aliquots containing 30 mL of male gametes (sperm) and 10 mL of female gametes (eggs) were collected in individual scintillation vials. A 5 µL aliquot of each egg sample was observed under a microscope for signs of fertilization (due to the potential risk of sperm-egg interaction before individuals were removed from the communal seawater bath). Eggs that showed the signs of being fertilized prior to experimental fertilization were excluded from the experiment. All others were pooled into a homogenous mixture to reduce variance in fertilization rates associated with female/male specific compatibility (Sherman et al., 2015; Eads et al., 2016). Sperm and eggs were counted using a hemocytometer and were standardized using dilution in filtered seawater; egg density to 50,000 eggs/mL and sperm to 10,000,000 sperm/mL.

As testing for recombination using PCR techniques couldn't be undertaken before sperm and egg samples began to deteriorate, fertilization trials were conducted 'blind' (i.e., at this timepoint it was unknown which sperm sample contained M-type or M^f-type mitochondria). From each sperm sample 1 mL was added to 4 mL of the homogenized egg mixture and allowed to interact for 1.5 h, at which point fertilization was halted by adding 0.5 mL of 10× buffered formalin. Once fertilization trials were underway, recombination detection was completed using the same screening methods described above. Eleven sperm samples had recombined mtDNA (M^f-type) and 25 had normal mtDNA (M-type).

A power analysis was conducted using G*Power v3.1.9.4 (Faul et al., 2009) to determine the minimum number of fertilized eggs required to detect significance between recombined and non-recombined individuals. The required sample size to achieve 95 % power for detecting a medium effect, with a significance criterion of $\alpha = 0.05$, was $n = 92$. Therefore, a minimum of $N = 100$ eggs were counted for each fertilization trial. Fertilization success was determined by counting the ratio of fertilized/unfertilized eggs from a randomly selected subsample of at least 100 eggs. Fertilized eggs were classified as those exhibiting either a polar body or dividing cells. Fertilization rate was calculated by comparing the ratio of cells exhibiting a polar body (early sign of fertilization) to dividing cells (mid-stage fertilization). The fertilization state of eggs was clearly visible under 200× magnification.

2.5. Data analysis

Statistical analyses were performed using the software program R v 4.1.0 (Rizzo, 2019). The data did not meet the assumptions of normality and homogeneity of variance needed for parametric tests; hence non-parametric Wilcoxon signed-rank tests were used in subsequent analyses to determine significance.

3. Results

3.1. Detection of recombination events and establishing the prevalence of recombined control region (M^f mitotype) in the studied samples

Phylogenetic relationships were determined by using maximum likelihood and distance-based approaches (neighbour joining), and by using the entire mitochondrial sequences and by removing the control region from the sequences. As there was very little difference in the position of the major inner nodes and such, in the topology of the four trees, the maximum likelihood tree with the full genome alignment is being presented in the main text of this manuscript. All other trees can be found in the Supplementary information (Figs. S1–S3).

All the mitogenomes confirmed to be M^f-type formed a clade together, clustering with a *M. chilensis* F-type reference sequence. All other mitogenomes were identified as F-type and clustered as such with *M. chilensis* and *M. galloprovincialis*. Removing the reference sequences did not change the topology of the tree (Supplementary Figs. S4–6). These phylogenetic relationships indicate the presence of two F-mitotypes present in the studied blue mussel specimens (branches marked red cluster with sequences from the Northern Hemisphere (*M. galloprovincialis* EF434638.2 and FJ890849) while sequences along the purple branches form a monophyletic clade with *M. chilensis* (KT966847.1) from the Southern Hemisphere, Fig. 2). One F-type individual (FH22-P) showed earlier divergence from the common ancestor of *M. trossulus*, *M. edulis* and *M. edulis platensis* (Fig. 2). Only one M-type mitogenome (ML15-W) clustered with male *M. galloprovincialis* sequences from the Northern Hemisphere (AY363687.2 and FJ890850.1).

