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Natural Heteroplasmy and Mitochondrial Inheritance in Bivalve Molluscs

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

Natural Heteroplasmy and Mitochondrial Inheritance in Bivalve Molluscs / Ghiselli F.; Maurizii M.G.; Reunov A.; Arino-Bassols H.; Cifaldi C.; Pecci A.; Alexandrova Y.; Bettini S.; Passamonti M.; Franceschini V.; Milani L. - In: INTEGRATIVE AND COMPARATIVE BIOLOGY. - ISSN 1540-7063. - STAMPA. - 59:4(2019), pp. 1016-1032. [10.1093/icb/icz061]

*Availability:*

This version is available at: <https://hdl.handle.net/11585/704182> since: 2019-12-06

*Published:*

DOI: <http://doi.org/10.1093/icb/icz061>

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Ghiselli F., Maurizii M.G., Reunov A., Ariño-Bassols H., Cifaldi C., Pecci A., Alexandrova Y., Bettini S., Passamonti M., Franceschini V., Milani L. (2019) Natural heteroplasmy and mitochondrial inheritance in bivalve molluscs. *Integrative and Comparative Biology*, 59,1016–1032

which has been published in final form at <https://doi.org/10.1093/icb/icz061>.

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Journal:	<i>Integrative and Comparative Biology</i>
Manuscript ID	ICB-2019-0088.R1
Manuscript Type:	Symposium article
Date Submitted by the Author:	n/a
Complete List of Authors:	Ghiselli, Fabrizio; University of Bologna, Biological, Geological, and Environmental Sciences Maurizii, Maria Gabriella; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Reunov, Arkadiy; Russian Academy of Sciences Far Eastern Branch Ariño Bassols, Helena; Universitat de Barcelona, Departament de Fisiologia i Immunologia Cifaldi, Carmine; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Pecci, Andrea; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Aleksandrova, Yana; Russian Academy of Sciences Far Eastern Branch Bettini, Simone; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Passamonti, Marco; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Franceschini, Valeria; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA) Milani, Liliana; University of Bologna, Department of Biological, Geological and Environmental Sciences (BiGeA)
Keywords:	mitochondrial heteroplasmy, doubly uniparental inheritance of mitochondria (DUI), <i>Ruditapes philippinarum</i> , germline, immunohistochemistry, OXPHOS



## Natural Heteroplasmy and Mitochondrial Inheritance in Bivalve Molluscs

*Fabrizio Ghiselli*<sup>1#</sup>, *Maria Gabriella Maurizii*<sup>1#</sup>, *Arkadiy Reunov*<sup>2</sup>, *Helena Ariño*<sup>3</sup>,  
*Carmine Cifaldi*<sup>1</sup>, *Andrea Pecci*<sup>1</sup>, *Yana Alexandrova*<sup>2</sup>, *Simone Bettini*<sup>1</sup>, *Marco*  
*Passamonti*<sup>1</sup>, *Valeria Franceschini*<sup>1</sup>, *Liliana Milani*<sup>1\*</sup>

<sup>1</sup>Department of Biological, Geological and Environmental Sciences (BiGeA), University of Bologna, 40126, Bologna, Italy

<sup>2</sup>National Scientific Centre of Marine Biology, Russian Academy of Sciences Far Eastern Branch, 690041, Vladivostok, Russia

<sup>3</sup>Departamento de Fisiología e Inmunología, Universitat de Barcelona, 08028, Barcelona, Spain

# *Equal contribution*

Author for correspondence

\*[liliana.milani@unibo.it](mailto:liliana.milani@unibo.it)

Running Title: Natural heteroplasmy in bivalves

Total number of words: 7,354

## Abstract

Heteroplasmy is the presence of more than one type of mitochondrial genome within an individual, a condition commonly reported as unfavourable and affecting mitonuclear interactions. So far, no study has investigated heteroplasmy at protein level, and whether it occurs within tissues, cells, or even organelles.

The only known evolutionarily stable and natural heteroplasmic system in Metazoa is the Doubly Uniparental Inheritance (DUI)—reported so far in ~100 bivalve species—in which two mitochondrial lineages are present: one transmitted through eggs (F-type) and the other through sperm (M-type). Because of such segregation, mitochondrial OXPHOS proteins reach a high amino acid sequence divergence (up to 52%) between the two lineages in the same species. Natural heteroplasmy coupled with high sequence divergence between F- and M-type proteins provides a unique opportunity to study their expression and assess level and extent of heteroplasmy. Here, for the first time, we immunolocalized F- and M-type variants of three mitochondrially-encoded proteins in the DUI species *Ruditapes philippinarum*, in germline and somatic tissues at different developmental stages. We found heteroplasmy at organelle level in undifferentiated germ cells of both sexes, and in male soma, while gametes were homoplasmic: eggs for the F-type and sperm for the M-type. Thus, during gametogenesis only the sex-specific mitochondrial variant is maintained, likely due to a process of meiotic drive. We examine the implications of our results for DUI proposing a revised model, and we discuss interactions of mitochondria with germ plasm and their role in germline development. Molecular and phylogenetic evidence suggests that DUI evolved from the common strictly maternal inheritance, so the two systems likely share the same underlying molecular mechanism, making DUI a useful system for studying mitochondrial biology.

**Keywords** - mitochondrial heteroplasmy, doubly uniparental inheritance of mitochondria (DUI), *Ruditapes philippinarum*, germline, immunohistochemistry, OXPHOS.

## Introduction

In most organisms, mitochondria are inherited maternally, a process known as Strictly Maternal Inheritance (SMI). Under SMI, the entire mitochondrial population derives from the egg, while sperm mitochondria are prevented from entering the zygote or

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2  
3 eliminated by the developing embryo through different mechanisms, for example by  
4 ubiquitination and degradation with proteases, or in lysosomes (Zhou et al. 2016; Punzi  
5 et al. 2018 and references therein). Moreover, each generation, between oogenesis  
6 and early embryogenesis, the mitochondrial population undergoes a drastic reduction  
7 in number, a process known as mitochondrial bottleneck (Bergstrom and Pritchard  
8 1998; Mishra and Chan 2014). SMI and mitochondrial bottlenecks are considered to  
9 be partly responsible for homoplasmy, namely the presence of only one mitochondrial  
10 haplotype in an individual (Bergstrom and Pritchard 1998; White et al. 2008). The  
11 opposite condition—by which different mitochondrial DNA (mtDNA) variants are  
12 present in the same individual—is defined heteroplasmy, and it is mostly known to be  
13 associated with unfavorable conditions, diseases, and ageing (see for example: Lane  
14 2011, 2012; Sharpley et al. 2012). Actually, heteroplasmy is much more common than  
15 previously thought, in both animals and plants (Kmiec et al. 2006; Woloszynska 2010),  
16 and how such genetic variation contributes to complex traits and diseases is still  
17 unclear (Dowling 2014). One of the reasons why assessing the effects of heteroplasmy  
18 is a difficult task is the large mtDNA copy number per cell/organelle: alleles do not  
19 show a phenotypic effect when present at mid-low frequency, so they drift in the  
20 mitochondrial gene pool without being subject to selection (“buffering”, or “threshold  
21 effect”: Rossignol et al. 2003; Ghiselli et al. 2013; Busch et al. 2014; Dowling 2014;  
22 Milani and Ghiselli 2015). Moreover, since one of the main functions of mitochondria,  
23 oxidative phosphorylation (OXPHOS), depends on mitonuclear matching (Lane 2011;  
24 Latorre-Pellicer et al. 2016), severe heteroplasmy—highly divergent haplotypes at high  
25 frequency—is likely an unfavourable, thus rare, condition. As a consequence, in most  
26 organisms, heteroplasmy represents a sort of “hidden genetic variation” that is difficult  
27 to study, and an exceptional model system could be extremely helpful.

28  
29 The only natural and evolutionarily stable heteroplasmic system known in Metazoa is  
30 the Doubly Uniparental inheritance (DUI) of mitochondria, so far described in ~100  
31 bivalve species (Gusman et al. 2016). In this system, two mitochondrial lineages are  
32 present: one transmitted through eggs (F-type) and the other transmitted through  
33 sperm (M-type). However, this is not a case of biparental (Mendelian) inheritance  
34 because F and M lineages are both transmitted uniparentally. Thus, conspecific F and  
35 M mtDNA show a remarkable nucleotide sequence divergence—ranging from 22% to  
36 39% in mytilids and reaching 43% in unionids—corresponding to an amino acid  
37 sequence divergence of mitochondrial OXPHOS proteins above 50% (see Zouros  
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2013 for a review). The available data suggest that DUI derived from SMI through a modification of the mechanisms of degradation and segregation of sperm mitochondria (Breton et al. 2007; Passamonti and Ghiselli 2009; Milani et al. 2015, 2016; Punzi et al. 2018). The DUI transmission route is as follows. Sperm carries only M-type mtDNA while eggs carry only F-type mtDNA. During fertilization, the spermatozoon transfers the M-type mtDNA to the egg, so the zygote is heteroplasmic. By fertilizing eggs with sperm stained with MitoTracker Green, it was possible to observe the distribution of paternal mitochondria in early embryos of *Mytilus* (Cao et al. 2004; Obata and Komaru 2005; Cogswell et al. 2006; Kenchington et al. 2009) and *Ruditapes philippinarum* (Milani et al. 2011, 2012). Two patterns were observed: the aggregated pattern, in which sperm mitochondria stay clustered together and localize in the middle of the first cleavage furrow, and the dispersed pattern, in which sperm mitochondria are scattered across blastomeres. In *Mytilus* it was possible to associate the aggregated pattern with male embryos, so it was hypothesized that the aggregate of sperm-derived mitochondria ends up in germline precursor blastomeres in males (Cao et al. 2004). It was also proposed that the midbody—a cytoplasmic structure formed by the compression of the microtubule spindle during the first cleavage—is involved in the positioning of sperm mitochondria in the region that eventually gives rise to germ cells (Milani et al. 2011). It is still unclear how these two different patterns originate. It was suggested that sperm mitochondria in DUI species are tagged with ubiquitin—similarly to what observed in mammals (Sutovsky et al. 2000)—or with some sort of molecular tag that in male embryos is inactivated/masked by maternal factors, allowing sperm mitochondria to invade the germline (see models proposed in: Ghiselli et al. 2012; Diz et al. 2013; Milani, Ghiselli, Nuzhdin, et al. 2013; Zouros 2013; Punzi et al. 2018). Another hypothesis concerns the preferential transmission of mitochondria having high membrane potential ( $\Delta\psi_m$ ) (Milani 2015). What happens next is even less clear.

A point that is well-established is the homoplasmy of gametes for the sex-specific mitochondrial lineage (F-type in eggs, M-type in sperm), strongly supported by the high sequence divergence that accumulates between the two lineages within the same species. Indeed, if the system were leaky or unstable, such divergence could not be reached. In *R. philippinarum*, qPCR analyses performed on mtDNA and mRNA targets (Ghiselli et al. 2011; Milani, Ghiselli, Iannello, et al. 2014) suggested that female somatic tissues are mostly homoplasmic for the F-type, while males are heteroplasmic. The extent of such somatic heteroplasmy in males depends on both tissue and species

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3 (see Zouros 2013). Ghiselli et al. (2011) hypothesized that the establishment of  
4 homoplasmic gametes starting from a condition of heteroplasmy could happen through  
5 three checkpoints. Checkpoint #1 would be the sex-linked distribution patterns of  
6 sperm mitochondria in early embryo development, checkpoint #2 would consist in the  
7 degradation of M-type mitochondria in females, and checkpoint #3 would be a strict  
8 selection of sex-specific mtDNA lineages during gametogenesis. Checkpoint #2 was  
9 hypothesized because M-type was rarely detected in *R. philippinarum* females after 24  
10 hours post fertilization (Ghiselli et al. 2011; Guerra et al. 2016).

