2014

Mitochondrial Replication from Embryogenesis to Early Adulthood, in DUI Species, Mytilus Galloprovincialis

Ellen Blenn Richardson
University of South Carolina - Columbia

MITOCHONDRIAL REPLICATION FROM EMBRYOGENESIS TO EARLY ADULTHOOD, IN DUI SPECIES, *MYTILUS GALLOPROVINCIALIS*

by

Ellen Richardson

Bachelor of Science
University of South Carolina, 2011

Submitted in Partial Fulfillment of the Requirements

For the Degree of Master of Science in

Biological Sciences

College of Arts and Sciences

University of South Carolina

2014

Accepted by:

Richard Showman, Director of Thesis

Thomas J. Hilbish, Reader

David Wethey, Reader

Lacy Ford, Vice Provost and Dean of Graduate Studies

ABSTRACT

Eukaryotes typically inherit mitochondria strictly maternally. There are however a group of bivalve molluscs that inherit different mitochondrial genomes from each parent. The paternally inherited mtDNA (M-type) is localized to, and dominates over the maternally inherited mtDNA (F-type) in the gonads of the male offspring, but is not normally retained in any tissue of the female offspring. This process is termed Double Uniparental Inheritance (DUI). Using quantitative PCR (qPCR), this study examines mtDNA replication compared to total DNA replication through embryonic stages of development and into early adulthood of the DUI species, *Mytilus galloprovincialis*. Results indicate that up through the early veliger stage of development, there is little replication of mtDNA. Between the early veliger and pediveliger stages, mtDNA replication increases dramatically, and then continues to replicate between pediveliger and 1mm spat. Rate of mtDNA replication slows after the pediveliger stage. This study also shows that mtDNA replication is not coupled to nuclear DNA replication, and that though replication of mtDNA increases between early veliger and pediveliger stages, nuclear DNA is replicating at a faster rate.

TABLE OF CONTENTS

ABSTRACT iii

LIST OF FIGURES v

CHAPTER 1: INTRODUCTION 1

CHAPTER 2: METHODS 8

2.1 SPAWNING OF MUSSELS 8

2.1 DNA EXTRACTION 9

2.1 QPCR 9

2.1 QUANTIFICATION OF TOTAL DNA 11

CHAPTER 3: RESULTS 13

CHAPTER 4: DISCUSSION 21

REFERENCES 25

LIST OF FIGURES

Figure 3.1 qPCR of Total mtDNA During Embryonic Stages of Development 16

Figure 3.2 Total mtDNA Replication from Trochophore to Spat.....16

Figure 3.3.Total DNA Content of Embryos.....18

CHAPTER 1

INTRODUCTION

Mitochondrial DNA (mtDNA) exists as a circular DNA structure with a genome distinct from that found in the nucleus. The mitochondrial genome as a whole is much smaller than the nuclear genome, consisting of approximately 15-20kb (Boore et al., 1999). Despite the rapid evolution of the mitochondrial genome, its gene content has been relatively conserved across animal species (Boore et al., 1997). In general, the mitochondrial genome codes for the proteins necessary for the subunits of the electron transport chain, rRNAs, and the tRNAs necessary for translation of those proteins, for a total of 37 genes (Boore et al., 1999). The majority of components found in the mitochondria however are encoded by nuclear genes (Shadel et al., 1997). Mitochondrial DNA (mtDNA) is also known to have an area of non-coding sequence known as the displacement loop (D-loop), or the control region (Shadel et al., 1997). All mitochondrial gene expression is regulated by promoters found in this region (Shadel et al. 1997). Regulation of these genes at these promoters is thought to be entirely by genes encoded by the nucleus (Scarpulla, 2006), so for this reason, communication and interaction between mtDNA and the nuclear DNA are necessary for proper mitochondria function and mtDNA replication. Mitochondria are also necessary for cell cycle progression, as low levels of ATP can signal a halt due to energy depletion (Mandal et al., 2005), also making it necessary for communication between the nucleus and the mitochondria.

Mitochondria are highly dynamic structures, and reproduce via the process of fission and fusion, with fission being the process of a single mitochondria dividing to provide two separate mitochondrion, and fusion of mitochondria occurring when two separate mitochondria come together to form a larger single mitochondria (Hales et al., 1997). The fission and fusing processes take place according to energetic need of the cell (Zorzano et al., 2010). Mitochondrial homeostasis is a complex process involving fission and fusion of mitochondria, replication of mtDNA and replication of nuclear DNA. It is known that all of these things contribute to mitochondrial reproduction, however the exact mechanisms, and the timing of mitochondrial reproduction throughout the cell cycle and development, as well as the coupling of mitochondria replication to mtDNA replication are generally not well understood (Martinez-Diez et.al., 2006).

Eukaryotes typically follow a pattern of strict maternal inheritance of mitochondria (Birky et al., 1995). Sometime during spermatogenesis, sperm mitochondria are tagged with ubiquitin, and once fertilization of the egg occurs, this targets the sperm mitochondria for proteolysis (Stuovsky et al., 1999). This is thought to be a mechanism that has evolved due to the fact that sperm produce high levels of reactive oxygen species upon fertilization, which are toxic to the mitochondria (Aitken et al, 1995). One exception consists of a group of bivalve molluscs, which can inherit mitochondria from both parents (Zouros et al., 1994). This process is termed Double Uniparental Inheritance (DUI), and has been identified in a number of different mollusc species, the most commonly studied being the members of the phylum *Mytilus* (Zouros et al., 1994). Each offspring inherits one mitochondrial genome from the mother, designated F-type, and one mitochondrial genome from the father, designated M-type

(Zouros et al., 1994). These two genomes are distinct from one another, with up to 40% sequence divergence in some species (Zouros 2013).

The two mitochondrial haplotypes have different functions in the organism, and although it is not clear how the function of the two genomes differ, it is becoming increasingly evident that the M-type mitochondria play a large role in spermatogenesis. The M-type mitochondria are transmitted to the egg upon fertilization via the sperm (Longo et.al., 1967). If the offspring is to be male, the M-type mitochondria aggregate together by the first cell division, and are localized to the D-blastomere by the second cell division (Cao et al., 2004). This blastomere and its progeny are thought to give rise to the germ line (Verdonk et al., 1983). In the mature male, the gonad is composed almost completely of M-type mitochondrial DNA (mtDNA), while the somatic tissues are composed almost completely of F-type mtDNA (Cao et al.,2004), indicating the M-type mtDNA functions specifically in the gonad and/or gametes. If the offspring is to be female, the sperm mitochondria are transmitted to the egg and have no apparent localization (Cao et al., 2004). They appear to randomly disperse within the zygote during cell division and the mature female tissues are all composed of F-type mtDNA (Cao et al., 2004). There are few exceptions to these rules, and these rules are not as well established in other DUI species (Milani et al., 2014), however in *Mytlids* this has proven to be the norm (Zouros et al., 1994).