When analysing all sequences altogether the number of base substitutions per site between sequences ranged between 0.003 and 0.255 in the full genomes analyses and between 0.003 and 0.065 when removing the control regions (Supplementary Tables 1–2). The M-type sequences were close to 6 times more different than the other sequences (>0.2 number of base substitutions per site between sequences) in the evolutionary divergence analyses conducted on both full mitochondrial sequences and when removing the control regions. As mentioned above, tree topology remained the same when we removed the reference sequences, and our F-type sequences formed two separate monophyletic groups (Supplementary Figs. S4–5). The average evolutionary divergence over sequence pairs within the two F-type clusters was $d = 0.01 \pm 0.005$ for both groups. The estimates of evolutionary divergence over sequence pairs between the two F-type groups was $d = 0.0578 \pm 0.00195$.

Across the studied individuals, three separate sites containing recombination events were detected. The first was in the control region of the mitogenome and was conclusive within 7 genomes across all detection methods. The second event was in the ND4 gene region within two genomes (FH23-W and ML15-W), and the third was identified in ATP8 within one genome (ML15-W). However, as these two events were only observed using one detection method, they were not accepted as conclusive. Manual observation of the aligned genomes was used to confirm the detected events, by looking for a crossover (recombination breakpoint) between male and female genomes (Fig. 3). The first event detected, within the control region, was confirmed using this observation. The second and third events, however, could be explained by a very variable gene region and large insertion in the male genome respectively. Comparing the full mitogenomes to reference sequences confirmed that within the recombined genomes the coding genes were most like the F-type genomes while the control region was a mixture of both M-type (before breakpoint) and F-type (after breakpoint).

The sex specific PCR primers confirmed the presence of 77 females and 154 males in our samples. Of the 154 male mussels tested with the recombination specific primers, 53 individuals presented as recombined (prevalence of 34.4 %) and the remaining 101 specimens were normal M-type (65.6 %).