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17 So far, no study has investigated heteroplasmy at the protein level, and whether it  
18 occurs within tissues, cells, or organelles. The high sequence divergence between F-  
19 and M-type proteins in DUI species provides a unique opportunity to produce specific  
20 antibodies, immunolocalize the two forms, and assess level and extent of  
21 heteroplasmy. We characterized the expression of three mitochondrially-encoded  
22 proteins in the DUI species *R. philippinarum* in germline and somatic tissues of both  
23 sexes at different developmental stages. The target proteins were: NADH  
24 dehydrogenase 5 (ND5), cytochrome b (CYTB), and cytochrome c oxidase subunit III  
25 (COX3), belonging to different complexes of the electron transport chain (I, III, and IV,  
26 respectively). We produced and tested specific antibodies to discriminate the two forms  
27 (F- and M-type) of each of these three OXPHOS proteins. After specificity verification  
28 using Western blot, we proceeded with immunohistochemistry and *in situ* visualization  
29 of the targets with both confocal and transmission electron microscopy (TEM).  
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39 Some of our results are consistent with the predictions drawn from the data available  
40 in literature—such as homoplasmy in eggs and sperm—but we also present some  
41 unexpected findings that are going to change the current view of the mechanisms  
42 underlying DUI. Indeed, the presence of both F and M protein variants in early germ  
43 cells cannot be explained by the current DUI models, so we propose a revision. Lastly,  
44 we emphasize the potential of this unusual system for future research in the field of  
45 mitochondrial biology.  
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## 52 **Materials and methods**

### 53 **Gonad anatomy and germline development**

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58 In bivalves, the gonad is a transient anatomical structure that consists of a series of  
59 connected tubules organized in sack-like structures named acini, in which germ cells  
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3 differentiate centripetally from the external border—a germinal epithelium defined  
4 “wall”—to the center (the “lumen”), where mature gametes accumulate (Milani, Ghiselli,  
5 Nuzhdin, et al. 2013; Milani et al. 2017, 2018). Acini are usually present at different  
6 stages of maturation, thus containing germ cells undergoing different phases of the  
7 differentiation process. During the maturation period, this tissue develops into a system  
8 of branching tubules connecting multiple acini, that are filled with mature gametes  
9 when the animal is ripe. The system of tubules merges into an excretory duct through  
10 which gametes are spawned; fertilisation is external and gametes are shed through  
11 the exhalant syphon (Gosling 2003).

12  
13 Bivalve reproductive cycle is deeply affected by environmental and trophic conditions  
14 and involves four main stages: 1) a spent phase, that is a period of sexual rest; 2) a  
15 phase of gonad redevelopment/reconstruction; 3) a phase of gonad  
16 ripening/maturation (gametogenesis); and 4) a single or more spawning events  
17 (Gosling 2003). Due to the lack of any secondary sexual characteristics, sex in bivalves  
18 can be assessed only by visualization of a gametic smear under an optical microscope.  
19 During the sexual rest, when there are no gonads/gametes, sex identification is not  
20 possible.

21  
22 In the Adriatic Sea, the spawning season of *R. philippinarum* occurs from late May to  
23 September (mostly between June and the end of August), then the animals enter the  
24 sexual rest until the following Spring. Clams, as *R. philippinarum*, are gonochoric, and  
25 gonads appear as a diffused, whitish mass closely associated to the digestive system.  
26 The specification of germ cells in bivalves takes place through a mechanism named  
27 preformation, by which germline determinants such as RNAs/proteins (e.g.: Vasa,  
28 Nanos, Piwi, Tudor), mitochondria, and mitochondrial components—the “germ  
29 plasm”— are stored in the egg and inherited by the progeny (Extavour and Akam 2003;  
30 Ghiselli et al. 2012; Milani, Ghiselli, Nuzhdin, et al. 2013; Solana 2013). Germ plasm  
31 components have been shown to be expressed in both multipotent cells and germ cells  
32 across diverse taxa, so Juliano et al. (2010) proposed the existence of a highly  
33 conserved germline multipotency program (GMP) operating in both types of cells.  
34 Spiralians show a highly conserved early development program, and studying the  
35 expression patterns of the germline gene set in several taxa, it was possible to trace  
36 the origin of gonads from the 4d mesentoblast, that gives rise to the visceral mesoderm  
37 and endodermal intestine (Fabioux et al. 2004; Henry et al. 2010; Lyons et al. 2012).  
38 In some bivalve species—including *R. philippinarum*—a strong association of the  
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3 intestinal epithelium with cells expressing Vasa was observed (Milani et al. 2017). It  
4 was then proposed that, at the beginning of the reproductive cycle, Primordial Stem  
5 Cells (PriSCs; see Solana 2013)—multipotent cells located in the intestinal  
6 epithelium—give rise to Primordial Germ Cells (PGCs) through an asymmetric cell  
7 division that produces a PriSC and a PGC. Then, PGCs migrate into the connective  
8 tissue originating Germinal Stem Cells (GSCs) that proliferate forming the gonad wall  
9 and gametogenesis begins. Finally, at the end of the reproductive period, gonads are  
10 reabsorbed and the animal enters the sexual rest period until, at the following  
11 reproductive season, the gonads are rebuilt from PriSCs associated with the intestinal  
12 epithelium and GSCs in the connective tissue (Milani et al. 2017, 2018). The scheme  
13 in Figure 1A summarizes the steps of germline development proposed for *R.*  
14 *philippinarum* and Figure 1B place the different germ cell types in an anatomical  
15 context.

### 26 27 **Sampling**

28  
29 Specimens of *R. philippinarum* were collected from the Northern Adriatic Sea, in the  
30 river Po delta region (Sacca di Goro, approximate GPS coordinates: 44°50'06"N,  
31 12°17'55"E). The sampling was performed at different times of the year to get clams at  
32 different stages of sexual maturity. More in detail: 1) 6 adult males and 6 adult females  
33 (shell size of 3-4 cm) were collected in May-June 2016 (ripe gonads); 2) 3 adult males  
34 and 3 adult females (shell size of 3-4 cm), and 5 juveniles (shell size ~1 cm) were  
35 collected in September 2016 (end of reproductive season); 3) 10 adult females were  
36 collected in November 2016 (approaching sexual rest); 4) 6 adults were collected in  
37 January 2016 (sexual rest). The sex of the latter specimens was unknown because of  
38 the absence of gonads, however, they were used for protocol optimization.

39  
40 The sex of the adults was assessed by observation of gonadic tissue smears under an  
41 optical microscope, then pieces of the body containing mostly gonads and portions of  
42 adjacent somatic tissues (e.g.: digestive tube and connective tissue) were collected  
43 and directly processed for immunohistochemistry or stored at -80°C for Western blot  
44 (WB) analysis. In the case of juvenile clams, the entire body was collected due to their  
45 small size and absence of a developed gonad.

### 56 57 **Design of M- and F-specific antibodies**

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3 M- and F-type mitochondrial OXPHOS sequences were downloaded from GenBank,  
4 aligned with ClustalW and compared to find highly divergent regions. We choose the  
5 proteins in which it was possible to find stretches of ~10-20 amino acids showing high  
6 sequence divergence between M and F forms, making such regions the most suitable  
7 to be used as immunogen peptides (Supplementary Fig. 1; Supplementary Table 1).  
8 Three proteins were chosen as best candidates for antibody production: ND5, CYTB,  
9 and COX3. Their sequences were sent to Davids Biotechnologie (Regensburg,  
10 Germany) where synthetic peptides were injected in rabbits for the production of the  
11 F-type antisera and in chicken for M-type antisera. Two different animals were needed  
12 for antibody production to allow the use of different secondary antibodies in order to  
13 perform the simultaneous staining of M and F targets (double staining). Supplementary  
14 Table 1 also reports the expected molecular weight and the concentration range used  
15 for each antibody in WB and immunohistochemistry protocols. The protein molecular  
16 weight expected for each OXPHOS protein was calculated using Compute pI/Mw  
17 (ExPASy) ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)).  
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### 31 **Western blot**

32 We ran WB of male and female gonad homogenates to determine the molecular weight  
33 of the protein bound by our antibodies. However, we could not establish *a priori* the  
34 presence/absence in the gonad of one or both the mitochondrial types (it was actually  
35 an issue we wanted to address), also, the samples contain portions of somatic tissues  
36 intimately wrapped around the gonad. So, the specificity for one OXPHOS protein  
37 type—a single band of the expected molecular weight—was determined by WB,  
38 instead, the sex-variant specificity was established by the immunological double  
39 staining, that localized only the sex-specific mitochondrial type in differentiated  
40 gametes.  
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48 Samples were homogenized using an Ultra Turrax T25 (Janke & Kunkel IKA-  
49 labortechnik) in RIPA buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1%  
50 detergent Tergitol-type NP-40 (nonyl phenoxyethoxyethanol), 0.25% Na-  
51 deoxycholate, 1 mM EDTA and protease inhibitors [1 mM PMSF and 1 mini tablet of  
52 protease inhibitor cocktail (Complete Mini, Roche) (1 mini tablet in 5 mL of buffer)] to  
53 limit the degradation of the sample. Then, samples were centrifuged at 7,500 xg for 10  
54 min at 4°C. The supernatant was collected and stored at -80°C. The amount of total  
55 proteins in the homogenates was quantified with Lowry method (Lowry et al. 1951).  
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3 Proteins were separated via 8.5% and 12% Sodium Dodecyl Sulphate -  
4 PolyAcrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970). Five  $\mu$ L of Bio-Rad  
5 Precision Plus Protein™ Dual Color Standards or Bio-Rad Prestained SDS-PAGE  
6 Standard Broad Range™ were also loaded for reference.  
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9  
10 For immunoblotting, proteins were electrically transferred to nitrocellulose membranes  
11 (Amersham™ Hybond™ Blotting Membranes, Buckinghamshire, UK). Before the  
12 incubation with the primary antibody, to prevent non-specific protein binding, unspecific  
13 sites were blocked with 5% dried skimmed milk (Bio-Rad Laboratories, Hercules, CA,  
14 USA), 3% Bovine Serum Albumin (BSA), in Tris-Buffered Saline (20 mM Tris base;  
15 137 mM NaCl) (pH 7.4) (TBS) with 0.1% Tween-20 (Sigma) (TBS-Tw), for 1 h 30 min  
16 at room temperature (RT), and subsequently washed for 30 min with TBS-Tw at RT.  
17

18 Then the membranes were incubated with primary antibodies overnight at 4°C, then  
19 for 1 h 30 min at RT. After rinsing for 30 min with TBS-Tw, the membranes were  
20 incubated with the secondary antibody for 1 h at RT. Finally, the membranes were  
21 washed for 20 min with TBS-Tw and 10 with TBS.  
22

23 The washed membranes were treated with ECL Western Blotting Detection Reagents  
24 (GE Healthcare) and exposed to Hyperfilm ECL (GE Healthcare). To verify antibody  
25 specificity, the synthetic peptides were added to the primary antibody solution at a 10X  
26 concentration before the incubation. This step was performed in order to chelate by  
27 competition every antigenic site of the primary antibody. In this way it is kept from  
28 binding its protein target and the specific bands is strongly attenuated.  
29

### 30 31 32 33 34 35 36 37 38 39 40 41 **Immunohistochemistry**

42 Immunohistochemistry was performed to localize the antibody reaction in somatic  
43 tissues and gonads of *R. philippinarum* males and females.  
44

45 A piece of the body (visceral mass) containing all the tissues of interest (intestine,  
46 connective and gonadic tissue) was excised and fixed in a solution of 3.7%  
47 paraformaldehyde, 0.1% or 0.25% (depending on sample size) glutaraldehyde in  
48 PIPES buffer 2X (piperazine-N,N'-bis(2-ethanesulfonic acid)) (160 mM K Pipes, 2 mM  
49 MgCl<sub>2</sub>, 10 mM EGTA, 0.4% Triton X-100) (pH 7) for 3 h-3 h 30 min (depending on  
50 sample size) at RT. The samples were washed in Phosphate Buffered Saline (PBS)  
51 (128 mM NaCl, 2 mM KCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>) (pH 7.2) every 10-15 min  
52 for 1 h. Then samples were embedded in 7% agar and sectioned (100-200  $\mu$ m  
53 thickness). The sections were post-fixed with increasing concentrations of methanol  
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3 (50–100%) and rehydrated in PBS or TBS (10 mM Tris–HCl, 155 mM NaCl) (pH 7.4)  
4 for 1 h.