The sperm and the egg contribute mitochondria to the zygote in unequal proportions. The sperm only contributes five, albeit large, mitochondria to the zygote (Longo et al., 1967), whereas the egg contributes tens of thousands (White et al., 2008). The number of copies of mtDNA in each mitochondria is unknown, and it stands to

reason that the sperm mitochondria may contain more mtDNA copies than the egg mitochondria due to possible fusion of many smaller mitochondria to form the 5 larger mitochondria that are transmitted to the egg upon fertilization (Zouros, 2013). However, even taking this into account, the amount of M-type mtDNA transmitted to the zygote is small in proportion to the F-type mtDNA transmitted by the egg (Zouros, 2013). If the replication rate between the two mtDNA haplotypes were the same, the F-type mtDNA would be chosen for replication more often than the M-type mtDNA, leading to a bottleneck effect in which the M-type mtDNA is lost. This cannot be what is occurring, since the ratio of M-type to F-type mtDNA in a mature male mussel (though very variable) can reach 1:1 (Sano et al., 2007). This would imply one of two things is occurring: either the M-type DNA is being preferentially amplified, or one of the two haplotypes is lost.

Molluscs are mosaic developers, and divide according to a spiral cleavage pattern. The first cleavage is unequal, resulting in two cells. The first is designated AB, and the second is CD, with the AB cell being considerably smaller than the CD cell. The second cleavage takes place in a spiral manner, forming equally sized A, B and C blastomeres, and a larger D blastomere, with the D-blastomere thought to give rise to the germ line (Verdonk et al., 1983). In DUI species, not only must the proper mtDNA haplotype be sorted into the correct cell lineage, the mtDNA must also remain purely F-type or M-type. This means that there must be a very complex sorting mechanism by which the mitochondria are sorted into cells in which they will function in DUI species. There also must be coordination between sorting, when mitochondria reproduce, and when mtDNA replicates. If fission and fusion of the mitochondria are occurring, it would give large

possibilities to recombination between the two genomes and mixing of the two mtDNA haplotypes in a single mitochondria. This is probably not what occurs given the distinct localization of the two haplotypes in the mature mussel tissues.

There are some arguments to be made about the role of M-type mtDNA being negligible. Studies performed on hybrid Mytlius species between *Mytlis edulis, Mytilus galloprovincialis*, and *Mytilus trossulus*, have shown what is known as DUI breakdown and mitochondrial genomes from the gonad of males of these hybrid species show sequences that resemble the F-type genome more so than the M-type genome (Weene et al., 1995; Hoeh et al., 1996; Saavedra et al. 1997; Quesada et al. 2003; Ladoukakis et al. 2002; and Burzynski et al. 2006). These sequences have been deemed "masculinized," with the control region of this masculinized sequence varying from a typical F-type mtDNA sequence by less than 3% (Hoeh et. al. 1997). One hypothesis is that the F-type mtDNA at one point invaded the germ line and took over the role of the M-type mtDNA, which may provide some evidence that the F-type mtDNA may be able to provide the same function as the M-type mtDNA in its absence (Hoeh et al. 1997). This masculinization event also provides evidence supporting the hypothesis that the mtDNA sequences themselves have directions for the route of transmission of the separate F and M-type mtDNA haplotypes (Zouros 2013). It is also not known whether replication of M-type mtDNA is causative or associative with the maleness of the offspring. It is known that males retain and replicate the M-type mtDNA, and that females do not (Cao et al., 2004), but retention and replication of M-type mtDNA may not be causative of the embryo becoming male. Female mussels have sex-ratio bias in that their offspring consists either of mostly males, mostly females, or a ratio of 50:50 males to females

(Zouros et al., 1994). A single female will continuously produce this ratio of male to female offspring despite the male it is mated with and daughters do not necessary have the same bias as their mothers (Kenchington et al. 2002). This indicates that something in the egg determines whether the offspring is male or female, and its control may be by genes encoded by the nucleus rather than the mitochondria (Kenchington et al., 2002). Thus, the egg may determine whether or not the zygote is male, and replication of M-type mtDNA is associated with maleness, not causative.

Put simply, the mature female mussel tissues normally consist of 100% F-type mtDNA and mature male tissues contain 100% F-type mtDNA in the somatic tissues, however males contain 100% M-type mtDNA in the germ cells (Cao et al., 2004). There have been studies measuring the proportion of F-type to M-type mtDNA in mature adult tissues that have shown trace amounts of M-type mtDNA detectable in somatic tissues of both male and female adults (Kyriakou et al., 2010, Obata et al., 2011). In female adults, trace amounts of M-type mtDNA would suggest that the M-type mitochondria get randomly dispersed in the zygote, and in mature mussels the M-type mtDNA is not destroyed, but is not subject to significant replication. It has also been hypothesized that "leakage" from the sperm mtDNA may occur in males, leading to somatic tissues being comprised of trace amounts of M-type mtDNA (Milani et al., 2012).

In order to have germ cells purely of M-type mtDNA, at least one copy must be localized to the cell destined to give rise to the germ line and either selective amplification of M-type mtDNA or loss of the F-type mtDNA must occur. Its well established that in male zygotes, the 5 aggregated sperm mitochondria enter the egg upon fertilization (Longo et al., 1967), however it is not known when the mitochondria

dissociate from one another. Studies have shown that in some embryos the sperm mitochondria are still aggregated together as late at as the trochophore stage of embryogenesis (Cao et al., 2004). If one assumes that the aggregate remains together until the germ line must mature, it could be that little to no fission and fusion of mitochondria occurs until this point, and little to no replication of mtDNA occurs either. If the M-type mtDNA has little function in the animal outside of spermatogenesis, it could be that until spermatogenesis occurs, no replication of mtDNA occurs. For these reasons is it necessary to study the replication of total mtDNA in DUI species.

This study will attempt to elucidate the replication rate of total mtDNA relative to nuclear DNA, in order to aid in the understanding of the sorting and reproduction of mtDNA in DUI species. Quantitative PCR (qPCR) will be used to analyze mtDNA replication across embryonic development and early adulthood in DUI species, *Mytilus galloprovincialis*, beginning with trochophore larvae (ca. 24 hours pos-fertlization), and comparing replication of mitochondrial DNA through the early veliger (D-stage; ca. 48 hours), and pediveliger (late stage veliger; ca. 7 days). The pediveliger stage is considered the final embryonic stage before metamorphosis and the formation of a juvenile mussel. Early juveniles (spat) measuring 1mm in length (ca. 70 days post fertilization) will also be used to monitor replication of mtDNA past the embryonic stages of development. Understanding mtDNA replication as a whole will aid in using these organisms as model systems for both germ line development and mutated mtDNA replication and inheritance.

CHAPTER 2

METHODS

Real-time, or quantitative PCR (qPCR) was used to monitor replication of total mtDNA (both F-type and M-type). Three different embryonic stages were used to track replication through embryonic development: trochophore, early veliger, and pediveliger embryos (listed in order from youngest to oldest). To monitor replication of total mtDNA post-embryogenesis, 24 individual spat, measuring 1mm in length were also studied with qPCR.