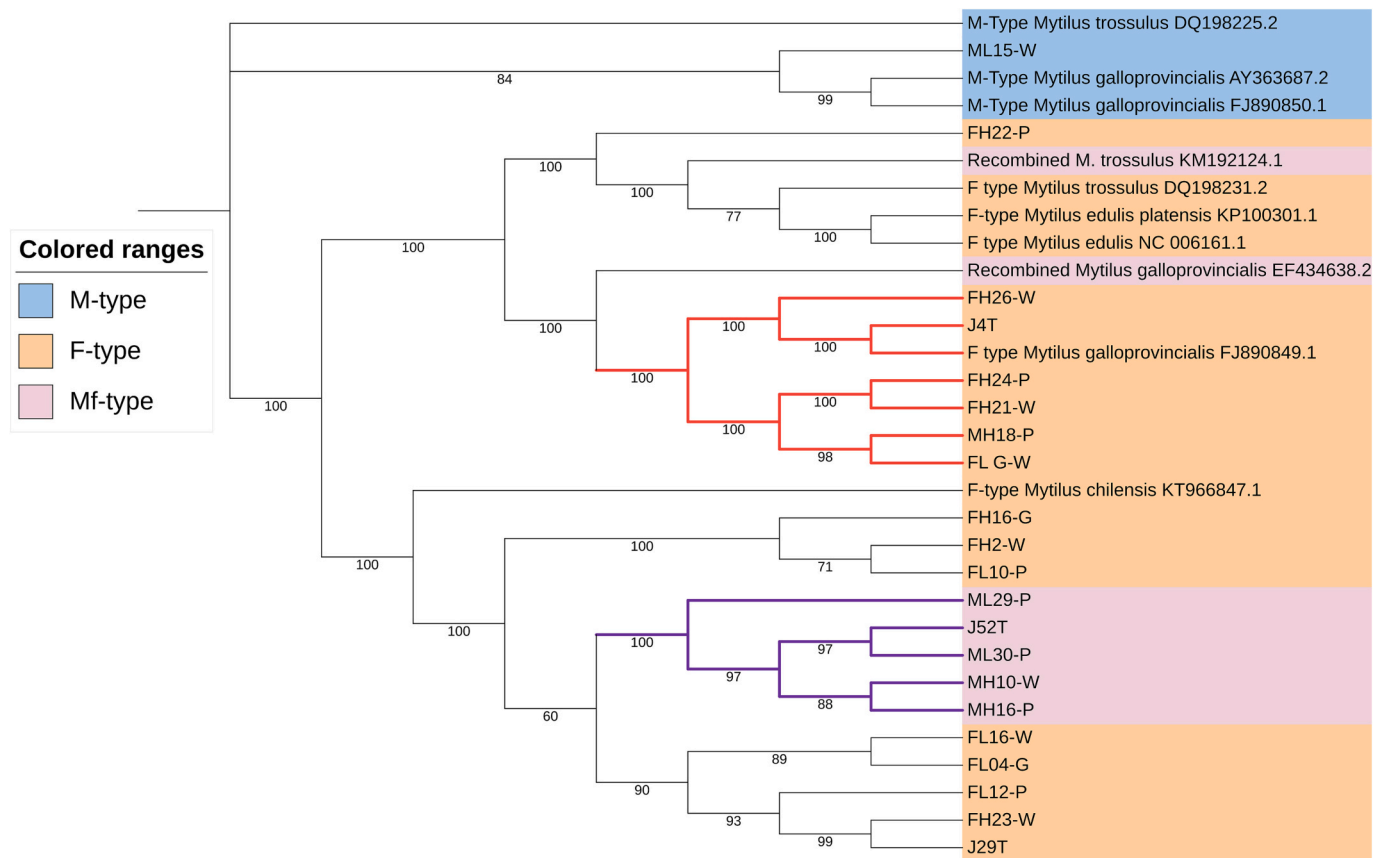


Fig. 2. Maximum likelihood phylogenetic tree based on whole mitochondrial genomes inferring the phylogenetic relationship between M-, F- and M^f-type mtDNA from the Australian blue mussel and closely related species (*M. galloprovincialis*, *M. edulis*, *M. platensis*, *M. chilensis* and *M. trossulus*). The phylogenetic tree was estimated using maximum likelihood method and Tamura-Nei model (Tamura and Nei, 1993). A bootstrap consensus tree inferred from 100 replicates was conducted and the branch partitions were reproduced to 50 % bootstrap replicates. Numbers along the branches indicate bootstrap values. Evolutionary analyses were conducted in MEGA X (Jukes and Cantor, 1969; Felsenstein, 1985; Kumar et al., 2018) and annotated using ITOL (Letunic and Bork, 2019). M-type samples are annotated by the blue colour, M^f-type by pink and F-type by orange. Red branches indicate specimens clustering with F-type Northern Hemisphere *M. galloprovincialis* and purple branches indicate specimens clustering with F-type Southern Hemisphere *M. chilensis*.

3.2. Fertilization trials

First, we analysed the sperm counts to determine whether the type of mtDNA affects the concentration of sperm produced (Fig. 4A). While mussels with a normal M mitotype provided a slightly higher average sperm count (3.1×10^7 sperm/ml $\pm 1.7 \times 10^7$ (S.D.)), however, due to the large variance among individuals, there was no significant difference between individuals carrying the M or M^f mitotypes (2.3×10^7 sperm/ml $\pm 2.2 \times 10^7$ (S.D.), $p = 0.37$). When comparing the fertilization rates (Fig. 4B) between sperm with M^f ($n = 11$, $59.8\% \pm 13.1$ (S.D.)) and M ($n = 25$, $55.3\% \pm 8.58$ (S.D.)) mitotypes, no significant difference was observed ($p = 0.17$). However, a larger standard deviation was detected in the fertilization success of males with the M^f mitotype.