5  
6 Unreacted aldehydes were reduced with 70 mM sodium borohydride (NaBH<sub>4</sub>) in TBS  
7 (pH 7.4) for 1 h 30 min at room temperature, followed by several washes in TBS with  
8 0.1% Triton X-100 (TBS-Tx 0.1%) for about 2 h.

9  
10 Antigen and epitopes were retrieved with 0.01% Pronase E (Merck) in PBS for 18 min  
11 at RT. Sections were rapidly washed with PBS in order to stop digestion, the last wash  
12 was with TBS-Tx 0.1%. The samples were permeabilized adding TBS-Tx 1% (pH 7.4)  
13 and left at 4°C overnight.

14  
15 Non-specific protein-binding sites were blocked with a preincubation buffer; i) for anti-  
16 Rph M antibodies: 10% Normal Goat Serum (NGS) and 1% BSA in TBS-Tx 0.1% (pH  
17 7.4) for 1 h 30 min, and ii) for anti-Rph F antibodies: 10% Normal Donkey Serum (NDS)  
18 and 1% BSA in TBS-Tx 0.1% (pH 7.4) for 1 h 30 min.

19  
20 Primary antibodies were diluted with 3% BSA in TBS-Tx 0.1% (pH 7.4) and section  
21 incubation was carried out for 72 h at 4°C, followed by washes with TBS-Tx 0.1% for  
22 26 h with several changes.

23  
24 The sections were incubated in the dark with secondary antibodies for 32 h at 4°C,  
25 and subsequently washed for 24 h with several changes in TBS-Tx 0.1% (pH 7.4). The  
26 secondary antibody was: i) for anti-Rph M antibodies: DyLight 550 Goat anti-Chicken  
27 IgG (IgY) (H+L), Cross Absorbed (1:500) (ThermoFisher Scientific), and ii) for anti-Rph  
28 F antibodies: Alexa Fluor® 488-AffiniPure Donkey antiRabbit IgG (H+L) Cross  
29 Absorbed (1:400) (Jackson ImmunoResearch). Antibodies were diluted with a dilution  
30 buffer (DI) (1% NGS for antibodies M-type and 1% NDS for antibodies F-type, 1% BSA  
31 in TBS-Tx 0.1%) (pH 7.3) for 1 h 30 min.

32  
33 The primary antibodies that showed the best reaction in the WB (for strength and  
34 specificity of the signal) were also used in a contemporary, double staining, in order to  
35 visualize M and F proteins on the same section.

36  
37 The nuclear counterstaining was performed with 1 mM TO-PRO®-3 nuclear dye (Life  
38 Technologies, Carlsbad, USA) in PBS (pH 7.2) for about 10 min in the dark at RT, then  
39 the dye was washed in TBS-Tx 0.1% (pH 7.4) for 30 min.

40  
41 All the immunostained sections were mounted in anti-fade mounting medium (2.5%  
42 1,4-diazabicyclo[2.2.2]octane, DABCO; Sigma), 50 mM Tris (pH 8) and 90% glycerol.  
43 Negative controls were also carried out: two controls testing secondary antibodies and  
44 a third control without any antibody. Sections were examined with a Leica TCS SL  
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3 confocal laser scanning microscope equipped with Ar/He/Ne lasers, using Leica  
4 confocal software.  
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### 8 **Transmission electron microscopy**

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10 TEM analysis was performed to find the ultrastructural localization of the antibody  
11 staining in specific cell types and organelles.  
12

13 Pieces containing gonadic tissue were excised from 3 males and 3 females and fixed  
14 in 4% paraformaldehyde and 0.5% glutaraldehyde in 0.1 M sodium cacodylate buffer  
15 (pH 7.4). After dehydrating the samples through increasing concentrations of ethanol,  
16 they were embedded into LR White Resin. The blocks were sectioned with an Ultracut  
17 Leica UC6 ultramicrotome (Leica Microsystems, Germany) using a diamond knife. Thin  
18 sections were mounted on formvar-coated nickel grids, and the grids were floated on  
19 drops of 1% BSA/0.01% Tween-20 in PBS for 1 h. This was followed by 1 h incubation  
20 with primary antibodies against *R. philippinarum* mitochondrial proteins. As primary  
21 antibodies, those showing the best performance in the previous analyses (WB and  
22 immunohistochemistry at confocal microscopy) were used (anti-ND5\_F, anti-CYTB\_M,  
23 anti-COX3\_M, anti-COX3\_F antibodies; Supplementary Table 1 for details). All of them  
24 were diluted 1:200 with PBS-0.05% Tween-20 (PBS-0.05% Tw). Next, sections were  
25 washed in PBS-0.05% Tw and incubated for 2 h at room temperature with a PBST  
26 solution containing a mixture of secondary antibody (1:50): 12-nm colloidal gold-  
27 conjugated goat anti-rabbit IgG antibody (111-205-144, Jackson) and 18-nm colloidal  
28 gold-conjugated goat anti-chicken IgG antibody (703-215-155, Jackson). As control,  
29 primary antibodies were omitted, and only secondary antibodies were used. Sections  
30 were washed three times in PBS-0.05% Tw, rinsed in distilled water, stained with  
31 uranyl acetate and lead citrate. Observation and imaging were performed with a Zeiss  
32 Libra 120 transmission electron microscope (Karl Zeiss Group, Germany).  
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## 49 **Results**

### 50 **Immunostaining shows heteroplasmy of OXPHOS proteins at tissue, cell, and** 51 **organelle level**

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53 Throughout all the experiments, the tissues analyzed were gonads with adjacent  
54 somatic tissues (gut, connective tissue, and muscle tissue), and both females and  
55 males were observed at different stages of gametogenesis.  
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3 The bands obtained with WB had a molecular weight comparable to the predicted  
4 protein weight, except in the case of F-type ND5 that resulted lighter than expected  
5 (Fig. 2; Supplementary Table 1). This discrepancy can be either due to a wrong  
6 annotation of the gene, or to post-transcriptional/translational editing of the nd5  
7 sequence. In any case, the ND5 protein seems to have a different weight in the two  
8 sexes.  
9

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12 At the confocal microscopy, the staining was localized in spots of ~ 500 nm, compatible  
13 with the dimension of mitochondria (e.g.: Fig. 3I,J).

14  
15  
16 Antibody specificity for F- and M-type mitochondrial variants was supported by the  
17 homoplasmy detected in differentiated gametes—oocytes positive for the F-type  
18 staining, spermatozoa for the M-type staining—observed at TEM with simultaneous  
19 staining with F- and M-type antibodies.  
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23 In control sections with only secondary antibody treatment, no staining was observed  
24 (Supplementary Fig. 2).  
25  
26

### 27 28 29 **F-type protein variants**

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31 In female samples, anti-F antibody staining was visible in somatic cells of the  
32 connective tissue and of the intestinal epithelium (batiprismatic cells) (Fig. 3O,P) and  
33 in muscle cells (Fig. 3Q). Also, anti-F antibody stained the cytoplasm of  
34 undifferentiated/early germ cells around developing oocytes (Fig. 3E,F insets) and the  
35 cytoplasm of oocytes at different phases of maturation (Fig. 3E,F).  
36  
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38  
39 In male samples, anti-F antibody staining was detected in somatic tissues  
40 (batiprismatic cells Fig. 3K-N, connective tissue Fig. 3K,L, and muscle cells Fig. 3J).

41  
42 A very strong staining was observed in cells with a small nucleus, located in the basal  
43 part of the intestinal epithelium, that can be identified as PriSCs (Fig. 3K-M).  
44 Differentiating male germ cells (spermatocyte and spermatids) showed anti-F staining,  
45 but spermatozoa did not (Fig. 3B,C).  
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### 51 52 **M-type protein variants**

53  
54 In male samples, anti-M staining was observed in batiprismatic cells, in the connective  
55 tissue and muscle cells (Fig. 3G-I). Anti-M staining was observed in male early germ  
56 cells, according to morphology and localization (Fig. 3A,C). Anti-M antibody staining  
57 was present in PriSCs located at the basal side of the gut epithelium, close to the  
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3 connective tissue (Fig. 3G), in PGCs/GSCs inside the connective tissue (Fig. 3H and  
4 inset).

5  
6 In female samples, no significant anti-M staining was observed neither in oocytes (Fig.  
7 3D) nor in somatic cells of the gut and connective tissue (Fig. 3P). Instead, the staining  
8 was observed in cells with a round nucleus localized around acini, these cells  
9 resembling undifferentiated/early germ cells (Fig. 3E,F).

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15 Summarizing, undifferentiated germ cells were positive for both anti-F and anti-M  
16 antibodies both in females (Fig. 3E,F and inset) and males (Fig. 3C).

### 17 18 19 20 **Double staining with anti-F and anti-M antibodies**

21  
22 The co-expression of the two variants was also verified with the contemporary staining  
23 with anti-F and anti-M antibody. The double staining was observed in PriSCs located  
24 at the basal pole of the intestine (Fig. 3K-N) and in PGCs/GSCs in the connective  
25 tissue (for example in Fig. 3H).

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Immuno-transmission electron microscopy highlighted the presence of both the  
mitochondrial protein variants (M+F) in undifferentiated/early germ cells, while  
gametes were homoplasmic (oocytes for the F type, spermatozoa for the M type) (Figs.  
4, 5).