2.1 Spawning of Mussels: In order to grow embryos to the trochophore stage, live *Mytilus galloprovincialis* mussels were obtained from Taylor Shellfish farms (Shelton, Washington.) Spawning of mussels was induced by placing individual mussels in separate dishes containing natural seawater at room temperature. Room temperature (22°C) is warm enough to induce spawning of ripe mussels. Eggs from a single female were collected and washed 2x with natural seawater that had been filtered through a 0.45uM filter. Sperm were collected with a pipette and added drop-wise to the eggs to allow for fertilization. Successful fertilization was followed by eye under a dissecting microscope. Embryos were then left to proceed through development until the embryos were between 2 and 4 cells in size. Once the embryos had reached the 2-4cell stage, the embryos were diluted 1:1000 in filtered seawater containing 10mg/ml gentamycin and kept in stirred culture at 22°C in 3 liter glass containers until they reached the

trochophore stage (ca. 24 hours.) Embryos were then washed in filtered sea water and fixed in 70% ethanol.

Taylor Shellfish Farms generously provided both early veliger and pediveliger embryos. The spawning date for these mussels was 11/14/12. Embryos were collected at the early veliger 2 days post-fertilization, pediveliger from the same spawning were collected at 7 days post fertilization, and spat from the same spawning were collected 70 days post fertilization. These larvae were shipped live on ice, washed in filtered sea water and fixed in 70% ethanol. All larvae were checked for viability (active swimming behavior and overall anatomical structure) before fixation.

2.2 DNA extraction: All embryos were fixed in 70%EtOH. They were washed 2X with TE buffer (10mM Tris-HCl pH 7.5, 1mM EDTA), and then resuspended in TE buffer. Single embryos were separated with a p20 pipette and suspended in 2uL of TE buffer. In order to extract DNA, 1uL of proteinase K (200ug/uL) was added to each sample. Spat were placed in 24uL of TE buffer, and 6uL of proteinase K (200ug/uL) was added to each sample to extract DNA. Samples were then incubated for 3 hours at 55°C, then at 95°C for 10min in order to inactivate the proteinase K.

2.3 qPCR: Quantitative PCR (qPCR)was run using a SensiFAST™ SYBR NO-ROX Kit (Bioline). Each reaction consisted of: 10uL of 2X Reaction mix, 0.27uL of forward and reverse primers (final concentration=0.4uM), and water up to 20uL after the addition of DNA. The reaction was run with the following 2-step cycling conditions: 1) 95°C for 3min, 2) 95°C for 5sec, 3) anneal (58°C for RS 233 and 234, 56°C for RS 219 and 220) for 30sec, 4) Steps 2-4 were repeated for 35 cycles. The melt curve was determined with

the following conditions: 1) 60°C for 5sec, and 2) 95°C for 5sec, with increment increases of 0.5°C for Step 1 with each subsequent read. All reactions were run with replicates, and with no-template controls. QPCRs were all run on a Bio-Rad CFX96 real-time PCR machine, and results were analyzed using Bio-Rad CFX Manager 3.0.

Primer set RS 233 and 234, amplifies a region of the mitochondrial D-loop identical in sequence in both M-type and F-type mtDNA. Primer set RS 219 and 220 amplifies a region of the male mitochondrial D-loop only found in the M-type mtDNA genome.

RS 233: 5'-GCC CTT CCG TTA GAA GAA GTA G-3'
RS 234: 5'-GCT CGA AAC CGC TAG ATA CAA-3'

RS 219: 5'-GGA ATA TAC GCA GAT AGT TGG AGA TAG-3'
RS 220: 5'-CCC TGG GAT TAC TCT GTT GTT C-3'

Primers were designed using PrimerQuest (Integrated DNA Technologies) and Genbank Accession ID NC_006886.2 for RS 233 and 234, and Accession ID AF188280 for RS 219 and 220.

In order to ensure that change in template concentration could be detected using qPCR, each set of samples (5 sets, each with 4 replicate samples) were set up such that each sample in a set contained one more embryo than the samples in the previous set, with the final sample set containing 5 embryos per sample.

qPCR threshold cycle values (Ct values) were obtained by the Bio-Rad CFX96 real-time PCR machine, when fluorescence reached levels above background. For each

sample, Ct values were linearized, using the following equation assuming a doubling of DNA with each amplification cycle. This linearized value is proportional to the amount of DNA in the original sample.

$$Ct\_Trans=(1/2)^{Ct}$$

Values were then normalized to the mean value of samples containing 3 trochophore larvae. Using a sample with 3 embryos ensured that the sample contained enough DNA to be reliably detected by the qPCR. The mean Ct of samples with 3 trochophore larvae was 20.7925. That number was transformed using the equation above, to get a Ct_Trans of $5.51x10^{-7}$. That value was then multipled by three, and all subsequent Ct values were normalized to this number, and deemed "trochophore equivalents" using the equation below.

$$\text{"trochophore equivalents"}=(Ct\_Trans/16.53x10^{-7})$$

Ct values for spat were adjusted to account for the fact that only 1/5 of the DNA extracted from the individual spat was used in the qPCR. Ct_Trans was multiplied by 5 before being converted to "trochophore equivalents." To compare these values to the mean values of 3 embryos per sample from qPCR at different embryonic stages of development, the values were then multiplied by 3 to adjust values to represent 3 spat.

2.4 Quantification of total DNA: Total DNA was quantified using a Qubit 2.0 Fluorometer (Invitrogen®) and Qubit™ dsDNA HS Assay Kit (Invitrogen®) according to kit specifications. In order to ensure that change in DNA concentration could be detected using this method, each set of samples (5 sets, each with 4 replicate samples) were set up such that each sample in a set contained one more embryo than the samples

in the previous set, with the final sample set containing 5 embryos per sample. The mean values of samples containing 3 embryos for each embryonic stage were analyzed in order to ensure there was enough DNA to detect reliable concentrations.

CHAPTER 3

RESULTS

To follow the process of total mtDNA replication during development, qPCR was performed using embryos at each of five developmental stages. In order to obtain a per embryo measurement, and to ensure that a change in template concentration could be detected, samples were initially set up so that each set of samples contained one more embryo than the previous set, with the final sample containing five embryos. Each set consisted of four replicates. Data sets from each stage of development were collected from a single qPCR run (Figure 3.1). This same set-up process was used in order to determine the amount of DNA present in a single embryo, and to make sure changes in DNA content was detectable using the Qubit 2.0 (Figure 3.2).