As there was no difference found within the fertilization success, we decided to investigate the rate of fertilization (Fig. 4C). Our hypothesis was that significantly higher numbers of dividing cells to polar bodies would indicate a faster rate of fertilization (i.e., sperm reaching and penetrating eggs faster). However, no significant difference was found between the two mitotypes in either the number cells with a polar body (M^f mitotype $32.2\% \pm 9.77$ (S.D.), normal M mitotype $30.6\% \pm 6.54$ (S.D.), $p = 0.43$) or the number of dividing cells (M^f mitotype $27.8\% \pm 6.81$ (S.D.), normal M mitotype $24.6\% \pm 5.81$ (S.D.), $p = 0.24$).

4. Discussion

Evolutionary theories predict that while the strict maternal

inheritance of mitochondria in eukaryotes prevents the coexistence of different mitochondrial haplotypes that can cause intergenomic conflicts (Cosmides and Tooby, 1981), it can also lead to the accumulation of mutations that may be deleterious in males but advantageous or neutral in females (Mother's curse) (Gemmell et al., 2004; Wade and Brandvain, 2009). In contrast, the doubly uniparental inheritance of mitochondria in bivalves allows the independent transmission of two mtDNA lineages through gametes, and as such the sex-specific evolution of mitotypes (Bettinazzi et al., 2020). The intraindividual presence of mtDNA variants in the gametes also facilitates the potential for mtDNA recombination (i.e., emergence of masculinized F-mitotypes) and concomitantly increases mitochondrial phenotype polymorphism (Milani and Ghiselli, 2015; Wu and Sloan, 2019).

Zbawicka et al. (2014) proposed that the tendency of species in *Mytilidae* to hybridize (Daguin et al., 2001; Westfall and Gardner, 2013; Ab Rahim et al., 2016; Michalek et al., 2016) may create "structural and evolutionary mitochondrial instability" and thus, foundations for recombination to occur. Taxonomic delineation of Australian *Mytilus* sp. has been contentious over the last decades (Popovic et al., 2020), but by using transcriptome-wide markers, a recent study showed that Tasmanian blue mussels represent the endemic Australian *M. planulatus* species, while the evolution of mussels in south-eastern Australia has been affected by two contemporary introduction events of two genetically divergent *M. galloprovincialis* lineages from the Mediterranean Sea and Atlantic Europe (Popovic et al., 2020). The latter study shows that both introductions have led to repeated hybridization and admixture between