## 61 62 63 64 65 66 67 68 69 70 **Discussion**

### 71 72 73 74 75 76 77 **Level and extent of heteroplasmy**

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In females, both variants were detected in PriSCs, in undifferentiated germ cells, and in early oocytes, but no M-type protein was observed in mature eggs and in the soma (Fig. 3). In males, both labelling were visible in PriSCs, undifferentiated germ cells, and in somatic tissues, but no F-type labelling was observed in spermatozoa (Fig. 3) (a summary of antibody localization in Supplementary Table 2; a scheme of the staining in Supplementary Fig. 3). Moreover, heteroplasmy was observed at the organelle level,



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3 namely both F- and M-type protein variants were present in the same mitochondrion  
4 (Figs. 4, 5).  
5

6 The expression pattern of F-type mitochondrial proteins in germline and somatic  
7 tissues of females is coherent with the results of qPCR analyses on mtDNA (Ghiselli  
8 et al. 2011). According to previous works, DUI females are generally homoplasmic for  
9 the F-type mtDNA (Ghiselli et al. 2011; Milani et al. 2012; Zouros 2013; Guerra et al.  
10 2016), so the consistent M-type staining in the undifferentiated germline lineage was  
11 unexpected. However, we got support to antibody specificity for a single variant by the  
12 immunological double staining on mature gamete ultrastructures (Figs. 4, 5), that  
13 showed only the sex-specific mitochondrial type, in accordance to the mtDNA gamete  
14 homoplasmy resulted from qPCR (Ghiselli et al. 2011).  
15

16 As reported in the literature, *R. philippinarum* males are heteroplasmic in somatic  
17 tissues, but homoplasmic for the M-type in gametes (Ghiselli et al. 2011; Milani,  
18 Ghiselli, Iannello, et al. 2014). In male samples, as expected, M-type antibodies  
19 stained both somatic tissues and germ cells (Fig. 3-5). F-type antibodies in males  
20 confirmed the mitochondrial heteroplasmy also at the protein level in somatic tissues,  
21 since F-type mitochondria were labeled in the intestinal epithelium, in the connective  
22 tissue, and in muscle cells (Fig. 3).  
23

### 24 **Implications for the DUI system**

25 The data obtained in this work allow, for the first time, to visualize the distribution of F-  
26 and M-type OXPHOS subunits, and provide new information about the DUI system,  
27 including some unexpected finding. The most surprising result is the presence of both  
28 F- and M-type proteins in PriSCs, PGCs, GSCs, and oogonia or spermatogonia, in  
29 males and females. This means that also females are heteroplasmic—even if to a  
30 lesser extent with respect to males—and that some of the hypotheses that have been  
31 made about the dynamics of the two mitochondrial lineages need to be revised. The  
32 checkpoint #1, which corresponds to the lineage-specific distribution pattern of M-type  
33 mitochondria in early embryos—dispersed in females, aggregated in males—was  
34 proposed to be the mechanism by which sperm mitochondria reach the germline in  
35 males, but not in females (Ghiselli et al. 2011). The present data show that M-type  
36 mitochondria reach the germline irrespectively from the sex of the individual, so the  
37 hypothesized function of the two distribution patterns needs to be reconsidered. A  
38 second checkpoint consisting in the selective degradation of M-type mitochondria in  
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3 females (analogously to what happens in some SMI organisms) was also proposed.  
4 There was already evidence that checkpoint #2 could be sometimes ineffective, given  
5 the finding of M-type-positive female somatic tissues (Ghiselli et al. 2011), but in the  
6 light of the results presented here, we think that its actual existence should be  
7 reconsidered, as well. Indeed, given the consistent finding of M-type proteins in female  
8 PriSCs and undifferentiated germ cells, either this checkpoint does not exist at all—  
9 meaning that there is no degradation mechanism of sperm mitochondria in this  
10 species—or its action, when successful, is limited to female somatic cells. Instead,  
11 these new results strongly support the existence of checkpoint #3, that is the  
12 segregation of sex-specific mtDNA lineages in mature gametes (F-type in eggs, M-  
13 type in sperm). This checkpoint was originally defined as “a filter for germline  
14 mitochondria, and it may operate when PGCs establish themselves (i.e., whenever  
15 PGCs do separate from somatic cells)” (Ghiselli et al. 2011), but such definition needs  
16 to be updated, because here we show that such filter is operating at a later stage, most  
17 likely during meiosis. In this light, the mechanism of segregation distortion in favor of  
18 F mitochondria in eggs and M mitochondria in sperm can be viewed as a case of  
19 meiotic drive. How is such distortion achieved?

20 Since the dawn of DUI research, one of the primary goals was to identify the genetic  
21 elements involved in the deviation from SMI. Bivalve genomics resources are still  
22 scarce and/or low-quality, but a fair amount of complete mitochondrial genomes of DUI  
23 species has been available for a while; for this reason, research has been focusing on  
24 finding such elements in the F- and M-mtDNAs. MtDNAs of bivalves are generally rich  
25 in unassigned regions (Ghiselli et al. 2013), namely regions of the genome that cannot  
26 be annotated. Several works that analyzed such regions in different species reported  
27 the presence of ORFans (open reading frames having no detectable sequence  
28 similarity to other known proteins; see Fischer and Eisenberg 1999), that became  
29 candidates for having a role in the DUI system (Breton et al. 2009; Breton, Ghiselli, et  
30 al. 2011; Breton, Stewart, et al. 2011; Ghiselli et al. 2013; Milani, Ghiselli, Guerra, et  
31 al. 2013; Milani, Ghiselli, Maurizii, et al. 2014; 2015, 2016; Mitchell et al. 2016). In a  
32 study on *R. philippinarum*, Milani et al. (2015) observed that a M-type-specific ORFan  
33 named RPHM21, is expressed only in a subset of PriSCs, while it is present in all the  
34 spermatozoa. The expression pattern observed for RPHM21 is consistent with that  
35 reported here for three M-type OXPHOS subunits: in males, M-type proteins are either  
36 absent or present together with F-type variants in cells expressing a germline  
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3 multipotency program (Juliano et al. 2010), but they become the only variant present  
4 in post-meiotic cells. A working hypothesis posits that, during spermatogenesis, germ  
5 cells carrying the M-type mtDNA and expressing RPHM21 gain some sort of  
6 advantage over the germ cells not expressing it. In particular, a faster proliferation of  
7 RPHM21-positive germ cells was proposed as a potential underlying mechanism, but  
8 another possibility that we would like to suggest here is that of RPHM21 being a “killer  
9 meiotic driver”, namely a selfish genetic element that can spread through a population  
10 by actively destroying competitors (Bravo Núñez et al. 2018).

11  
12 A mechanism of meiotic drive involving killer ORFs produced by mitochondria or  
13 chloroplasts is well known in numerous species of gynodioecious plants: it is the  
14 Cytoplasmic Male Sterility (CMS; reviewed in Chase 2007). In species with CMS, novel  
15 mitochondrial ORFs produce chimeric proteins that cause male sterility. In rice, for  
16 example, ORF79 protein—which is toxic when expressed in *Escherichia coli*—  
17 accumulates in microspores (spores that will develop into male gametophytes). Two  
18 nuclear genes can restore male fertility by blocking ORF79 via endonucleolytic  
19 cleavage or degradation (Wang et al. 2006).

20  
21 In the future, it would be interesting to look for similarities between the DUI and the  
22 CMS systems—if any—to track down the molecular mechanisms behind DUI. So far  
23 only two bivalve mitochondrial ORFans have been analyzed more in depth, and each  
24 found to actually produce a protein: a F-type specific ORFan in the freshwater mussel  
25 *Venustaconcha ellipsiformis* (Breton, Stewart, et al. 2011), and the above-mentioned  
26 M-type specific RPHM21 in *R. philippinarum* (Milani, Ghiselli, Maurizii, et al. 2014).  
27 Comparative *in silico* analyses found high sequence divergence across bivalve  
28 ORFans, but similar structural features (Milani, Ghiselli, Guerra, et al. 2013; Mitchell et  
29 al. 2016), something that might indicate a shared function. Much more work is needed  
30 to understand the function of these novel mitochondrial proteins and to elucidate the  
31 molecular mechanisms of DUI, but we can advance a new working hypothesis taking  
32 into account the findings reported here.

### 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50 51 52 53 **A revised model for DUI**

54 Gametes are homoplasmic for the respective sex-specific mitochondrial lineage (F-  
55 type in eggs, M-type in sperm), and upon fertilization the embryo is heteroplasmic.  
56 According to the previous model of DUI, such heteroplasmic condition persists only in  
57 males, where the cluster of sperm mitochondria reaches the 4d blastomere and then  
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3 the gonad (checkpoint #1, aggregated pattern). In females, sperm mitochondria are  
4 dispersed (checkpoint #1, dispersed pattern) and degraded (checkpoint #2). The  
5 observations reported here show that M-type mitochondria can reach the cells that  
6 inherit the germ plasm in both males and females. How? In oocytes of several model  
7 animals (see for example: Wilding et al. 2001; Cox and Spradling 2003; Zhang et al.  
8 2008; Zhou et al. 2010; Bilinski et al. 2017), it was reported that mitochondria showing  
9 a high  $\Delta\psi_m$  are preferentially imported into the Balbiani Body, a cytoplasmic structure  
10 that includes the germ plasm (Kloc et al. 2004). Accordingly, Milani (2015) proposed  
11 that sperm mitochondria—if not degraded or excluded from the embryo—can reach  
12 the germ plasm because of their higher  $\Delta\psi_m$ , a mechanism that might explain why M-  
13 type mitochondria invade the germline in DUI organisms.

14  
15 A point that is difficult to explain is the different occurrence and extent of heteroplasmy  
16 between female and male somatic tissues. Females with heteroplasmic soma have  
17 been reported, but they seem to be much rarer than males, for which somatic  
18 heteroplasmy is instead a common condition—with M-type being often dominant in  
19 some male tissues of *R. philippinarum* (Ghiselli et al. 2011). The available data do not  
20 allow us to formulate clear hypotheses, however, it is possible that the different pattern  
21 of mitochondrial segregation plays a role in this.

22  
23 In contrast, we have now fairly more information about what happens next in the  
24 germline: in both the sexes, the switch between heteroplasmy and homoplasmy occurs  
25 during meiosis, and this seems to be the only existing—or the only effective—  
26 checkpoint (checkpoint #3). DUI then appears to be based on a process of meiotic  
27 drive that happens either in both oogenesis and spermatogenesis, or only in  
28 spermatogenesis. In the first case, we would have two drivers—the F-type in  
29 oogenesis and the M-type in spermatogenesis—while in the second case the driver  
30 would be the M-type, and its spread would be successful in spermatogenesis but not  
31 in oogenesis.