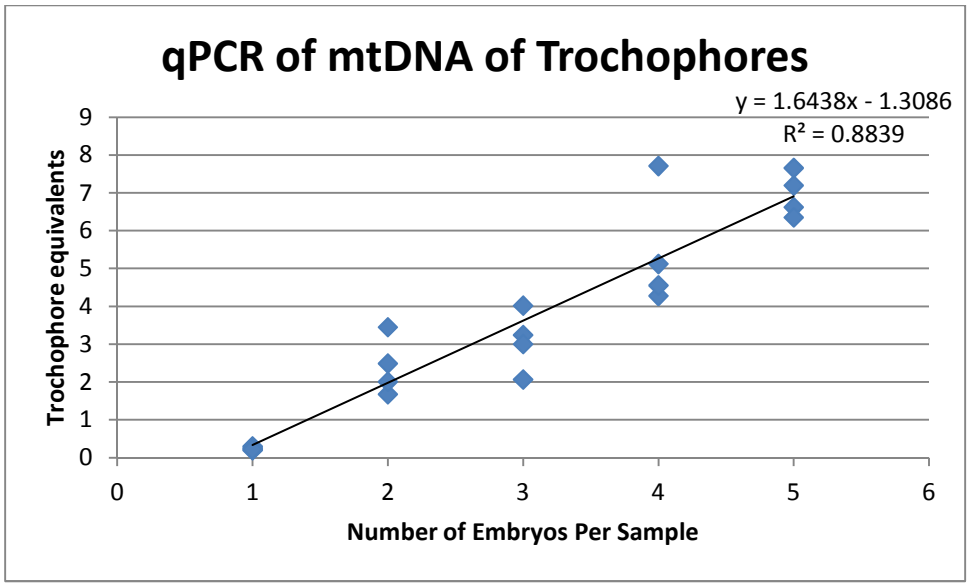
Results from both the qPCR (Figure 3.1) and quantification of the amount of DNA in a single embryo (Figure 3.3) display characteristics of a positive linear correlation. Based on these results the most reliable values came from samples containing 3 embryos. For qPCR results from each stage, the mean "trochophore equivalent" value for samples containing 3 embryos were graphed according to age (Figure 3.2). The mean of 3 trochophore embryo samples is equal to 3.08 trochophore equivalents. The mean of 3 early veliger embryo samples corresponds to 3.16 trochophore equivalents, indicating a lack of mtDNA replication between these two stages. The mean of 3 pediveliger embryo samples however, corresponds to 21.36

trochophore equivalents. This shows that during the early development of the mussel there is no significant mtDNA replication. However in the five days between the early veliger (48 hours post fertilization) and pediveliger stage (7 days post-fertilization) total mtDNA has clearly undergone some replication.

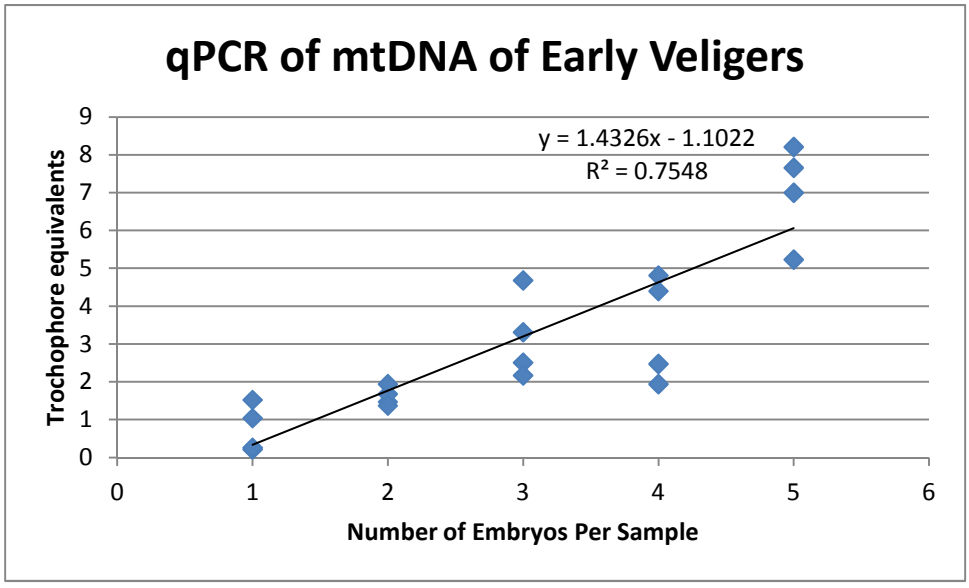
The pediveliger stage in *Mytilus* occurs just prior to metamorphosis and settlement of young adult mussels. To determine if there is a significant change in the amount of mtDNA associated with 1mm young adults (spat; 70 days post-fertilization) 24 individual spat were also measured for total mtDNA content using qPCR. At this late stage in development, M-type mitochondrial signal is easily detected using qPCR. Each individual 1mm adult was measured for both total mtDNA (Figure 3.2), and M-type mtDNA (data not shown). Individuals were deemed male if the M-type mtDNA signal was reliably detected at a Ct value of less than 30, and female if the Ct value for the male mtDNA was 32 or greater (background noise level.) Of the 24 individuals tested, 7 were male, and 17 were female. The mean value of total mtDNA for both the male individuals and female individuals was determined (Figure 3.2) Based on these values there is no significant difference between total mtDNA in female versus male 1 mm adult mussels. The mean value of all individuals, regardless of sex, is 80.19 trochophore equivalents. The increase in total mtDNA from pediveliger to 1mm spat is 3.75-fold while the increase between early veliger and pediveliger is 6.76-fold. The total net increase in mtDNA between early veliger and 1mm spat is 25.38. Given that the time between veliger and pediveliger is 5 days and between pediveliger and 1mm spat is 63 days, this means that the highest rate of mtDNA replication is occurring between the early veliger

and the pediveliger stage prior to settlement. There is continued slower replication of total mtDNA replication following settlement and growth of the young adult.

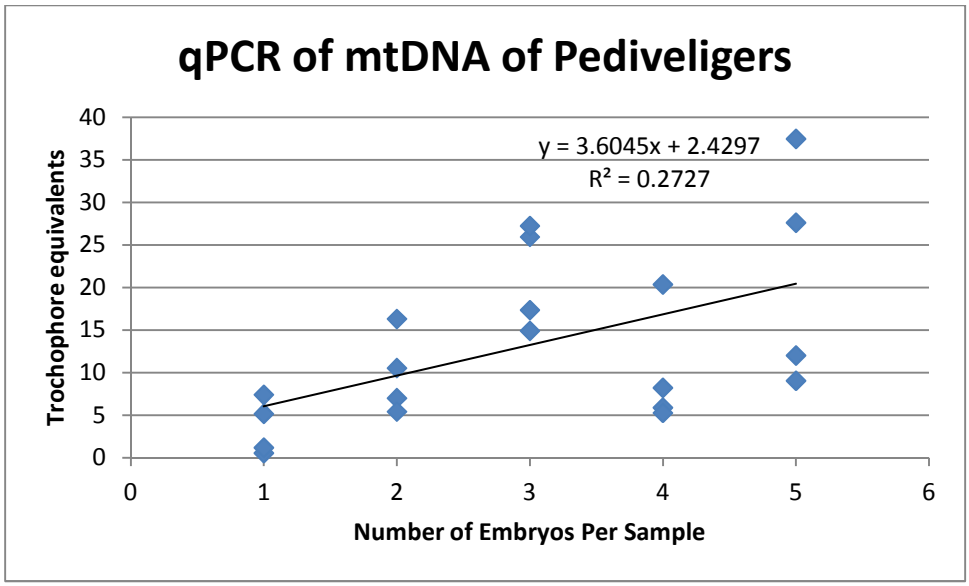
In order to compare total DNA (genomic plus mitochondrial) with mtDNA replication during embryonic development, the increase in total DNA (quantitatively mostly genomic DNA) was determine for each of the three stages examine (Figure 3.3). Again using the mean value of 3 embryos per sample, total DNA was measure using a Qubit 2.0 Fluorometer (Invitrogen®). A comparison was then made between the rate of increase in total DNA versus that of the mtDNA. Between the zygote and the 128 cell trochophore (Zouros, 2013) there is an expected 7-fold increase in genomic DNA, added to which is the egg mtDNA present at fertilization was then compared among each stage. The mean value of samples containing 3 embryos for trochophores is 0.68ng. For early veligers the mean value of three embryos is 0.62ng, and for pediveligers the mean value is 13.2ng (Figure 3.3f). The increase in total DNA correlates with the rapid seven cell divisions occurring during the first 24 hours of early development. Over the next 24 hours there is no significant increase in the amount of total DNA, consistent with the absence of any mtDNA replication and suggesting that this period represents a pause during which cell rearrangement and differentiation is occurring following mid-blastula transition. When one examines the relative amount of total DNA between early veliger and pediveliger there is a 21.29-fold increase in total DNA content as cell division resumes and growth proceeds over the next seven days in the feeding veliger.