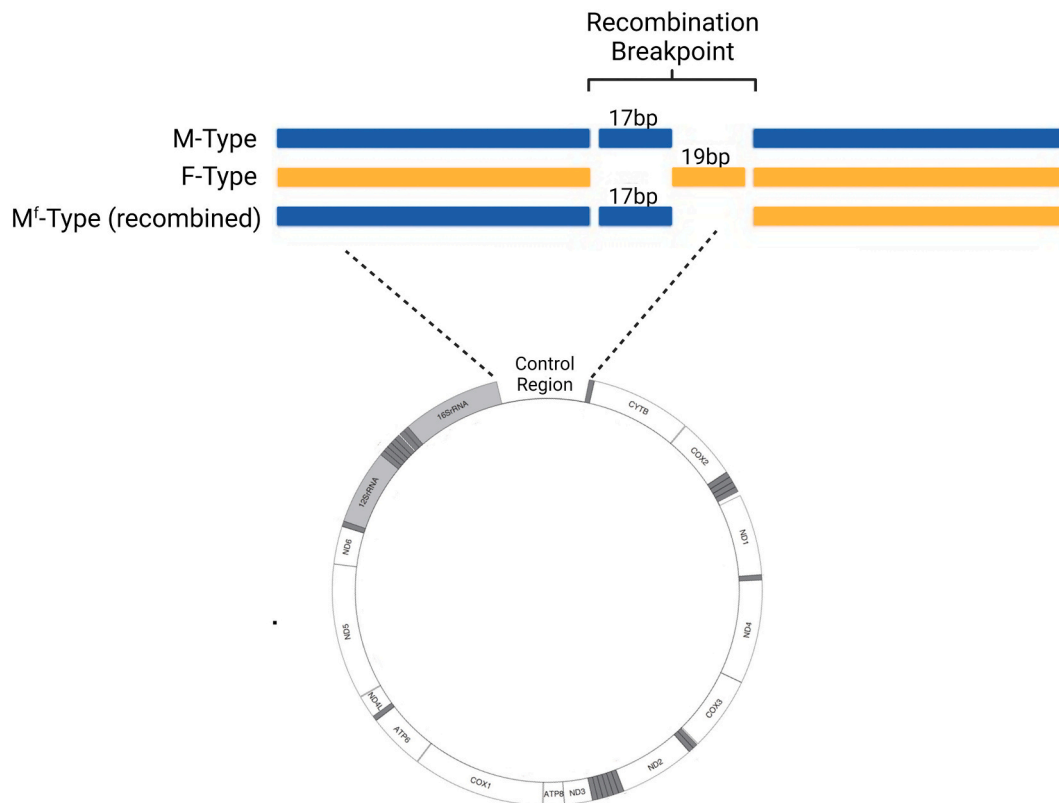


Fig. 3. Recombination breakpoint example. Diagram illustrating the recombination event occurring within the control region of *Mytilus* sp. mitochondrial DNA. Blue boxes indicate M-type mtDNA and orange boxes indicate F-type mtDNA. Gene identities: ND1–6 and ND4L, NADH dehydrogenase subunits 1–6, 4L; COX1–3, cytochrome c oxidase subunits I–III; Cytb, cytochrome b; ATP6/8, ATP synthase subunit 6 and 8; 12S and 16S, small and large subunits of ribosomal RNA (light grey); transfer RNA genes (dark grey). Created with [Biorender.com](https://biorender.com).

endemic *M. planulatus* and the introduced *M. galloprovincialis* populations. Interestingly, our phylogenetic analyses revealed the presence of two female mitotypes in the studied samples. While some of our sequences clustered with those from the Northern Hemisphere, others grouped with a *M. chilensis* sequence from the Southern Hemisphere. These results may indicate an introduction of mussels also from Southern seas but may be an artefact due to sequence availabilities from GenBank. It is also possible that sequences that group with the *M. chilensis* represent the endemic *M. planulatus* species in Australian waters. Further studies would be necessary to validate whether Southern genotypes have been introduced to Australia or our sequences originate from the endemic species. Despite recombination events known to occur within Northern Hemisphere populations (Rawson and Hilbish, 1998; Filipowicz et al., 2008a; Filipowicz et al., 2008b), in this study recombination events were only detected in mussels clustering with the Southern Hemisphere species. As most of the M-type and F-type mitotypes cluster with *M. galloprovincialis*, while all M^f-type sequences cluster with *M. chilensis*, it could be possible that the observed patterns are not due to hybridization but rather due to DUI and recombination. Based on Zbawicka et al. (2014) and Bageřová Poláková et al. (2021), it is however also possible that the recombination events may have occurred because of hybridization. Species introductions and widespread admixture accompanied by introgression (Curat et al., 2008) may be the underlying evolutionary force of the observed recombination events in Australian blue mussels. Introgression between *M. planulatus* and *galloprovincialis* could have resulted in cytonuclear incompatibility, and such in the destabilization of mtDNA, as suggested by Zbawicka et al. (2014) in *Mytilus* mussels from the Baltic Sea.

Paternal transmission of mtDNA allows selective forces to directly impact male mitochondria and to select for genomes and sperm performance that influence reproductive success. Previous studies

conducted on bivalves found metabolic remodelling between F- and M-mitotypes (Bettinazzi et al., 2019) and swimming performance differences between M and M^f-mitotypes (Jha et al., 2008), with the recently masculinized mtDNA potentially having greater fitness (that could explain the replacement of normal M-type over evolutionary time (Everett et al., 2004)). Population level studies conducted in the Northern Hemisphere have however so far shown relatively low prevalence of the recently masculinized mtDNA (<35 %), questioning the selective advantage of the M^f-mitotype. Not only is the M-type evolving at a faster rate, with more relaxed selection (Zouros, 2013), but it has also been known to contain gene inversions, gene localizations and an extension of the Cytochrome c oxidase subunit II gene that are not present in the F-type genome (Doucet-Beaupré et al., 2010). It is likely that DUI originated once early on in the history of bivalves, with multiple masculinization events (a cycle of M^f-type becoming the normal M-type which then recombines once again) having complicated the interpretation of the origins of the unusual system (Hoeh et al., 1996; Stewart et al., 2009).