32  
33 There are several types of meiotic drive, encompassing a broad range of mechanisms  
34 that can act at different stages of meiosis (Lindholm et al. 2016). Bivalve molluscs such  
35 as *R. philippinarum* and *Mytilus* produce the so-called class II oocytes, in which meiosis  
36 is arrested in prophase I, reinitiated, and secondarily arrested in metaphase I before  
37 the extrusion of the first polar body (Colas and Dubé 1998); meiosis is reactivated and  
38 completed after fertilization. Mitochondria of late oocytes in female acini showed only  
39 the F-type labelling, so if the drive mechanism operates in both the sexes (two-drivers  
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3 hypothesis) and at the same stage of gametogenesis, it is probably acting in prophase  
4 I. Further possibilities are: *i*) the drive occurs in both sexes but with different  
5 mechanisms at different stages of meiosis; *ii*) the drive occurs only in males (one-driver  
6 hypothesis) but the different dynamics of meiosis during oogenesis (e.g.: meiotic  
7 arrest, asymmetry) prevent the M-type-linked driver from being successful in females.  
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### 10 11 12 13 **Mitochondria, germline, and sex**

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15 There is a tight interconnection between mitochondria and germ plasm, and  
16 mitochondria have been shown to be actively involved in germline formation, especially  
17 through the interaction with other elements of the Balbiani body. Not only the Balbiani  
18 body has been proposed as the structure responsible for the selection of germline  
19 mitochondria, but there is evidence of a contribution of mitochondrial material (e.g.:  
20 ribosomes and proteins) to the germ plasm (Kobayashi et al. 1993; Reunov et al. 2000,  
21 2018; Amikura et al. 2001, 2005; Isaeva 2001; Reunov 2004; Ninomiya and Ichinose  
22 2007; Bilinski et al. 2017). Reunov et al. (2019) investigated the activity of germ plasm  
23 and associated structures during the mitosis-to-meiosis shift in *R. philippinarum*, and  
24 observed an interplay between germ plasm and mitochondrial material during the  
25 triggering of meiosis onset in both sexes. More in detail, the Authors found that the  
26 shift from mitosis to meiosis in both females and males involves a phase in which a  
27 VASA-positive substance enters the mitochondria that are disassembled and release  
28 their content in the germ plasm. We think that the drive process is occurring at this  
29 stage (prezygotene-pachytene) or shortly after. During this phase, mitochondrial  
30 elements (ribosomes, F- and M-specific factors such as RPHM21, other ORFans,  
31 RNAs, etc.; Milani et al. 2016; Pozzi et al. 2017) could interact among each other, with  
32 the germ plasm, and with the nucleus, contributing to germline development and  
33 influencing the mitochondrial inheritance process.  
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37 Overall, there is an increasingly large portion of the evolutionary literature advocating  
38 a role for mitochondria in the origin of sex (Lane and Martin 2010; Hadjivasiliou et al.  
39 2013; Radzvilavicius and Blackstone 2015; Garg and Martin 2016), and the strong  
40 interactions between mitochondria and germ plasm observed in a wide range of  
41 organisms is clearly consistent with such view. Given the evident modifications of  
42 mitochondria around germ plasm and nucleus in proximity of the mitosis-to-meiosis  
43 transition, and the clear contribution of mitochondrially-derived material in germline  
44 development, it is tempting to speculate that broken mitochondrial membranes and  
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3 mitochondrial content may be necessary to activate meiosis. Taking the speculation  
4 even further, we might hypothesize that mitochondria, in some conditions, can trigger  
5 either spermatogenesis or oogenesis, thus being responsible of sexual differentiation.  
6 This might seem an outrageous conjecture, but it is worth noting that some  $\alpha$ -  
7 proteobacteria—the closest living relatives of mitochondria—can distort the sex ratio  
8 of their host.  
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### 15 **Evolutionary significance of DUI**

16 The biological function, if any, and the evolutionary significance of DUI are still  
17 unknown: it is not clear whether the tight linkage between the type of mtDNA inherited  
18 and the sex of the individual transmitting it is coincidental or causal. In the first case,  
19 sex and mitochondrial type would be just associated, meaning that the driver(s)  
20 evolved the ability to spread in a specific meiotic environment  
21 (spermatogenesis/oogenesis), but no specific functions or adaptive roles at the level  
22 of the whole individual are responsible for the origin of the system. After its origin, DUI  
23 might have been maintained for such an extensive evolutionary time (hundreds of  
24 millions of years) because of some advantageous “side-effects”, like as having both  
25 mtDNAs under selection; indeed, DUI is the only known biological system in which a  
26 mtDNA can be under selection for male functions (Ghiselli et al. 2013; Milani and  
27 Ghiselli 2015; Milani et al. 2016; Skibinski et al. 2017). In the second case,  
28 mitochondrial type could be causally linked to sex, namely mitochondria could be  
29 involved in the process of sexual differentiation. If we consider that there is no sexual  
30 dimorphism in clams and that the difference between females and males lies in the  
31 type of gametogenesis carried out by the animal, the above-hypothesized manipulation  
32 of meiosis by a mitochondrial driver would actually become a sex-differentiation  
33 system. Needless to say, this is an intriguing, yet speculative, possibility.  
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### 50 **Broader implications and future perspectives**

51 After 25 years since its discovery, and despite 115+ papers published by ~20 different  
52 research groups around the world, DUI is still mostly overlooked, or considered just a  
53 curious feature limited to a few species, a transient phenomenon, or both. We argue  
54 that this could not be farther from the truth: the DUI system is evolutionarily stable (e.g.:  
55 it dates back 200+ million years in Unionids; see Breton, Ghiselli, et al. 2011) and its  
56 distribution across bivalve species is consistent (100+ species reported so far). Most  
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3 importantly, it represents a precious system to study several aspects of mitochondrial  
4 biology and evolution, thanks to its unique features. Of course, the condition of natural  
5 heteroplasmy with highly divergent mtDNAs opens up the possibility for performing  
6 observations and experiments that would not be possible in other organisms, or that  
7 would require very expensive manipulation techniques and/or breeding programs.  
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10 In the last few years, mitonuclear interactions have gained a great deal of attention in  
11 the field of life science, and the central role of mitochondria in almost every aspect of  
12 eukaryote life has been increasingly acknowledged. The DUI system can be extremely  
13 helpful in studying mitonuclear interactions and mitonuclear coevolution, and one of  
14 the first questions that come to mind is: “How can the nuclear genome deal with two  
15 very different mtDNAs?”. We still do not know. Sex-specific alleles/paralogs/splice  
16 forms interacting each with the corresponding mtDNA? Protein structure robustness  
17 (i.e.: high divergence at the mtDNA level but very similar protein structures)? Relaxed  
18 mitonuclear match due to lower metabolic needs and/or physiological adaptations to  
19 hypoxia/anoxia? Actually, since bivalve molluscs have anaerobically functioning  
20 mitochondria—namely facultatively anaerobic mitochondria (see class 2 mitochondria  
21 in Müller et al. 2012)—and because of their adaptations to infaunal and intertidal  
22 environment (Sokolova 2018), it is possible that their tolerance to mitochondrial  
23 heteroplasmy is much higher in respect with other animals. Another interesting and  
24 difficult topic that DUI could help unveiling is fission-fusion dynamics, again thanks to  
25 natural heteroplasmy with highly divergent variants.  
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29 A research area that could benefit even more from exploiting DUI as model system is  
30 that of germline development. The features discussed in this work—interactions of  
31 mitochondria with germ plasm and Balbiani body—are evidently widespread across a  
32 large range of biodiversity, and this means that the study of DUI animals can help to  
33 draw more general conclusions by means of comparative approaches.  
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### 39 **Author contributions**

40 L.M. conceived the study.

41 M.G.M., H.A., C.C., A.P., A.R., Y.A., S.B., and L.M. performed the experiments.

42 F.G., M.G.M., A.R., and L.M. analyzed the data.

43 F.G., M.G.M., and L.M. wrote the manuscript.

44 H.A., C.C., M.P., and V.F. revised the manuscript.

45 All authors approved the final version of the manuscript.  
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## Acknowledgements

We gratefully thank Justin Havird and Geoffrey Hill for organizing the Symposium "Beyond the powerhouse: integrating mitonuclear evolution, physiology, and theory in comparative biology", and all the participants for their interesting and exciting contributions. We also thank two anonymous Reviewers for their valuable contribution to the improvement of the manuscript.

## Funding

This work was supported by the Italian Ministry of Education, University and Research (MIUR) SIR Programme grant No. RBSI14G0P5 funded to L.M., MIUR FIR2013 Programme grant No. RBFR13T97A funded to F.G., "Ricerca Fondamentale Orientata" (RFO) funding from the University of Bologna to F.G., M.G.M., L.M., and the Canziani bequest funded to F.G. and M.P.

Travel and support funds for attending the 2019 meeting of the Society for Integrative and Comparative Biology came from the National Science Foundation (IOS-1839203), the Company of Biologists (EA1694), The Crustacean Society, and the Divisions of Comparative Physiology and Biochemistry, Invertebrate Zoology, and Phylogenetics and Comparative Biology.

## Figure captions

**Fig. 1. Germline formation in *R. philippinarum*.** **A:** The scheme represents the suggested process of germline differentiation based on the results previously obtained on the species (Milani et al. 2017, 2018). Primordial Stem Cells (PriSCs); Primordial Germ Cells (PGCs); Germinal Stem Cells (GSCs). **B:** Histological view of a female sample with indication about the localization of germ cell types. Hematoxylin and eosin staining following the method in Bettini et al. (2012).

**Fig. 2. Western blot.** Western Blot of female and male gonadic extracts using anti-F and anti-M-type antibodies, respectively. Numbers indicate the molecular weight in kilodaltons of the adjacent band. Molecular weight standard lanes (St). The standards used are: Bio-Rad Precision Plus Protein™ Dual Color Standards (with a band of 75 kDa, pink) or Bio-Rad Prestained SDS-PAGE Standard Broad Range™. The bands



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3 detected with the three Ab-F (**A**) and the three Ab-M (**B**) are indicated by an arrowhead.  
4 A clear reduction of these F and M bands is visible in WB in which the anti-F-type (**C**)  
5 and the anti-M-type (**D**) antibodies are utilized after incubation with the corresponding  
6 synthetic peptide at a concentration of at least 10X compared to that of the primary  
7 antibodies. **E,F**) Example of WB optimization obtained for a couple of antibodies used  
8 in the double staining. F-ND5 lane, F-type antibody on female homogenate (RIPA  
9 buffer Ab1 1: 5,000). M-CYTB lane, M-type antibody on male homogenate (RIPA  
10 buffer; Ab1 1: 500 in 1% dried skimmed milk). Other details for antibody production  
11 and usage are reported in Suppl. Fig. 1 and Suppl. Table 1.  
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21 **Fig. 3. Immunolocalization of M- and F-type mitochondrial proteins in germ cells**  
22 **(A-F) and in somatic tissues (G-Q) of *R. philippinarum* at confocal microscope.**

23 Colors: anti-M-type, red; anti-F-type, yellow; TO-PRO®-3 nuclear dye, green.

24 Abbreviations in alphabetical order: batiprismatic cells of the intestinal epithelium, bc;  
25 basal membrane, bm; cytoplasm, c; connective tissue cells, ct; intestinal lumen, il;  
26 muscle cells, mc; nucleus, n; spermatozoa, s; spermatocytes, sc; spermatogonia, sg.  
27 PriSCs indicated by arrowhead. Differentiating germ cells show in the cytoplasm  
28 mitochondrial-size spots stained with one or the other type of antibody. **A-C: Male**  
29 **germ cells. A:** Anti-M antibody staining. In sc and sg. **B:** Anti-F antibody. In male germ  
30 cells. **C:** Anti-M/-F antibody double staining. In male germ cells. **D-F: Female germ**  
31 **cells. D:** Anti-M antibody staining. No staining is observed in late oocytes (one is  
32 circled in dotted line; chromosomes are visible at the periphery of n). **E, F:** Anti-M/-F  
33 antibody double staining. Only F-type mitochondria are visible in late oocytes. Both  
34 anti-M and anti-F stained spots are present in early germ cells at the periphery of the  
35 acini (early germ cells magnified in the insets). **G-N: Male soma. G-I:** Anti-M antibody  
36 staining. **G:** In ct, in bc (top right inset) and in putative PriSCs (lower left inset). **H:** Low  
37 labelling in ct and strong labelling in PriSCs (inset). **I:** In some males, mc are strongly  
38 labelled with M-type antibody. **J:** Anti-F antibody. In other males, mc are F-type  
39 antibody labelled. **K-N:** Anti-M/-F antibody double staining. **K, L:** F-type mitochondria  
40 in bc; low F-type staining in ct. At higher magnification, a clear double staining is visible  
41 in PriSCs located close to bm (insets). **M:** Two PriSCs (circled) near bm with a  
42 prevalence of yellow (on the left) or red spots (on the right). **N:** A prevalent F-type  
43 staining is visible in bc, while some PriSCs (one shown) show a prevalence of M-type  
44 staining. **O-Q: Female somatic tissues. O:** Anti-F antibody. Present in ct and bc. **P,**  
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3 **Q:** Anti-M/-F antibody double staining. **P:** Only F-type mitochondria in ct and bc. **Q:**  
4 Only F-type mitochondria in mc. See also Suppl. Figs. 2,3 and Suppl. Table 2.  
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8 **Fig. 4. Female germline of *R. philippinarum* with double immunostaining at TEM.**