qPCR of mtDNA of Trochophores
y = 1.6438x - 1.3086
R² = 0.8839
Trochophore equivalents
Number of Embryos Per Sample
0
1
2
3
4
5
6
7
8
9

3.1a.


qPCR of mtDNA of Early Veligers
y = 1.4326x - 1.1022
R² = 0.7548
Trochophore equivalents
Number of Embryos Per Sample
0
1
2
3
4
5
6
7
8
9

3.1b.



3.1c.

Figure 3.1. Quantitative PCR (qPCR) was run on total mtDNA using embryos of each embryonic stage of development. Five samples were run with each subsequent sample containing one additional embryo than the previous sample. Ct values were transformed to graph the results linearly, and then normalized such that mean value of samples with 3 trochophores represents 1, and values are shown in "3 Troch Equivalents." Results from each stage show a linear increase as a single embryo is added to the sample.

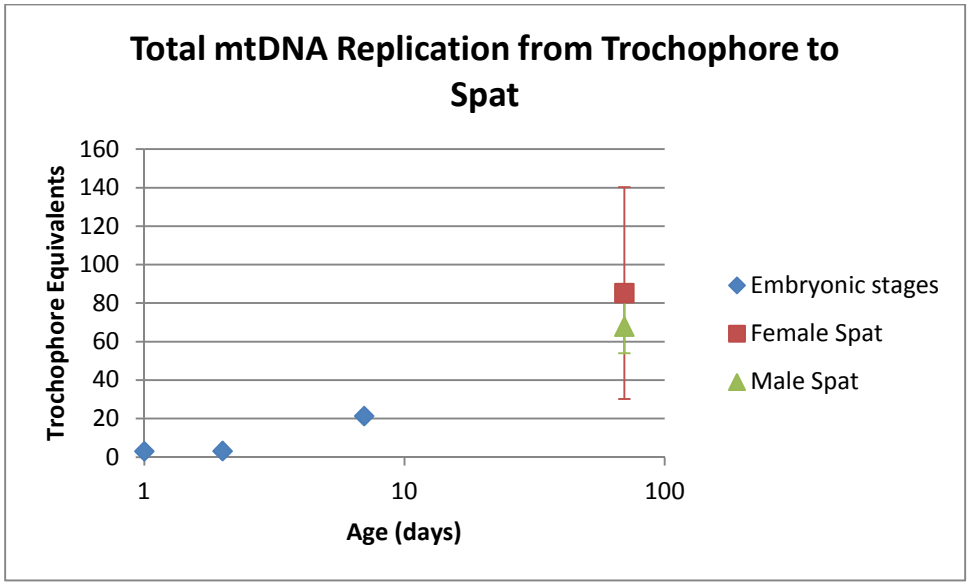
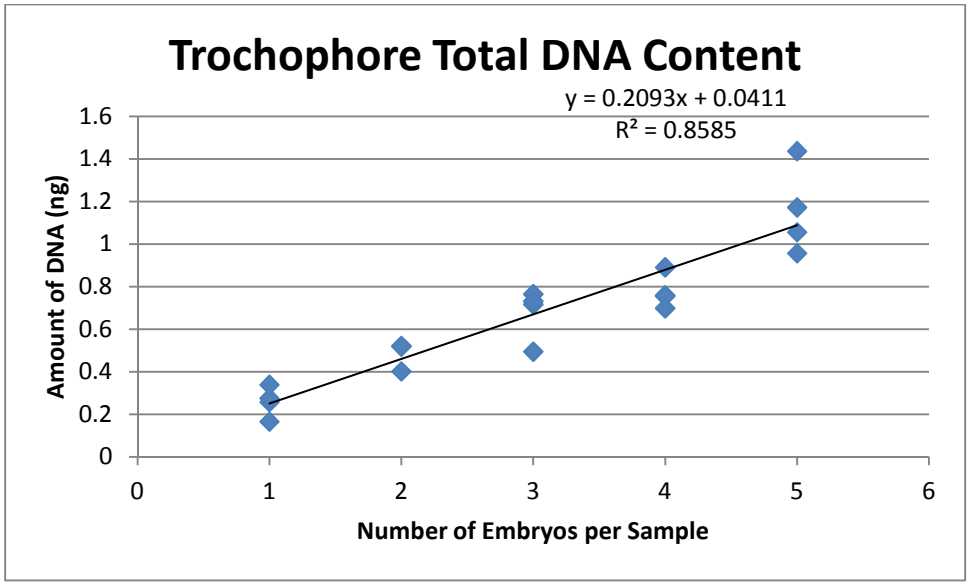
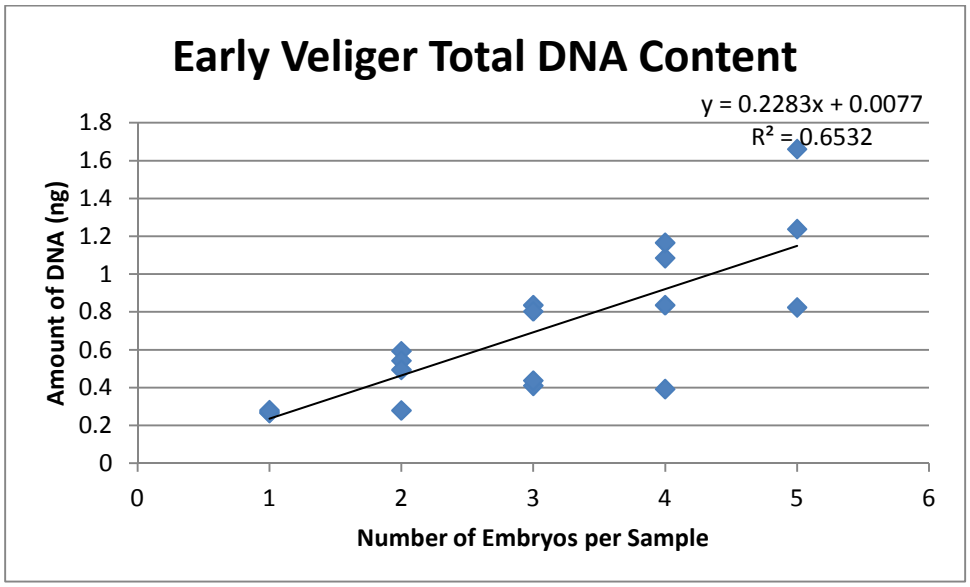