We show that the position of the recombination events in the studied specimens from the Southern Hemisphere are like those observed on the Northern Hemisphere. Moreover, akin to previous studies (Mizi et al., 2006; Filipowicz et al., 2008a; Smith et al., 2022), recombination events occurred predominantly in the non-coding control region. The process of a maternally inherited genome becoming paternally inherited due to the incorporation of sequences from the M-type suggests that the M-type genome is responsible for the reversal of transmission (Cao et al., 2009). As the M-type control region is known to contain protein factors that only occur in the male gonad (Kyriakou et al., 2015), these events have transferred from the M-type into the F-type and, as a result, reversed its transmission from maternal to paternal. Kyriakou et al. (2015) proposed that this protein complex protected the mtDNA genome from

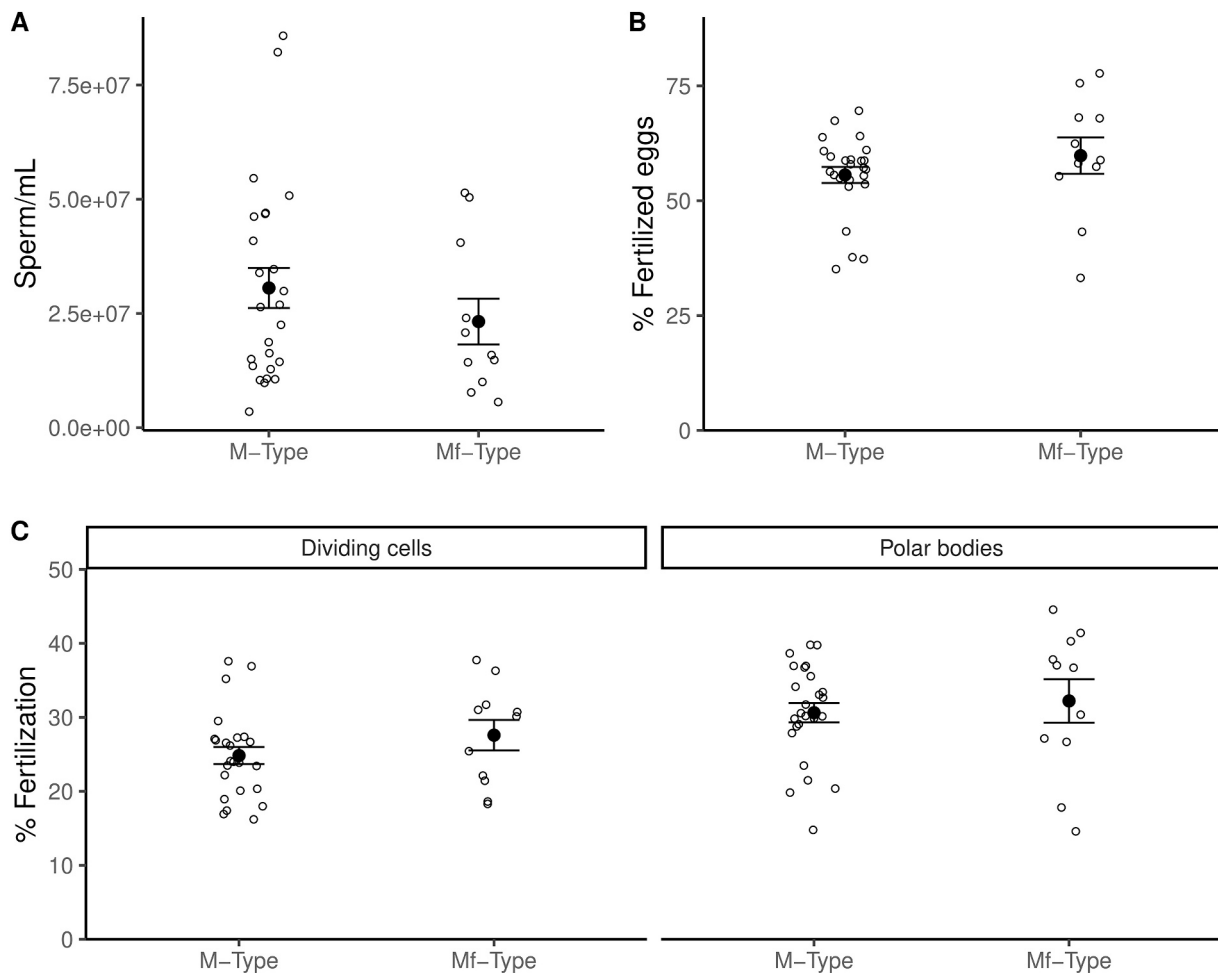


Fig. 4. Sperm counts (A) and fertilization success (B–C) of the two mitotypes (M-type and M^f-type) of blue mussels (*Mytilus* sp.). The effects of mussel mitotype on sperm abundance (A), fertilization success through the percentage of fertilized eggs (B) and percentage of fertilization indicated by dividing cells and cells with polar bodies (C) depending on mitotype were analysed. All analysis showed non-significant results ($p > 0.05$) when comparing the two male mitotypes (M-type and M^f-type).

degradation during spermatogenesis, allowing the process of paternal transmission to occur.

The method we used also indicated other possible recombination points (although with low confidence, i.e., in ND4 and ATP8 genes). While previous studies have also reported recombination events in the vicinity of the control region (Zbawicka et al., 2014) and in the COX3 gene (Ladoukakis and Zouros, 2001), we believe that the detected break points in the ND4 and ATP8 genes in our study are artefacts of the program not being able to differentiate the high divergence rate between M- and F-type mtDNA (Zouros et al., 1994; Cotton, 2001; Rawson, 2005; Zbawicka et al., 2014). The program may also mistake the presence of extra genetic material – such as the recently discovered transmissible cancers (Metzger et al., 2015; Metzger et al., 2016; Riquet et al., 2017; Yonemitsu et al., 2019) for recombination. The prevalence of M^f mitotype in Australian blue mussels was very