9 **A,B** and **E,F:** anti-F\_ND5 (12 nm) and anti-M\_CYTB (18 nm). **C,D** and **G,H:** anti-  
10 F\_COX3 (12 nm) and anti-M\_COX3 (18 nm). Early germ cells (**A,C**) have mitochondria  
11 with both types of labelling (F- and M-type); **B,D:** magnification of the mitochondria  
12 in the white boxes in **A** and **C**, respectively (12 nm and 18 nm dots, arrow and arrowhead,  
13 respectively). Late oocytes (portions in **E,G**) have mitochondria with only F-type  
14 labelling (12 nm) (**F,H:** magnification of the mitochondria in the white boxes in **E** and  
15 **G**, respectively). Nuclei, n. Scale bars: left column 2  $\mu\text{m}$ ; right column 0.2  $\mu\text{m}$ .  
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24 **Fig. 5. Male germline of *R. philippinarum* with double immunostaining at TEM.**

25 **A,B** and **F:** anti-F\_ND5 (12 nm) and anti-M\_CYTB (18 nm). **C,D** and **G:** anti-F\_COX3  
26 (12 nm) and anti-M\_COX3 (18 nm). Early germ cells (**A,C**) have mitochondria with both  
27 types of labelling (F- and M-type); **B,D:** magnification of the mitochondria in the white  
28 boxes in **A** and **C**, respectively) (12 nm and 18 nm dots, arrow and arrowhead,  
29 respectively). Spermatozoa (**E**) have mitochondria (m) with only M-type labelling (18  
30 nm), magnified in (**F,G**). Nuclei, n. Scale bars: left column 2  $\mu\text{m}$ ; right column 0.2  $\mu\text{m}$ .  
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38 **Supplementary Fig. 1. Alignments of F- and M-type of mitochondrial proteins**  
39 **(ND5, CYTB and COX3)**  
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43 **Supplementary Fig. 2. Control sections for the immunological analyses in which**  
44 **the primary antibodies were omitted (-Ab1)**  
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48 **Supplementary Fig. 3. Schematic representation of mitochondrial segregation**  
49 **during germline differentiation in the DUI species *R. philippinarum***  
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53 **Supplementary Table 1. Characteristics of primary antibodies and OXPHOS**  
54 **proteins**  
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58 **Supplementary Table 2. Summary of the localization of anti-OXPHOS staining in**  
59 **female and male tissues**  
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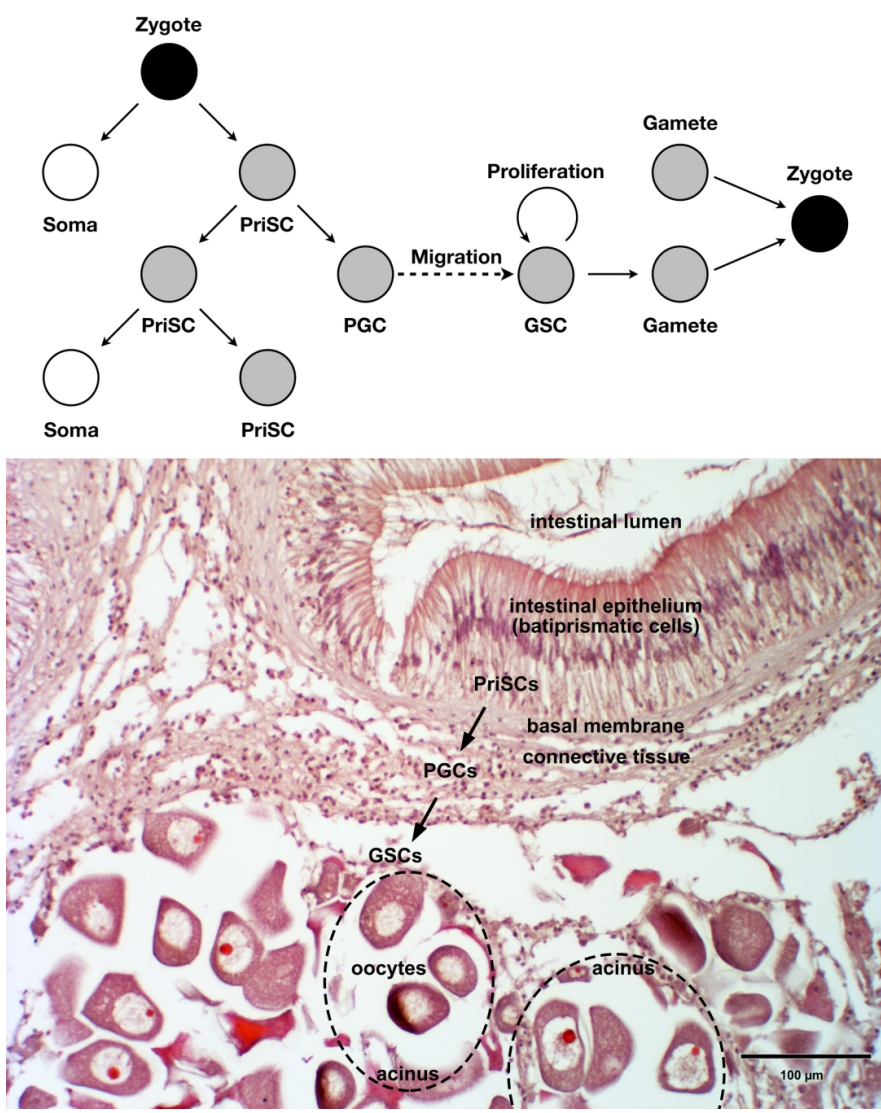


Fig. 1. Germline formation in *R. philippinarum*. A: The scheme represents the suggested process of germline differentiation based on the results previously obtained on the species (Milani et al. 2017, 2018). Primordial Stem Cells (PriSCs); Primordial Germ Cells (PGCs); Germinal Stem Cells (GSCs). B: Histological view of a female sample with indication about the localization of germ cell types. Hematoxylin and eosin staining following the method in Bettini et al. (2012).

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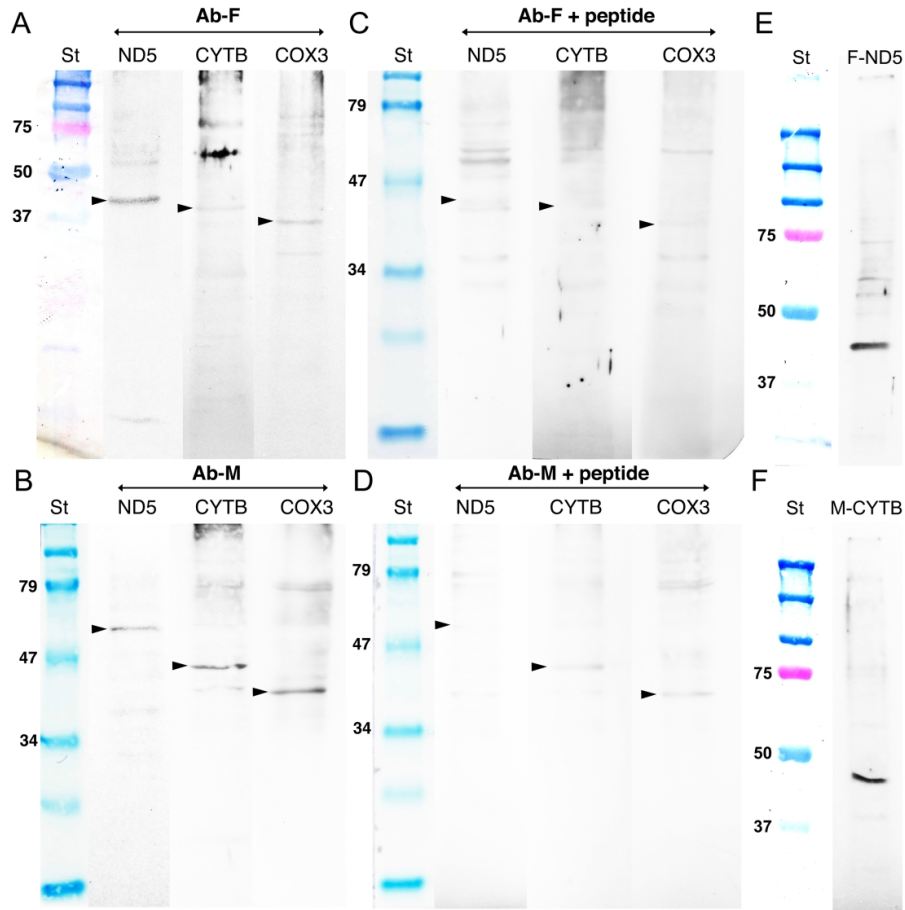


Fig. 2. Western blot. Western Blot of female and male gonadic extracts using anti-F and anti-M-type antibodies, respectively. Numbers indicate the molecular weight in kilodaltons of the adjacent band. Molecular weight standard lanes (St). The standards used are: Bio-Rad Precision Plus Protein™ Dual Color Standards (with a band of 75 kDa, pink) or Bio-Rad Prestained SDS-PAGE Standard Broad Range™. The bands detected with the three Ab-F (A) and the three Ab-M (B) are indicated by an arrowhead. A clear reduction of these F and M bands is visible in WB in which the anti-F-type (C) and the anti-M-type (D) antibodies are utilized after incubation with the corresponding synthetic peptide at a concentration of at least 10X compared to that of the primary antibodies. E,F) Example of WB optimization obtained for a couple of antibodies used in the double staining. F-ND5 lane, F-type antibody on female homogenate (RIPA buffer Ab1 1: 5,000). M-CYTB lane, M-type antibody on male homogenate (RIPA buffer; Ab1 1: 500 in 1% dried skimmed milk). Other details for antibody production and usage are reported in Suppl. Fig. 1 and Suppl. Table 1.