Figure 3.2. The mean values of samples containing 3 embryos were graphed according to "3 Troch Equivalents" against days post fertilization. The trochophore larvae are represented at 1 day post fertilization, the early veligers are 2 days post fertilization, the

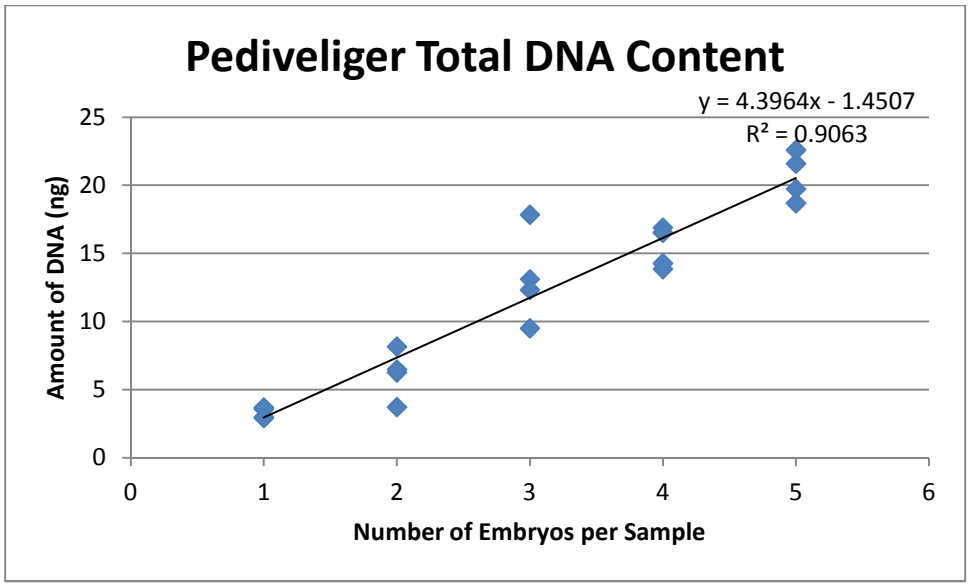
pediveligers are 7 days post fertilization, and the spat are 70 days post fertilization. The mean value of 3 trochophore larvae is 1, while the mean value of 3 early veliger larvae is also 1. The mean value of 3 pediveliger larvae is 7. When taking standard deviation into account (represented by error bars), there is no difference in total mtDNA between male and female spat. The mean value of all individuals is 26.73 "3 Troch Equivalents". There is a 7-fold increase in mtDNA from trochophore and early veligers to pediveliger. There is a 26.73-fold increase in mtDNA from early veliger to 1mm spat, and a 3.81-fold increase from pediveliger to 1mm spat.



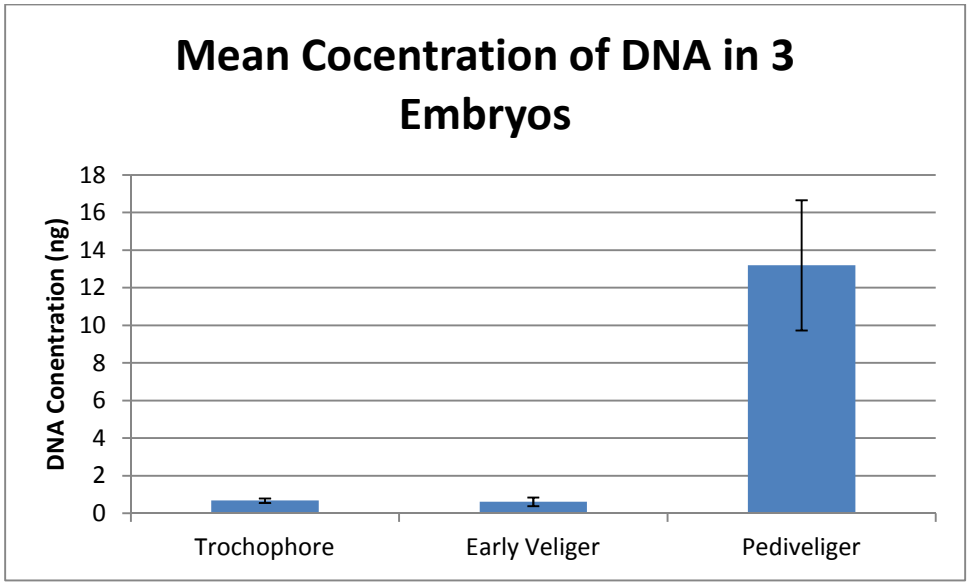
3.3a.



3.3b.



3.3c.



3.3d.

Figure 3.3a-c: Embryos were measured for total DNA content, five samples were run with each subsequent sample containing one additional embryo than the previous sample. As each additional embryo is added to the sample, the DNA concentration increases linearly. Fig 3d graphs the mean value of samples containing 3 embryos for each stage. The mean value of samples containing 3 embryos for trochophores is 0.68ng, for early veliger is 0.62ng, and for veligers is 13.2ng. Taking standard deviation into account (represented by error bars), there is no change between trochophores and early veligers,

however, between early veligers and pediveligers there is a 21.29-fold increase in total DNA content.

CHAPTER 4

DISCUSSION

A small number of members of the Bivalvia (Mollusca) exhibit a unique form of mitochondrial inheritance referred to as Double Uniparental Inheritance (DUI) of mitochondria (Zouros et al., 1994). In these species the male, through the sperm, provides the zygote with 5 large mitochondria containing only M-type mtDNA while the female egg has a very large number of small mitochondria containing only F-type mtDNA (Longo et al., 1967; Cao et al., 2004). The initial ratio of M to F type is very low but by the adult state the entire germ line of the male is M-type while the bulk of the somatic tissue is F-type (Cao et al., 2004). The absolute ratio is quite variable but can often reach 1:1 or even greater (Sano et al., 2007). In the female the M-type apparently fail under normal conditions to replicate and the female germ-line remains F-type, thus maintaining the pattern of DUI (Stewart et al., 1995). The change in ratio of M to F type mtDNA requires that at some point between early development and the formation of gametes that there be a selective amplification of the M-type mtDNA relative to the F-type (Zouros et al., 1994). This study examines where between fertilization and the young adult stage total mtDNA amplification first occurs.