similar to those observed in *Mytilus trossulus* in the Baltic Sea, 35 % in our study and 36 % in *Mytilus trossulus* (Burzyński et al., 2003) however, 0–24.1 % recombination was in *Mytilus edulis* across South-west Nova Scotia, Canada (Stewart et al., 2018), indicating species specific variations and/or environmental factors influencing the likelihood and maintenance of recombination events.

Apart from swimming speed, other fitness traits, such as endurance, longevity and gamete level selection (egg driven mate-choice and/or sperm competition) (Breton et al., 2007; Everett et al., 2004) could influence fertilization success, and as such the adaptive advantage of

mitotypes. Therefore, we investigated whether sperm containing normal M or M^f mitotypes affected fertilization success in *Mytilus* sp., a sessile, benthic gonochoristic and broadcast spawning bivalve.

The mitotype of the sperm does not appear to influence average sperm count, fertilization success or fertilization rate. These results were surprising since the two mitotypes have the same fertilization success, even prevalence of the two mitotypes was expected in the studied population. The equal fertilization success of sperm with the two mitotypes, but the lower prevalence of M^f mitotype in the adult population, indicate either post gametic adaptive advantage of the normal mitotype, or the disadvantage of the recently masculinized mtDNA. Although, due to the stability of fertilization success between the two mitotypes it can also be hypothesized that the prevalence of M^f-type to M-type mitochondria may also remain stable. Previous studies have shown gamete level mate choice in *M. galloprovincialis*, where chemo attractants released by eggs influenced sperm swimming trajectory, speed (Evans et al., 2012; Oliver and Evans, 2014) and physiology (Kekäläinen and Evans, 2017), and ultimately influenced offspring survival, and fitness (Oliver and Evans, 2014). Furthermore, by using microsatellite primers and sequencing the female-type (F-type) COX1 mtDNA, Lymbery et al. (2017) have shown fertilization preference by *M. galloprovincialis* eggs for males genetically similar at the mtDNA, but dissimilar at the nuclear loci. Unfortunately, in their study, Lymbery et al. (2017), refrained from sequencing the COX1 mtDNA gene directly from sperm, and as such, it is not possible to decipher whether selection for cytonuclear compatibility