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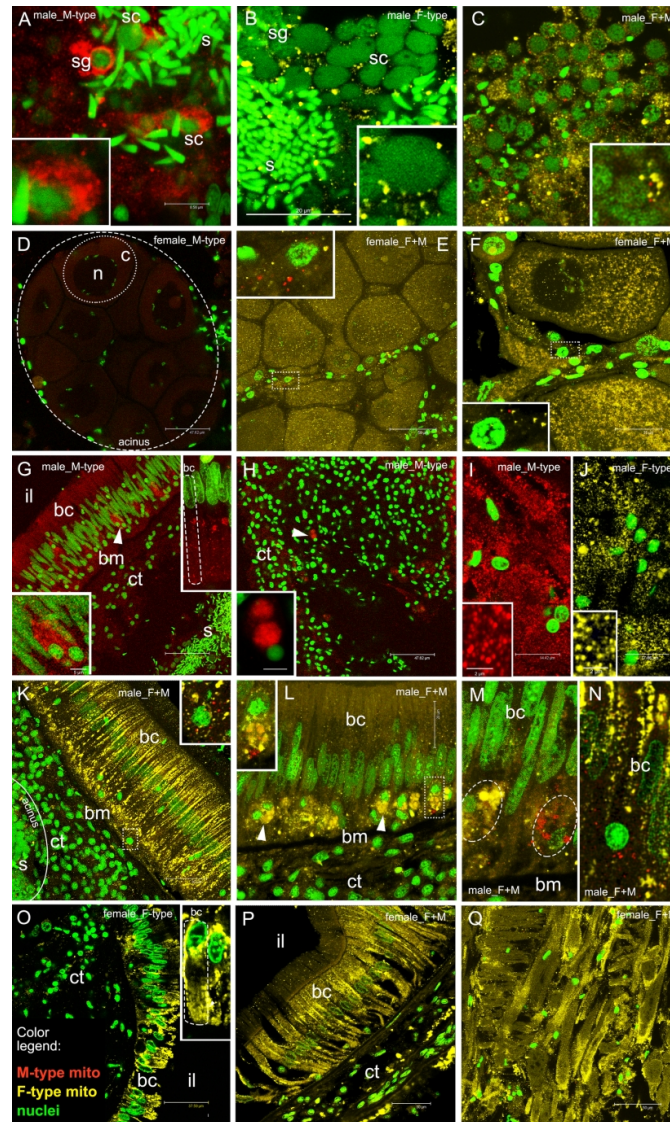


Fig. 3. Immunolocalization of M- and F-type mitochondrial proteins in germ cells (A-F) and in somatic tissues (G-Q) of *R. philippinarum* at confocal microscope.

Colors: anti-M-type, red; anti-F-type, yellow; TO-PRO®-3 nuclear dye, green.

Abbreviations in alphabetical order: batiprismatic cells of the intestinal epithelium, bc; basal membrane, bm; cytoplasm, c; connective tissue cells, ct; intestinal lumen, il; muscle cells, mc; nucleus, n; spermatozoa, s; spermatocytes, sc; spermatogonia, sg. PriSCs indicated by arrowhead. Differentiating germ cells show in the cytoplasm mitochondrial-size spots stained with one or the other type of antibody. A-C: Male germ cells. A: Anti-M antibody staining. In sc and sg. B: Anti-F antibody. In male germ cells. C: Anti-M/-F antibody double staining. In male germ cells. D-F: Female germ cells. D: Anti-M antibody staining. No staining is observed in late oocytes (one is circled in dotted line; chromosomes are visible at the periphery of n). E, F: Anti-M/-F antibody double staining. Only F-type mitochondria are visible in late oocytes. Both anti-M and anti-F stained spots are present in early germ cells at the periphery of the acini (early germ cells magnified in the insets). G-N: Male soma. G-I: Anti-M antibody staining. G: In ct, in bc (top right inset) and in putative PriSCs (lower left inset). H: Low labelling in ct and strong labelling in PriSCs (inset). I: In some males, mc



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3 are strongly labelled with M-type antibody. J: Anti-F antibody. In other males, mc are F-type antibody  
4 labelled. K-N: Anti-M/-F antibody double staining. K, L: F-type mitochondria in bc; low F-type staining in ct.  
5 At higher magnification, a clear double staining is visible in PriSCs located close to bm (insets). M: Two  
6 PriSCs (circled) near bm with a prevalence of yellow (on the left) or red spots (on the right). N: A prevalent  
7 F-type staining is visible in bc, while some PriSCs (one shown) show a prevalence of M-type staining. O-Q:  
8 Female somatic tissues. O: Anti-F antibody. Present in ct and bc. P, Q: Anti-M/-F antibody double staining.  
9 P: Only F-type mitochondria in ct and bc. Q: Only F-type mitochondria in mc. See also Suppl. Figs. 2,3 and  
10 Suppl. Table 2.

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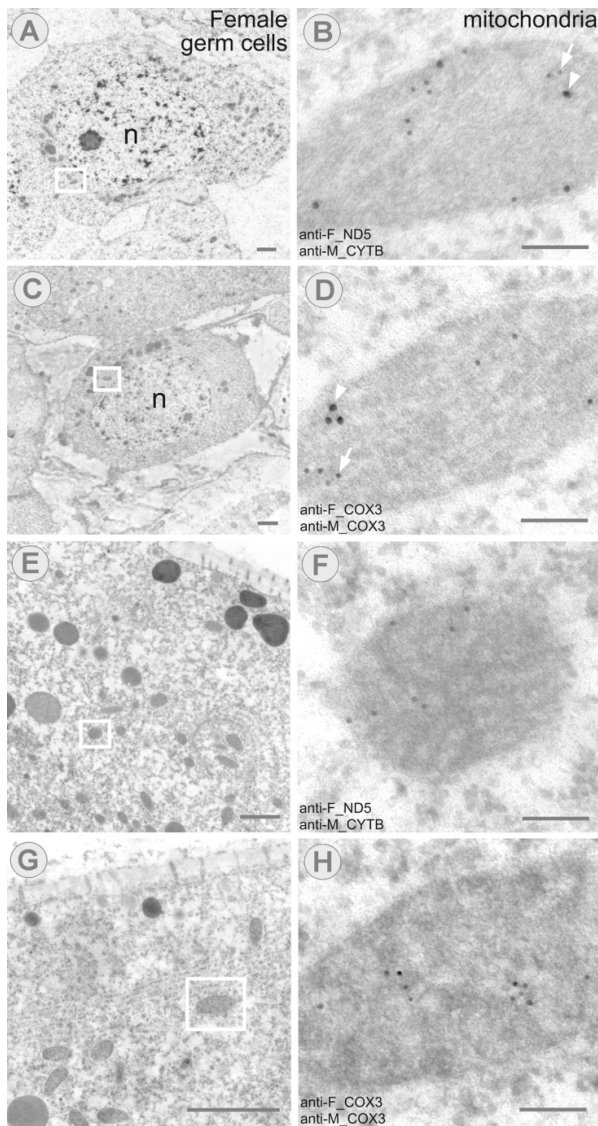


Fig. 4. Female germline of *R. philippinarum* with double immunostaining at TEM. A,B and E,F: anti-F\_ND5 (12 nm) and anti-M\_CYTB (18 nm). C,D and G,H: anti-F\_COX3 (12 nm) and anti-M\_COX3 (18 nm). Early germ cells (A,C) have mitochondria with both types of labelling (F- and M-type); B,D: magnification of the mitochondria in the white boxes in A and C, respectively (12 nm and 18 nm dots, arrow and arrowhead, respectively). Late oocytes (portions in E,G) have mitochondria with only F-type labelling (12 nm) (F,H: magnification of the mitochondria in the white boxes in E and G, respectively). Nuclei, n. Scale bars: left column 2  $\mu$ m; right column 0.2  $\mu$ m.

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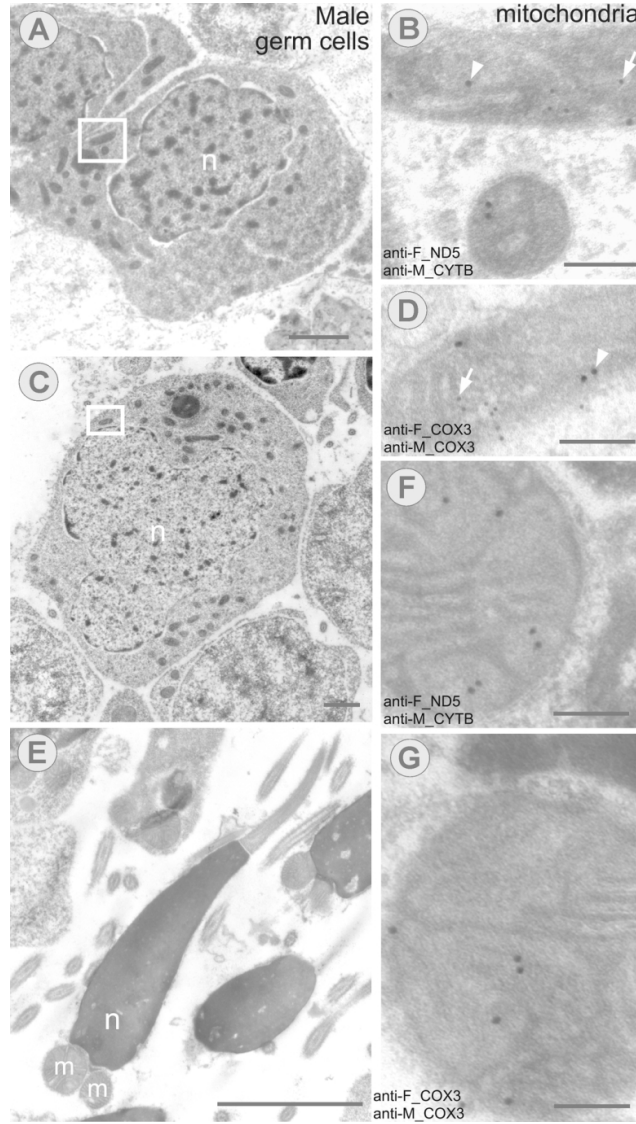


Fig. 5. Male germline of *R. philippinarum* with double immunostaining at TEM. A,B and F: anti-F\_ND5 (12 nm) and anti-M\_CYTB (18 nm). C,D and G: anti-F\_COX3 (12 nm) and anti-M\_COX3 (18 nm). Early germ cells (A,C) have mitochondria with both types of labelling (F- and M-type); B,D: magnification of the mitochondria in the white boxes in A and C, respectively) (12 nm and 18 nm dots, arrow and arrowhead, respectively). Spermatozoa (E) have mitochondria (m) with only M-type labelling (18 nm), magnified in (F,G). Nuclei, n. Scale bars: left column 2  $\mu$ m; right column 0.2  $\mu$ m.

88x157mm (300 x 300 DPI)

**Supplementary Table 1. Characteristics of primary antibodies and OXPHOS proteins**

Expected reactivity (*in silico* calculation) of the antibodies and the dilutions used in Western blot and immunohistochemistry. OXPHOS protein expected and obtained molecular weight.