Using embryos at the trochophore stage as a standard we have converted PCR Ct values early stage veligers (48 hours post-fertilization) and pediveligers (7 days post-fertilization) to a standard mtDNA and then compared the total amount of mtDNA in the

three stages of embryogenesis and also to a 1mm post-settlement 70 day old young adult (Figures 2.1 and 2.2). The amount of mtDNA present in the trochophore and the early veliger stages (24 and 48 hours post-fertilization) is statistically identical, indicating that no significant amount of mtDNA is being synthesized during these early stages of embryogenesis. The period between early veliger and pediveliger (48 hours and 7 days post fertilization respectively) shows a large increase in mtDNA replication with a 6.76-fold increase in total mtDNA. This rate of increase, assuming that it is linear, is approximately a doubling time of between 1.5 and 2 days. Between the pediveliger stage and the 1mm young larva morphogenesis and settlement occurs (Bayne, 1965). A comparison of the pediveliger and the 1mm young larva (age difference of 63 days) shows a 3.75 fold increase in total mtDNA. Again assuming that the mtDNA replication rate is linear during this period, one sees a doubling rate of approximately 16.5 days or a little over a 10-fold decrease over that seen between early veliger and pediveliger stage.

By comparison, nuclear DNA is rapidly doubling between the zygote and the trochophore stage while the mtDNA is not replicating. While both nuclear DNA and mitochondrial DNA are not replicating between the trochophore and the early veliger stage, between the early veliger and pediveliger stages nuclear DNA is replicating at an even faster rate than the mtDNA, with a 21.29-fold increase in total DNA content. This confirms previous observations that mtDNA replication is not directly coupled to nuclear DNA replication. After the pediveliger stage, the mtDNA continues to replicate but at a decreased rate relative to the replication between early veliger and pediveliger stages. Results from previous studies also support this data. A recent study by Milani et. al. 2012 in the clam DUI species, *Ruditapes philippinarum* used qPCR to analyze mtDNA

replication up to the 32-cell stage of development, and found that little mtDNA replication occurred relative to nuclear DNA replication (Milani et al., 2012). Similar low levels or mtDNA replication during early stages of embryogenesis are also seen in several other non-DUI species including fishes (Wang et al., 1992), sea urchins (Matsumoto et al., 1974), frogs (Chase et al., 1972), and nematodes (Tsang et al., 2002).

The large number of mitochondria in the eggs of both normal and DUI species (White et al., 2008; Longo et al., 1967) suggests that there is more than sufficient mitochondrial capacity to allow the embryo to pass through early embryogenesis without the need for additional mitochondrial DNA replication. In DUI exhibiting species of molluscs, however, not only must the organism increase the total number of mitochondria as growth occurs, but it must also (a) segregate the M-type mitochondria into the germ line through some as yet unknown mechanism, but it must also selectively increase the M-type mtDNA to account for the decreased ratio of F to M-type seen in the adult (Zouros et al., 1994). This cell-sorting component is especially interesting in the case of a DUI species like *M. galloprovincialis* where during the early cleavage stages sperm mitochondria are aggregated together and localized to a specific cell during the first division in males (Cao et al., 2004). Though the mechanism by which this sorting occurs is not known, one idea is that the M-type mtDNA localized in specific male mitochondria, fails to undergo any replication until it is assigned to cells committed to the germ line, thus insuring proper sorting of the M-mtDNA only to germ line cells (Cao et al., 2004). The point at which this sorting is complete and the replication of the M-mtDNA can commence is not known but the studies reported here indicate that there is a point between the early veliger and the pediveliger stage when mtDNA replication

become highly active. Further studies need to be done in regards to this to determine if this replication is associated with the M-type, the F-type or both haplotypes of the mtDNA . Preliminary qPCR studies using male specific qPCR primers show that M-type mtDNA can be detected at low levels in the pediveliger stage and in high quantities in the early larval stages while they are below the detection of qPCR in earlier stages of development.

In humans, mutations in mtDNA can give rise to a number of diseases including neurodegenerative diseases, those related to aging, and cancer (Reddy et al., 2011; Taylor et al., 2005). These diseases often worsen with age, and severity can vary depending on the ratio of mutated:wild-type mtDNA inherited (Chan et al., 2006). It is not known how a mutation in a single copy of mtDNA, in a cell where 10^5-10^6 copies of mitochondrial DNA are present (White et al., 2008), can out-replicate wild-type mtDNA thus resulting in phenotypic expression of a disease state. In DUI species the fact that the M-type mtDNA replicates to a point at which it dominates an entire tissue and often exceeds the total amount of F-mtDNA makes organisms exhibiting a DUI form of mitochondrial inheritance a good model for studying selective amplification of one of two types of mtDNA.

REFERENCES

Aitken, R. J., Jones, K. T. and Robertson, S. A. (2012), Reactive Oxygen Species and Sperm Function—In Sickness and In Health. *Journal of Andrology, 33*: 1096–1106. doi: 10.2164/jandrol.112.016535

Birky, C. W. J., 1995. Uniparental inheritance of mitochondrial and chloroplast genes: mechanisms and evolution. *Proc Natl Acad Sci USA*, *92*, 11331-11338.

Boore, J. L. (1999). Animal mitochondrial genomes. *Nucl. Acids Res.*, *27*, 1767-1780. doi: 10.1093/nar/27.8.1767Shadel 1997

Burzynski, A., Zbawicka, M., Skibinski, D.O.F. & Weene, R. (2006). Doubly uniparental inheritance is associated with high polymorphism for rearranged and recombinant control region haplotypes in Baltic *Mytilus trossulus. Genet, 174*, 1081-1094.

Cao, L., Kenchington, E., & Zouros, E., 2004. Differential segregation patterns of sperm mitochondria in embryos of the blue mussel (*Mytilus edulis*). *Genetics, 166*, 883-894.

Garrido-Ramos, M., Stewart, D., Sutherland, B., & Zouros, E. 1998., The distribution of male-transmitted and female-transmitted mitochondrial DNA types in somatic tissues of blue mussels: Implications for the operation of Doubly Uniparental Inheritance of mitochondrial DNA. *Genome, 41*, 818-824. doi: 10.1139/g98-081

Hales, K. G., & Fuller, M. T. (1997). Developmentally regulated mitochondrial fusion mediated by a conserved, novel, predicted GTPase. *Cell, 90,* 121-129. doi: 10.1016/S0092-8674(00)80319-0

Hoeh, W. R., Stewart, D. T., Saavedra, C., Sutherland, B. W., & Zouros, E. (1997). Phylogenetic evidence for role-reversals of gender-associated mitochondrial DNA in *Mytilus* (bivalvia: *Mytilidae*). *Mol Biol Evol,14,* 959-967. doi: 10.1093/oxfordjournals.molbev.a0258

Hoeh, W.R., Stewart, D.T., Sutherland, B. W., & Zouros, E. (1996) Multiple origins of gender-associated mitochondrial DNA lineages in bivalves (Mollusca: Bivalvia). *Evolution, 50,* 2276-2286

Kenchington, E., MacDonald, B., Cao, L., Tsagkarakis, D., & Zouros, E. (2002). Genetics of mother-dependent sex ratio in blue mussels (*Mytilus* spp.) and implications for Doubly Uniparental Inheritance of mitochondrial DNA. *Genetics, 161,* 1579-1588.