was based on the presence of M^f mitotype. In our study the normal M and M^f mitotypes (the first being entirely male genotype and the latter being mostly female genotype) had similar fertilization success, indicating the lack of cryptic female choice for either of the mitotypes (Rosengrave et al., 2016; Kustra and Alonzo, 2023). However, it is important to note that in an experimental context, the conditions may be too artificial and may not replicate the complexity of the natural environment. Also, the sample size presented in this study does not allow the detection of potentially small differences between two groups of mussels. However, such small differences, which would be in the order of a few percent, are unlikely to have a high effect on the population dynamic of the species. These results, however, do rule out a large effect differences between the recombined and non-recombined individuals.

As previous studies have shown, gamete fertilization success does not always correlate with offspring survival (Gilchrist and Partridge, 1997; García-González and Simmons, 2007; Evans et al., 2012), and post-fertilization stressors (such as ocean acidification (Ventura et al., 2016), water temperature changes and pollution (Ventura et al., 2016)) may influence and alter larvae viability (Gallo et al., 2020). Future studies should conduct similar sperm competition assays to those by Lymbery et al. (2017), but by using males with normal and M^f mitotypes, to investigate whether female gamete choice for cytonuclear compatibility is driven by selection for F-type coding genes in sperm mitochondria. In addition, future experimental studies mimicking different environmental conditions (e.g., altered temperature, salinity, and pollutant levels) would be necessary to determine the post gametic fitness advantage of sperm carrying the normal or M^f mitotypes.

Climate change and rapid expansion of anthropogenic activities have been accelerating the deterioration of aquatic and terrestrial ecosystems over the past century (Frumkin and Haines, 2019). Rising water temperatures and chemo—and physical pollution of marine ecosystems appear to interfere with the reproductive mechanisms and events of a wide variety of species (reviewed in (Gallo et al., 2020)). The prevalence of recombination in our study, along with those conducted on the Northern Hemisphere Mytilidae since the 1990s (Burzyński et al., 2003), indicates that over the last 20 years, sperm carrying the normal M mitotype may have higher fitness over the M^f mitotype in *Mytilus* populations on both the Northern and Southern Hemispheres. Long-term studies are needed to determine whether under changing climatic and pollution conditions the frequency of the masculinized mitotype will eventually rise and replace the normal mitotype.

CRediT authorship contribution statement

Georgina Bramwell: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Aaron G. Schultz:** Conceptualization, Methodology, Supervision, Writing – original draft, Writing – review & editing, Visualization. **Geordie Jennings:** Data curation, Formal analysis, Methodology, Writing – review & editing, Project administration. **Urmi Nishat Nini:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Caitlin Van-beek:** Data curation, Investigation, Methodology, Writing – review & editing. **Peter A. Biro:** Formal analysis, Funding acquisition, Resources, Writing – review & editing. **Christa Beckmann:** Formal analysis, Funding acquisition, Methodology, Resources, Writing – review & editing. **Antoine M. Dujon:** Conceptualization, Formal analysis, Methodology, Supervision, Writing – review & editing. **Frédéric Thomas:** Funding acquisition, Resources, Supervision, Writing – review & editing. **Craig D.H. Sherman:** Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Supervision, Writing – review & editing. **Beata Ujvari:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All sequencing data generated in this study has been uploaded to Genbank under accession numbers: OR574991–OR575008.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2023.169491>.

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