Expected reactivity ( <i>in silico</i> calculation)					
Protein	Peptide	Antigenicity	Solubility	Epitope	double immuno
	n rabbit				
Rph F ND5	LWQSGVKNMFMGMSNKVLK	medium	medium	medium	X
Rph F CYTB	YNVVSITSGFSSHSLK	medium	low	medium	
Rph F COX3	FFHSEMGMVPLLMLLS	low	low	medium	X
Protein	Peptide	Antigenicity	Solubility	Epitope	
	n chicken				
Rph M ND5	FIGSKVINSISMVFRNKIWE	low	medium	medium	
Rph M CYTB	LWEKVSKNVEKKVEK	good	good	good	X
Rph M COX3	YFSIFILVHSIIS	low	low	medium	X
Concentration in Western Blot and Immunohistochemistry					
Protein	Peptide	Western Blot	WB optimized (*)	Immuno	Immuno opt
	n rabbit				
Rph F ND5	LWQSGVKNMFMGMSNKVLK	1:100 – 1:100000	1:200-5,000(+2%dsm)	1:20 – 1:10000	1:200
Rph F CYTB	YNVVSITSGFSSHSLK	1:100 – 1:100000	1:200+2%dsm	1:20 – 1:10000	1:30
Rph F COX3	FFHSEMGMVPLLMLLS	1:100 – 1:100000	1:200+2%dsm	1:20 – 1:10000	1:100
Protein	Peptide	Western Blot	WB optimized (*)	Immuno	Immuno opt
	n chicken				
Rph M ND5	FIGSKVINSISMVFRNKIWE	1:100 – 1:100000	1:250+1%dsm	1:20 – 1:10000	1:200
Rph M CYTB	LWEKVSKNVEKKVEK	1:100 – 1:100000	1:500+1%dsm	1:20 – 1:10000	1:300
Rph M COX3	YFSIFILVHSIIS	1:100 – 1:100000	1:250+1%dsm	1:20 – 1:10000	1:150
OXPHOS protein molecular weight					
Protein	length AA	kDa calculated (**)	kDa obtained (WB)		
Rph F ND5	542	60.47 - 60.46	~45		
Rph F CYTB	416	46.57 - 46.56	~44		
Rph F COX3	292	34.04 - 34.03	~37		
Protein	length AA	kDa calculated (**)	kDa obtained (WB)		
Rph M ND5	563	62.78 - 62.78	~62		
Rph M CYTB	441	49.61 - 49.60	~46		
Rph M COX3	298	34.57 - 34.56	~40		

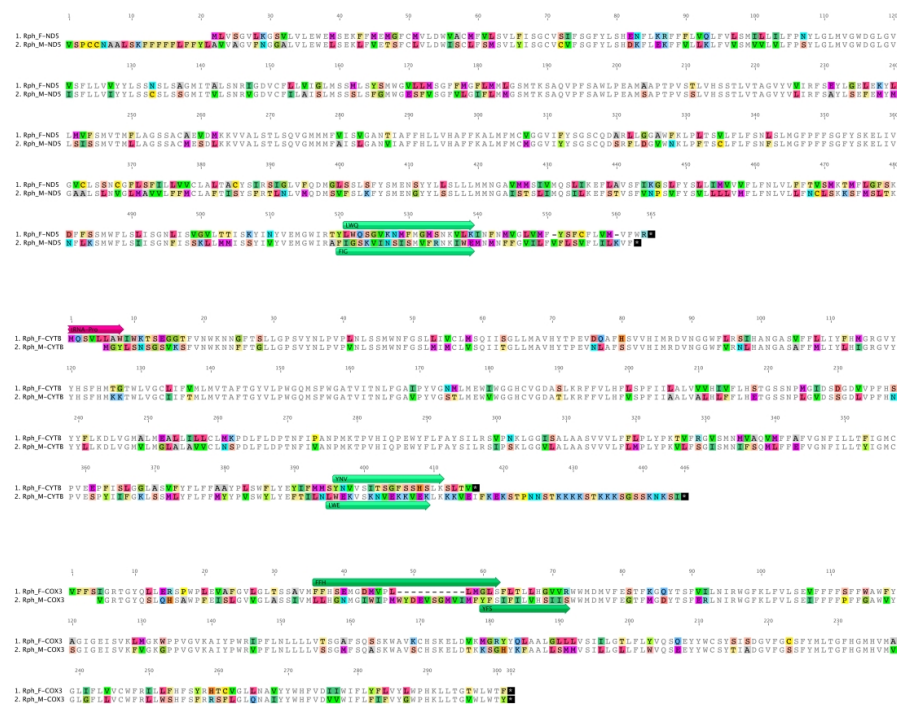
Note: (\*) for 30-40 µg; (\*\*) [http://www.bioformat.cs.org/sms/prot\\_mw.htm](http://www.bioformat.cs.org/sms/prot_mw.htm) - [http://web.expasy.org/compute\\_p/](http://web.expasy.org/compute_p/); length AA: from GenBank annotation; dsm = dried skimmed milk.



Supplementary Table 2. Summary of the localization of anti-OXPHOS staining in female and male tissues

	undifferentiated/ early germ cells	oocytes	spermatozoa	batiprismatic cells (gut)	connective tissue	muscle cells	PriSCs	PGCs/GSCs
<b>F-TYPE</b>								
<i>Anti-F_ND5</i>								
females	X	X		X	X		X	X
males				X	X			
<i>Anti-F_CYTB</i>								
females	X	X			X		X	X
males	X			X	X	X	X	X
<i>Anti-F_COX3</i>								
females	X		X		X		X	X
males	X					X		
<b>M-TYPE</b>								
<i>Anti-M_ND5</i>								
females	X						X	X
males	X		X					
<i>Anti-M_CYTB</i>								
females	X						X	X
males	X		X	X			X	
<i>Anti-M_COX3</i>								
females	X						X	X
males	X		X	X	X	X	X	X

Supplementary Fig. 1.  
Alignments of F- and M-type of mitochondrial proteins (ND5, CYTB and COX3).  
The peptides used for immunization and antibody production are in green.  
The "disagreements" in amino acids between the two aligned sequences are in colors.



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**Supplementary Fig. 2.**

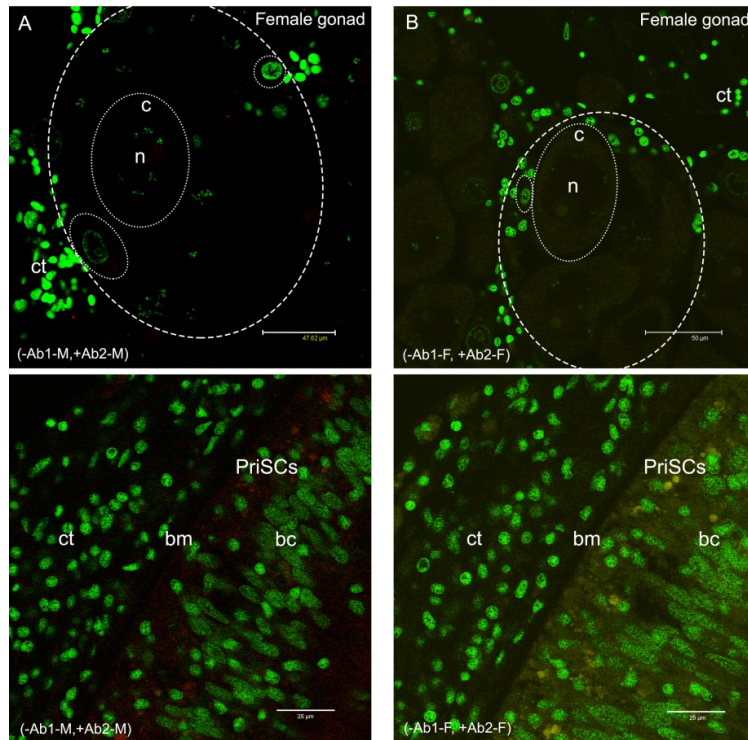
**Control sections for the immunological analyses in which the primary antibodies were omitted (-Ab1).**

**A:** Female section of a portion of gonadic and connective tissues with only secondary anti-M antibody (DyLight 550). No staining is present in oocytes at different stages of differentiation (dotted circles) (c: cytoplasm; n: nucleus) inside an acinus (dashed oval) and in the somatic cells of the connective tissue (ct; lower left).

**B:** Female section of a portion of gonadic and connective tissues (ct) with secondary anti-F antibody (Alexa Fluor 488). No staining is present in late and early oocytes (dotted circles) inside an acinus (dashed oval) and in the cells localized at the acinus wall.

**C-D:** Female sections of a portion of intestinal epithelium (batiprismatic cells, bc) and adjacent connective tissue (ct) with anti-M secondary antibody (DyLight 550) (C), and anti-F secondary antibody (Alexa Fluor 488) (D). With both the antibodies, no staining is evident in batiprismatic cells and in the connective cells. The slight marking observed in Primordial Stem Cells (PriSCs) located in the intestinal epithelium near the basal membrane (bm) can be considered not significant if compared to that obtained with the corresponding primary antibodies (see the main text).

Red: Ab2 M; yellow: Ab2 F; green: TO-PRO®-3 nuclear dye.



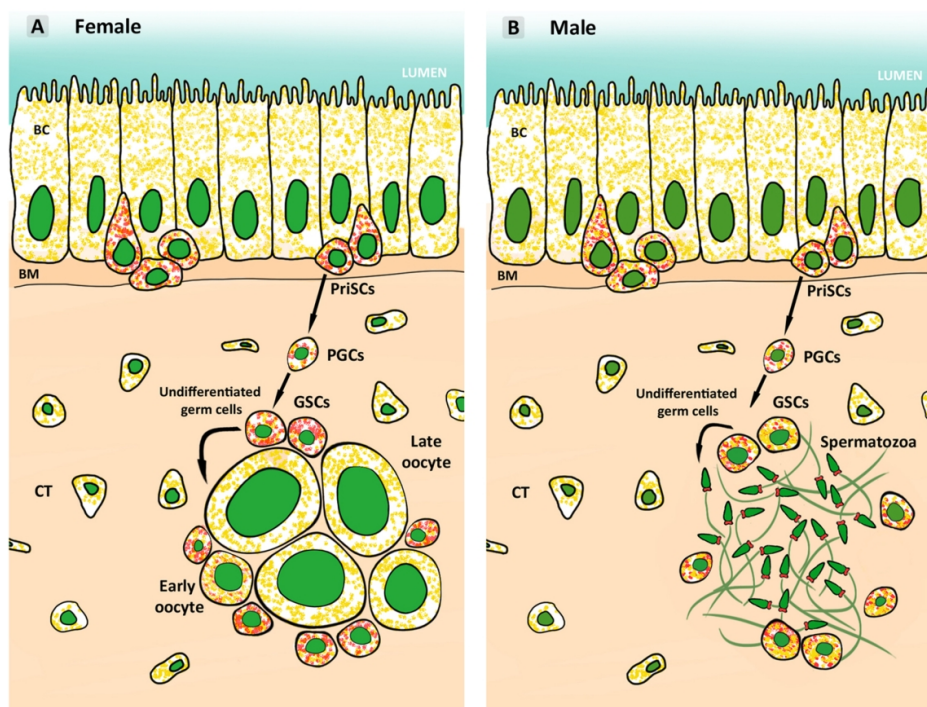
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## Supplementary Fig. 3.

**Schematic representation of mitochondrial segregation during germline differentiation in the DUI species *R. philippinarum*.**

Simplification of the hypothesized process leading to homoplasmic gametes. While Primordial Stem Cells (PriSCs) appear to contain both the types of mitochondria (F and M), during germ cell differentiation there is the loss of the non sex-specific mitochondrial type. Primordial Germ Cells (PGCs); Germinal Stem Cells (GSCs); batiprismatic cells of the intestinal epithelium (BC); basal membrane (BM).

Red: M-type mitochondria; yellow: F-type mitochondria; green: nuclei.



170x167mm (300 x 300 DPI)