Kyriakou, E., Zouros, E., & Rodakis, G. C, 2010. The atypical presence of the paternal mitochondrial DNA in somatic tissues of male and female individuals of the blue mussel species *Mytilus galloprovincialis BMC Research Notes, 3,* 222. doi: 10.1186/1756-0500-3-222

Longo, F. J., & Dornfeld, E. J., 1967. The fine structure of spermatid differentiation in the mussel, *Mytilus edulis*. *J Ultrastruc Res., 20,* 462-480.

Loudakakis, M., Saavedra, C., Magoulas, A., & Zouros, E. (2002). Mitochondrial DNA variation in a species with two mitochondrial genomes: The case of *Mytilus*

galloprovincialis from the Atlantic, the Mediterranean and the Black Sea. *Molecular Ecology, 11*, 755-769.

Mandal, S., Guptan, P., Owusu-Ansah, E., & Banerjee, U. (2005). Mitochondrial regulation of cell cycle progression during development as revealed by the tenured mutation in drosophila. *Dev. Cell*, *9*, 843-854. doi: 10.1016/j.devcel.2005.11.006

Martı´nez-Diez M, Santamarı´a G, Ortega A´D, Cuezva JM (2006) Biogenesis and dynamics of mitochondria during the cell cycle: Significance of 3'UTRs. *PLoS ONE, 1*, e107. doi:10.1371/journal.pone.0000107

Matsumoto, L., Kasamatsu, H., Piko, L., & Vinograd, J., 1974. Mitochondrial DNA replication in sea urchin oocytes. *J Cell Biol*, *63*, 146-159.

Milani, L., Ghiselli, F., & Passamonti, M. (2012). Sex-linked mitochondrial behavior during early embryo development in *Ruditapes philippinarum* (bivalvia veneridae) a species with the Doubly Uniparental Inheritance (DUI) of mitochondria. *J. Exp. Zool. (Mol. Dev. Evol.)*, *318*, 182-189.

Milani, L., Ghiselli, F., Iannello, M., & Passamonti, M., 2014. Evidence for somatic transcription of male-transmitted mitochondrial genome in the DUI species *Ruditapes philippinarum*. *Curr Genet*, Forthcoming 2014, doi: 10.1007/s00294-014-0420-7

Obata, M., Sano, N., & Komaru, A., 2011. Different transcriptional ratios of male and female transmitted mitochondrial DNA and tissue-specific expression patterns in

the blue mussel, *Mytilus galloprovincialis*. *Develop. Growth Differ.*, *53*, 878-886. doi: 10.1111/j.1440-169X.2011.01294.x

Quesada, H., Stuckas, H., & Skibiniski, D.O.F. (2003). Heteroplasmy suggests paternal co-transmission of multiple genomes and pervasive reversion of maternally into paternally transmitted genomes of mussel (*Mytilus*) mitochondrial DNA. *Journal of Molecular Evolution, 57*, 138-147

Reddy, P., & Reddy, T., 2011. Mitochondria as a therapeutic target for aging and neurodegenerative diseases. *Curr Alzheimer Ress, 8,* 393-409. doi: 10.2174/156720511795745401

Saavedra, C., Reyero, M.I., & Zouros, E. (1997). Male-dependent Doubly Uniparental Inheritance of mitochondrial DNA and female-dependent sex-ratio in the mussel *Mytilus galloprovincialis.* Genet, 145, 1073-1082

Sano, N., Obata, M., & Komaru, A. (2007). Quantitation of the male and female types of mitochondrial DNA in a blue mussel, *Mytilus galloprovincialis*, using real-time polymerase chain reaction assay. *Develop. Growth Differ., 49,* 67-72. doi: 10.1111/j.1440-169x.2007.00904.x

Scarpulla, R. C. (2006), Nuclear control of respiratory gene expression in mammalian cells. *J. Cell. Biochem., 97:* 673–683. doi: 10.1002/jcb.20743

Stewart, D. T., Saavedra, C., Stanwood, R. R., Ball, A. O., & Zouros, E., 1995. Male and female mitochondrial DNA lineages in the blue mussel (*Mytilus edulis*) species group. *Mol Biol Evol, 12*, 735-747.

Sutovsky , P., Moreno, R. D., Ramalho-Santos, R., Dominko, T., Simerly, C., & Schatten, G. (2000). Ubiquitinated sperm mitochondria, selective proteolysis, and the regulation of mitochondrial inheritance in mammalian embryos. *Biology of Reproduction, 63*, 582-590. doi: 10.1095/biolreprod63.2.582

Taylor, R., & Turnbull, D., 2005. Mitochondrial DNA mutations in human disease. *Nat Revs Genet, 6*, 389-402. doi: 10.1038/nrg1606

Tsang, W. Y., & Lemire, B. D., 2002. Mitochondrial genome content is regulated during nematode development. *Biochem Biophys Res Commun, 291*, 8-16. doi: 10.1006/bbrc.2002.6394

Verdonk, N.H., & Van Den Biggelaar, J.A.M. 1983. Early development and the formation of the germ layers. *The Mollusca*, 3, 91-122.

Wang, G., & Yan, S., 1992. Mitochondrial DNA content and mitochondrial gene transcriptional activities in the early development of loach and goldfish. *Int J Dev Biol, 36*, 477-482.

Weene, R., & Skibinski, D.O.F. 1995. Mitochondrial DNA heteroplasmy in European populations of the mussel *Mytilus edulis. Marine Biology, 122*, 619-624

White, D. J., Wolff, J. N., Pierson, M., & Gemmell, N. J., 2008. Revealing the hidden complexities of mtDNA inheritance. *Mol Ecol, 17*, 4925-4942. doi: 10.1111/j.1365-294X.2008.03982.x

Zorzano, A., Liesa, M., Sebastian, D., Segales, J., & Palacin, M. (2010). Mitochondrial fusion proteins: Dual regulators of morphology and metabolism. *Seminars in Cell & Developmental Biology, 21*, 566-574. doi: 10.1016/j.semcdb.2010.01.002

Zouros, E., 2013. Biparental inheritance through uniparental transmission: The Doubly Uniparental Inheritance (DUI) of mitochondrial DNA. *Evol Biol*, *40*, 1-31. doi: 10.1007/s11692-012-9195-2

Zouros, E., Oberhouser Ball, A., Saavedra, C., & Freeman, K. R,. 1994. An unusual type of mitochondrial DNA inheritance in the blue mussel *Mytilus*. *Proc Natl Acad Sci USA*, *91*, 7463-7467.